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Identifying Mechanism of Traversal of Corneal Epithelial Cells by Pseudomonas aerugnosa

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Identifying Mechanisms of Traversal of Corneal Epithelial Cells by *Pseudomonas aeruginosa*

By

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Pseudomonas aeruginosa keratitis is a sight-threatening complication of contact lens wear. Ordinarily, the ocular surface is effective against microbial infiltration through a variety of mechanical, anatomical, and immunological defense mechanisms which protect the cornea. However, contact lens wear, ocular injury and/or surgery predispose individuals to bacterial keratitis. In the case of contact lens wear, the Gram-negative bacterium *Pseudomonas aeruginosa* is the most frequently isolated causative agent. As the number of antimicrobial compounds effective against *P. aeruginosa* decreases, due to of the acquisition and spread of antibiotic resistance, there is a growing need for novel therapeutic approaches corneal infection. The fact that *P. aeruginosa* can cross the corneal epithelium into the stroma to cause disease in contact lens wearers suggest a compromise in host defense. However, little is known of the mechanisms by which bacteria reach the stroma after adherence to corneal epithelial cells and how contact lenses increase the incidence of *P. aeruginosa* infection. Our broad theoretical model to explain the pathogenesis of contact lens related *P. aeruginosa* infection is that contact lens wear, because it provides a surface for biofilm formation, enables bacteria to persist at the ocular surface for long enough to adapt to defense factors that otherwise limit their ability to penetrate the corneal epithelium. Candidate corneal epithelial defense factors that might limit their ability to penetrate and which could also provide the driving force for bacterial adaptation include epithelial expressed antimicrobial peptides. Corneal epithelial cells are known to express a number of antimicrobial peptides capable of killing *P. aeruginosa*; including hBD 1-3 and cathelicidin LL-37, and it is known that exposure to antimicrobial peptides can induce differential gene expression in bacteria. In this dissertation, the hypothesis that was explored addressed only specific components of the broad theoretical model: That under normal circumstances, antimicrobial peptides expressed by the corneal epithelium limit *P. aeruginosa* translocation of the epithelium, but that prolonged exposure to corneal epithelia induces differential bacterial gene expression which enhances epithelial traversal.

To the loving memories of Rose Fanny Augustin and Marcel Joseph Augustin

To my family and friends who have been by my side throughout this journey, thank you.

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CHAPTER 1.

INTRODUCTION.

1.1 Background.

Pseudomonas aeruginosa is a Gram-negative rod shape bacterium that is able to exhibit both an intracellular and extracellular lifestyle (52, 54, 117). *P. aeruginosa* is ubiquitous environmental bacterium capable of adapting and thriving in many ecological niches, from water and soil to plant and animal tissues. This bacterium is non-pathogenic in healthy individuals, however, once an infection with *P. aeruginosa* has been established it is difficult to treat due to the organism's intrinsic resistance to a variety of antimicrobial agents, including β-lactams, macrolides and quinolones [2,26]. In 2000, the complete *P. aeruginosa* sequence was published and this provided insight into the basis of the versatility of this bacterium (176). It revealed one of the largest bacterial genomes sequenced: 6.3 mega base pairs with 5570 predicted open reading frames and revealed a high number of regulatory genes as well as genes involved in catabolism, transport, efflux of organic compounds and several different secretion and motility systems for *P. aeruginosa*. These regulators facilitate rapid response to environmental conditions, including coordinating expression of specific virulence systems (61, 100, 185). Due to a multiplicity of virulence factors *P. aeruginosa* possesses these findings reflects its pathogenic ability resulting in this bacterium to cross several layers of epithelium (traversal), resulting in rapid destruction of the epithelium through the use of bacterial virulence factor (42, 71, 73). Making it one of the major causative agents of mortality and morbidity in nosocomial infections worldwide.

1.2. Human Infections caused by *P. aeruginosa*.

P. aeruginosa is an opportunistic bacterium that can exploit preexisting epithelial cell injury to establish infection. The three prevalent human diseases caused by *P. aeruginosa* include: (i) acute infections in burn wounds (ii) chronic lung infections in cystic fibrosis patients and (iii) contact lens-associated microbial keratitis (22, 48, 141).

1.2.1. Infection of Burn Wounds.

The first line of human defense against microbial infection is the skin, composed of epithelial and specialized immune cells; breech of this protective layer often results in infection. Many pathogens, such as *P. aeruginosa*, have developed strategies to circumvent the barrier function of an intact epithelium. In the case of burn wound injury, destruction of skin and the ubiquitous occurrence of *P. aeruginosa* provide a suitable environment for bacterial growth and invasion due to the fact that burn wounds are highly exudative, presenting a moist nutrient-rich environment for growth (29). Highly problematic is the high emergence of antibiotic resistant strains of *P. aeruginosa* making it a challenge to eradicate this bacterium from individuals suffering from burn wounds prior to healing.

In the pathogenesis of burn wound infections, depressions of local host cellular and humoral immune responses are pivotal factors contributing to *P. aeruginosa* infections (22, 29, 117). Burn wounds result in an immunocompromised host with endogenous decreased levels of Immunoglobulin G, IgG (44). Immunoglobulins are a natural component of the functional immune system that promote the opsonization and

phagocytosis of bacteria, neutralization of bacterial toxins, and the complement induced lysis of bacteria. *P. aeruginosa* takes advantage of the immunocompromised host to establish infection and does so by secreting a variety of virulence factors at the site of the burn wound. This bacterium produces a number of cell-associated (i.e. adhesions, alginate, pili, flagella, and lipopolysaccharide) and extracellular (i.e. elastase, exoenzyme S, exotoxin A, hemolysins, iron-binding proteins, and protease) virulence factors that mediate a number of processes to allow dissemination (22, 29).

1.2.2. Cystic Fibrosis.

Cystic fibrosis (CF) is an autosomal recessive disorder due to mutation of the gene encoding cystic fibrosis transmembrane-conductance regulator (CFTR). The CFTR protein is a member of the ATP binding cassette (ABC) family of transporters. The highly conserved motif that defines the ABC family of proteins includes a membrane-spanning domain, containing six membrane-spanning peptides, followed by a nucleotide-binding domain (NBD). The NBD is responsible for ATP binding and hydrolysis which supplies energy to drive the opening and closing of chloride ion channels. The N-terminal NBD (NBD-1) hydrolyzes one molecule of ATP to open the channel; this is followed by the C-terminal NBD (NBD-2) hydrolyzing of a second molecule of ATP to close the channel addition to the two protein domains already described (membrane-spanning domain and NBD), CFTR contains a regulatory (R) domain, which modulates channel activity (Lyczak *et al*., 2002). Over 1,800 naturally occurring mutations have been identified in the CFTR gene (Cystic Fibrosis Mutation database; http://www.genet.sickkids.on.ca/app). The phenotypes generated by these mutations display a range of disease severity (141). In healthy individuals, chloride ions are secreted by the sweat glands in an isotonic solution; the ions are then reabsorbed in a CFTR-dependent manner before the secretions reach the skin surface. In CF patients, due to CFTR mutations, sweat gland secretions have a higher level of chloride ions. Normally airway epithelia are covered with a biphasic mucus layer composed of a lower more fluid layer and an upper viscous layer required for microorganism clearance through ciliary motion. CF patients exhibit a lack of stratification of their mucus layer leading to impaired mucocilliary escalator activity and decreased microbial clearance.

CF patients are susceptible to infection by a wide variety of microbes. Ultimately, 80 to 95 % of these patients succumb to respiratory failure due to bacterial infection and airway inflammation, predominantly chronic infection by *P. aeruginosa* (118). While the use of antimicrobial chemotherapy and chemoprophylaxis have reduced the onset of morbidity and early mortality of CF patients, the intrinsic ability of *P. aeruginosa* to develop biofilms that are resistant to antimicrobials underlies the inability to eradicate this organism from the lungs of CF patients. It is not well understood why *P. aeruginosa* is the prominent infecting agent in CF lungs, however several hypotheses relating to the underlying molecular defects in CF patients have been suggested (33). It is likely that multiple factors of *P. aeriuginosa* also contribute to its ability to dominate the CF lung. The massive destruction of airway epithelia and eventual lung function loss due to extensive damage cause by neutrophil infiltration may provide *P. aeruginosa* suitable niche to exploit host (33, 117, 118). In addition, the interaction of *P. aeruginosa* with

CFTR protein is thought to be a critical step in bacterial internalization into airway epithelium allowing *P. aeruginosa* to avoid the host immune response (170).

1.2.3. Contact Lens Related Microbial Keratitis.

Ordinarily, the ocular surface is thought to be effective against microbial infiltration through a variety of mechanical (e.g. blinking), anatomical (e.g. conjunctival epithelia), and immunological defense mechanisms (e.g. IgA, antimicrobial peptides; (42, 59, 126). However, contact lens is a major risk factor for the development of this corneal infection, which was previously thought to only be associated with ocular injury. For instance, 56% of patients with microbial keratits in the United States are associated with contact lens wear (169). Of the microbial organisms capable of inducing ketatitis, *Pseudomonas aeruginosa* is considered the most devastating of the pathogens involved, result in many instances in vision loss (47, 71).

The higher risk of infections among contact lens users has been thought to be attributed to synergistic effects that occur with use of contact lens including, corneal hypoxia and subsequent damage to the corneal epithelial cells, as well as providing a surface for the bacteria to colonize and persist. This imperfection in the corneal epithelium greatly increases the incident of infection, for instance, under normal circumstances, *P. aeruginosa* is unable to adhere and colonize healthy corneal cells using *ex-vivo* cultures (53). However, this adherence is increased when the epithelial cells are damaged. In fact, it's proposed that the initial step of development of microbial keratitis for *P. aeruginosa* is binding to, then traversal of, the damage multilayered corneal epithelial barrier to cause infection. Consequently, bacterial entry into the underlying stroma rapidly results in corneal destruction through a combination of bacterial virulence factor expression (e.g. exoenzymes), and host cellular inflammatory and immune responses (42). Poor responses to antibiotic therapy e.g., increased incidence of resistant isolates, necessitate development of new therapeutic approaches to prevent *P. aeruginosa* from accessing the stroma by preventing bacterial traversal through the corneal epithelium after they adhere.

Contact lens effects on the corneal epithelium have also been shown, and this could aid in the involvement of the pathogenesis of lens-related infection. Our lab has published studies done *in vitro* showing that exposure of corneal epithelial cells to soft contact lenses can block the up-regulation of the antimicrobial peptide human β-defensin-2 in response to *P. aeruginosa* antigens through effects on JNK/AP-1 signaling (122). Further studies, show hypoxia and extended lens wear have also been linked to increased *P. aeruginosa* binding to exfoliated corneal epithelial cells (46, 105, 114). All these studies suggest that contact lens effects on the corneal epithelium (through hypoxia or otherwise) could influence corneal susceptibility to *P. aeruginosa* infection. However, the relationship between these lens-induced epithelial effects and actual susceptibility to infection has not been well defined.

1.3. Virulence Factors.

P. aeruginosa utilizes numerous virulence factors to establish these severe infections. These virulence factors are diverse and complex often eliciting irreversible damage.

Once *P. aeruginosa* encounters an immunocompromised individuals (e.g. acute infection in burn wounds or chronic lung infection in CF patients), especially in nosocomial settings, it is easy to imagine that because of the availability of such an arsenal of virulence factors and its ubiquitous nature, it has became one of the most dangerous opportunistic pathogens. Prominent virulence factors include adhesion molecules such as lipopolysaccharide (LPS), biofilm formation, quorum sensing (QS), and protein secretion and export apparatus systems.

1.3.1. Lipopolysaccharide (LPS).

Lipopolysaccharide (LPS) molecules are located in the cell wall of Gram-negative bacteria. The location of LPS in the outer membrane permits interaction of LPS with the external environment. Thus LPS plays an important structural role and also mediates interaction with the environment (163). LPS consists of three parts: a hydrophobic lipid A region, a central core oligosaccharide region, and a repeating polysaccharide portion referred to as O-antigen or O-polysaccharide. The lipid A region (also referred to as endotoxin) is responsible for the extensive immune response upon infection with *P. aeruginosa*. This response includes activation and recruitment of specialized immune cells, which can result in sepsis shock syndrome and in severe cases death of the host. *P. aeruginosa* LPS produces two forms of O-antigen, known as A-band (homopolymer) and B-band (heteropolymer). The variable O-antigen chains are the basis of antigenic identification of *P. aeruginosa* serotypes. The A-band O-antigen region is composed of D-Rhamnose (D-Rha) residues arranged as trisaccharide repeating units bound by α1-2, α1-3, α1-3 linkages. The A-band D-Rhamnose polysaccharide is composed of approximately 70 D-Rha residues, which are equivalent to 23 repeating units (163). The B-band O-antigen is composed of ≥ 50 repeating di- to -pentasaccharide units with various monosaccharide side chains that mask the A-band polysaccharide (1). Interestingly, the production of alternative forms of LPS is regulated by the gene cluster *alg*ACD, which is also responsible for alginate production in *P. aeruginosa* biofilms (143).

P. aeruginosa is known to express virulence factors differentially during infection. These phenotypic changes have been observed in pulmonary infections of CF patients (90). A change in LPS from a “rough” (lacking O-antigen) to “smooth” (containing O-antigen) morphology in *P. aeruginosa* strains has been associated with chronically infected patients (90). This alteration occurs once *P. aeruginosa* is well established within the lungs of CF patients and correlates with poor lung function. *P. aeruginosa* isolates from chronically infected CF patients either lack B-band O-antigen or express smaller amounts, while the levels of A-band O-antigen are maintained. A study in two other Gram-negative bacterial species, *Yersinia* spp. and *Shigella* spp, demonstrated that alteration in LPS components increases invasion and/or the secretion of effector proteins of the TTSS (21, 189). This strongly suggests that LPS structure plays an important role in modulating expression of virulence factors in these species, however to date this critical aspect has not been addressed in *P. aeruginosa* keratitis infections.

1.3.2. Biofilm Formation.

Bacteria living in the environment can form structural microbial communities called biofilm. Biofilm generation involves the formation of these communities (usually a heterogeneous population) encased in a protective exopolysaccharide (EP) matrix. The biofilm development proceeds in three major steps: (i) attachment of the planktonic cell (free-swimming) forms to the surface, (ii) maturation of a biofilm structure, (iii) partial degradation of biofilm, release and dispersal (35). The formation of biofilm in itself allows microbes to survive in hostile environment and colonize new niches by various dispersal mechanisms (66, 120, 158). In addition, microbes existing within these biofilms have been shown to exhibit cooperative gene expressions, which involve changes in surface adhesion molecules, antibiotic resistance, nutrient acquisition, and virulence factors which promote its survival against hostile conditions (140). Manipulation of these signals combined with considerable environmental adaptability allows microbes, such as *P. aeruginosa*, to be a formidable pathogen. Such microbial communities are ubiquitous in natural environments, but can also be found in industrial and clinical settings contributing to the hazardous and costly problem seen in hospital settings (100). One of the most important features of microbial biofilms is their tolerance to antibiotics and components of the host immune system. Although antimicrobial agents may decrease the number of bacteria in biofilms, they will not completely eradicate the bacteria, which may have important clinical consequences in the form of relapses of infections.

Biofilms contain distinct subpopulations of cells that exhibit differential physiological states. An increasing body of evidence suggests that the prevailing physiological states of biofilm cell subpopulations directly relate to their susceptibility and tolerance phenotypes dependent on the antimicrobial compound used. Conventional antimicrobial agents that are known to interfere with fundamental physiological processes of bacterial cells, such as replication (e.g. ciprofloxacin), or translation (e.g. tetracycline, tobramycin, and gentamicin), were found to specifically kill the metabolically active cells in the top layer of biofilms, whereas cells of low metabolic activity survived the treatment (Figure 1; 18, 74, 140, 144, 187).

Studies on *P. aeruginosa* biofilms reveal the mechanisms that contribute to tolerance include restricted antimicrobial diffusion, differential physiological activity, induction of specific tolerance mechanisms, and persister cell formation. The *P. aeruginosa* EP matrix contains mainly polysaccharides, proteins, and nucleic acids (26, 56, 88, 190, 191). The composition of the matrix depends on the environmental conditions, the age of the biofilm, and the particular *P. aeruginosa* strain forming the biofilm. Evidence has been provided that *P. aeruginosa* induces the synthesis of EP matrix components in response to environmental signals sensed by two component response regulators such as LadS, RetS, and GacS (61, 152, 184, 203). The matrix components involved in biofilm formation include glucose rich Pel polysaccarides, mannose rich Psl polysaccharides, alginate, CdrA protein, Cup fimbria, type IV pili, lectins, and eDNA (66).

The exopolysaccharide matrix of *P. aeruginosa* is predominantly composed of alginate, an acetylated polysaccharide composed of monomers of β-1,4-linked l-guluronic and d-mannuronic acids, and is mainly produced by *P. aeruginosa* in chronic

infections of lungs of CF patients (62). Its physical and chemical properties play a important roles in protecting *P. aeruginosa* (62). Biofilms formed by an alginate-overproducing strain were shown to exhibit a highly structured architecture and were significantly more resistant to the antibiotic than biofilms formed by a nonmucoid strain (74). In addition, regulation of rhamnolipid and eDNA has recently been shown to play an important role in biofilm persistence by *P. aeruginosa*. Antimicrobial activity was significantly enhanced on mucoid *P. aeruginosa* biofilms by the addition of both alginate lyase and DNase, suggesting that alginate and eDNA act synergistically as a physical barrier for antibiotics diffusion (8). Also of some interest is that rhamnolipid production by *P. aeruginosa* plays a role in the tolerance of *P. aeruginosa* biofilms toward immune cells (7, 23, 91). Purified *P. aeruginosa* rhamnolipids were shown to be able to destroy polymorphonuclear neutrophilic leukocytes (PMNs) via necrosis (91).

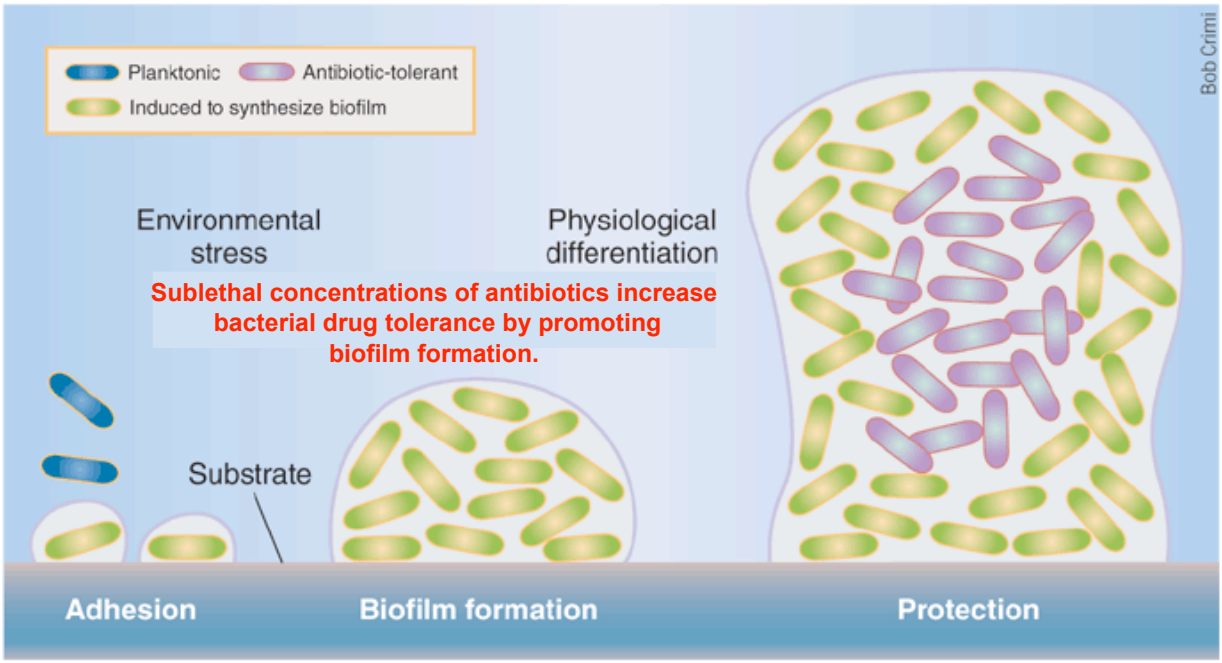


Figure 1. Environmental stress induces planktonic bacteria (blue) to form a biofilm. In the model system of *P. aeruginosa* challenged with tobramycin, the aminoglycoside antibiotic is the stress. Of the cells induced to produce biofilm (green), a subpopulation (purple) develops tolerance of chemical challenges by virtue of physiological differentiation and/or the altered microenvironment. Adopted by O'Toole 2005 publication (140). Copyright 2005 by Nature Publishing Group. Reprinted with permission.

1.3.3. Quorum Sensing.

Quorum sensing (QS) is a population density dependent cell-to-cell communication system that permits coordinate gene expression within the bacterial population. Quorum sensing in *P. aeruginosa* is mediated through the *las* system, which consists of the transcriptional activator LasR and the AHL synthase LasI, that directs the synthesis of

N-3-oxo-dodecanoyl-homoserine lactone (3-oxo-C_{12}-HSL) and the *rhl* system, which consists of the transcriptional regulator RhlR and the AHL synthase RhlI, that directs the synthesis of *N*-butanoyl-homoserine lactone (C_4-HSL) (93). A third system, a quinolone signal 2-heptyl-3-hydroxy-4-quinolone (PQS), acts as a bridge between *las* and *rhl* system (93). PQS controls the expression of RhlR and RhlI (34, 128, 150). The transcription of genes required for PQS synthesis is positively regulated by LasR but under negative control of the *rhl* system (34). These quorum sensing signal molecules accumulate intracellularly as the bacterial density increase towards stationary phase, in which these signal molecules can freely diffuse through the bacterial membrane. In *P. aeruginosa* cells are freely permeable to C_4-HSL, active transport via the MexAB-OprM multidrug efflux pump is involved in the secretion of 3-oxo-C_{12}-HSL (149).

The QS system in *P. aeruginosa* was found to be essential for the expression of specific virulence factors by effecting the expression of a broad spectrum of genes (Figure 2), these included elastase, alkaline protease, exoenzyme S, neuraminidase, haemolysin, lectins, pyocyanin, rhamnolipids, hydrogen cyanide or oxidative stress-responsive enzymes catalase and superoxide dismutase (68, 107, 146, 148, 151). These virulence factors are all crucial for the pathogenesis of *P. aeruginosa* to establish and maintain the infection. Mutants defective in quorum sensing are typically compromised in their ability to establish a successful infection (165, 166, 182).

Quorum sensing plays an important role in pathogenesis of *P. aeruginosa* and is known to control expression of specific virulence factors associated with chronic infection in the lungs of CF patients (185). In addition, direct involvement with quorum sensing signaling molecules in the lungs of CF patients have been found to play a role in *P. aeruginosa* ability to maintain persistence in the lungs. For example, in Cystic Fibrosis sputum it was shown to contain mRNA for the major regulators of quorum sensing in sufficient amounts to drive heterogonous expression of AHL-dependent fusion reporter genes (40, 172). In addition, the sputum, bronchoalveolar lavage fluid, and mucopurulent fluid from the airways were shown to contain PQS, indicating that this quorum sensing signaling molecule is also produced *in vivo* in the lungs of CF patients infected by *P. aeruginosa* (63).

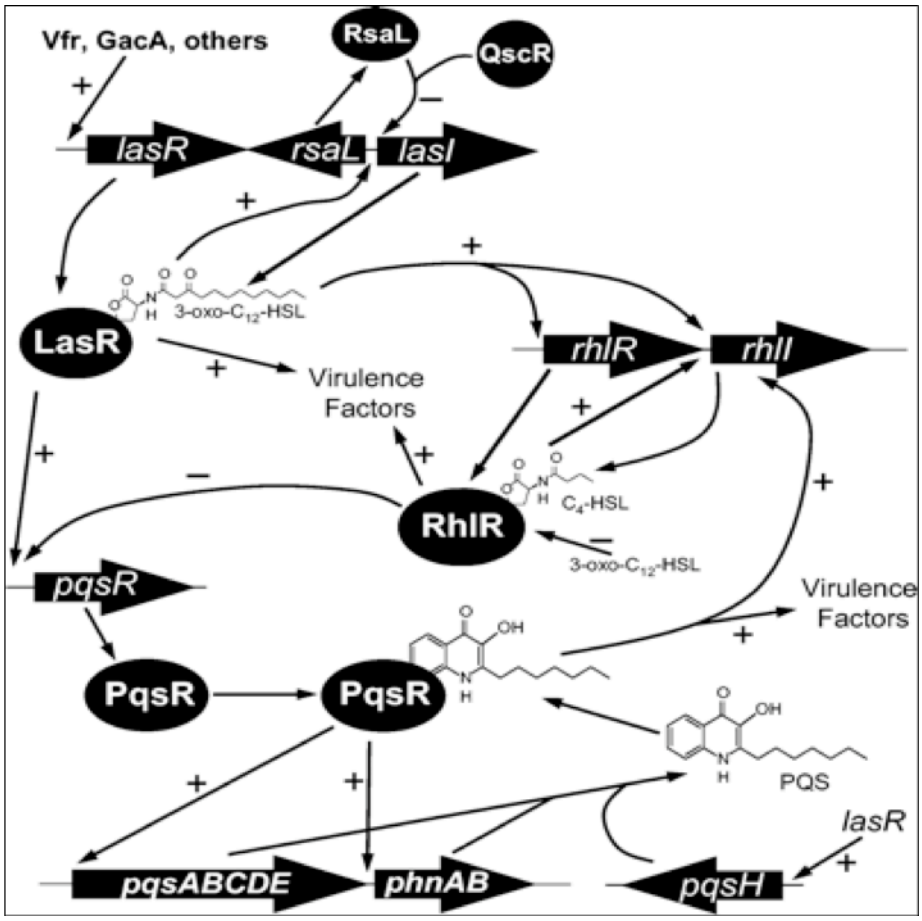


Figure 2. *P. aeruginosa* cell-to-cell signaling hierarchy (185). + and – symbols represent positive and negative effect respectively. Copyright 2005 by American Society of Microbiology. Reprinted with permission.

1.3.4. Protein Secretion Systems and Export Apparatus.

Bacterial pathogens are usually distinguished from their non-pathogenic relatives by the presence of pathogenicity genes, often organized in clusters, termed pathogenicity islands (85). As a result, despite the large variety of symptoms and diseases in humans, distantly related pathogens harbor closely related virulence genes (181). One such case is with the secretion system and export apparatus in many Gram-negative bacterial species, including *P. aeruginosa*. Six different classes of secretion systems have been described, which are identified as type I secretion system (T1SS) up to type VI secretion system (T6SS) (37). These secretion systems secrete proteins (e.g. protease, phospholipase, AB toxins, and ExoS) that are toxic to host cells by releasing them into the extracellular medium or directly into the host cell cytoplasm through a

“needle-like” complex (25, 85). The export of these proteins (sometime termed exoproteins or effectors) via the secretion system can be further divided into two main pathways, a Sec/Tat-dependent or Sec-independent. In Gram-negative bacteria, protein secretion is achieved after crossing two hydrophobic barriers, the inner membrane (IM) and the outer membrane (OM), which are separated by an aqueous peptidoglycan containing periplasm (P). The Sec pathway facilitates the translocation of pre-proteins (i.e. unfolded), whereas the Tat pathway promotes the secretion of folded proteins across the bacterial IM, secretion of the proteins across the OM is facilitated by the T2SS and T5SS. Proteins using these pathways are directed to the Sec or Tat pathway using a cleavable N-terminal signal peptide. In contrast, T1-, T3-, T4-, and T6 - secretion system span both the IM and OM allowing for direct into the extracellular medium (i.e. T1SS) or directly into the host cell cytoplasm through a “needle-like” complex (i.e. T3SS –T6SS) and making these systems Sec-independent mechanisms (25). In the case of *P. aeruginosa*, it possesses five (Figure 3; excluding T4SS) of the six secretion systems mechanisms (176), making this bacterium a formidable pathogen to manipulate eukaryotic host cells for its advantage.

For *P*. *aeruginosa*, there are two T1SS, the Apr and the Has system. The Apr system consists of AprD (ABC transporter), AprE (an adaptor protein), and AprF (Outer Membrane Factor; OMF) and is involved in the extracellular secretion of the alkaline protease AprA and AprX, a protein of unknown function (36, 65). Like most secreted proteases, AprA is a recognized virulence factor involved in various *Pseudomonas* infections. Howe and Igleswski (79) showed using mutant strains deficient in alkaline protease production in *P. aeruginosa* cytotoxic strain PA103 demonstrated a decrease in disease severity. In other experiments (64), showed that AprA contributed to the initial binding of *P. aeruginosa* to mouse corneal epithelium. The other T1SS is involved in utilization of iron and requires the Has system. The secreted protein HasAp is a haemophore, which binds to haemoglobin, consists of HasD (ABC transporter), HasE (an adaptor protein), and HasF (OMF) (113, 119). Due to the low availability of iron nutrients within the host cell during invasion, the use of HasAp enables *P. aeruginosa* to overcome iron depletion, making HasAp production a crucial step in the early development of pathogenesis (188).

Considered the most versatile system used by *P. aeruginosa* to secrete a wide range of exoproteins with diverse activities into the extracellular milieu, the T2SS is one reason this bacterium is able to produce such diverse and often overwhelming infections. In *P. aeruginosa*, the T2SS is encoded by *xcpP*-*Q* and *xcpR*-*Z*, as well as *xcpA/pilD* operons. The exoproteins secreted by this system include (but not limited too), proteolytic enzymes such as the elastase LasB, which is the major extracellular protease that degrades elastin, a major component in the lung ((142). Protease IV, a secreted serine protease, that has been shown to degrade surfactant protein D (SP-D), which is involved in several immune functions of alveolar macrophage and regulation of bacterial clearance in the cornea (5, 6, 135), resulting in acute respiratory infection and keratits caused by *P. aeruginosa* (39, 121). Exotoxin A (AB toxin), which inhibits elongation factor 2 through ADP-ribosylation resulting in proteins synthesis inhibition and cell death (38, 72).

Maybe the most studied of the secretion system in Gram-negative bacteria is the T3SS. This secretion system mediates the direct injection of cytotoxic proteins, termed

effectors, directly into the host cytosol (201). In *P. aeruginosa*, five distinct operons (*pscN-pscU, popN-pcrR, pcrG-popD, exsC-exsD, pscB-pscL*) are located within a pathogenecity island are involved in the biogenesis and the translocation machinery. The genes encoding the effectors are scattered elsewhere in the chromosome. T3SS regulation is mediated by ExsA, a member of the AraC family of transcriptional activators (117), however other global regulatory systems involving cAMP biosynthesis, two-component systems RetS/LadS/Gac-Rsm (61, 134, 184, 203), and a variety of stresses can influence the T3SS. To date there are four known effectors: exoenzyme S (ExoS), exoenzyme T (ExoT), exoenzyme U (ExoU), and exoenzyme Y (ExoY) that are known to be actively translocated via a PopB/D pore. Most clinical isolates do not posses all four exoenzyme genes. In a typical cohort 100% encode *exoT*, 89% *exoY*, 72% *exoS* and 28% have *exoU* (164). ExoS and ExoT are bifunctional enzymes that include a GTPase-activating (GAP) function within the N-terminal domain and an ADP-ribosyltransferase activity within the C-terminal domain (19). They confer anti-phagocytotic capacities to *P. aeruginosa* mainly through their action on the actin cytoskelon. ExoY is an adenylate cyclase that leads to cyclic AMP (cAMP) accumulation in the host cell (200), which has profound effects on the cell morphology (2). ExoU is a potent phospholipase A_2 activity responsible for acute cytotoxicity and lung tissue damages (167).

The T5SS is the simplest of the secretion pathway studied in *P. aeruginosa*. There are two subtypes of T5SS that exist: the autotransporters (T5aSS) and a two-partner secretion system (T5bSS). EstA is the only characterized autotransporter in *P. aeruginosa* (193), however there are thought to be putative proteins (PA0328 and PA3535) in *P. aeruginosa* PAO1 strain (176). EstA has been shown to be involved in the production of rhamnolipids (glycolipid), and studies done with an *estA* defective mutant was shown to be deficient in twitching, swarming, and swimming motilities, and thus in biofilm formation (192). The two-partner secretion mechanisms are very similar to that of the autotransporter, except for the OM β barrel protein (Figure 2). Six TPS clusters have been identified in *P. aeruginosa* PAO1 strain (176). Five are complete, *tps1*: *PA0040–PA0041*; *tps2*: *PA0690–PA0692*; *tps3*: *PA2462–PA2463*; *tps4*: *PA4540–PA4541*; *tps5*: *PA4624–PA4625*; and one of them is composed of an orphan *tpsA* gene: *tpsA6* (*PA4082*). The TpsB4 transporter (PA4540) named LepB is required for the secretion of the TpsA4 (PA4541) protein, LepA. LepA is a protease that activates transcription factor NF-κB for host inflammatory and immune responses through digestion of human specific receptors (PAR-1, -2, or -4). LepA was detected in the extracellular medium of *P. areruginosa* keratitis clinical isolates, but not of the laboratory strain PAO1 (97). However, the relevance in pathogenesis has yet to be determined.

The most recently discovered secretion pathway in *P. aeruginosa* is the T6SS. The genome of the *P. aeruginosa* PAO1 strain contains 3 loci encoding T6SS components, called *HSI-I*, *HSI-II* and *HSI-III* (80, 133). The secretion of Hcp1 is so far the only example of a T6SS substrate in *P. aeruginosa*. Protein secretion through this system is achieved by the serine-threonine phosphorylase/kinase pair PpkA/PppA acting on the FHA (fork-head associated) domain protein Fha1, which acts as a core scaffolding protein for the system, recruiting the ATPase protease ClpV protein to the T6SS assembly via an unknown mechanism. Five substrates of this system, proteins Tse1–3 (Type six exported 1–3), and the VgrG1 and VgrG3 proteins, which are

coregulated with the secretory apparatus and secreted under tight posttranslational control, have been identified (78). The role of HSI-I (and the others) on *P. aeruginosa* virulence and persistence during infections is still under study. What is known, is that Hcp1 protein can be detected in the sputum of cystic fibrosis patients (133).

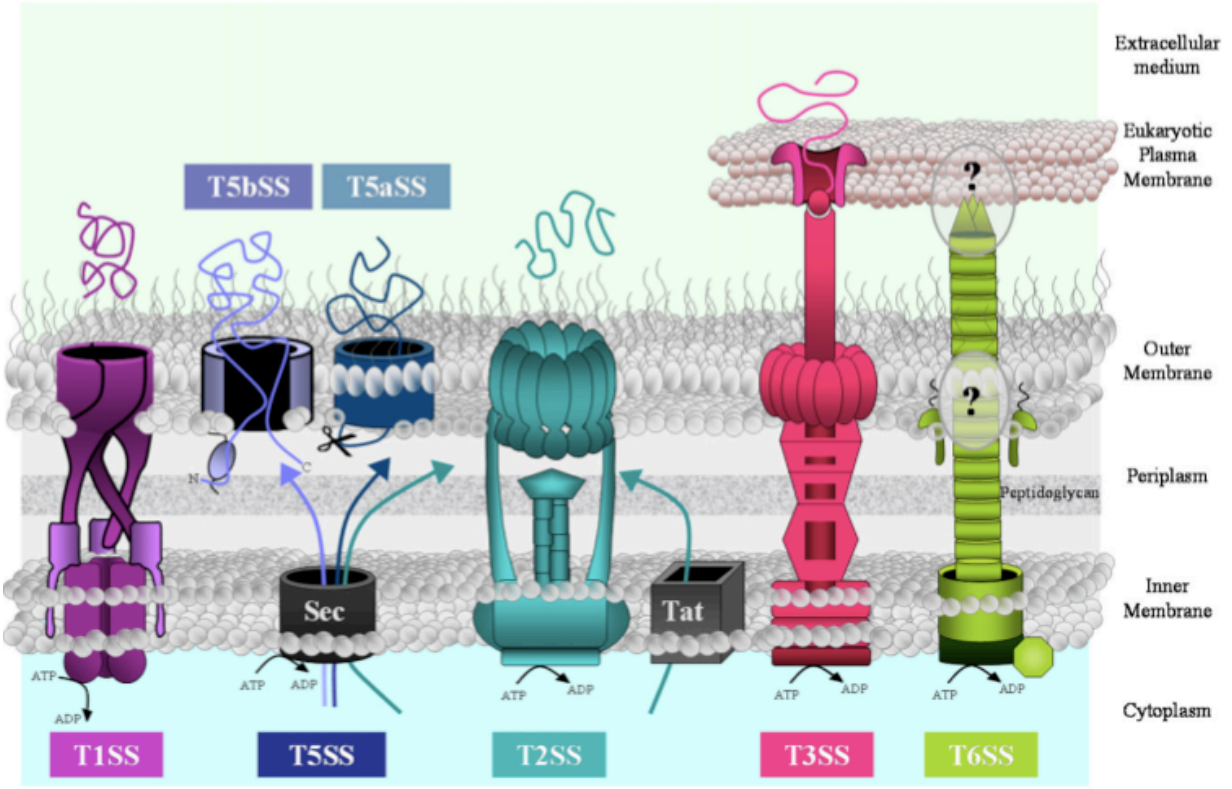


Figure 3. Schematic representation of the different secretion systems found in *Pseudomonas aeruginosa* strain PAO1 (25). Five of the six secretion pathways found in Gram-negative bacteria are present in *P. aeruginosa*. Protein transport across the bacterial envelope can be subdivided into Sec-independent and Sec/Tat-dependent pathways. Type II (T2SS)- and type V (T5SS) secreted exoproteins are firstly exported to the periplasm by the Sec or the Tat system before crossing the outer-membrane by their dedicated secretion pathway. In contrast, type I, type III, and type VI (T1SS, T3SS, T6SS) exoproteins are directly taken over in the cytoplasm by their cognate secretion machinery. Copyright 2010 by John Wiley & Sons, Inc. Reprinted with permission.

1.4. Specific Aims.

Our broad theoretical model to explain the pathogenesis of contact lens related *P. aerugoinosa* is that contact lens wear, providing a surface which bacteria can persist in biofilm formation, enables *P. aeruginosa* to persist at the ocular surface long enough to adapt to defense factors that otherwise limit their ability to penetrate the corneal epithelium. While epithelial-expressed antimicrobial peptides (AMPs) are logical candidate defense factors (and have been shown important in clearing bacteria that have gained access to the corneal stroma), a role for AMPs in actual epithelial barrier function during health has only been assumed. Related to this, *in vitro* studies have shown gene expression in bacteria can be impacted by exposure to AMPs to promote survival. The hypothesis tested here investigated specific components of the broad theoretical model: That under normal circumstances, antimicrobial peptides expressed by the corneal epithelium limit *P. aeruginosa* traversal of the epithelium, but prolong exposure to corneal epitheilia induces differential bacterial gene expression which then enhances bacterial capacity for epithelial traversal. To address the hypothesis, the following specific aims were proposed:

Specific Aim 1: Determine if known corneal epithelial expressed antimicrobial peptides modulate *P. aeruginosa* traversal

Specific Aim 2: Identify bacterial genes that differentially expressed when *P. aeruginosa* acquires the capacity to traverse corneal epithelium.

Specific Aim 3: Determine which differentially expressed genes modulate traversal of corneal epithelial cells.

CHAPTER 2.

Role of Defensins in Corneal Epithelial Barrier Function against *P. aeruginosa* Traversal

2.1. ABSTRACT.

Studies have shown that epithelial-expressed antimicrobial peptides (AMPs), e.g. β-defensins, play a role in clearing bacteria from mouse corneas already infected with *Pseudomonas aeruginosa.* Less is known about the role of AMPs in allowing the cornea to resist infection when healthy. We previously reported that contact lens exposure, a major cause of *P. aeruginosa* keratitis, could inhibit upregulation of human β-defensin 2 (hBD2) by corneal epithelial cells in response to *P. aeruginosa* antigens *in vitro*. Here, we studied the role of AMPs in maintaining the corneal epithelial barrier to *P. aeruginosa* penetration using both *in vitro* (human) and *in vivo* (mouse) experiments. Results showed that pre-exposing human corneal epithelial multilayers to bacterial antigens in a culture supernatant (known to upregulate AMP expression) reduced epithelial susceptibility to *P. aeruginosa* traversal up to 6-fold ($p < 0.001$). Accordingly, siRNA knockdown of any one of four AMPs expressed by human epithelia promoted *P. aeruginosa* traversal by more than 3-fold ($p < 0.001$). Combination knockdown of AMPs further enhanced susceptibility to bacterial traversal by ~8-fold ($p < 0.001$). *In vivo* experiments showed that loss of mBD-3*,* the mouse ortholog of hBD-2, enhanced corneal susceptibility to *P. aeruginosa*. The uninjured ocular surface of mBD-3 (-/-) mice showed reduced capacity to clear *P. aeruginosa*, and their corneal epithelia were more susceptible to bacterial colonization, even when inoculated *ex vivo* to exclude tear fluid effects. Together, these *in vitro* and *in vivo* data show functional roles for AMPs in normal corneal epithelial barrier function against *P. aeruginosa*.

2.2. INTRODUCTION.

The opportunistic bacterial pathogen *Pseudomonas aeruginosa* is capable of infecting numerous tissues in the human body, including the airways (nosocomial/ventilator-associated pneumonia), the urinary tract, and the cornea (86, 117, 175). The pathogenesis of *P. aeruginosa* infections is complex, but in most instances, *P. aeruginosa* (like other microbial pathogens) must overcome single or multilayered epithelial barriers to establish infection. For example, in the cornea, a multilayered epithelium protects the underlying stroma. Indeed, *P. aeruginosa* corneal infection does not occur in the absence of full-thickness epithelial injury or contact lens wear (110, 135, 180). For this reason, much of what we understand about host-microbe interactions *in vivo* has been derived from experimental models that deliberately by-pass the epithelial barrier (71, 179). How that multilayered epithelium maintains a barrier to microbial traversal during health has not been well studied. Yet there is likely much to learn, considering that these cells are highly vulnerable to *P. aeruginosa* virulence strategies when grown *in vitro* (49, 51). Understanding the molecular details of how epithelial barrier function is modulated will provide a foundation for studies aimed at understanding how it becomes compromised by contact lens wear or other risk factors. Similar knowledge gaps exist for epithelia that line our other body surfaces.

Factors that might enable otherwise vulnerable epithelial cells to form a resistant barrier *in vivo* could include extra-epithelial molecules, e.g. factors in tear fluid or the basement membrane, or epithelial cell-derived factors differentially expressed in the *in*

vivo environment. Potential candidates include secretory IgA (125) or surfactant proteins, e.g. SP-D (103, 138, 139), mucin glycoproteins (55, 59), tight-junctions/epithelial polarity (49, 95, 174), and epithelial-derived antimicrobial peptides (AMPs) which can inhibit or kill microbes, e.g. cationic AMPs including human β-defensins (hBD)-1, -2, -3 and the cathelicidin LL-37 (17, 84, 108, 126, 130). To date none of these have been directly tested for their involvement in limiting epithelial traversal by adherent bacteria. For multilayered corneal epithelium, we have found that addition of tear fluid, or growth of the cells on basement membrane proteins, are each protective against *P. aeruginosa* traversal (4, 104). Little else has been published on this topic for these or other epithelial cell types.

The cornea expresses several AMPs, some expressed constitutively and others upregulated in response to microbial antigens (58, 126, 127, 130). AMPs are known to have diverse functions including direct antimicrobial activity, phagocyte chemotaxis, and contributions to wound healing. Alone or in combination, they are thought to help protect the cornea from microbial pathogens. However, their relative contributions have only been studied during active infection. For example, for *P. aeruginosa* infections enabled using a scarification method, studies have found that murine defensins mBD-3 (the ortholog of hBD-2) and mBD-4 promote disease resolution (197), while flagellin-mediated cathelicidin-related antimicrobial peptide (CRAMP) expression, reduced disease severity (101).

In this study, we hypothesized that corneal antimicrobial peptides also participate in protecting healthy cornea (i.e. resistant epithelium) against *P. aeruginosa*. This was tested using human corneal epithelia grown as multilayers *in vitro* and also non-scarified mouse corneas.

2.3. MATERIALS AND METHODS.

2.3.1. Bacteria and Preparation of Culture Supernatant. *Pseudomonas aeruginosa* strain PAO1 (51) (expressing GFP on the pSMC2 plasmid) was used unless otherwise stated (135). Bacteria were grown on trypticase soy agar (BD Biosciences, CA) supplemented with carbenicillin (300 μg/mL) at 37 °C for ~16 h then resuspended in KGM-2 without antibiotics at a concentration of ~10^8 colony forming units (cfu)/ml (OD at 650 nm of ~ 0.1). This plasmid is stably retained by *P. aeruginosa* without antibiotic selection even after 48 h *in vivo* (109). Inocula were then prepared by diluting this suspension in KGM-2 to a final concentration of ~10^6 cfu/ml for use in most experiments. *In vivo* experiments involved a higher inoculum of ~10^9 cfu in 5 μl (~10^{11} cfu/ml). Viable counts were used to confirm inoculum size. In some experiments, corneal epithelial cells were pre-treated with a PAO1 culture supernatant, prior to inoculation with whole bacteria of strain PAO1. Supernatant was prepared by growing strain PAO1 in trypticase soy broth (BD Biosciences) with shaking at 250 rpm at 37 °C for ~16 h, sub-culturing into fresh media and allowing growth to mid-logarithmic phase (OD at 650 nm of ~0.7). That culture was centrifuged at 10,000 rpm (~10,000 x g) at 4°C for 50 min, the supernatant was then removed and filtered through a 0.2-μm membrane filter (Corning, Inc., NY) to remove whole bacteria, but leaving PAMPs including lipopolysaccharide (LPS), flagellin, and pilin and other extracellular

components (122, 130). Culture supernatant was prepared as a batch, and frozen in aliquots (-20 ^{o}C). When needed for experiments, an aliquot of supernatant was defrosted and diluted (1:5) in KGM-2 medium.

2.3.2. Cell Culture. Telomerase-immortalized human corneal epithelial (HCE) cells were maintained in KGM-2 media (Lonza; MD) containing 0.15 mM $CaCl_2$ (low calcium) in T75 tissue flasks (Falcon Labware; BD Biosciences, CA) [5% CO_2 at 37°C] and passaged when cells reached 80 % confluence. Polarized multilayers of these epithelial cells were generated by seeding 5.0 x 10^4 HCE cells/ml onto 12-well collagen-coated tissue culture inserts (3.0 μM pore size; Corning, Inc., NY) in KGM-2 media containing 1.15 mM $CaCl_2$ (high calcium) as previously described (162). Briefly, cells were submerged in high calcium KGM-2 media on both apical (upper) and basal (lower) compartments. The media was changed on alternate days for up to 4 days. To induce epithelial differentiation, media was removed from the apical compartment exposing cells to an air-liquid interface (air-lifting). Media in the basal compartment was changed every day during air-lifting for 7 days. Trans-Epithelial Resistance (TER) readings were collected using an EVOM meter (World Precision Instruments Inc., Sarasota, FL). Cells were used for experiments when TER measurement was ≥ 200 Ω/cm^2.

2.3.3. *In vitro* Traversal Assay. Traversal assays involved similar methodology to that described previously (3, 4, 104). Briefly, air-lifted HCE cells were cultured on 3.0 μm pore-size filters as described above (see Figure 4). PAO1 inocula (10^6 cfu in 1 mL of media, unless otherwise stated) were carefully added to the apical compartment, and incubated with the cells [5% CO_2 at 37°C] for up to 8 h. Apical and basal compartments were sampled at 4 h and/or 8 h post-inoculation to determine numbers of viable bacteria. In control experiments (not shown), sampling of the basal compartment at 0 h did not result in recovery of viable bacteria. Uninfected corneal epithelial cells were sham-inoculated as controls. Internalization of PAO1 by the HCE cells was quantified using gentamicin survival assays by removing media from both apical and basal compartments, washing with phosphate-buffered saline (PBS), then immersing the inserts in high-calcium KGM-2 media supplemented with gentamicin (200 μg/ml, Lonza, MD) for 1 h to kill extracellular bacteria. Inserts were then washed once with PBS, treated with 0.25% (v/v) non-ionic surfactant (Triton X-100; Sigma, MO) in PBS for 2 min, and vortexed to further disrupt eukaryotic membranes. Viable counts were performed on the cell lysate. All traversal and internalization assays were performed in triplicate. TER controls included EGTA-treated (disrupted tight-junctions) and sham-inoculated (intact tight-junctions) cells. In control experiments (not shown) fluorescein isothiocyanate (FITC)-conjugated inulin (3.5 kDa; 100 μg/ml in culture medium) was added to the apical compartment of uninfected epithelial cells during the experimental procedure to measure epithelial permeability. Aliquots of basal medium were taken and fluorescence measured (absorbance at 490 nm) using a standard ELISA plate reader. No significant changes in epithelial permeability were observed over an 8 h time period.

2.3.4. RNA Interference. Target-specific knockdown of antimicrobial peptides was performed using small interfering RNA (siRNA) for hBD 1-3 or LL-37 with an appropriate scrambled control (Santa Cruz Biotechnology, CA) using Lipofectamine RNAiMAX

(Invitrogen, CA) transfection according to manufacturer's instructions (see supplemental data; Table S1). siRNA transfection was started 2 days prior to bacterial inoculation (traversal assay) by adding siRNA (8 μM) to apical and basal compartments. On the day of infection 4 μM siRNA was added to the apical compartment only. Knockdown efficiency of each antimicrobial peptide was tested using Western immunoblot.

2.3.5. SDS-PAGE and Western Immunoblot. Corneal epithelial cells collected from the culture inserts were pooled and resuspended in lysis buffer [1% sodium dodecyl sulfate (SDS) and 50 mM Tris-HCL pH 8.0] and boiled for 3 min. Culture supernatant was concentrated using a precipitation method with 10 % Trichloroacetic acid (TCA)/ acetone solution. Protein concentration was determined for both supernatant and corneal epithelial cells using DC protein assay kit (BioRad, CA) with bovine serum albumin as standards. Standardized concentrations of each fraction were added to Tricine sample buffer (BioRad, CA). Total proteins were separated at equal concentrations from scramble control and siRNA treated hBD 1-3 or LL-37 samples and the control peptide (2 μg; Abcam; MA) using a 10 – 20 % Tris-Tricine/Peptide gel (BioRad, CA) under denaturing conditions. Proteins were transferred onto polyvinylidene difluoride (PVDF; BioRad, CA) membranes using standard tank-blot buffer and conditions (100 mV, 2 h). Membranes were then blocked overnight at 4 °C using 5 % non-fat dry milk prepared in TPBS (1 X PBS containing 0.05 % Tween-20; Fisher Scientific, PA). Membranes were incubated with primary rabbit anti-human hBD 1-3 or LL-37 antibodies (1/500 diluted in PBS-T containing 1 % non-fat milk; Santa Cruz Biotechnology, CA) for 2 h, followed by goat anti-rabbit IgG peroxidase secondary antibody (1/5000 diluted in PBS-T containing 1 % nonfat milk; Abcam; MA) for 2 h at 4 °C, washed three times for 10 min each with PBS-T, and developed using Amersham ECL Plus (GE Healthcare, NJ) according to manufacturer's instructions.

2.3.6. Real-Time RT-PCR. Total RNA was isolated from corneal epithelial cells using Trizol (Invitrogen, CA) according to manufacturer's instructions. Specific primer sets for target genes have been previously described and are shown in Table 1. Total RNA was checked for integrity and the absence of DNA by PCR prior to cDNA synthesis using 1 μg of total RNA and the GeneAmp RNA PCR Kit (Applied Biosystems, CA) according to manufacturer's instructions. mRNA transcript concentrations were measured using SYBR Green PCR Master Mix (Applied Biosystems, CA). Reactions were carried out using Step One Plus Real Time PCR System (Applied Biosystems, CA) under the following conditions: 95 °C for 10 min followed by 40 cycles of 95 °C for 30 sec, 58 °C for 1 min and 72 °C for 30 sec. The data acquisition step was set at 58 °C with a final melt curve analysis to ensure amplification of a single product. Transcript levels of each gene were normalized to that of internal control β-actin and expression calculated using the $2^{-\Delta\Delta CT}$ method (115). The absence of DNA contamination was verified using negative controls in which reverse transcriptase was omitted from the master mix.

2.3.7. Murine Models. Wild-type C57BL/6 mice (6 to 8 weeks old) and age-matched mBD-3 (-/-) gene knockout mice were used. The mBD-3 (-/-) mice were obtained from the Mutant Mouse Regional Resource Center (MMRRC) at the University of California,

Davis, CA. The MMRRC provided the control wild-type mice with a genetic background matching that of the mBD-3 (-/-) mice.

To study the impact of AMPs in defense against epithelial traversal by bacteria, eyeballs were removed and immersed in PBS (5 mL) to exclude tear fluid, before superficial blotting with tissue paper (Kimwipe) to enable susceptibility to bacterial adhesion. They were then inoculated with 5 μl of bacterial suspension containing ~10^9 cfu for either 4.5 or 7.5 h before processing for confocal microscopy as described below.

The impact of AMPs in bacterial clearance from the living healthy murine ocular surface was quantified using a null-infection model as previously described (135). Briefly, after induction of anesthesia (intraperitoneal infection with 21 mg/ml ketamine, 2.4 mg/ml xylazine, and 0.3 mg/ml acepromazine), 5 μl of bacterial suspension containing ~10^9 cfu was applied to the healthy ocular surface without prior manipulation of the eye. At 3 h or 6 h post-inoculation, ocular surface washes were collected (by capillary action) from the lateral canthus after 4 μl of PBS had been added to the surface of the eye. Viable counts were used to determine the number of viable bacteria in the collected samples. All experiments involving animals were conducted under a protocol approved by the Animal Care and Use Committee of the University of California, Berkeley, CA.

2.3.8. Microscopy. Cultured corneal epithelial cells were imaged by immunofluorescence microscopy with deconvolution. Briefly, Transwell-grown epithelial cells were fixed in neutral-buffered 4% (v/v) paraformaldehyde, embedded in Tissue-Tek frozen O.C.T. compound (Sakura Finetek Inc., CA) and frozen in liquid nitrogen. Samples were cross-sectioned at 10 μm thickness and mounted onto glass microscope slides, and incubated at 37°C overnight to dry the samples. After permeabilization with saponin (0.005 % wt/v), samples were blocked using goat serum and BSA in PBS (3 % wt/vol each), then incubated overnight at 4°C with rabbit antisera raised against *P. aeruginosa* strain PAO1 (157) diluted 1:10,000 in blocking buffer (unless GFP-labeled bacteria were used). Samples were then washed twice in PBS at room temperature for 10 min each, blocked again (5 min), and incubated for 1 h at room temperature with goat polyclonal anti-rabbit IgG (Abcam, MA) diluted 1:5000 in blocking buffer. After two more PBS washes, samples were labeled with rhodamine-phalloidin according to manufacturer instructions (Invitrogen, CA). After two more PBS washes, samples were mounted using vectashield DAPI embedding medium (Vector Laboratories Inc., CA), and viewed with an immunofluorescence microscope (Olympus IX-70).

Whole eyes that had been challenged *ex-vivo* with PAO1-GFP were examined by confocal microscopy. After bacterial exposure, eyes were rinsed with PBS three times to remove non-adherent bacteria. Each eyeball was attached to a 12 mm round glass cover-slip with instant glue to maintain an upright position (i.e. cornea facing upwards), then placed in a 47 mm Petri dish filled with tissue culture medium (i.e. KBM) to completely cover the ocular surface. Eyes were imaged using a Zeiss 510 AxioImager META/NLO confocal and 2-photon microscope equipped with Spectra-Physics MaiTai laser (Molecular Imaging Center, UC Berkeley) (63X/0.95 water-dipping objective). Corneal cells were imaged without chemical fixing and labeling by using a 633 nm laser to obtain reflection of all cells (live or dead). GFP -expressing bacteria

were viewed using wavelength of 488 nm. Images were captured with a 2-channel non-descanned detector (NDD) and processed with the Zeiss LSM Image Browser. Measurements for distribution of PAO1-GFP were done using NIH Image J 1.42q software (http://rsb.info.gov/ij). Three or more random fields of each eye were imaged from the corneal surface through the entire epithelium in 0.5 μm steps (confirmed by reflection of cells during image acquisition).

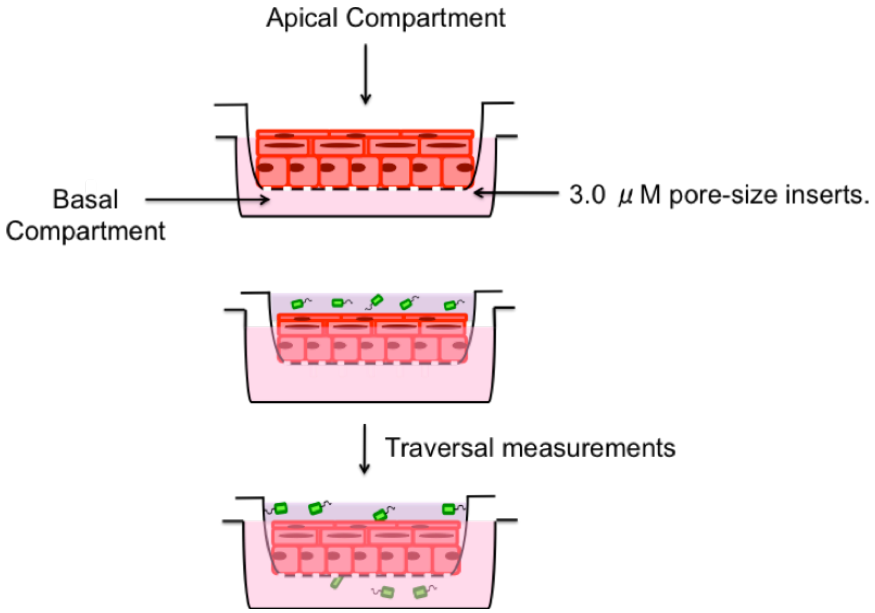
2.3.9. Statistical Analysis. Data were expressed as a mean ± standard deviation (SD) for *in vitro* traversal assays, or a median with upper and lower quartiles for *in vivo* clearance assays. Statistical significance between groups was determined using ANOVA (with Fisher PLSD post-hoc analysis) for multiple group comparisons, or an unpaired Student's *t*-Test for two groups of normally distributed data. Otherwise a non-parametric Mann-Whitney test was used to compare two groups. P values of < 0.05 were considered significant.

2.4. RESULTS.

2.4.1. *P. aeruginosa* traverses telomerase immortalized multilayered human corneal epithelium without disrupting TER.

The capacity of *P. aeruginosa* invasive strain PAO1 to traverse human corneal epithelial cells grown as multilayers on Transwell filters was explored. This was done by performing viable counts of the lower (basal) media compartment at various times up to 8 h after the addition of bacteria to only the upper (apical) compartment. As expected from previous studies using rabbit corneal epithelia (3, 4, 104), some bacteria traversed the multilayered human corneal epithelia as early as 2 h, with increasing traversal to the basal compartment observed over the remainder of the 8 h period (Figure 5). As we have previously shown for rabbit corneal epithelial cells (3, 4, 104), *P. aeruginosa* traversal occurred without significant reduction to the TER of the cells. After 8 h, the TER of uninfected cells was 231 ± 1 Ω/cm^2 compared to 227 ± 4 Ω/cm^2 for infected cells ($p > 0.05$, *t*-Test). In control experiments (not shown), bacterial growth rates in apical and basal compartments were found to be similar. Increased bacterial viability in the basal compartment with time likely reflects a combination of traversal and growth.

A.



B.

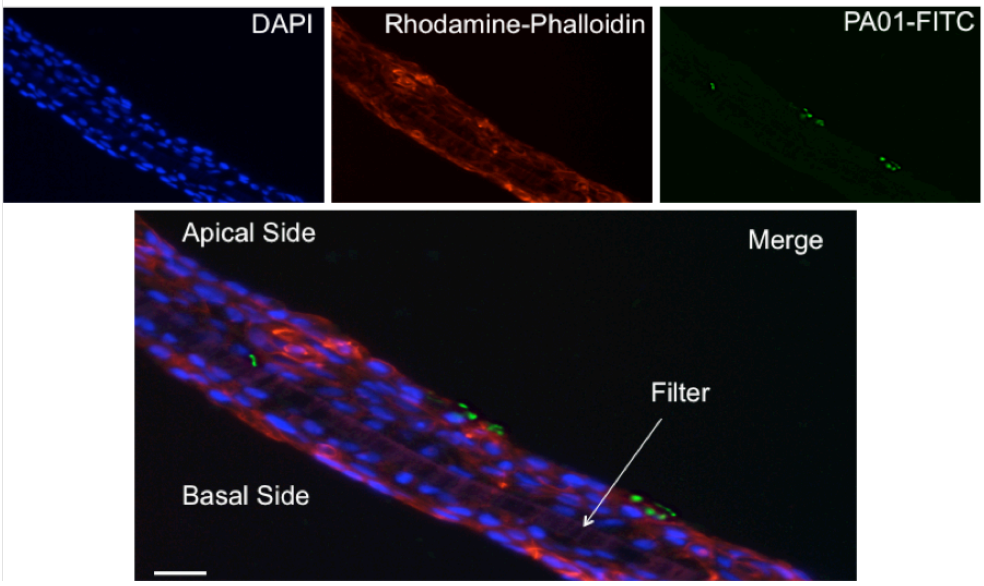


Figure 4. A schematic diagram (A) and immunofluorescence microscopy pictures (B) illustrating the *in vitro* traversal model. Human corneal epithelial cells were grown air-lifted on Transwell tissue culture inserts (3 μm pore-size) under high-calcium conditions (1.15 mM) to induce a polarized multilayered epithelium**.** To measure bacterial traversal, bacteria were added only to the apical compartment, and bacteria traversing to the basal compartment enumerated at various times post-inoculation. Fluorescence microscopy using rhodamine-conjugated phalloidin (red) to label the actin cytoskeleton, and DAPI (blue) to label cell nuclei, showed that the cells formed a multilayered epithelium to which *P. aeruginosa* (FITC-labeled using antibody to PAO1, green) can adhere and traverse (B). Magnification ~1000x. Bar = 10μm.

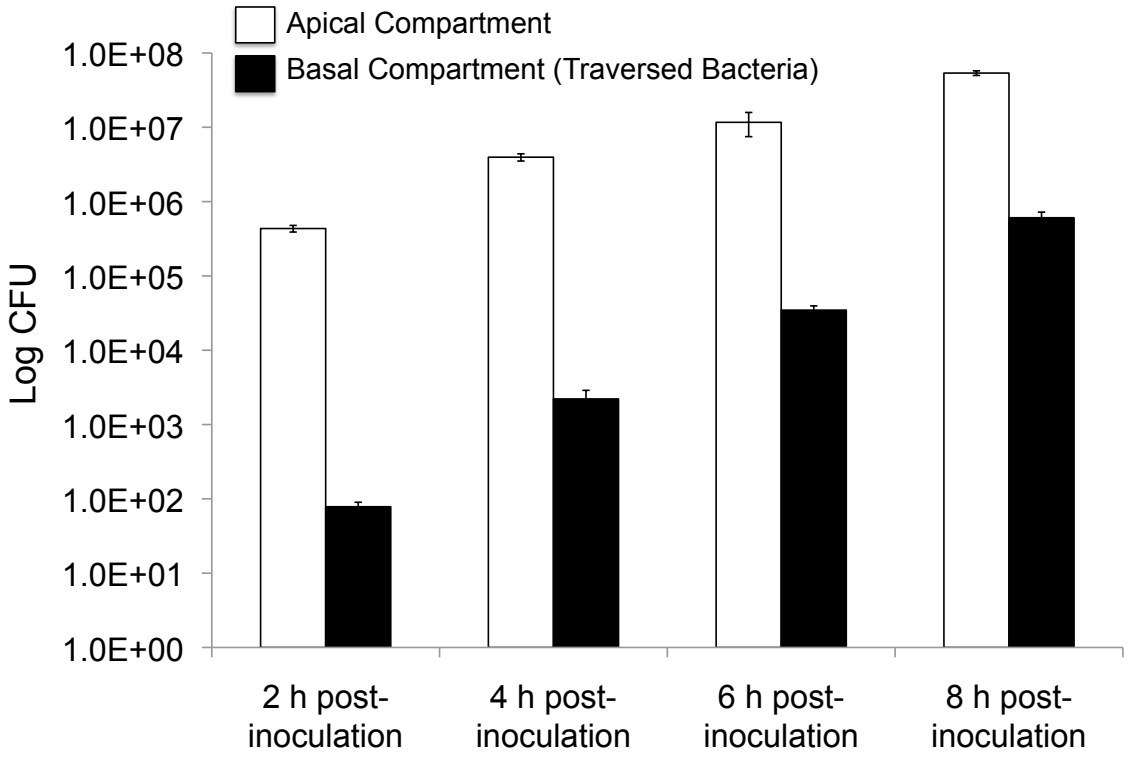


Figure 5. *P. aeruginosa* traversal of multilayered human corneal epithelial cells *in vitro*. PAO1 (10^6 cfu) were added to the apical compartment and viable counts taken from the apical (white bar) and basal (black bar) compartments at 2, 4, 6, and 8 h post-inoculation. *P. aeruginosa* traversal was detected at each time-point. One of three independent experiments is shown.

2.4.2. Pre-exposure of multilayered human corneal epithelia to *P. aeruginosa* culture supernatant reduces bacterial traversal.

P. aeruginosa culture supernatant contains bacterial antigens (e.g. LPS, flagellin, pilin) some of which are known to activate innate host defenses, including the upregulation of antimicrobial peptide expression (58, 122, 130). We explored the impact of culture supernatant pre-exposure on corneal epithelial susceptibility to *P. aeruginosa* traversal. Corneal epithelial cells were pre-treated with PAO1 culture supernatant (1:5 dilution in KGM-2 tissue culture medium, see methods) for 4, 8, and 12 h and compared to media-treated controls for susceptibility to bacterial traversal at 8 h post-inoculation with ~10^6 cfu bacteria (in the continued presence of the supernatant). All pre-treatment durations (4, 8, and 12 h) reduced epithelial susceptibility to traversal by up to ~ 6.5 fold at 8 h (Figure 6C; $p = 0.0001$ for each comparison of pre-treated versus cells treated with media only). TER remained stable throughout experiments, remaining within 6 % of baseline values. A ~ 1.4 fold decrease in bacterial numbers was observed in apical compartments after supernatant treatments, although differences from controls were not significant (Figure 6A). Bacterial internalization into epithelial cells could provide a

possible explanation for decreased PAO1 traversal (3). However, gentamicin survival assays showed that bacterial internalization was also significantly reduced for supernatant-treated cells by ~ 3.5 fold at 8 h (Figure 6B. $p < 0.001$). Continued presence of supernatant was required for these effects. Removal of supernatant from corneal epithelial cells after pre-treatment, and replacement with KGM-2 medium before bacterial inoculation, resulted in the loss of supernatant pre-treatment effects (not shown), suggesting that protection against traversal involved supernatant-induced factors secreted from the epithelial cells.

2.4.3. Knockdown of hBD-1, hBD-2, hBD-3 or LL-37 antimicrobial peptides using siRNA enhances *P. aeruginosa* traversal of multilayered human corneal epithelia.

Since AMPs can impact the viability of *P. aeruginosa* (82), and because pre-exposure to *P. aeruginosa* antigens enhances AMP expression by corneal epithelia (58, 122) while increasing resistance to bacterial traversal, we hypothesized that AMPs participate in epithelial defense against bacterial traversal. Small interfering RNA (siRNA) was used to knockdown four different AMPs known to be expressed by human corneal epithelium [human β-defensins (hBD 1-3)] and the cathelicidin LL-37). Western immunoblot confirmed the effects of siRNA treatment in reducing AMP peptide expression (Figure 7A), which was confirmed by real-time RT-qPCR (Figure 7B). Epithelial cells were then pre-treated with bacterial culture supernatant (as above) for 4 h to provide a stimulus for AMP expression then inoculated with *P. aeruginosa* (10^6 cfu). Bacterial traversal was increased by ~ 3-fold in siRNA-treated epithelia compared to scrambled siRNA controls (Figure 8B; $p < 0.0001$ in each instance). Combination knockdown of multiple antimicrobial peptides resulted in further enhancement of bacterial traversal more than 8-fold (Fig. 8B; $p < 0.0001$). Importantly, AMP knockdown increased the number of viable bacteria recoverable from the apical compartment where the inoculum had been added at the beginning of the experiment (Figure 8A; $p < 0.003$ in all instances), showing that AMP knockdown promoted bacterial survival/growth at that location. The magnitude of the increased apical viable counts were similar to that of increased traversal (basal viable counts) for each sample set, suggesting that the mechanisms were linked. While bacterial traversal caused some reduction of TER in these experiments (~ 9.0 %, scrambled siRNA compared to 9.5 – 13% for sequence specific siRNA), there were no significant effects of siRNA on TER as compared to untreated controls either before or after bacteria had traversed the epithelial cells.

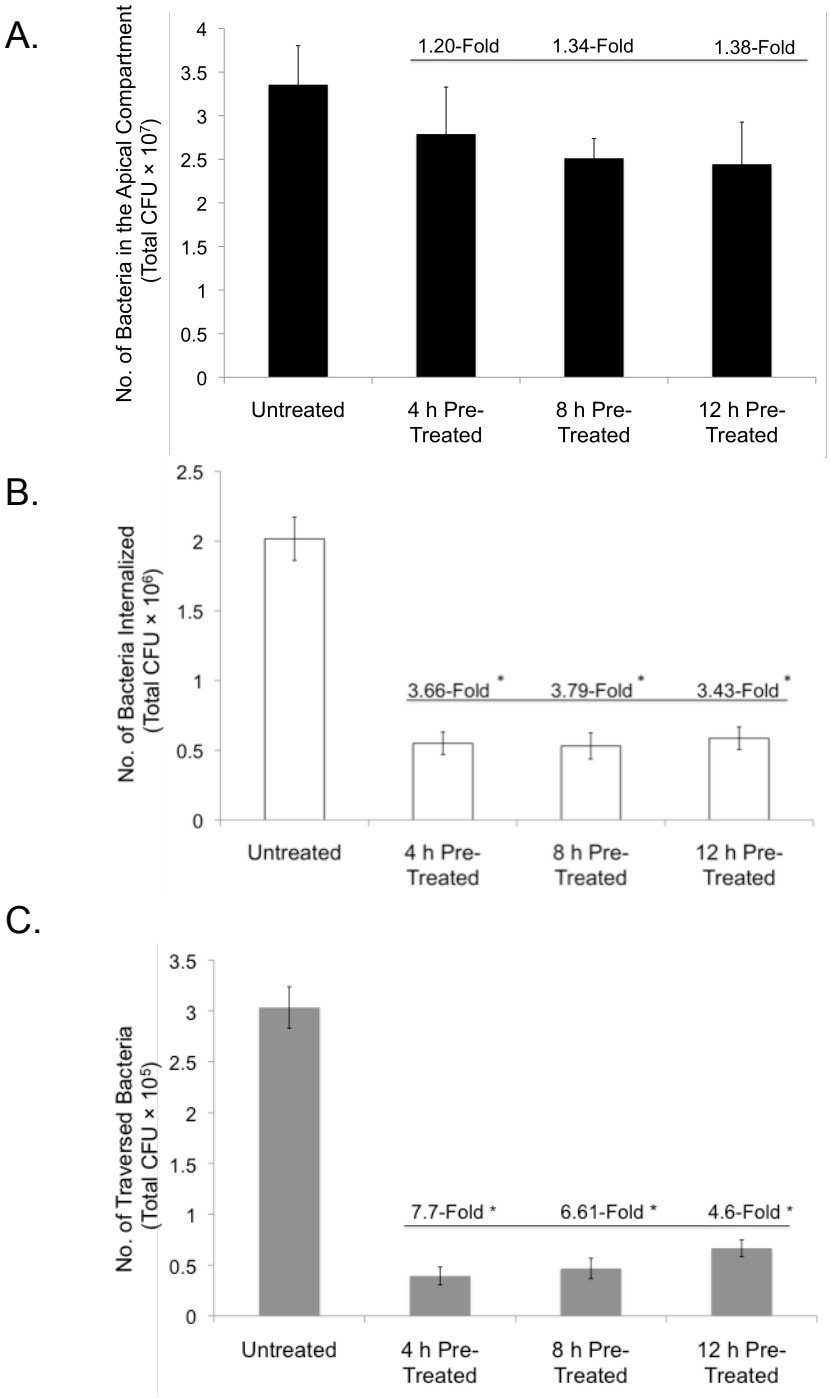


Figure 6. *P. aeruginosa in vitro* traversal assay using multilayered human corneal epithelia which were pre-exposed to PAO1 culture supernatant (1:5 dilution in KGM-2 medium) for 4, 8, or 12 h before inoculation with 10^6 cfu of strain PAO1 for 8 h in the continued presence of the supernatant. Controls were treated with KGM-2 medium only. Viable counts were taken from the apical compartment (A), internalized bacteria were assessed by gentamicin survival (B), and viable traversed bacteria (basal compartment) were counted (C). *$p < 0.001$ in each instance versus untreated controls (ANOVA, with Fisher PLSD post-hoc analysis). A representative of three experiments is shown. Note the X-axis represents different supernatant pretreatment times prior to PAO1 inoculation.

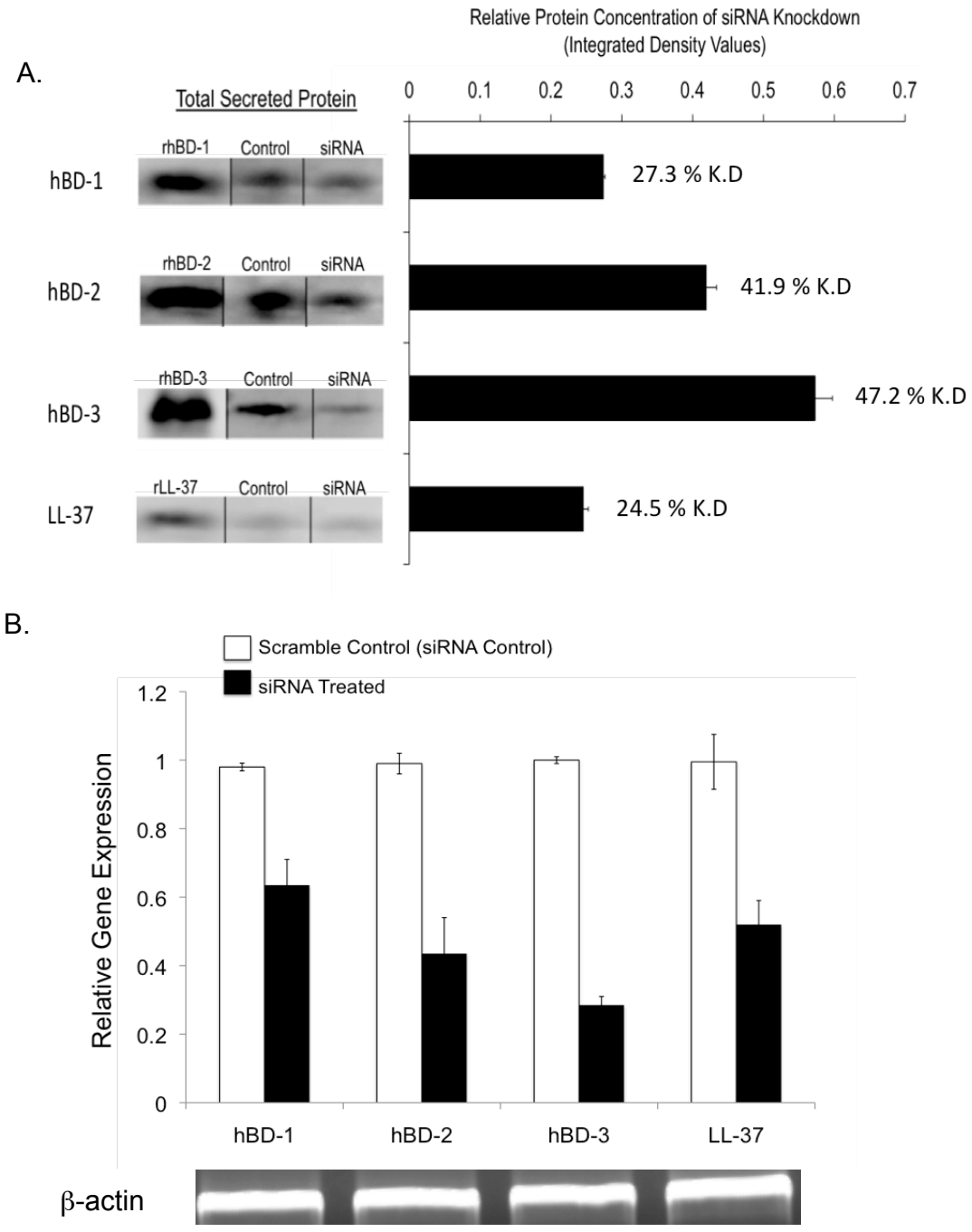
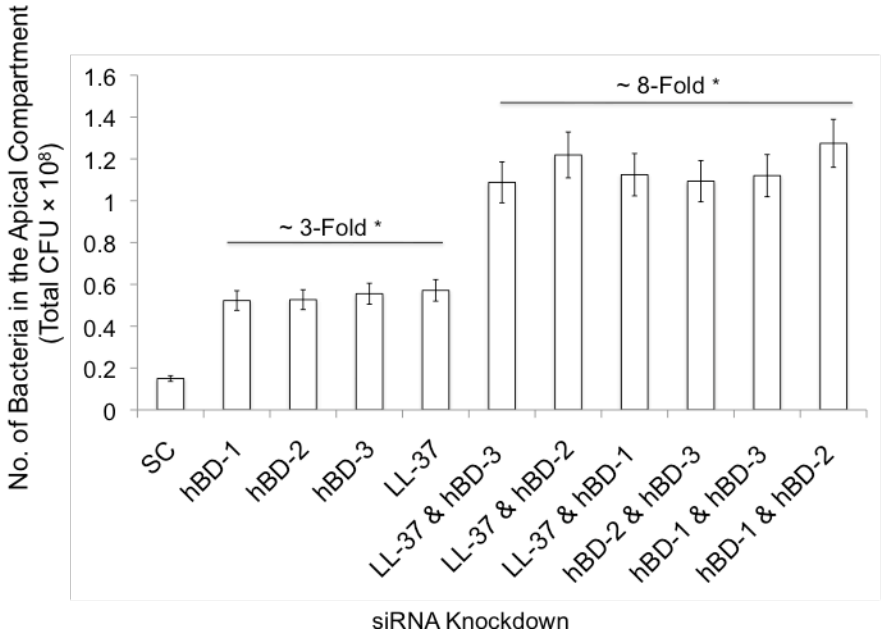


Figure 7. (A) Immunoblot analysis of secreted hBD 1-3 or LL-37 peptide expression by human corneal epithelial cells after siRNA treatment versus scrambled siRNA controls. 2 μg of recombinant (r) positive control peptide was included in each experiment (left panel). The right panel shows relative knockdown (KD) of each peptide (i.e. the integrated density of immunoblot bands normalized to the scrambled siRNA controls for each peptide). (B) Gene expression levels of hBD 1-3 or LL-37 in siRNA-treated human corneal epithelial cells as measured by RT-qPCR, and normalized to β-actin show the successful siRNA knockdown. Data is representative of three similar experiments each using six pooled culture inserts.

A.



B.

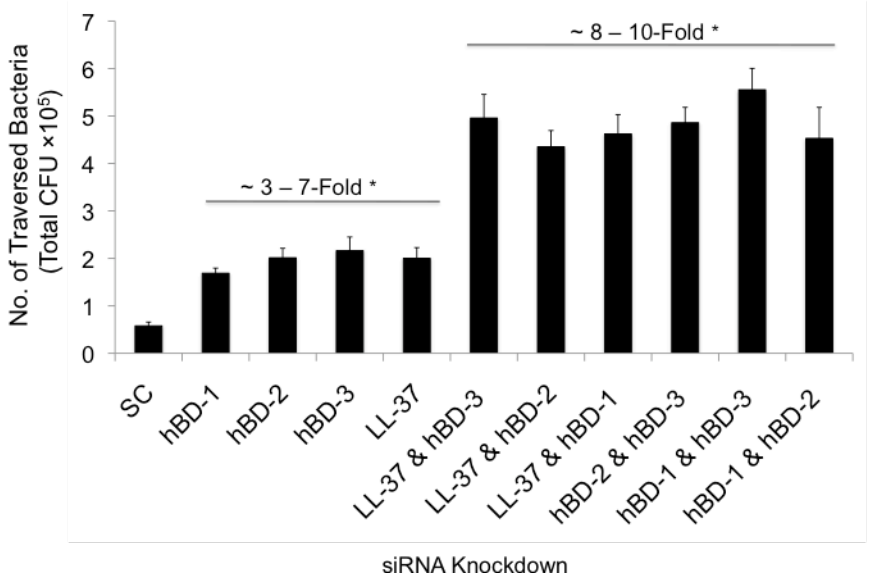


Figure 8. *P. aeruginosa* traversal of human corneal epithelia *in vitro* after siRNA knockdown of AMPs. Cells were treated with siRNA specific to hBD 1-3 or LL-37 or with a scrambled siRNA control (SC), stimulated with bacterial culture supernatant (1:5 in KGM-2 medium) for 4 h prior to inoculation with 10^6 cfu of PAO1 in the continued presence of the supernatant. Viable counts of the apical (A) and basal (B) compartments showed significant increases in bacterial survival in the apical compartment, and in bacterial traversal to the basal compartment, in siRNA-treated cells versus scrambled controls at 8 h post-inoculation. Effects on bacterial survival and traversal were greater when AMPs were knocked-down in combination. *$p < 0.001$ (*t*-Test) in each instance compared to scrambled siRNA controls. Results represent one of three independent experiments.

2.4.4. *P. aeruginosa* colonizes the corneas of mBD-3 deficient, but not wild-type, mice *ex vivo*.

Since *in vitro* studies showed a role(s) for AMPs in preventing *P. aeruginosa* traversal of corneal epithelia, we began to test the role of AMPs in preventing bacterial colonization of non-scarified corneas *in vivo*, using AMP-gene knockout mice. To ensure that bacterial inocula were matched for wild-type and AMP mutant corneas throughout the assays, epithelial susceptibility to bacteria was examined using eyeballs *ex vivo* to exclude tear fluid. This was done because some tear fluid AMPs could impact bacterial viability or ability to adhere/traverse. This also served to exclude known effects of tear fluid on AMP efficacy (83). mBD-3 (murine ortholog of hBD-2) was chosen for study given its recently reported contributions to resolution of *P. aeruginosa* corneal infections established by scratch injury (197). Enucleated whole eyeballs of mBD-3 (-/-) C57BL/6 mice, and wild-type controls, were challenged with 5 μl of bacterial suspension containing ~10^9 cfu of PAO1-GFP for 4.5 or 7.5 h. Corneal epithelia were imaged without fixation or labeling using 2-photon and confocal microscopy (see methods). *Ex-vivo* the mBD-3 (-/-) mice showed increased susceptibility to *P. aeruginosa* adherence 4.5 h post-inoculation (Figure 9A) compared to wild-type, the latter showing little or no attachment (Figure 9B). Adherent bacteria did not penetrate the corneal epithelium of either mBD-3 (-/-) or wild-type mice (Figure. 9A, B). Differences in adherence, and the lack of epithelial traversal, were clearly seen when bacterial numbers on inoculated corneas were quantified (Figure 9C). Adherent bacteria still did not penetrate the corneal epithelium of either mutant or wild-type eyes after 7.5 h (Figure 10). At this later time point, differences in bacterial adherence between mBD-3 (-/-) and wild-type mice were no longer apparent, with wild-type corneas also showing susceptibility to bacterial adhesion (Figure 10).

2.4.5. mBD-3 (-/-) mice show delayed clearance of *P. aeruginosa* from the ocular surface *in vivo*.

The ability mBD-3 (-/-) to clear PAO1 from the healthy ocular surface after *in vivo* inoculation, (i.e. in the presence of tear fluid and other *in vivo* factors) was explored using a null-infection model which utilizes an uninjured cornea (135). As shown in Figure 11, the ocular surface of mBD-3 (-/-) mice were colonized by significantly more viable bacteria at both 3 and 6 h post-inoculation with ~10^9 cfu of *P. aeruginosa* (~190-fold at 3h, and 32-fold at 6 h; $p < 0.03$ for both comparisons).

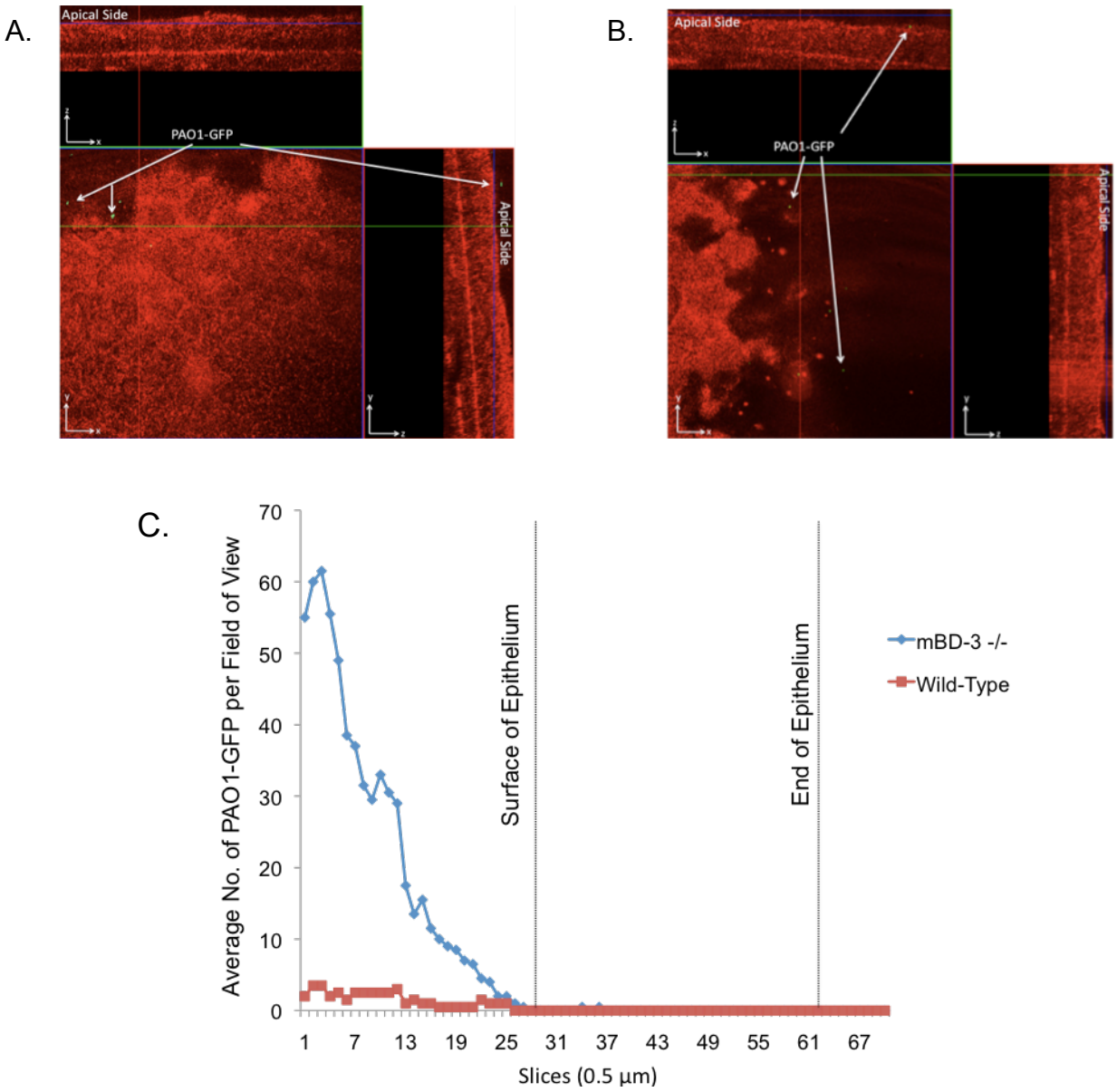


Figure 9. Confocal reflection microscopy of mBD-3 (-/-) (A) or wild-type (B) C57BL/6 mouse eyes at 4.5 h after inoculation *ex vivo* with ~10^9 cfu *P. aeruginosa* strain PAO1-GFP (green). Eyeballs were carefully enucleated, rinsed with PBS, then tissue paper blotted before bacterial challenge (see Methods). Z-stack images were split into an orthogonal view to show x, y, and z planes of the corneal epithelium. Bacteria showed greater adherence to mBD-3 (-/-) corneas (red), but did not traverse the epithelium. These differences were clearly shown by quantifying bacterial distribution over the corneal epithelium (C). (Red = reflection of corneal epithelial cells at 633 nm). Magnification ~1000x.

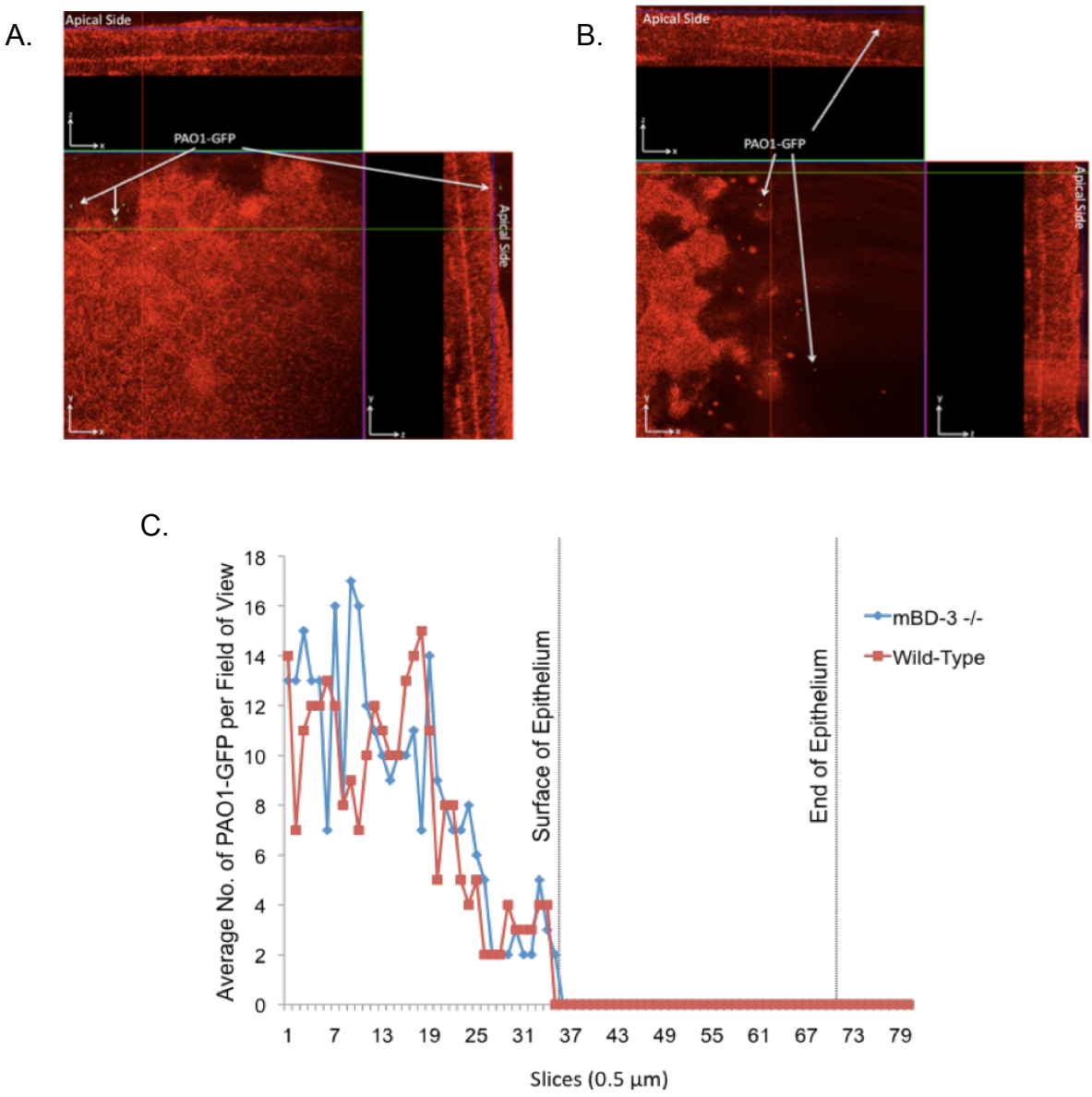


Figure 10. Confocal reflection microscopy of mBD-3 (-/-) (A) or wild-type (B) C57BL/6 mouse eyes at 7.5 h after inoculation *ex vivo* with ~10^9 cfu *P. aeruginosa* strain PAO1-GFP (green). Eyeballs were carefully enucleated, rinsed with PBS, then tissue paper-blotted before bacterial challenge (see Methods). Z-stack images were split into an orthogonal view to show x, y, and z planes of the intact corneal epithelium. After 7.5 h, bacteria showed equal levels of adherence to mBD-3 (-/-) and wild-type corneas, but still did not traverse the epithelium. These data were confirmed by quantifying bacterial distribution over the corneal epithelium (C). (Red = reflection of corneal epithelial cells at 633 nm). Magnification ~1000x.

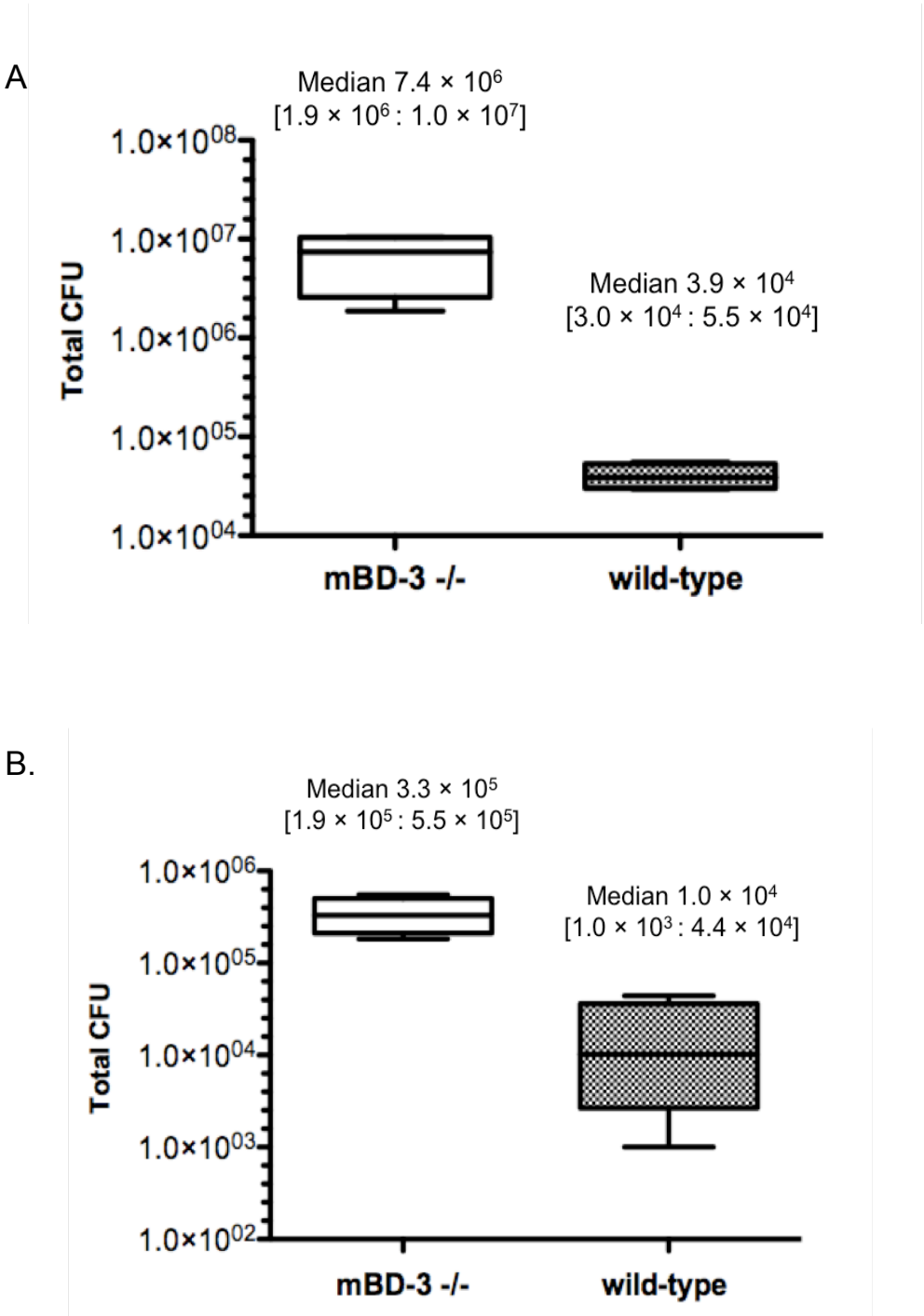


Figure 11. Viable *P. aeruginosa* strain PAO1 in the tear fluid of mBD-3 (-/-) versus wild-type C57BL/6 mice at 3 h (A) or 6 h (B) post-inoculation with ~10^9 cfu bacteria *in vivo* using the null-infection clearance model (n= 6 - 8 mice per group) (135). Data are expressed as a median [lower quartile: upper quartile]. *p < 0.03, Mann Whitney test. One of three independent experiments is shown.

2.5. DISCUSSION.

The results of this study show that antimicrobial peptides (AMPs), specifically human β-defensins (hBD-1, 2, and 3) and the human cathelicidin LL-37, contribute to the ability of human corneal epithelia to resist traversal by *P. aeruginosa in vitro*. They also show that murine β-defensin mBD-3 (equivalent of hBD-2 in humans) contributes to clearance of *P. aeruginosa* from the healthy mouse eye *in vivo,* and additionally limits *P. aeruginosa* adhesion to the corneal epithelial surface. These data suggest that in addition to their role in innate defense responses to ocular injury and infection (101, 197), AMPs are also involved in maintaining normal resistance to infection when the tissue is healthy.

Previous studies investigating mechanisms of *P. aeruginosa* traversal have mostly been done using epithelial cell monolayers grown on permeable filters, e.g. MDCK cells (14, 15, 75, 76) and some have found concomitant loss of TER, suggesting disruption of epithelial tight junctions is involved. Rhamnolipid-mediated disruption of tight junctions was associated with *P. aeruginosa* traversal of primary cultured multilayered human airway epithelia (205), as was the type III secreted effector ExoS for monolayers of airway epithelia (173). However, our results with corneal epithelial multilayers differ in that they show *P. aeruginosa* traversal can occur without TER disruption, which we've previously shown using rabbit corneal epithelial cells (3, 104). Thus, reduced TER is not a pre-requisite for allowing *P. aeruginosa* to penetrate through cell multilayers, nor is TER loss necessarily a consequence of bacterial penetration when it does occur.

It is possible that *P. aeruginosa*-mediated disruptions in epithelial cell polarity involving expression of basolateral membrane proteins on apical cell surfaces (98) could impact traversal of *P. aeruginosa* observed in the present study. *P. aeruginosa*-induced cell polarity changes, which we have shown can occur without disruption of tight junctions (98), would promote internalization of bacteria into epithelial cells (49). Growth factor-mediated polarity changes could have the same effect (50). We have previously shown that traversal of multilayered rabbit corneal epithelia by invasive *P. aeruginosa* involves twitching motility, which we also found involved in exit of epithelial cells after internalization (3). How cell internalization (and therefore cellular exit) participates in cellular traversal mechanisms of *P. aeruginosa* has not yet been elucidated. However, it is of interest that our data show correlations (in both directions) between internalization and traversal; i.e. pre-exposure to *P. aeruginosa* antigens *reduced* susceptibility to both phenomena at 8 h (Figure 6 B, C). Considering how the number of intracellular bacteria was impacted, the data suggest either that cells became more resistant to bacterial entry, or that they were more efficient at killing intracellular bacteria. However, since recovery of viable intracellular bacteria by gentamicin assays depends on how many bacteria are internalized, not just how many survive after internalization, both could be simultaneously impacted with their effects potentially masking one another. Nevertheless, the data continue to support a role for internalization in *P. aeruginosa* traversal of multilayered epithelia, and that its potential roles could include both contributions to, and defense against, traversal.

It is of interest that siRNA knockdown of multiple AMPs *in vitro* showed additive effects on promoting *P. aeruginosa* traversal. Additive or synergistic effects for antimicrobial peptides have been shown with respect to their antimicrobial activity (27, 136), and in their contributions to resolution of murine *P. aeruginosa* corneal infections

(197). Since AMPs can have broad-spectrum antimicrobial activity, which includes activity against *P. aeruginosa* (57, 83, 126, 132), it is likely that at least part of the mechanism for AMP protection against *P. aeruginosa* traversal involves killing of bacteria in the *in vitro* assays. Indeed, the data showed increased bacterial viability at the apical site of inoculation after AMP knockdown *in vitro* and knockout *in vivo*.

However, defensins also have autocrine and paracrine effects on mammalian cells, and can influence epithelial wound-healing and cytokine expression (58, 126, 132). Thus, it remains possible that the role of AMPs in protection against *P. aeruginosa* traversal *in vitro* also involves indirect effects *via* other epithelial-derived factors or multilayer-integrity.

The data demonstrated a role for the mouse ortholog of hBD-2 in clearing bacteria from the healthy mouse eye *in vivo*, despite the fact that tear fluid has been shown to interfere with its antimicrobial function (83). In a previous study, we found SP-D can also contribute to bacterial clearance from the healthy ocular surface (135), yet it has weak antimicrobial activity against the strains tested. It is possible that factors involved in clearance at the ocular surface work additively or synergistically to increase their antimicrobial activity against *P. aeruginosa* (83, 195), thereby also countering the negative impact of potential modulators such as tear fluid.

Pretreatment of cells with bacterial culture supernatant, containing multiple bacterial factors with potential to activate TLR-mediated signaling (20, 147), reduced cell susceptibility to *P. aeruginosa* traversal. We, and others, have shown that AMP expression in epithelial cells can be upregulated by that supernatant or by purified factors contained within it (58, 102, 122, 130). The siRNA data presented in this study show that multiple epithelial expressed AMPs participate in protecting against traversal. Thus, it is likely that culture supernatant induces defense against traversal via AMP upregulation. However, the culture supernatant could have a plethora of other effects on the innate immunity of these cells beyond AMP upregulation, and some of these might also modulate their susceptibility to *P. aeruginosa* traversal. Potential candidates include surfactant-protein D, which we previously showed is upregulated by either *P. aeruginosa* LPS or flagellin, and inhibits *P. aeruginosa* invasion of corneal epithelial cells (138, 139). Mucin glycoproteins can be upregulated by bacterial antigens, and can prevent *P. aeruginosa* attachment to epithelial surfaces (55, 69, 129). Further studies will be needed to explore the impact of these and other epithelial expressed factors in epithelial defense against bacterial traversal.

While knocking down hBD-2 in human corneal epithelial cells *in vitro* promoted bacterial traversal, knockout of its ortholog (mBD-3) in mice promoted bacterial adherence, but not their traversal. While this could be because mBD-3 plays no role in preventing bacterial traversal after adherence, it is more likely to reflect functional redundancy with other traversal defense factors. Indeed, the siRNA data showed additive effects among AMPs for cells grown *in vitro* suggesting functional redundancy exists. There may also be compensatory upregulation of other protective factors in the mBD-3 gene knockout animals. While we were unable to prove directly that mBD-3 protects mouse corneas against bacterial traversal, other data did show it participates in maintaining ocular surface resistance to bacteria during health; i.e. in clearing bacteria from the healthy ocular surface, and protecting the corneal epithelium against bacterial adherence; even when experiments were done ex vivo to compensate for differences in

clearance. The mechanism for these protective roles *in vivo* could involve killing of approaching bacterial cells (83), but effects on epithelial expression of other antimicrobial/anti-adherence factors, or even on resident dendritic cells of the cornea, cannot be ruled out.

In summary, the capacity to cross the epithelial multilayers is likely to be a critical virulence strategy for both opportunistic and outright pathogens that infect body surfaces in the absence of overt tissue injury. Yet much of what has been learned about epithelial defense against microbes has been derived from one-on-one bacteria-cell interaction studies using and epithelial cells grown as monolayers on plastic or glass, with relevance to barrier function only assumed. This study adds to a growing body of literature suggesting that a complex set of events occur when microbes attempt to cross the epithelial cell multilayers. Further studies to elucidate mechanisms involved in epithelial traversal by bacteria, the barriers that normally protect us against it during health, and how these are compromised during susceptibility, using models that enable this critical step in pathogenesis to be studied directly, will enhance our understanding of microbial keratitis, and of other epithelial-associated infections.

CHAPTER 3.

Bacterial Genes Impacted in *P. aeruginosa* invasive strain PAO1 during Traversal of Multilayered Human Corneal Epithelial Cells.

3.1. ABSTRACT.

Ordinarily, the corneal epithelium is a protective barrier to microbial infection, yet contact lens wear can predispose individuals to infectious keratitis. The mechanism by which contact lens wear enable epithelial susceptibility to microbes is not well understood. We have previously reported, using *in vivo* rodent models, that adaptation to epithelial host defense at the ocular surface is required for disease onset, beginning only after 1-2 weeks. In this study, we tested the hypothesis that prolong exposure to corneal epithelia increases *P. aeruginosa* capacity to adapt to and overcome epithelial barrier function that would otherwise limit penetration using our *in vitro* traversal model. We also explored how epithelial traversal impacts bacterial gene expression, as a first step towards identifying bacterial genes that contribute to penetration (and traversal) when the epithelial barrier is overcome. Results showed bacteria that had traversed corneal epithelial cells (pre-conditioned) acquired an enhanced capacity for subsequent traversal (~ 2 log; p = 0.0001). Microarray analysis identified genes in naïve PAO1 and pre-conditioned PAO1 differentially expressed after traversal, which included genes for membrane transport, transcriptional regulators, and a number of genes encoding hypothetical proteins. Transposon mutants in ~90 genes were examined for traversal capacity. Of these, 21 showed loss of traversal function and 10 showed enhanced traversal capacity to penetrate corneal epithelial cells, including one know to negatively regulate exotoxin A.

3.2. INTRODUCTION.

Pseudomonas aeruginosa can cause various opportunistic infections, most involving epithelial and soft tissue, e.g. cornea, skin, airways. The capacity to cross single or multilayer's of epithelial cells is likely to be a critical virulence strategy for those pathogens that infect exposed mucosal surfaces in the absence of overt tissue injury (117). The capacity of *P. aeruginosa* to elicit such infections is due to the versatility of virulence factors this bacterium possesses, as well as its ubiquitous nature (176). These factors are diverse and complex ranging from regulatory genes for cell wall composition e.g. *wxx1/2*, diffusion of small hydrophobic molecules e.g. *oprG* (183), iron-uptake e.g. *fpvA* (155), drug efflux pumps e.g. *mexA/B* (156), and numerous secretion system and apparatus e.g. *exsA* (32). While there is a breadth of knowledge on the virulence factors associated, little is known on the co-regulation between disparate virulence systems during the first stages of pathogenesis (i.e. invasion, intracellular survival and traversal of the epithelium) and how this contributes to disease progression. Dissecting apart early events that contribute to pathogenesis is import if we are to develop means to prevent infections that enable epithelial susceptibility to microbes.

Ordinarily, the corneal epithelium is a protective barrier to microbial infection. The ocular surface maintains resistance to microbial infection by expression of several corneal epithelial derived defense factors e.g. mucins, surfactant proteins-D, β-defensins-2 and cathelicidin LL-37 (5, 12, 126, 135), all known to have antimicrobial activities against *P. aeruginosa*. However, contact lens wears, ocular injury, or surgery are major risk factor for the development of microbial keratitis (48, 96, 178), an acute

infectious disease that can result in vision lost. In order to cause keratitis, *P. aeruginosa* must first overcome these epithelial derive defenses and traverse the multilayered corneal epithelium into the underlying stroma. Having done so, the stroma can be rapidly destroyed through a combination of bacterial virulence factor, host inflammatory and immune response (71).

Many pathogens have developed strategies to circumvent the barrier function of an intact epithelium; breech of this protective layer often results in infection. *P. aeruginosa*, like many other microbial pathogens, can overcome monolayer or multilayered host epithelial barriers to establish an infection. Although, *P. aeruginosa* has been traditionally classified as an extracellular pathogen, our research both *in vitro* and *in vivo*, have demonstrated that *P. aeruginosa* has the capacity to invade cells (41, 52, 54, 94). Currently, what is know is *P. aeruginosa* invasion of epithelial cells is mediated by the outer-core LPS region which interacts with lipid-rich membrane rafts and includes the interaction with cystic fibrosis transmembrane-conductance regulator (CFTR) on host epithelial cells (99, 202). Other surface antigens, including flagelin and pili (45, 204), can also mediate this process by binding to asialo-GMI (30). Previous studies investigating mechanism of *P. aeruginosa* traversal (including the invasion process) have mostly been done using epithelial cell monolayers grown on permeable filters, e.g. MDCK cells (14, 15, 75).

To date, detailed studies to investigate the molecular mechanism and co-regulation between different virulence systems of *P. aeruginosa* for entry, intracellular survival, and traversal of human epithelial cells and its contribution to disease progression have not been carried out. Epithelial cells are the first cell type that many bacteria encounter during the pathogenesis process. By investigating how epithelial invasion and traversal impacts *P. aeruginosa* gene expression, we can better understand the relevance of specific bacterial virulence traits on the early development of *P. aeruginosa* keratitis infections (or any other epithelial derived infections). Here, we tested the hypothesis that *P. aeruginosa* has the capacity to adapt to corneal epithelial host defense factors that would otherwise limit their ability to traverse. In addition, we explored how epithelial traversal impacts bacterial gene expression during this process.

3.3. MATERIAL AND METHODS.

3.3.1. Bacterial Strains and Growth Conditions. *P. aeruginosa* PAO1 wild-type strain and all the *P. aeruginosa* transposon insertion mutants used were from the comprehensive *P. aeruginosa* transposon mutant library at the University of Washington Genome Center (89). The strain ID (unique identifier) is given on the tables (Tables 1-5 and supplemental data; Tables S3-4), and further information on these mutants can be found at (http://www.genome.washington.edu/UWGC/Pseudomonas/index.cfm). All transposon mutant strains were grown on trypticase soy agar (BD Biosciences, CA) supplemented with tetracycline, (20 μg/mL) at 37 °C for ~16 h then resuspended in KGM-2 without antibiotics at a concentration of ~10^8 colony forming units (cfu/ml) (OD at 650 nm of ~ 0.1). Inocula were then prepared by diluting this suspension in KGM-2 to a final concentration of ~10^6 cfu/ml for use.

3.3.2. Cell Culture. Telomerase-immortalized human corneal epithelial (HCE) were maintained as previously described in section 2.3.2.

3.3.3. *In vitro* Traversal Assay. Traversal assay was performed as previously described in section 2.3.3.

3.3.4. RNA Extraction. Total RNA extraction was isolated from bacterial strains collected from the apical (naïve) and/or basal (pre-conditioned) compartments (pooled from a total of 120 Transwells) using the *in vitro* traversal assay 8 h post-inoculation (see figure 4), with Trizol (Invitrogen, CA) according to manufacturer's instructions. Total RNA was check for integrity and the absence of DNA by PCR prior to cDNA synthesis in which RNA samples contaminated with genomic DNA were treated with DNase (RQ1 RNase-free DNase; Promega, CA). PCR reactions were carried out using MJ Mini Personal Thermal Cycler using 16S ribosomal RNA gene (27F 5'-AGAGTTTGATCCTGGCTCAG-3'; 1492R 5'-GGTTACCTTGTTACGACTT -3') under the following conditions: 95 °C for 10 min followed by 40 cycles of 95 °C for 30 sec, 58 °C for 1 min and 72 °C for 30 sec. RNA quality was checked on a 1% agarose gel.

3.3.5. Microarray Target Preparation and Hybridization. cDNA and Microarray preparation was done at the Functional Genomics Laboratory at University of California, Berkeley. First-strand cDNA was synthesized as described in the Affymetrix expression technical manual. Briefly, cDNA was purified using the MinElute™ Purification kit (Qiagen, CA), fragmented with DNaseI (Qiagen, CA), and 3′-end-labelled using the GeneChip® DNA Labeling Reagent (Affymetrix, CA). DNA fragmentation and cDNA suitability was assessed using a Bioanalyser and a 'Test3' array (100 housekeeping genes) respectively, prior to hybridization. Hybridization was done in a GeneChip Hybridization Oven 320 for 16 h at 50 °C. The GeneChips were scanned on a GeneChip® Scanner 3000 7G at 532 nm for excitation and 570 nm for emission. Raw image files was converted to CEL (intensity) and CHP (expression) data files using the Microarray Suite (MAS 5.0).

3.3.6. Microarray Data Analysis. Probeset data files were normalized together and expression values were determined using the Robust Multi-chip Average (RMA) method as implemented in RMA Express (http://stat-www.berkeley.edu/~bolstad/RMAExpress/RMAExpress.html). Subsequent analysis was done using the R statistical computing package (http://www.r-project.org) and the Bioconductor libraries (http://www.bioconductor.org). Cluster analysis was done using the Gene Functional Classification (81) tool from the Database for Annotation, Visualization and Integrated Discovery (DAVID; http://david.abcc.ncifcrf.gov/home.jsp).

3.3.7. Statistical Analysis. Data expresses a mean ± standard deviation (SD) for *in vitro* traversal assays. Gene list from the microarray analysis was generated by comparing gene expression profile of PAO1 exposed to corneal epithelial cells that were naïve to traversal (Naïve PAO1) or those that traversed the multilayered epithelial cells (Pre-conditioned PAO1). Baseline reference for each condition was PAO1 grown in media only (see supplemental data for full list; Table S3-S4). To identify genes that

were impacted by the traversal process as oppose to cell contact, the pre-conditioned PAO1 group was compared to naïve PAO1 (Table 1), following the pre-screen procedure mentioned above. Genes in the microarray analysis were considered differentially regulated if the relative change (*n*-fold) was ≥ 2.0. Statistical significance between groups was determined using unpaired Student *t*-Test. P-value of ≤ 0.05 was considered significant.

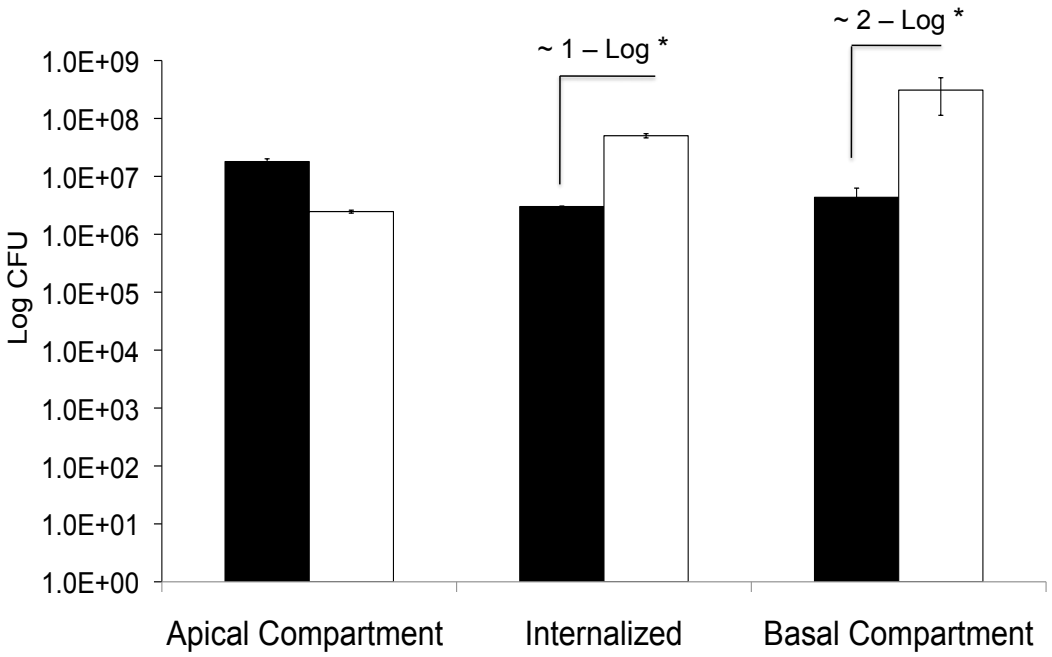
3.4. RESULTS.

3.4.1. The ability of *P. aeruginosa* to traverse is increased with subsequent traversal events of multilayered human corneal epithelia.

In vitro, exposure of *P. aeruginosa* to sub-lethal of AMPs has been shown to alter bacterial gene expression in a manner that increases their resistance to killing AMPs (171). Given that our data showed that AMPs participate in epithelial cell defense against bacterial (i.e. siRNA knockdown of AMPs increased basal viable counts, Figure 8A), and that this correlated with effects on bacterial survival/replication (i.e. siRNA knockdown also increased apical viable counts, Figure 8B), we next tested the hypothesis that *P. aeruginosa* might be capable of adapting to cellular defenses against traversal. This would be of interest to know, since susceptibility to *P. aeruginosa* infection is commonly associated with situations that have potential to increase contact time between bacteria and epithelial cells (i.e. contact lens wear). We compared bacteria that had previously traversed (i.e. pre-conditioned bacteria) multilayered corneal epithelial cells to PAO1 naïve the traversal process. In our *in vitro* traversal assay, viable counts were done to confirm that the inoculum added to cells was similar for the test and control groups (data not shown).

Pre-conditioned PAO1 were found to be ~ 2 log more efficient at subsequent traversal (i.e. basal compartment) of healthy multilayered corneal epithelial cells compared to naïve PAO1 (i.e. apical compartment; Figure 12A; $p < 0.0001$). Gentamicin survival assays showed that these pre-conditioned bacteria were also better at internalizing corneal epithelial cells (Figure 12A; $p < 0.001$). Interestingly, pre-conditioned, but not naïve bacteria, exhibited mass aggregation in the media inside the basal compartment after traversal which was clearly visible without magnification (see photo in Figure 12B), suggesting up-regulation of genes involved in establishing bacterial communities (i.e. biofilm). Trans-Epithelial Resistance readings (TER; which measures tight-junction integrity and cell polarity) were interestingly reduced multilayered corneal cell culture infected with pre-conditioned bacteria as compared to cells infected with naïve bacteria (see supplemental data; Table S2, $p < 0.05$) that pre-conditioned bacteria had acquired the capacity to kill cells and/or disrupt cell-to-cell junctions.

A.



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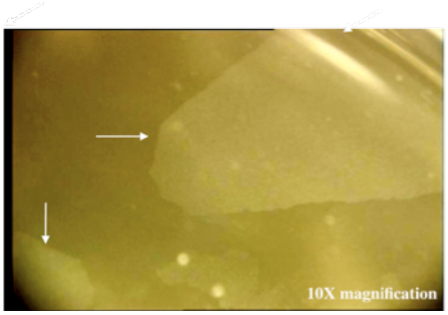


Figure 12. Traversal of human corneal epithelial cells by naïve (black bars) or pre-conditioned (white bars) *P. aeruginosa* strain PAO1 (inoculum of 10^6 CFU). Values able reflect the viable counts of bacteria in the basal compartment at 8 h post-inoculation (A) (*p < 0.001 versus naïve PA01). Pre-conditioned bacteria, i.e. those that had previously traversed the corneal epithelial cells, formed dense aggregates (arrows) in the basal media after a second traversal (8 h post-inoculation) (B). Results represent one of three independent experiments.

3.4.2. Identifying genes in *P. aeruginosa* that are differentially expressed in response to traversal of multilayered human corneal epithelia.

Since a change in traversal was observed by *P. aerugnosa* invasive strain PAO1 and the visible morphological changes following the subsequent traversal events, we proposed that *P. aeruginosa* adapted to corneal epithelial host factor(s), which would otherwise limit its traversal. To better identify which genes are differentially expressed by *P. aeruginosa* upon traversal, a transcript profile using microarray analysis was constructed. Gene list from the microarray analysis was generated by comparing gene expression profile of PAO1 exposed to corneal epithelial cells that were naïve to traversal (naïve PAO1) or those that traversed the multilayered epithelial cells (pre-conditioned PAO1). Baseline reference for each condition was PAO1 grown in media only (see supplemental data for full gene list; Table 2S-3S). Genes in the microarray analysis were considered differentially regulated if the relative change (*n*-fold) was ≥ 2.0. Statistical significance between groups was determined using unpaired Student *t*-Test with p-values of ≤ 0.05 was considered significant.

Total bacterial RNA was harvested from PAO1 collected from the apical (i.e. naïve PAO1) or basal (i.e. pre-conditioned PAO1) compartment and pooled from a total of 120 Transwell from the *in vitro* traversal assay 8 h post-inoculation (see figure 4). The resultant cDNAs were hybridized to Affymetrix Genechip® *P. aeruginosa* Genome microarrays. In the statistical analysis of normalized probsets, 264 ORFs were found to be upregulated by naïve bacteria, with 234 ORFs by pre-conditioned PAO1. Overall, the two sets had 139 upregulated ORFs in common. Our analysis also identified a total of 164 ORFs repressed, 85 ORFs by naïve PAO1 and 79 ORFs by pre-conditioned PAO1, with 51 ORFs being shared between the two (the complete gene list is in Table S2-S3). To determine which differential expressed genes correlated with enhanced traversal capacity, the pre-conditioned PAO1 group was compared to naïve PAO1 (Table 1), following the pre-screen procedure mentioned above. Interestingly, the largest functional group (~ 55 %) of the total number of upregulated ORFs involved encoded hypothetical proteins.

Also of some interest, were the genes that were exclusively found expressed (based on the normalized probsets list generated above) in either naïve or pre-conditioned PAO1 (Table 2-5). A total of 125 upregulated ORFs exclusively by naïve PAO1, in which 34 ORFs were repressed and a total of 95 upregulated ORFs by pre-conditioned PAO1, with 28 ORFs repressed during traversal process. When analyzed by function using Database for Annotation, Visualization and Integrated Discovery (DAVID (81); http://david.abcc.ncifcrf.gov/home.jsp), genes upregulated by naïve PAO1 encoded proteins in nearly every functional category (Table 2): including a large fraction of hypothetical proteins [38], membrane proteins and transport [23], conserved hypothetical proteins [17], metabolism [12], transcriptional regulators [11], secretion factors and apparatus [9], putative enzymes [4], and proteins involved in adaptation and protection [2]. Repressed genes included several for hypothetical proteins, cell wall/LPS/capsule, and amino acid biosynthesis (Table 3). In comparison, genes up-regulated by pre-conditioned PAO1 showed similar categories as that of naïve, however with a difference in hierarchy amongst the functional groups (Table 4): hypothetical proteins [19], conserved hypothetical proteins [16], metabolism [12], transcriptional

regulators [10], membrane proteins [8], proteins involved in adaptation and protection [4], putative enzymes [4], chemotaxis [2], and secretion factors and apparatus [1]. Repressed genes included several for hypothetical proteins, membrane proteins, and putative enzymes (Table 5).

Table 1. Genes impacted by pre-conditioned PAO1 compared to those naïve to traversal of multilayered human corneal epithelial cells based on microarray analysis.

Upregulated

PA No./Gene[a]	Known/Predicted Function	Functional Group	P-value	Fold-Change[b]
PA3572	Hypothetical	Unknown	0.0003	4.5
PA2501	Hypothetical	Unknown	0.0002	3.92
PA3732	Conserved Hypothetical	Unknown	0.002	3.6
arcD	Arginine/Ornithine Antiporter	Amino Acid Biosynthesis and Metabolism	0.0003	3.13
ibpA	Heat Shock Protein	Chaperones and Heat Shock Proteins	0.009	2.91
PA2754	Conserved Hypothetical [DUF883 super family]	Unknown	0.006	2.82
PA0404	Conserved Hypothetical [UPF0081]	Unknown	0.05	2.54
aer	Aerotaxis receptor	Adaptation and Chemotaxis	0.02	2.53
PA1789	Hypothetical	Unknown	0.004	2.42
PA3229	Hypothetical	Unknown	0.02	2.38
PA3006	Transcriptional Regulator	Transcriptional Regulators	0.001	2.35
PA1746	Hypothetical	Unknown	0.005	2.28
rsaL	Regulatory protein RsaL	Transcriptional Regulators	0.002	2.27
atpF	ATP synthase B chain	Energy Metabolism	0.009	2.27
PA2411	Probable Thioesterase	Adaptation and Protection	0.04	2.19
PA5475	Hypothetical	Unknown	0.002	2.13
PA5232	Conserved Hypothetical [HlyD family secretion]	Unknown	0.0001	2.1
hasAp	Heme Acquisition Protein	Transport of Small Molecules	0.0008	2.08
phaF	Polyhydroxyalkanoate Synthesis Protein	Central intermediary metabolism	0.005	2.04
PA0713	Hypothetical	Unknown	0.001	2.02

Downregulated

PA No./Gene[a]	Known/Predicted Function	Functional Group	P-value	Fold-Change[b]
PA0239	Hypothetical	Membrane proteins	4.00E-05	1.96

a. PA gene number, known and predicted functions, and functional groupings were obtained from http://www.pseudomonas.com. Genes represented are those impacted by traversal (i.e. pre-conditioned PAO1) compared to cell contact only (i.e. naïve PAO1).

b. Genes in the microarray analysis were considered differentially regulated if the relative change (*n*-fold) was ≥ 2.0, following the pre-screen procedure mentioned in material and methods section 3.3.7. Statistical significance between groups was determined using unpaired Student *t*-Test were p-value of ≤ 0.05 was considered significant.

Table 2. Genes upregulated by PAO1 naïve to traversal of multilayered human corneal epithelial cells relative to those grown in media only.

PA No./Gene[a]	Known/Predicted Function	Functional Group	P-value	Fold-Change[b]
mtlY	Xylulose Kinase	Carbon compound catabolism	0.0016	3.07
PA0221	Probable Aminotransferase	Putative Enzymes	0.0003	2.97
PA0136	Probable ATP-binding Component of ABC transporter	Membrane Protein	0.0002	2.92
PA0192	Probable TonB-Dependent Receptor	Energy Metabolism	0.0012	2.87
nosF	NosF protein	Energy Metabolism	0.0002	2.82
PA0069	Probable TonB-Dependent Receptor	Membrane Protein	0.0002	2.81
cupB4	Chaperone	Chaperones	0.0008	2.76
PA2149	Hypothetical Protein	Unknown	0.0003	2.66
PA3425	Hypothetical Protein	Unknown	0.0004	2.64
PA1268	Hypothetical Protein	Unknown	0.0003	2.63
madL	Malonate Transporter	Membrane Protein	0.0054	2.6
opdK	Histidine Porin	Membrane Protein	0.0012	2.6
PA0148	Probable Adenosine Deaminase	Nucleotide Biosynthesis and Metabolism	0.0024	2.56
PA4860	Probable Permease of ABC Transport	Membrane Protein	0.0025	2.55
PA0345	Hypothetical Protein	Unknown	0.0002	2.54
oprJ	Multidrug Efflux Outer Membrane Protein	Membrane Protein	0.0002	2.51
PA0066	Conserved Hypothetical Protein [isoleucine patch superfamily]	Unknown	0.0003	2.5
PA0174	Conserved Hypothetical Protein [CheD domain]	Unknown	0.0039	2.5
PA1228	Hypothetical Protein	Unknown	0.0035	2.46
PA0325	Probable Permease of ABC Transporter	Membrane Protein	0.0002	2.46
treA	Periplasmic Trehalase Precursor	Carbon Compound Catabolism	0.0003	2.46
PA1394	Hypothetical Protein	Unknown	0.0004	2.45
PA5465	Hypothetical Protein	Unknown	0.0003	2.44
PA1888	Hypothetical Protein	Unknown	0.0002	2.41
lipH	Lipase Modulator Protein	Protein Secretion/Export Apparatus	0.0056	2.4
PA1879	Hypothetical Protein	Unknown	0.0004	2.4
PA2916	Hypothetical Protein	Unknown	0.0003	2.37
PA1221	Hypothetical Protein	Unknown	0.0004	2.37
PA3378	Conserved Hypothetical Protein [PhnI domain]	Transport of Small Molecules	0.0008	2.36
PA4193	Probable Permease of ABC Transporter	Membrane Protein	0.0002	2.35
PA4121	Conserved Hypothetical Protein [FAA Hydrolase Superfamily]	Unknown	0.0352	2.35
ampR	Bacterial Regulatory Helix-Turn-Helix Protein [LysR Family]	Transcriptional Regulator	0.0005	2.33
PA3501	Hypothetical Protein	Unknown	0.001	2.32
PA0213	Hypothetical Protein	Unknown	0.0008	2.32
PA4824	Hypothetical Protein	Unknown	0.0008	2.3
PA3383	Binding Protein Component of ABC Phosphonate Transporter	Transport of Small Molecules	0.0033	2.3
madM	Malonate Transporter	Transport of Small Molecules	0.0005	2.29
PA2274	Hypothetical Protein	Unknown	0.0006	2.29
napD	Periplasmic Nitrate Reductase	Energy Metabolism	0.0003	2.29
PA3444	Conserved Hypothetical Protein [FMNH2-Dependent Monooxygenase]	Putative Enzymes	0.004	2.28
phzB1	Probable Phenazine Biosynthesis	Secreted Factors	0.003	2.28
PA1351	Probable Sigma-70 Factor	Transcriptional Regulator	0.0015	2.26
PA5381	Hypothetical Protein	Unknown	0.0003	2.26
PA0274	Hypothetical Protein	Unknown	0.0022	2.25
PA0113	Probable Cytochrome C Oxidase Assembly Factor	Energy Metabolism	0.0021	2.25
PA2881	Probable Two-Component Response Regulator	Transcriptional Regulator	0.0005	2.24
PA3412	Hypothetical Protein	Unknown	0.0011	2.23
PA0163	Probable Transcriptional Regulator	Transcriptional Regulator	0.0008	2.22

a. PA gene number, known and predicted functions, and functional groupings were obtained from http://www.pseudomonas.com. Genes represented are those impacted exclusively by naïve PAO1.

b. Baseline reference was PAO1 grown in media only. Genes in the microarray analysis were considered differentially regulated if the relative change (*n*-fold) was ≥ 2.0. Statistical significance between groups was determined using unpaired Student *t*-Test with p-values of ≤ 0.05 was considered significant.

Table 2 continued. Genes upregulated by PAO1 naïve to traversal of multilayered human corneal epithelial cells relative to those grown in media only.

PA No./Gene[a]	Known/Predicted Function	Functional Group	P-value	Fold-Change[b]
lipA	Lactonizing Lipase Precursor	Secreted Factors	0.006	2.22
PA4878	Probable Transcriptional Regulator	Transcriptional Regulator	0.002	2.22
rpsO	30S Ribosomal Protein S15	Translation	0.0005	2.21
PA0147	Probable Oxidoreductase	Putative Enzymes	0.0008	2.2
PA2331	Hypothetical Protein	Unknown	0.0013	2.19
antC	Anthranilate Dioxygenase Reductase	Carbon Compound Catabolism	0.0088	2.19
PA1412	Hypothetical Protein	Unknown	0.0015	2.18
PA2178	Hypothetical Protein	Unknown	0.0008	2.18
PA4107	Hypothetical Protein	Unknown	0.0005	2.18
PA2360	Hypothetical Protein	Unknown	0.0005	2.16
exbB1	Transport Protein	Transport of Small Molecules	0.0018	2.16
PA5481	Hypothetical Protein	Unknown	0.0025	2.16
phzG2	Probable Pyridoxamine 5'-Phosphate Oxidase	Secreted Factors	0.0033	2.16
PA2919	Hypothetical Protein	Unknown	0.0123	2.15
ampC	Beta-Lactamase Precursor	Adaptation and Protection	0.003	2.15
PA3896	Probable 2-Hydroxyacid Dehydrogenase	Putative Enzymes	0.0005	2.15
PA3463	Conserved Hypothetical Protein [UPF0270]	Unknown	0.0215	2.15
PA5517	Conserved Hypothetical Protein	Membrane Protein	0.005	2.14
PA1226	Probable Transcriptional Regulator	Transcriptional Regulator	0.0075	2.13
PA2312	Probable Transcriptional Regulator	Transcriptional Regulator	0.004	2.13
cttP	Chemotactic Transducer for Trichloroethylene	Adaptation and Protection	0.001	2.13
PA1408	Hypothetical Protein	Unknown	0.005	2.13
bdhA	3-Hydroxybutyrate Dehydrogenase	Carbon Compound Catabolism	0.001	2.12
PA2315	Hypothetical Protein	Unknown	0.007	2.12
PA2910	Conserved Hypothetical Protein	Membrane Protein	0.002	2.12
cupA3	usher CupA3	Motility and Attachment	0.0018	2.12
PA3934	Conserved Hypothetical Protein [Oligopeptide Transporter OPT Family]	Membrane Protein	0.0032	2.12
PA4187	Probable Major Facilitator Superfamily (MFS) Transporter	Antibiotic Resistance and Susceptibility	0.01	2.11
PA4830	Hypothetical Protein	Unknown	0.0008	2.11
tssJ1	Protein Secretion by the Type VI Secretion System	Protein Secretion/Export Apparatus	0.0008	2.11
PA0467	Conserved Hypothetical Protein	Unknown	0.0005	2.11
PA3937	Probable ATP-Binding Component of ABC	Transport of Small Molecules	0.002	2.11
PA4089	Probable Short-Chain Dehydrogenase	Putative Enzymes	0.004	2.11
pcaQ	Transcriptional Regulator	Carbon Compound Catabolism	0.0006	2.11
PA1909	Hypothetical Protein	Unknown	0.045	2.1
PA4135	Probable Transcriptional Regulator	Transcriptional Regulator	0.001	2.1
pchE	Dihydroaeruginoic Acid Synthetase	Secreted Factors	0.0015	2.1
PA0132	Beta-Alanine-Pyruvate Transaminase	Amino acid Biosynthesis and Metabolism	0.0017	2.09
PA4134	Hypothetical Protein	Unknown	0.0027	2.09
PA2184	Conserved Hypothetical Protein [DUF892]	Unknown	0.0006	2.09
PA4104	Conserved Hypothetical Protein [DoxX Domain]	Unknown	0.002	2.08
ppkA	Serine/Threonine Protein Kinase	Protein Secretion/Export Apparatus	0.001	2.07
PA2914	Probable Permease of ABC Transporter	Membrane Protein	0.0053	2.07
PA4879	Conserved Hypothetical Protein [AsmA Family]	Unknown	0.0016	2.07
PA4038	Hypothetical Protein	Unknown	0.0187	2.06
PA1259	Hypothetical Protein	Unknown	0.005	2.06
PA3424	Hypothetical Protein	Unknown	0.01	2.06
PA4171	Probable Protease	Secreted Factors	0.0025	2.06

a. PA gene number, known and predicted functions, and functional groupings were obtained from http://www.pseudomonas.com. Genes represented are those impacted exclusively by naïve PAO1.

b. Baseline reference was PAO1 grown in media only. Genes in the microarray analysis were considered differentially regulated if the relative change (*n*-fold) was ≥ 2.0. Statistical significance between groups was determined using unpaired Student *t*-Test with p-values of ≤ 0.05 was considered significant.

Table 2 continued. Genes upregulated by PAO1 naïve to traversal of multilayered human corneal epithelial cells relative to those grown in media only.

PA No./Gene[a]	Known/Predicted Function	Functional Group	P-value	Fold-Change[b]
PA3411	Hypothetical Protein	Unknown	0.003	2.05
PA0194	Hypothetical Protein	Unknown	0.0044	2.05
PA4873	Probable Heat-Shock Protein	Chaperones	0.0018	2.05
pcaD	Beta-Ketoadipate Enol-Lactone Hydrolase	Carbon Compound Catabolism	0.0021	2.05
nosY	ABC-Type Transport System involved in Multi-Copper Enzyme Maturation	Membrane Protein	0.0239	2.05
PA0115	Conserved Hypothetical Protein [GNAT Family]	Unknown	0.0014	2.05
PA0366	Probable Aldehyde Dehydrogenase	Putative Enzymes	0.0042	2.04
PA1274	Conserved Hypothetical Protein [Nitroreductase Family]	Unknown	0.0133	2.04
fepD	Ferric Enterobactin Transport Protein	Membrane Protein	0.0014	2.04
PA0522	Hypothetical Protein	Unknown	0.0021	2.04
znuC	Zinc Transport Protein	Transport of Small Molecules	0.0038	2.04
PA0056	Probable Transcriptional Regulator	Transcriptional Regulator	0.0069	2.04
pslM	Hypothetical Protein	Unknown	0.0008	2.03
PA2336	Hypothetical Protein	Unknown	0.0019	2.03
PA2296	Hypothetical Protein	Unknown	0.0013	2.03
PA0191	Probable Transcriptional Regulator	Transcriptional Regulator	0.0013	2.03
tagF1	Protein Secretion by the Type VI Secretion System	Secreted Factors	0.0009	2.03
ppiC2	Peptidyl-Prolyl Cis-Trans Isomerase C2	Translation	0.0014	2.03
PA2086	Probable Epoxide Hydrolase	Carbon Compound Catabolism	0.0008	2.03
PA0370	Conserved Hypothetical Protein	Unknown	0.0023	2.02
PA0810	Probable Haloacid Dehalogenase	Carbon Compound Catabolism	0.0006	2.02
hasD	Transport Protein	Protein Secretion/Export Apparatus	0.0037	2.02
PA2055	Probable Major Facilitator Superfamily (MFS) Transporter	Antibiotic Resistance and Susceptibility	0.0008	2.02
vgrG1	Protein Secretion by the Type VI Secretion System	Protein Secretion/Export Apparatus	0.0007	2.02
PA0095	Protein Secretion by the Type VI Secretion System	Protein Secretion/Export Apparatus	0.0011	2.01
PA2135	Probable Transporter	Membrane Protein	0.001	2.01
PA0226	Probable CoA Transferase, Subunit A	Carbon Compound Catabolism	0.0007	2
PA1211	Hypothetical Protein	Unknown	0.0055	2
PA2123	Probable Transcriptional Regulator	Transcriptional Regulator	0.0064	2

a. PA gene number, known and predicted functions, and functional groupings were obtained from http://www.pseudomonas.com. Genes represented are those impacted exclusively by naïve PAO1.

b. Baseline reference was PAO1 grown in media only. Genes in the microarray analysis were considered differentially regulated if the relative change (*n*-fold) was ≥ 2.0. Statistical significance between groups was determined using unpaired Student *t*-Test with p-values of ≤ 0.05 was considered significant.

Table 3. Genes repressed by PAO1 naïve to traversal of multilayered human corneal epithelial cells relative to those grown in media only.

PA No./Gene[a]	Known/Predicted Function	Functional Group	P-value	Fold-Change[b]
PA2501	Hypothetical Protein	Unknown	0.0002	3.53
PA3278	Hypothetical Protein	Unknown	0.0008	3.11
PA5232	Conserved Hypothetical Protein [HlyD Family Secretion]	Unknown	0.0002	2.83
PA3572	Hypothetical Protein	Unknown	0.0004	2.62
hasAp	Heme Acquisition Protein	Transport of Small Molecules	0.0009	2.62
PA0713	Hypothetical Protein	Unknown	0.0012	2.59
psrA	Transcriptional Regulator	Transcriptional Regulators	0.0012	2.53
PA3352	Hypothetical Protein	Unknown	0.0012	2.52
fabG	3-Oxoacyl-[Acyl-Carrier-Protein] Reductase	Fatty Acid and Phospholipid Metabolism	0.0002	2.5
clpP2	Protease Subunit of ATP-Dependent Clp Proteases	Translation	0.0003	2.5
PA0938	Hypothetical Protein	Unknown	0.0006	2.44
arcD	Arginine/Ornithine Antiporter	Amino Acid Biosynthesis	0.0004	2.37
rimM	16S rRNA Processing Protein	RNA processing and Degradation	0.0012	2.29
rpsB	30S Ribosomal Protein S2	Translation	0.0047	2.25
rpsP	30S Ribosomal Protein S16	Translation	0.0042	2.23
trpS	Tryptophanyl-tRNA Synthetase	Amino Acid Biosynthesis	0.0005	2.18
PA2973	Probable Peptidase	Translation	0.0008	2.18
ccoP1	Cytochrome C oxidase	Energy Metabolism	0.0006	2.15
katA	Catalase	Adaptation and Protection	0.0007	2.12
wbpK	Probable NAD-Dependent Epimerase	Cell Wall / LPS / Capsule	0.0025	2.12
PA1093	Hypothetical Protein	Unknown	0.0008	2.11
tyrZ	Tyrosyl-tRNA Synthetase 2	Amino Acid Biosynthesis	0.0008	2.11
PA0981	Hypothetical Protein	Unknown	0.0015	2.1
wbpI	UDP-N-Acetylglucosamine 2-Epimerase	Cell Wall / LPS / Capsule	0.0012	2.1
PA3284	Hypothetical Protein	Unknown	0.0033	2.09
PA0664	Hypothetical Protein	Unknown	0.001	2.07
gltX	Glutamyl-tRNA Synthetase	Translation	0.0023	2.06
atpF	ATP Synthase B Chain	Energy Metabolism	0.0098	2.06
rsaL	Regulatory Protein	Secreted Factors	0.0018	2.03
PA2562	Hypothetical Protein	Unknown	0.0008	2.02
cmk	Cytidylate Kinase	Nucleotide Biosynthesis and Metabolism	0.0008	2.01
arcC	Carbamate Kinase	Amino Acid Biosynthesis	0.0006	2
PA1746	Hypothetical Protein	Unknown	0.0055	2

a. PA gene number, known and predicted functions, and functional groupings were obtained from http://www.pseudomonas.com. Genes represented are those impacted exclusively by naïve PAO1.

b. Baseline reference was PAO1 grown in media only. Genes in the microarray analysis were considered differentially regulated if the relative change (*n*-fold) was ≥ 2.0. Statistical significance between groups was determined using unpaired Student *t*-Test with p-values of ≤ 0.05 was considered significant.

Table 4. Genes upregulated by PAO1 during traversal of multilayered human corneal epithelial cells relative to those grown in media only.

PA No./Gene[a]	Known/Predicted Function	Functional Group	P-value	Fold-Change[b]
PA4611	Hypothetical Protein	Unknown	1.00E-04	5.95
PA3229	Hypothetical Protein	Unknown	0.0006	3.84
PA3819	Conserved Hypothetical Protein	Membrane Protein	0.0013	2.64
PA4463	Conserved Hypothetical Protein	Structure and Biogenesis	0.0001	4.23
PA3731	Conserved Hypothetical Protein [PspA/IM30 family]	Unknown	0.0012	2.37
PA1198	Conserved Hypothetical Protein [NLP/P60 family]	Unknown	0.0025	3.96
grpE	Heat Shock Protein	Chaperones & Heat Shock Proteins	0.0004	3.76
PA0404	Conserved Hypothetical Protein [UPF0081]	Unknown	0.0001	3.45
PA3031	Conserved Hypothetical Protein [DUF903]	Unknown	0.0078	3.45
PA5446	Hypothetical Protein	Unknown	0.0001	3.39
algP	Alginate Regulatory Protein	Transcriptional Regulators	0.0012	3.21
etfB	Electron Transfer Flavoprotein Beta-Subunit	Energy Metabolism	0.0002	3.06
PA0506	Probable Acyl-CoA Dehydrogenase	Putative Enzymes	0.0001	3.07
ftsH	Cell Division Protein	Cell Division	0.0007	2.96
azu	Azurin Precursor	Energy Metabolism	0.0003	2.97
lptF	Lipotoxon F	Membrane Protein	0.0002	2.94
hslV	Heat Shock Protein	Chaperones & Heat Shock Proteins	0.0013	2.87
PA1592	Hypothetical Protein	Unknown	0.0031	2.86
hslU	Heat Shock Protein	Chaperones & Heat Shock Proteins	0.0019	2.84
amrZ	Alginate and Motility Regulator Z	Transcriptional Regulators	0.0039	2.77
algQ	Alginate Regulatory Protein	Transcriptional Regulators	0.0003	2.77
PA2883	Hypothetical Protein	Unknown	0.0018	2.74
rpoH	Sigma Factor	Transcriptional Regulators	0.0005	2.73
PA0329	Conserved Hypothetical Protein [DUF1508]	Unknown	0.0035	2.65
PA3691	Hypothetical Protein	Unknown	0.0007	2.64
galU	UTP-Glucose-1-Phosphate Uridylyltransferase	Central Intermediary Metabolism	0.0008	2.57
clpB	ClpB Protein	Post-Translational Modification	0.0005	2.48
oprI	Outer Membrane Lipoprotein	Membrane Protein	0.0007	2.45
PA0456	Probable Cold-Shock Protein	Adaptation and Protection	0.0109	2.44
trxA	Thioredoxin	Post-Translational Modification	0.0025	2.41
PA1041	Probable Outer Membrane Protein	Membrane Protein	0.0012	2.4
PA3788	Hypothetical Protein	Unknown	0.0008	2.39
glnA	Glutamine Synthetase	Amino Acid Biosynthesis and Metabolism	0.0019	2.38
PA3748	Conserved Hypothetical Protein	Membrane Protein	0.0005	2.37
PA0836	Acetate kinase	Putative Enzymes	0.0005	2.36
PA0433	Hypothetical Protein	Unknown	0.0007	2.35
algU	Sigma Factor	Transcriptional Regulators	0.0118	2.33
PA3182	6-Phosphogluconolactonase	Central Intermediary Metabolism	0.0014	2.33
hfq	Host Factor I Protein	RNA Processing and Degradation	0.0017	2.32
cc4	Cytochrome C4 Precursor	Energy Metabolism	0.0081	2.31
adhA	Alcohol Dehydrogenase	Energy Metabolism	0.0009	2.3
PA4607	Hypothetical Protein	Unknown	0.0024	2.27
PA3040	Conserved Hypothetical Protein [DUF883]	Unknown	0.001	2.27
PA3764	Conserved Hypothetical Protein [SBP Bac 3 Family]	Unknown	0.0006	2.25
ahpC	Alkyl Hydroperoxide Reductase Subunit C	Adaptation and Protection	0.0071	2.25
PA3458	Probable Tanscriptional Regulator	Transcriptional Regulators	0.0006	2.25
PA1676	Hypothetical Protein	Unknown	0.0017	2.25
PA3306	Hypothetical Protein	Unknown	0.0006	2.24

a. PA gene number, known and predicted functions, and functional groupings were obtained from http://www.pseudomonas.com. Genes represented are those impacted exclusively by pre-conditioned PAO1.

b. Baseline reference was PAO1 grown in media only. Genes in the microarray analysis were considered differentially regulated if the relative change (*n*-fold) was ≥ 2.0. Statistical significance between groups was determined using unpaired Student *t*-Test with p-values of ≤ 0.05 was considered significant.

Table 4 continued. Genes upregulated by PAO1 during traversal of multilayered human corneal epithelial cells relative to those grown in media only.

PA No./Gene[a]	Known/Predicted Function	Functional Group	P-value	Fold-Change[b]
PA5424	Conserved Hypothetical Protein	Membrane Protein	0.0045	2.23
zwf	Glucose-6-Phosphate 1-Dehydrogenase	Energy Metabolism	0.0007	2.22
liuR	Regulator of Liu Genes	Transcriptional Regulators	0.0007	2.22
bkdA2	2-Oxoisovalerate Dehydrogenase (Beta subunit)	Amino Acid Biosynthesis and Metabolism	0.0013	2.21
PA0623	Probable Bacteriophage Protein	Transposon or Plasmid	0.0051	2.21
rpsQ	30S Ribosomal Protein S17	Post-Translational Modification	0.0194	2.2
PA1677	Conserved Hypothetical Protein [Isochorismatase family]	Unknown	0.009	2.19
PA5463	Hypothetical Protein	Unknown	0.016	2.19
PA3665	Hypothetical Protein	Unknown	0.001	2.19
PA4870	Conserved Hypothetical Protein [DksA/TraR C4-Type Zinc Finger]	Unknown	0.001	2.18
tssC1	Protein Secretion by the Type VI Secretion System	Protein Secretion/Export Apparatus	0.001	2.17
PA4223	Probable ATP-binding Component of ABC transporter	Membrane Protein	0.0006	2.16
PA2491	Probable Oxidoreductase	Putative Enzymes	0.0014	2.16
tpbA	Protein Tyrosine Phosphatase	Motility & Attachment	0.0006	2.16
ygdP	Nudix Hydrolase	Nucleotide Biosynthesis and Metabolism	0.0025	2.16
arcA	Arginine Deiminase	Amino Acid Biosynthesis and Metabolism	0.001	2.16
PA5148	Conserved Hypothetical Protein [Bacterial Fe(2+) Trafficking]	Unknown	0.002	2.15
groEL	Chaperone	Chaperones & Heat Shock Proteins	0.002	2.15
PA4061	Probable Thioredoxin	Energy Metabolism	0.001	2.14
PA3529	Probable Peroxidase	Adaptation and Protection	0.0053	2.13
PA0943	Hypothetical Protein	Unknown	0.0021	2.13
oprG	Outer membrane protein	Membrane Protein	0.009	2.12
dnaK	Modification and Repair	Adaptation and Protection	0.0044	2.11
PA4465	Conserved Hypothetical Protein [P-loop ATPase Protein Family]	Unknown	0.001	2.11
xseB	Exodeoxyribonuclease VII Small Subunit	Adaptation and Protection	0.0037	2.1
PA0462	Hypothetical Protein	Unknown	0.0062	2.1
PA4844	Probable Chemotaxis Transducer	Chemotaxis	0.0125	2.09
thiE	Thiamin-Phosphate Pyrophosphorylase	Biosynthesis of Cofactors	0.0124	2.09
rmf	Ribosome Modulation Factor	Post-Translational Modification	0.0078	2.08
PA1429	Probable Cation-Transporting P-type ATPase	Membrane Protein	0.0016	2.08
pilH	Twitching Motility Protein	Motility & Attachment	0.0024	2.08
nadE	NH3-Dependent NAD Synthetase	Amino Acid Biosynthesis and Metabolism	0.0013	2.07
PA4523	Hypothetical Protein	Unknown	0.0037	2.06
chpA	Component of Chemotactic Signal Transduction System	Chemotaxis	0.009	2.06
gntR	Transcriptional Regulator	Transcriptional Regulators	0.0021	2.06
PA1244	Hypothetical Protein	Unknown	0.0308	2.05
PA5312	Probable Aldehyde Dehydrogenase	Putative Enzymes	0.0114	2.04
PA4578	Hypothetical Protein	Unknown	0.0044	2.04
PA3880	Conserved Hypothetical Protein [DGC domain]	Unknown	0.0013	2.03
PA5436	Probable Biotin Carboxylase	Central Intermediary Metabolism	0.0123	2.02
PA2140	Probable Metallothionein	Central Intermediary Metabolism	0.0008	2.02
gbdR	Bacterial Regulatory Proteins with ArgR regulatory Signature	Transcriptional Regulators	0.0007	2.02
flgM	Flagellar Assembly	Transcriptional Regulators	0.003	2.01
PA1333	Hypothetical Protein	Unknown	0.0434	2.01
htpG	Heat Shock Protein	Heat Shock Protein	0.0017	2.01
nirS	Nitrite Reductase Precursor	Energy Metabolism	0.0017	2
PA4819	Probable Glycosyl Transferase	Putative Enzymes	0.0089	2

a. PA gene number, known and predicted functions, and functional groupings were obtained from http://www.pseudomonas.com. Genes represented are those impacted exclusively by pre-conditioned PAO1.

b. Baseline reference was PAO1 grown in media only. Genes in the microarray analysis were considered differentially regulated if the relative change (*n*-fold) was ≥ 2.0. Statistical significance between groups was determined using unpaired Student *t*-Test with p-values of ≤ 0.05 was considered significant.

Table 5. Genes repressed by PAO1 during traversal of multilayered human corneal epithelial cells relative to those grown in media only.

PA No./Gene[a]	Known/Predicted Function	Functional Group	P-value	Fold-Change[b]
PA3399	Hypothetical Protein	Unknown	0.0054	3.02
PA5216	Probable Permease of ABC Iron Transporter	Membrane Protein	0.0002	2.85
PA0986	Conserved Hypothetical Protein [Transposase]	Transposon	0.0004	2.72
PA2599	Conserved Hypothetical Protein [SsuA Family]	Unknown	0.0016	2.44
PA1509	Hypothetical Protein	Unknown	0.0016	2.4
PA4718	Hypothetical Protein	Unknown	0.0009	2.39
PA3467	Probable Major Facilitator superfamily (MFS) Transporter	Membrane Protein	0.0004	2.36
PA2947	Hypothetical Protein	Unknown	0.0037	2.34
PA3513	Hypothetical Protein	Unknown	0.0173	2.21
nqrB	Na+ Translocating NADH	Energy Metabolism	0.0051	2.18
PA2602	Hypothetical Protein	Unknown	0.0007	2.1
leuC	3-Isopropylmalate Dehydratase Large Subunit	Amino Acid Biosynthesis	0.0011	2.09
PA5093	Probable Histidine/Phenylalanine Ammonia-Lyase	Putative Enzymes	0.0012	2.08
PA0483	Probable Acetyltransferase	Putative Enzymes	0.0007	2.08
PA3132	Probable Hydrolase	Putative Enzymes	0.0021	2.07
ygbP	4-Diphosphocytidyl-2-C-Methylerythritol Synthase	Biosynthesis of Cofactors	0.0014	2.06
PA1519	Probable Transporter	Membrane Protein	0.0011	2.05
PA1108	Probable major facilitator superfamily (MFS) transporter	Membrane Protein	0.0007	2.05
PA2840	Probable ATP-Dependent RNA Helicase	RNA Processing and Degradation	0.0013	2.05
PA1668	Hypothetical Protein	Unknown	0.0065	2.05
PA2671	Hypothetical Protein	Unknown	0.0014	2.04
glpT	Glycerol-3-Phosphate Transporter	Membrane Protein	0.0012	2.02
PA0663	Hypothetical Protein	Unknown	0.0048	2.02
PA3799	Conserved Hypothetical Protein [Ribosome-Associated GTPase]	Unknown	0.0031	2.02
PA4712	Hypothetical Protein	Unknown	0.0012	2
PA0864	Probable Transcriptional Regulator	Transcriptional Regulators	0.0021	2
PA4571	Probable Cytochrome C	Energy Metabolism	0.0049	2

a. PA gene number, known and predicted functions, and functional groupings were obtained from http://www.pseudomonas.com. Genes represented are those impacted exclusively by pre-conditioned PAO1.

b. Baseline reference was PAO1 grown in media only. Genes in the microarray analysis were considered differentially regulated if the relative change (*n*-fold) was ≥ 2.0. Statistical significance between groups was determined using unpaired Student *t*-Test with p-values of ≤ 0.05 was considered significant.

3.4.3. Functional analysis of bacterial genes in *P. aeruginosa* differentially expressed after traversal of multilayered human corneal epithelia.

Transposon mutants, in a *P. aeruginosa* PAO1 background, were screened for traversal capacity (8 h post-inoculation with ~10^6 cfu bacteria) to determine which differentially expressed genes modulate traversal of multilayered human corneal epithelial cells (Figure 13). Mutants in ~ 90 genes were chosen based on transcript profile from microarray analysis (how to explain some weren't in list). The criteria for those chosen were based on the maximum fold-change differences (upregulated or downregulated), selected from Tables 1-5. Also, functional group characteristics, as well as availability (89) determined which genes were screened. Traversal capacity was normalized to the values of wild-type PAO1 parental strain (dashed line indicated fold-change differences). Of the mutants tested, the majority showed similar traversal efficiency to wild-type PAO1, however 21 of the screened mutants showed loss of traversal function including one known to be involved in virulence factor regulation (i.e. *vfr*). To eliminate auxotroph, growth curves were done in minimal liquid media to measure growth rate (data not shown). Also of interest, 10 mutants showed enhanced traversal capacity to penetrate cells, including 1 known to negatively regulate exotoxin A (i.e. *ptxS*).

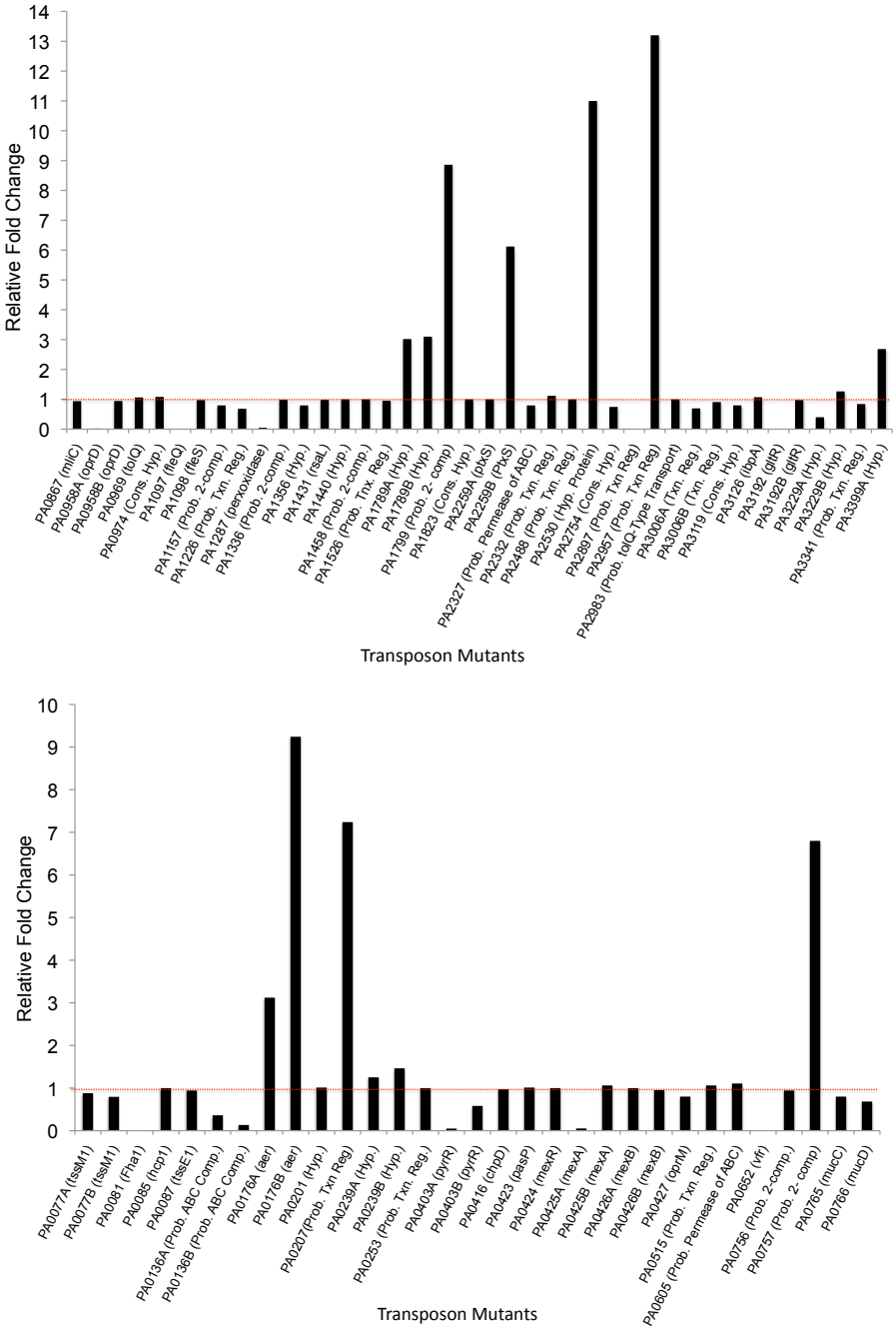


Figure 13. Transposon mutants in *P. aeruginosa* invasive strain PAO1 background screened for traversal of multilayered corneal epithelia cells *in vitro*. Bacterial strains (~10^6 cfu) were added to the apical compartment and viable counts taken from basal compartment (i.e. traversed bacteria) 8 h post- inoculation. Mutants were normalized to the values of wild-type PAO1 parental stain. Dashed lines indicate similar efficiency as wild-type parent. Experimental data represents one experiment done in duplicates wells. Gene names are indicated in parenthesis. Hyp, hypothetical protein; Con. Hyp, conserved hypothetical protein; Prob. Txn. Reg., probable transcriptional regulatory; Prob. 2-comp., probable two-component regulatory system.

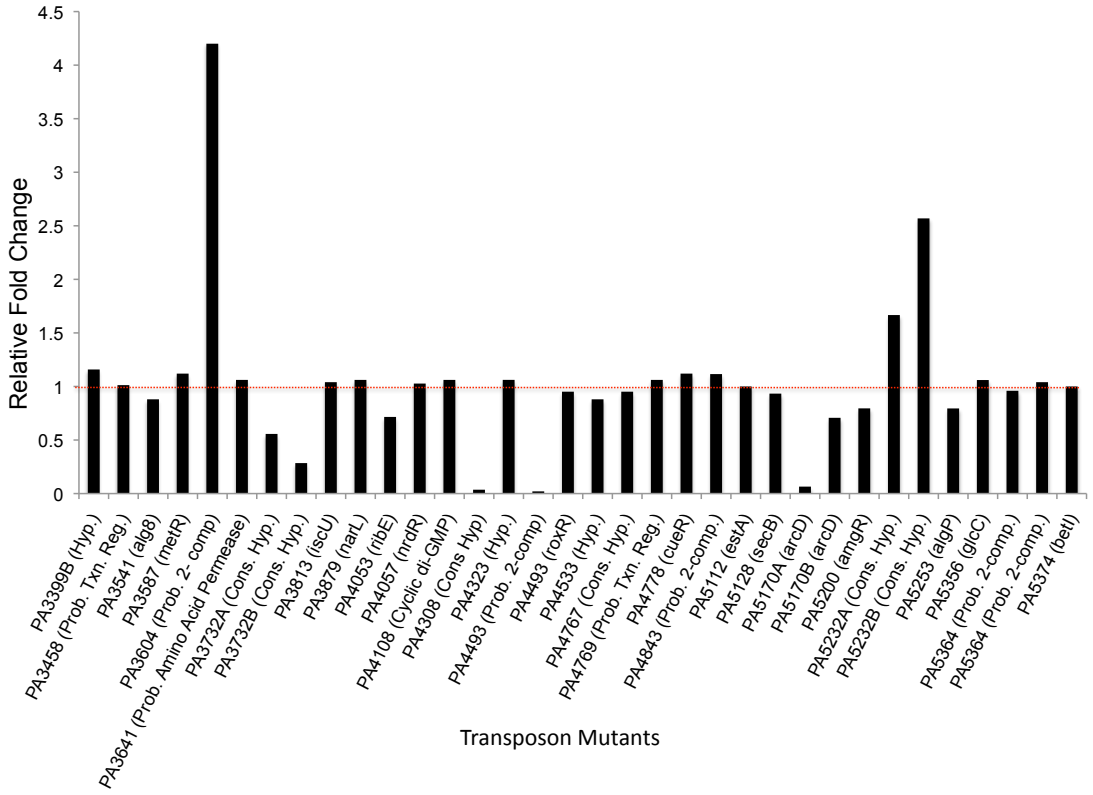


Figure 13 continued. Transposon mutants in *P. aeruginosa* invasive strain PAO1 background screened for traversal of multilayered corneal epithelia cells *in vitro*. Bacterial strains (~10^6 cfu) were added to the apical compartment and viable counts taken from basal compartment (i.e. traversed bacteria) 8 h post- inoculation. Mutants were normalized to the values of wild-type PAO1 parental stain. Dashed lines indicate similar efficiency as wild-type parent. Experimental data represents one experiment done in duplicates wells. Gene names are indicated in parenthesis. Hyp, hypothetical protein; Con. Hyp, conserved hypothetical protein; Prob. Txn. Reg., probable transcriptional regulatory; Prob. 2-comp., probable two-component regulatory system.

3.5. DISCUSSION.

The data presented in this report demonstrated an enhanced traversal capacity by bacteria following subsequent traversal using our *in vitro* traversal assay, suggesting that *P. aeruginosa* can adapt to the environment of multilayered epithelium. We believe that the adaptability, ubiquitousness, and pathogenicity of *P. aeruginosa* are closely related. The significance of "sheet" formation (Figure 12B) induced by traversal experience is not clear, but the appearance resembles biofilm formation, known to assist bacteria in survival, adaptation and virulence (67, 186). Successful adaptation of any bacterial organism depends on its ability to sense changes in their environment and respond to these challenges (i.e. host defense factors). *P. aeruginosa* genome contains numerous sensor regulator systems, which are able to facilitate rapid response to environmental conditions (176), many of which are controlled by membrane-bound two-component response regulators. Two-component systems have been shown to be responsible for reciprocal regulation of virulence determinants specific to chronic (i.e. biofilm-associated) or acute (i.e. planktonic-associated) infection by *P. aeruginosa* (13, 61, 184, 199, 203), suggesting that these regulators modulate virulence gene expression in response to changes mode of lifestyles (60, 61, 116, 186, 194, 201, 203).

Since a change in traversal efficiency was observed with subsequent exposure of multilayered corneal epithelial cells by PAO1, we examined how traversal could impact gene expression. Previous studies investigating mechanism of *P. aeruginosa* traversal have mostly been done using epithelial cell monolayers grown on permeable filters, e.g. MDCK cells (14, 15, 75). Our data suggest that the traversal process itself contributes to a complex set of events when *P. aeruginosa* attempt to cross the epithelial cell multilayers that ultimately determine the outcome. Control experiments done using the *in vitro* traversal model demonstrated that exposure to epithelial cell alone does not contribute to the phenotype observed by PAO1 subsequent traversal (see supplemental data Figure S1).

Genes impacted by the traversal process as oppose to cell contact, i.e. the pre-conditioned PAO1 compared to naïve PAO1, identified a large number of functional groups (~ 55 %) encoding hypothetical proteins (Table 1). Of interest, were results showing that this process impacted RsaL expression. The RsaL protein is known to repress transcription of the *lasI* gene, encoding the 3-oxo-C_{12}-HSL quorum sensing molecule (159). Recent studies by Rampioni *et al*., (160), showed that *P. aeruginosa* strains deficient in RsaL hypersecretor of various virulence factors (i.e. pyocyanin, lactase, hemolysins) and in addition, mutants in *rsaL* exhibited an inability to form biofilms. In addition, rhamnolipids (*rsaIAB* operon is invovled in biosythesis) can disrupt tight-junctions of multilayered human airway epithelia to allow paracellular bacterial transport (205). Taken together, it is possible that during the traversal process, *P. aeruginosa* cells expressing high levels of RsaL can better adapt to the hostile epithelium environment (i.e. secretion of antimicrobial factors) with greater efficiency by orchestrating a rapid transition between virulence traits to promote survival and dissemination.

In investigating genes that were impacted by the traversal process as oppose to cell exposure we looked at genes that were exclusively found expressed (based on the normalized probsets list generated) in either naïve or pre-conditioned PAO1 (Table 2-5).

While our microarray data showed previously implicated genes involved in traversal of other epithelia, e.g. *pilU* (3), our results showed novel roles for previous identified virulence genes, e.g. *aer*, a aerotaxis receptor (Table 1), as well as a large number of genes encoding hypothetical proteins. When analyzed, upregulated genes found exclusively by PAO1 naïve to traversal (based on full gene list following the pre-screened procedure) encoded proteins in various functional groups (Table 2-3), with a good portion of these involved membrane-bound transporters that confer resistance to natural substances produced by the host activity and antibiotic resistance. These included: ATP-binding cassette components, e.g. PA0136, multidrug efflux pumps, e.g. OprJ, probable major facilitator superfamily (MFS), e.g. PA4187, and β-lactamase, e.g. AmpR-C. While it is well known that *P. aeruginosa* is difficult to eradicate once infection is established due to its intrinsic resistance to a variety of antimicrobials by use of β-lactamase and efflux pumps (153, 177), our data showed that previously implicated genes involved in traversal of epithelial cell monolayers, e.g. MexAB-OprM drug efflux pump (76) did not impact expression during cell exposure nor traversal. However, this does not preclude the involvement of MexAB-OprM in the traversal process (as demonstrated in Figure 13) but suggest other key players may contribute to towards resistance and modulate traversal of human multilayered epithelia. Also impacted by traversal of PAO1, alginate regulatory system, e.g. *algPQ*. It is generally accepted that chronic CF lung infection is established upon conversion of *P. aeruginosa* from the non-mucoid to the mucoid phenotype (i.e. biofilm formation). The occurrence of mucoid *P. aeruginosa* in the lungs is associated with poor prognosis, deterioration of lung function, and increased tissue damage (186). Thus, alginate is one of the most significant virulence determinants in the context of chronic CF airway disease (77). This reinforces that biofilm plays a major role in establishing a suitable niche for *P. aeruginosa* adaptation of epithelium defense factors to contribute to early stages of pathogenesis.

In summary, *P. aeruginosa* posses several virulence traits that contribute to its pathogenesis. These include resistance to antimicrobial agents, ability to form biofilms, and metabolic versatility. It is likely that *P. aerugnosa* traversal *of* multilayered corneal (or other) epithelia induces multiple phenotypes and by passaging bacteria that have already traversed we enriched for those best suited to the process. Our microarray data reveled many genes involved in traversal of *P. aeruginosa*, many of which encoded hypothetical proteins and previous virulence factors novel to the process. Of course, further studies will be needed to elucidate the function and mechanisms of these newly identified genes and their involvement in epithelial traversal.

CHAPTER 4.

A Mutation in *Pseudomonas aeurginosa* PA4308 Gene Leads to Increase Cytotoxicity and Traversal of Human Corneal Epithelia by Effecting ExoS Secretion.

4.1. ABSTRACT.

Pseudomonas aeruginosa is one of the major causative agents of mortality and morbidity in hospitalized patients due to a multiplicity of virulence factors associated with both chronic and acute infections. The type III secreted system (T3SS) of *P. aeruginosa* is an important virulence mechanism associated with clinical outcome. T3SS mediates the direct injection of effector proteins directly into the host cytosol. One of the most commonly expressed effectors, ExoS, interferes with cytoskeleton integrity ultimately causing cell death. The principle structure components of the T3SS and regulation have been investigated in detail. However, here we report the identification of a novel gene in *P. aeruginosa* PAO1 strain encoding a hypothetical protein, PA4308, which mutation in the gene leads to increase cytotoxicity (~ 19.5% compared to PAO1; $p < 0.0001$) of human corneal epithelia due to early secretion of ExoS. Accordingly, PA4308 mutation demonstrated an increase in traversal capacity of multilayered human epithelia *in vitro* by 2-fold ($p < 0.01$). Enhance susceptibility of corneal epithelia was also monitored by intracellular viability (~2-fold; $p < 0.01$). Domain analysis reveled that PA4308 belongs to members of the P-loop NTPase domain superfamily responsible for diverse cellular activities.

4.2. INTRODUCTION.

Pseudomonas aeruginosa is an opportunistic pathogen responsible for 10 – 15 % of nosocomial infections worldwide (24). These infections include: ventilator-associated pneumonia, contact lens related keratitis, and acute infections in burn wounds (29, 47, 164). Interactions between *P. aeruginosa* and epithelial cells are thought to be pivotal in the development of most infections. Like many other pathogens, *P. aeruginosa* can exploit and manipulate host cells due to its arsenal of virulence factors it possess to establish infections (176), one of which, is the type III secretion system (T3SS). This secretion system mediates the direct injection of cytotoxic proteins, termed effectors, directly into the host cytosol (201).

In *P. aeruginosa*, thirty-six genes within five distinct operons are located within a pathogenecity island are involved in the biogenesis and the translocation machinery (70); the genes encoding the effectors are scattered elsewhere in the chromosome. The transcriptional regulator ExsA mediates expression of the T3SS machinery and its effectors (117). To date there are four known effector proteins described in *P. aeruginosa*, exoenzyme S (ExoS), exoenzyme T (ExoT), exoenzyme U (ExoU), and exoenzyme Y (ExoY). ExoS and ExoT are bifunctional enzymes that include a GTPase-activating (GAP) function within the N-terminal domain and an ADP-ribosyltransferase activity within the C-terminal domain (19). They confer anti-phagocytotic capacities to *P. aeruginosa* mainly through their action on the actin cytoskeleton. ExoY is an adenylate cyclase that leads to cyclic AMP (cAMP) accumulation in the host cell (200), which has profound effects on the cell morphology (2). ExoU is a potent phospholipase A_2 activity responsible for acute cytotoxicity and lung tissue damages (167). Effectors are actively translocated into the target host by the use of PopB, PopD and PcrV. PopB and PopD are secreted by the “needle-like” complex and forms pores in the plasma membrane of

the target host (70). PcrV has been shown to be an integral component of the translocation apparatus of the T3SS, mediating the delivery of effector proteins into target host cells (137). Isogenic mutants of *P. aeruginosa* lacking the genes for *pcrV* or *popD* are unable to damage eukaryotic cells (9). In addition, active and passive immunization against PcrV improves acute lung injury and mortality of mice infected with cytotoxic *P. aeruginosa* (43).

Here we report the identification of a novel gene in *P. aeruginosa* PAO1 strain encoding a hypothetical protein, PA4308, which mutation in this gene leads to increase cytotoxicity of human corneal epithelia by modulating secretion of ExoS. Domain analysis reveled that PA4308 belongs to members of the P-loop NTPase domain superfamily, included are ATPases Associated Activities (AAA+), which are associated with diverse cellular functions (87, 92, 168). Members of the P-loop NTPases domain superfamily are an abundant class of proteins characterized by a conserved nucleotide-binding phosphate (NTP; typically ATP or GTP) motif. P-loop NTPases generally rely on Walker A and Walker B motifs to bind the β - and - γ phosphates of NTP and Mg^{2+} cation, respectively. Most P-loop proteins cleave the β - γ phosphate bond to provide energy to induce conformational changes in other molecules. These domains are implicated in nearly all biochemical and mechanical processes, including translation, trancscription, replication and repair, intracellular trafficking, and membrane transport (112).

4.3. MATERIAL AND METHODS.

4.3.1. Bacterial Strains and Growth Conditions. *Pseudomonas aeruginosa* strains used in this study were as followed: PAO1 parental wild-type (obtained from the Mutant Library at the University of Washington Genome Center) and PA4308 (chromosomal gene deletion in sequence position 4833374-4834864 in a PAO1 background). Bacterial strains were grown on trypticase soy agar (TSA; BD Biosciences, CA) at 37°C for ~16 h then resuspended in KGM-2 (unless otherwise stated) without antibiotics at a concentration of ~10^8 cfu/ml [OD at 650 nm of ~ 0.1]. Inocula were then prepared by diluting this suspension in KGM-2 to a final concentration of ~10^6 cfu/ml for use in most experiments. Cytotoxicity experiments involved a higher bacterial inoculum of ~10^7 cfu/ml. Viable counts were used to confirm inoculum size for each experiment. For analysis of Type III secretion and production, overnight cultures were grown on TSA plates as described above prior to being diluted in trypitcase soy broth (TSB; BD Biosciences, CA) to a final concentration of OD at 650 nm of ~ 0.1 and used to inoculate in fresh medium grown under inducing or non-inducing conditions (13). Cultures were then incubated at 37°C with vigorous shaking at 250 rpm until they reached early-log [OD at 600 nm of ~ 0.3] or late-log / early stationary growth phase [OD at 600 nm ~ 1.6]. Cultures were then harvested by centrifugation and supernatants concentrated using Amicon Ultra Centricon tubes (Millipore, MA).

4.3.2. Chromosomal Gene Deletion of PA4308 in *Pseudomonas aeruginosa* PAO1. A non-polar chromosomal gene deletion in *P. aeruginosa* PAO1 was constructed using primers that directly joined the flanking region of the target gene (see supplemental

data; Table S1). Regions flanking *PA4308* gene were PCR amplified and ligated into pEXG2 (a suicide vector derivative of pEX18Gm) according standard molecular cloning techniques (28). The resultant plasmid was introduced into *P. aeruginosa* PAO1 by electroporation and merodiploid transformants were identified by their resistance to both carbenicillin and gentamicin. To force resolution, resistant colonies were patched onto TSA supplemented with 5 % sucrose; gentamicin sensitivity and sucrose resistance identified mutant clones. Deletion of *PA4308* was confirmed using PCR with PA4308 upstream*5*' and PA4308 downstream*3'* primers.

4.3.3. Cell Culture. Telemerase-immortalized human corneal epithelial cells were maintained as previously described in section 2.3.2 unless otherwise stated.

4.3.4. *In vitro* Traversal Assay. Traversal assay was performed as previously described in section 2.3.3.

4.3.5. Motility Assays. *Swimming*. Media used for this assay contained M9 minimal broth medium supplemented with 0.3% (w/v) granulated agar (Difco, TX). Swim plates were inoculated with bacteria from an overnight culture in LB agar (1.5% w⁄v) plates at 37°C with a sharp sterile toothpick. The plates were then wrapped with parafilm to prevent dehydration and incubated at 37°C for 24 h. ***Twitching***. Media for this assay was made using LB broth [10 g/L tryptone, 5 g/L yeast extract 10 g/L NaCl; Difco, TX] solidified with 1% (w/v) granulated agar. Plates were then briefly dried and bacterial strains were stab inoculated with a sharp sterile toothpick to the bottom of the Petri dish from an overnight culture grown on LB agar (1.5%, w/v) plate. After incubation at 37°C for 24 h, the zone of motility was measured.

4.3.6. Intracellular Survival/Replication Assays. Internalization and survival of *P. aeruginosa* was quantified using gentamicin survival assays with telomerase-immortalized human corneal epithelial cells (HCE) as previously described (11). Briefly, HCE were cultured onto 24-well tissue culture plates by seeding 5.0 x 10^4 HCE cells/ml until cells reached 90% confluence in KGM-2 without antibiotic. Bacterial inocula (~10^6 cfu/ml) were incubated with the cells [5% CO_2 at 37°C] for up to 8 h. Following 3 h incubation, wells were washed twice with PBS and treated with KGM-2 media supplemented with gentamicin (200 μg/ml, Lonza, MD) for 1 h to kill extracellular bacteria or an additional 5 h to determine intracellular survival. Cells were then washed once with PBS, treated with 0.25% (v/v) non-ionic surfactant (Triton X-100; Sigma, MO) in PBS for 15 min incubated in 5% CO_2 at 37°C to further disrupt eukaryotic membranes. Viable counts were performed on the cell lysate using trypticase soy agar. Control experiments (data not shown) confirmed no significant differences in the growth rates of wild-type and mutant stains within cell culture media over an 8 h time period and susceptible to killing by gentamicin.

4.3.7. Cytotoxicity Assay. Human corneal epithelial cells were cultured as previously described above and seeded into a 96-well flat bottom tissue culture plates at a density of 2.5 X 10^5 cells per well and incubated [5% CO_2 at 37 °C] for 18 h until cells reached 100% confluence. The cells were then rinsed twice with PBS, and inoculated with *P.*

aeruginosa strains resuspended in KGM-2 media (without phenol red or antibiotics; PromoCell, Germany) at a concentration of ~ 10^7 cfu/ml to each well. Uninfected HCE were sham-inoculated as negative controls. At 8 h post-inoculation, 100 μl of each supernatant was removed and placed in a new 96-well flat bottom tissue culture plate and centrifuged at low speed (3,000 rpm), to pellet any cells carried over. Lactate dehydrogenase activity of 50 μl of the resulting supernatant was assayed using the CytoTox96 Non-Radioactive Cytotoxicity Assay Kit according to manufacture's instructions (Promega, CA).

4.3.8. SDS-PAGE and Western Immunoblot. Immunblot analysis against ExoS was performed as previously decribed (13).

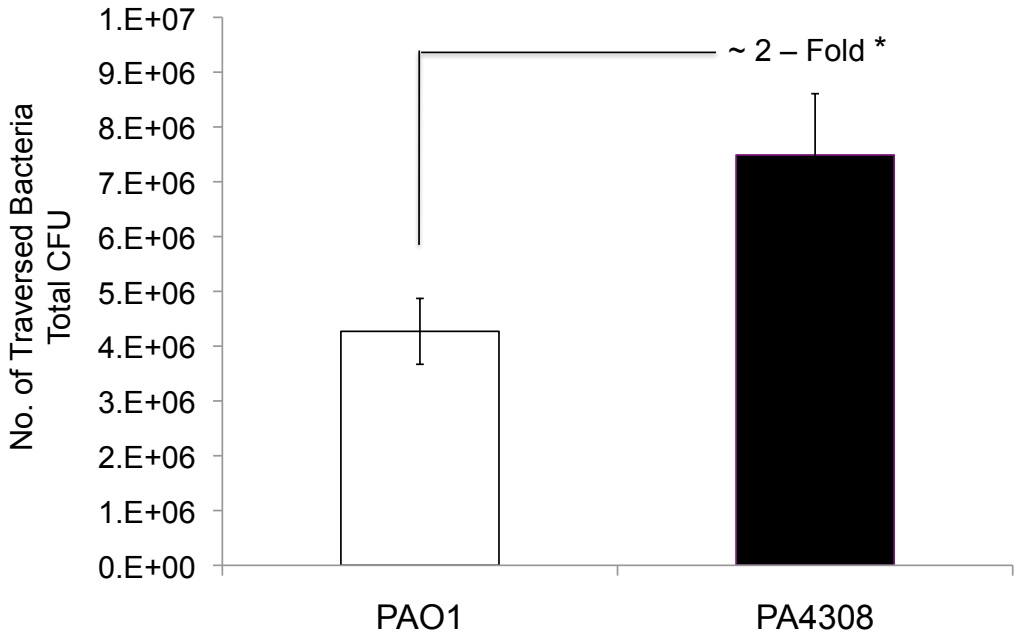
4.3.9. Statistical Analysis. Data were expressed as a mean ± standard deviation (SD) for all experiements. Statistical significance between two groups was determined using unpaired Student's *t*-Test. P values of of ≤ 0.05 was considered significant.

4.4. RESULTS.

4.4.1. A novel gene in *P. aeruginosa* demonstrates a role in traversal of human multilayered corneal epithelia.

We had previously screened a set of isogenic transposon mutants (Figure 13) in *P. aeruginosa* PAO1 to test which differentially expressed genes contributes to traversal of multilayered human corneal epithela; as a first step towards determining bacterial virulence traits impacted during early development of *P. aeruginosa* keratitis (or any other epithelial derived infections). Of the genes investigated, a mutation in a gene encoding a hypothetical protein, *PA4308TM* (TM denotes transpson mutant) in *P. aeruginosa*, reduced susceptibility to traversal by ~ 2-Fold at 8 h post-inoculation (see supplemental data; Figure S2; $p < 0.01$). Domain analysis using pFAM revealed that PA4308 belongs to members of the P-loop NTPase domain superfamily, which members include ATPases Associated Activities (AAA+), which are associated with diverse cellular functions (87, 92, 168). Construction of a non-polar deletion in this gene however produced a differenent phenotype from *PA4308TM* (possibly due to frame-shift events), however we continued with further analysis. All subsequent experiements were performed with the non-polar mutant, *P. aeruginosa* PAO1 isogenic mutant strain, PA4308. This mutant strain was ~ 2-Fold more efficient at traversal of multilayered corneal epithelia *in vitro* in comparision to PAO1 parental strain at 8 h post-inoculation (Figure 14A; $p < 0.01$). Growth curves confirmed that the imapct on traversal capacity by isogenic mutant strain *PA4308* was not due to an increase in growth rate (data not shown). Swimming and twitching motility assays were done to determine if the increased traversal by the *PA4308* mutant was due to effects on flagella or type IV pili (Figure 14B). After 24 h incubation at 37°C, the twitching zone (% WT ± SEM) for PAO1 was in 100 ± 5 compared to 97 ± 2, $p = 0.6129$. In experiments measuring swimming motility, PAO1 diffusion zone was 100 ± 3 and PA4308 was 94 ± 5, $p = 0.5772$.

A.



B.

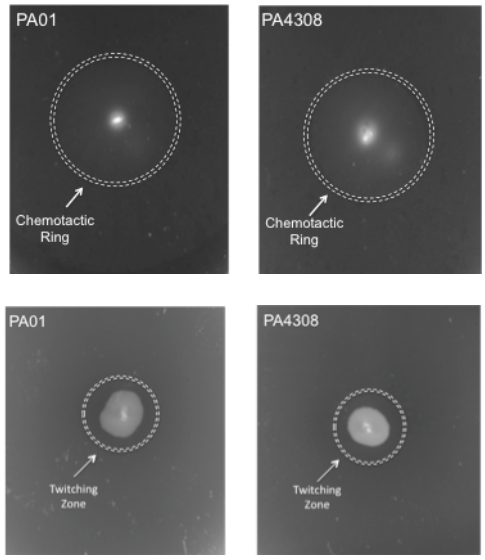


Figure 14. *P. aeruginosa* PAO1 and isogenic *PA4308* mutant traversal of multilayered human corneal epithelia cells *in vitro* (A). PAO1 or isogenic mutant strain PA4308 (~10^6 cfu), were added to the apical compartment and viable counts taken from basal compartment (i.e. traversed bacteria) 8 h post- inoculation. * $p < 0.01$, student *t*-Test. One of two independent experiements is shown. Swimming and twitching motility phenotypes (B) were acessed to determine motility efficiency of PAO1 or PA4308. Bacteria from overnight culture were inoculated by toothpick onto M9 media supplemented with 0.3% (swimming motility) or 1.5% (twitching motility) agar. The denser and smaller zone represents surface colony growth while dashed circle represents diffusion zone.

4.4.2. Mutation of *P. aeruginosa* PA4308 results in decreased intracellular viability within corneal epithelial cells.

Here we assessed the role *PA4308* on intracellular survival and replication of *P. aeruginosa* within the corneal epithelia (grown as monloayers), using gentimicin survival assay as described previously (11). As expected, *P. aeruginosa* PAO1 wild-type strain were able to survive and replicate within corneal epithelail cells after initial internalization. In contrast, *PA4308* mutant strains showed reduced internalization (~ 2-Fold at 8 h; $p < 0.01$) despite having similar viable counts to PAO1 at the ealier time-point (Figure 15). One possible explaination why *PA4308* mutants were found to have lower viablity counts at the later time-point, is that they were able to damage the corneal epithelia thereby exposing internalized bacteria to gentimicin treatment. Another, is that *PA4308* mutation effects the type III secretion system (T3SS) of *P. aeruginosa*. We have previoulsy shown that PAO1 deficient in *exsA*, a positive transcriptional regulator for T3SS, resulted in alteration in intracellular viability and trafficking (11).

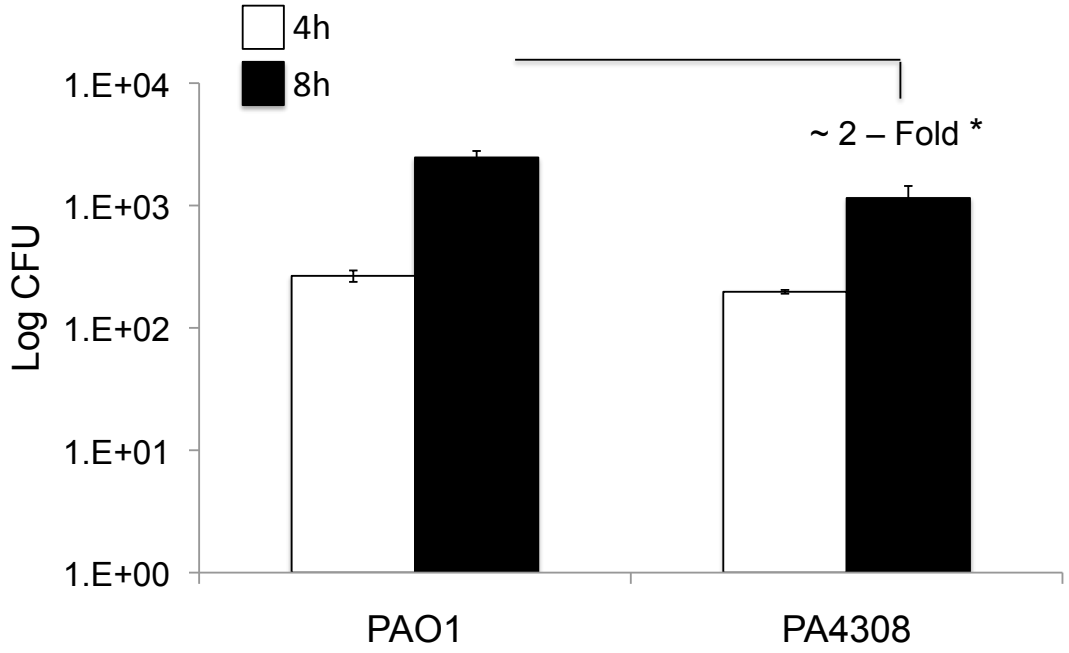


Figure 15. Intracellular survival and replication of *P. aeruginosa* PAO1 and isogenic PA4308 mutant within human corneal epithelial cells grown as monolayers. PAO1 or PA4308 (~10^6 cfu) were added to cell culture and following 3 h incubation, cells were treated with media supplemented with gentamicin (200 μg/ml) for 1 h (to kill extracellular bacteria) or an additional 5 h (to determine intracellular survival) represented by 4 h (white bars) and 8 h (black bars) time points, respectively. * $p < 0.01$, student *t*-Test. One of three independent experiments is shown.

4.4.2. Mutation of *P. aeruginosa* PA4308 leads to increased cytotoxicity to human corneal epithelia due to early secreation of ExoS.

Since T3SS is known to impact the intracellular viability of *P. aeruginosa*, and because *PA4308* mutation increased traversal of multilayered corneal epithelia, we looked at whether *P. aeruginosa PA4308* mutation showed defects in type III effector protein production and secretion (Figure 16). Culture supernatants of PAO1 or PA4308 isogenic mutant strains were grown in the presence or absence of T3SS-inducing medium (13) and harvested during early-log (Figure 16A) or late-log / early stationary (Figure 16B) growth phase. Analysis of supernatant derived from isogenic mutant *PA4308* reveled an abundance of secreted effector toxin exoenzyme S (ExoS) in comparision to PAO1 when grown under inducing conditions during early-log growth phase (Figure 16A). The additional band seen (~ 26 kDa ladder) does not correlate to any known T3SS effector proteins, however it may be one of the translocon PcrV or a T3SS chaperone. Further analysis is needed to determine band identity. Conversely, supernatant harvested at late-log / early stationary from both PAO1 and *PA4308* mutant strain exhibited similar ExoS secretion profile (Figure 16B). The identity of ExoS was confirmed by immunoblot analysis using antibodies against ExoS (lower panel Figure 16B). To determine if the difference in ExoS secretion observed for isogenic mutant *PA4308* compared to PAO1 correlated with an increase in cell death, cytotoxicity was determined using corneal epithelial cells as described in Material and Methods. Consistent with the protein profile analysis (Figure 16), *PA4308* mutants exhibited an increase in cytotoxicity (~ 2.5-Fold; equaling to a 19.5 % increase) compared to PAO1 (Figure 17; $p < 0.0001$). Thus, the observation of reduced intracellular survival seen previoulsy were do to the effects of ExoS damaging the epithelia thereby exposing bacteria to gentamicin.

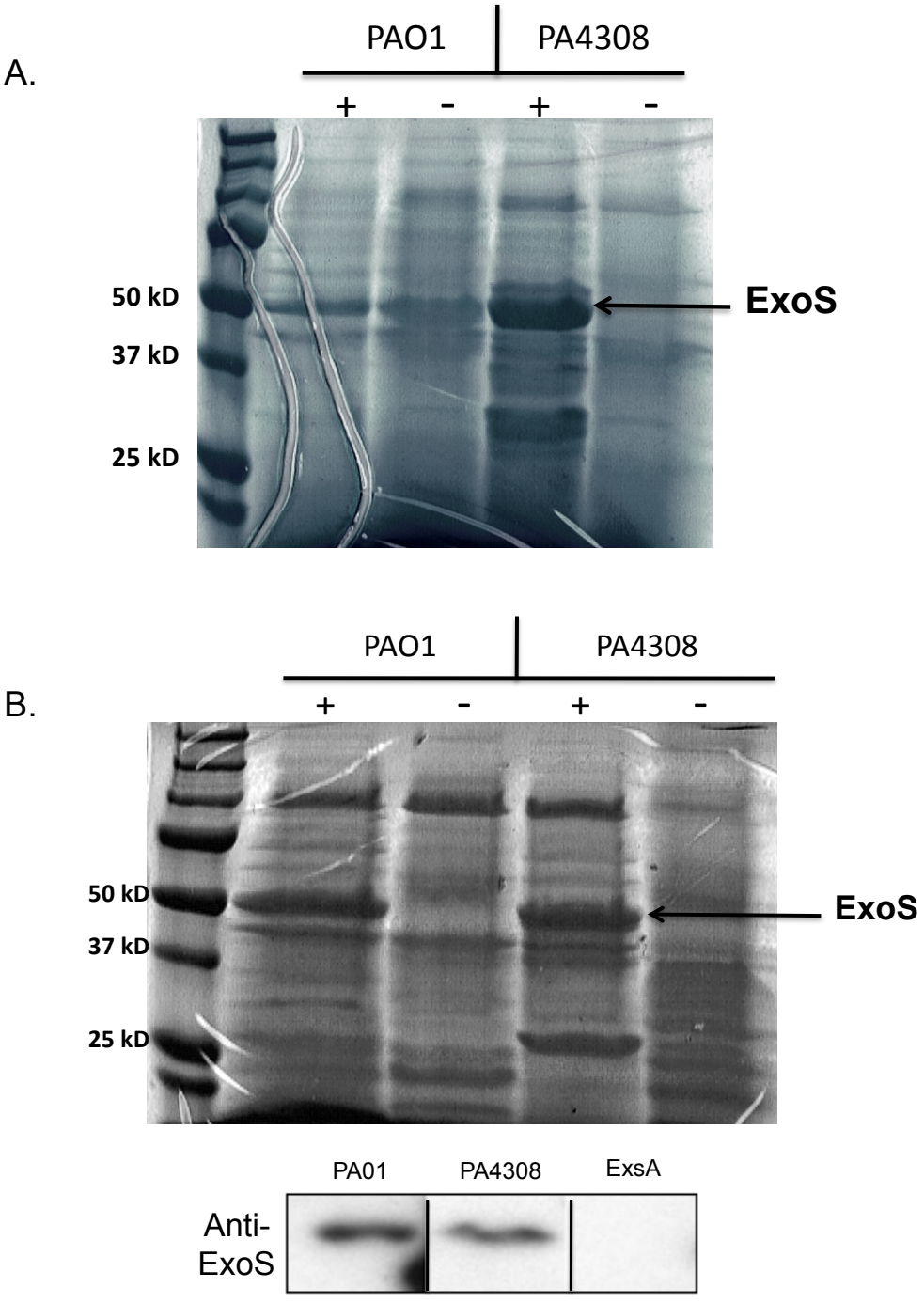


Figure 16. Effects of *P. aeruginosa* PA4308 gene mutation on type III secreted proteins. Culture supernatants of PAO1 or PA4308 were grown in the presence (+) or absence (-) of TTSS-inducing (calcium-deplete) medium. Cultures were harvested during early-log (A) or late-log / early stationary (B) growth phase followed by staining with Coomassie blue. Immunoblot analysis on was performed on culture supernatants of PAO1 and PA4308 to confirm the identity of ExoS (bottom panel Figure B).

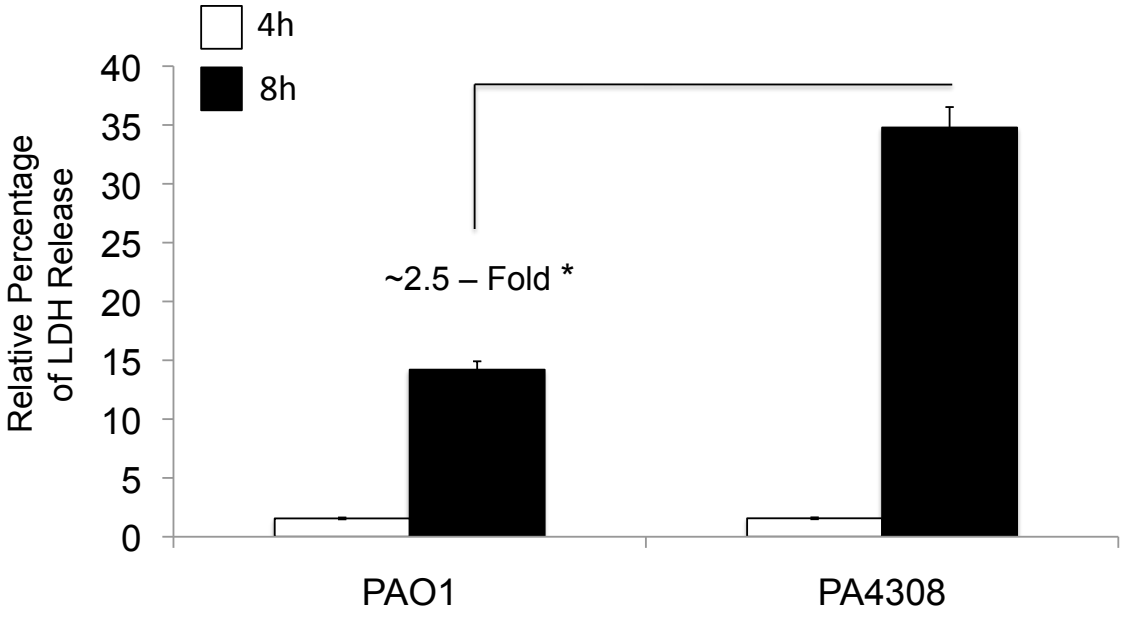


Figure 17. Human corneal epithelial cells cytotoxicity in response to *P. aeruginosa* PAO1 and isogenic PA4308 mutant. PAO1 or PA4308 (~ 10^7 cfu) were added to cell culture and following 4 h (white bars) or 8 h (black bars) post-inoculation, cell culture was measured for release of latcate dehydrogenase (LDH). Cytotoxicity is expressed as a percentage of the toatal amount of LDH relase from epithelial cells treated with 1 % triton X-100. * $p < 0.001$, student *t*-Test. One of three independent experiments is shown.

4.5. DISCUSSION.

The opportunistic pathogen *P. aeruginosa* cause a diverse range of disease ranging from superficial acute skin infections to chronic lung colonization of patients with cystic fibrosis (CF). Early stages of infection and colonization require the production of a range of virulence factors, including T3S effectors, which function to aid the bacteria in invasion and traversal of host epithelia. In contrast, a transition to chronic infection requires repressing some of these same virulence factors, adopting a biofilm-associated life-style, due to the fact they are no longer in close proximity to host cells. The results of this study show a novel gene in *P. aeruginosa* PAO1 strain, *PA4308*, functions in preventing premature secretion of type III secretion effector protein, ExoS. This suggests that PA4308 may function as a negative regulator for the T3SS. Cell contact-dependent delivery of effector proteins is a core feature of type III secretion system machinery. How this process is regulated is key in the pathogenesis of *P. aeruginosa* during different stages of infectivity (13, 66).

Type III secretion in *P. aeruginosa* is regulated at two levels: direct transcription of T3SS and initiation of secretion by other virulence systems. Both are equally important in allowing T3S components to be produced at high levels when they are most needed, during cell host contact. In most cases the T3SS complex is initiated by the regulation of ExsA, the positive regulator of T3SS (117). ExsA induces the T3SS genes including it's own promoter in a 'switching-mechanism" involving ExsC, ExsD, and ExsE. Under inducing conditions (i.e. low calcium conditions or cell contact), ExsA is associated with ExsD (anti-activator), which inhibits ExsA function. ExsC (anti-anti-activator) can inhibit the activity of ExsD by distrupting the interaction between ExsA-ExsD; thereby allowing ExsA to liberated and activates T3SS gene expression. During non-inducing conditions (i.e. high calcium), ExsC would be bound to ExsE (due to the high-affinity interaction) preventing the binding to ExsD and activating ExsA. Under inducing conditions, ExsE would normally be depleted (161). ExsA-dependent T3SS transcription is also modulated by two global two-component regulatory systems, which both are controlled by environmental stress, the intracellular cyclic AMP (cAMP) system and the Gac regulatory system. The cAMP pathway influences T3SS gene expression by acting on *vfr* regulation (201). In the Gac system, response sensor proteins RetS and LadS mediate reciprocal regulation of the type III secretion system during different phases of infectivity (152). The exact details of how PA4308 is acting on ExoS regulation remains to be elucidated. However, it is possible that PA4308 is acting at the transcriptional level within this hierarchy of regulation. It is also possible that PA4308 modulates the switch between ExsE-C thereby releasing ExsA. Other possibilities include actions downstream of RetS or LadS regulatory sensors; the fact that secretion of ExoS occurred during different growth phase suggests a role in environmental cue modulating PA4308 activity. Of course further experiments are needed to determine the regulation of this gene during different environmental stress and how this may correlate to T3SS.

While the T3SS regulation is well documented, it is however still unclear how secretion is prevented until contact with a host cell membrane. A key question unanswered is how effector secretion is prevented prior to cell contact, or how cell contact results in differential secretion of these translocators and effector protein is still

unclear. Previous studies investigating mechanisms of *P. aeruginosa* T3SS effector secretion regulation, have proposed several models to explain how the effector secretion is regulated (111, 137), many of which involve the needle tip complex translocon proteins, PopB, PopD and PcrV. Among the translocons studied, PcrV has been shown to be an integral component of the translocation apparatus of the T3SS, mediating the delivery of effector proteins into target host cells. Mutation in this protein has resulted in consititutive expression of the type III effector protein ExoS (161). In addition, recent work has shown that PcrV-PcrG is able to controls effector secretion by influencing the conformation of the needle apparatus (111), by shifting the effector secretion from an "off" conformation to an "on" and vice versa. It is possible that *P. aeurginosa* PA4308 acts downstream and aids PcrV-PcrG in this conformational change of the needle, mediate through the P-loop ATPase activity. Members of the P-loop NTPases domain superfamily are an abundant class of proteins characterized by a conserved nucleotide-binding phosphate (NTP; typically ATP or GTP) motif. P-loop NTPases generally rely on Walker A and Walker B motifs to bind the β - and - γ phosphates of NTP and Mg^{2+} cation, respectively. Most P-loop proteins cleave the β - γ phosphate bond to provide energy to induce conformational changes in other molecules. Supporting this hypotheisis is work done on other systems, HrpR-S ATPases were shown to trigger the type III secretion system in *Pseudomonas syringae* by regulating HrpV-HrpS operon, which functions are inhibiting and regulating components in the secretion system (92). Other work done in *Shigella flexneri* showed that Spa47 ATPase is required for transit of the needle components by initiating a conformational change to the needle complex (124). The data presented in this study imply a possible role for ATPase PA4308 in regulating the differential expression of ExoS. However much work is needed in dissecting the mechanistic role on how PA4308 modulates the T3SS in *P. aeruginosa*.

Analysis of supernatant derived from isogenic mutant *PA4308* reveled an abundance of secreted effector toxin ExoS in comparision to PAO1 during planktonic-associated growth (i.e. early-log growth phase). *P. aeruginosa* is known to elicit both acute (i.e. planktonic-associated) and chronic (i.e. biofilm-associated) infections, with reciprocal regulation to genes specific to each. The severity of these infections can be attributed to this inverse relationship, with T3SS regulation being implicated. It maybe that PA4308 may act upon certain environmental cues that cause this transition from chronic to acute infection by *P. aeruginosa*. Many *P. aeruginosa* isolates from the airways of older CF patients, carry *mucA* mutations and overproduce alginate, resulting in a mucoid phenotype (123). A study done by Wu *et al*. recently found that expression of the T3SS genes was repressed in a *mucA* mutant, suggesting that emergence of the mucoid phenotype and loss of T3SS expression coincides with the acquisition of *mucA* mutations (198). The mechanism linking expression of the T3SS genes and alginate production, however, remains unclear. Other studies have shown the role of LadS in promoting biofilm formation, a *ladS* mutant correlated with increased cytotoxicity relative to wild type by overexpessing ExoT (184). In contrast, RetS primes *P. aeruginosa* for acute infections by inducing T3SS genes and normal piliation, but repressing genes that promote biofilm growth (61, 203).

Regardless of where *P. aeruginosa* PA4308 fits in the hierarchy of T3SS regulation, it is of note that work done here gives us an insight on the role of T3S

effector protein ExoS and the relationship between invason and traversal. We previously reported that *P. aeruginosa* utilizes a novel intracellular survival strategy within epithelial cells that involves trafficking of bacteria to plasma membrane bleb niches, which is driven by the ADP-ribosylating domain activity of ExoS (10, 11). In addition, work done on traversal on lung epithelial cells grown as monolayers showed permeability of epithelia was also associated with the ADP-ribosylating domain of ExoS (173). We showed that a mutation in *PA4308* gene, which hypersecretes ExoS during early-log phase, displayed an increase in traversal capacity of multilayered human epithelia *in vitro*. It also showed that ExoS was involved in the internalization of *P. aeruginosa* (Figure 15), and that the increase in traversal seen could reflect an increase in susceptibility of corneal epithelia to invasion during later time-points. Thus, our data obtained continue to support a correlation between ExoS and intracellular survival and traversal of *P. aeruginosa*.

In summary, in this study we report the identification of a novel gene in *P. aeruginosa* PAO1 strain, PA4308, which demonstrates a role in regulating early secretion of ExoS. This gene is a strong candidate for playing a role monitoring transition from acute to chronic by regulating certain virulence factors that promote different stages of infectivity. Understanding the regulation involved and which factors facilitate these signals are of great importance in recognizing the cooperation between these two different stages and their role in infection. Manipulation of these signals could then provide a means to convert antimicrobial-resistant chronic infections to susceptible planktonic cells. Such a strategy, coupled with immuno-therapy (e.g. anti-PcrV IgG) can provide immerse benefits to protect the host from type III secretion by *P. aeruginosa*.

CHAPTER 5.

CLOSING REMARKS.

Pseudomonas aeruginosa typically does infect healthy eye; ordinarily, the corneal epithelium is a protective barrier to microbial infection, yet contact lens wear can predispose individuals to infectious keratitis (48, 106, 154). The mechanism by which contact lens wear enable epithelial susceptibility to microbes is not well understood. As the number of antimicrobial compounds effective against *P. aeruginosa* decreases, due to the acquisition and spread of antibiotic resistance, there is a growing need for novel therapeutic approaches.

Currently, research aimed at understanding *P. aeruginosa* keratitis has centered mostly on pathology and immunology after disease initiation using murine scarification models that deliberately bypass epithelial barriers (31, 109, 125, 196). While these studies are informative, it does not allow us to study host factors that would normally protect the eye from developing infections. Epithelial cells are the first cell type that many bacteria encounter during the pathogenesis process, including the ocular surface. Interactions between *P. aeruginosa* and epithelial cells are thought to be key in the development of most infections. Our research differed in that we aimed at understanding ocular epithelial defenses and how they maybe compromised through contact lens wear (or other predisposing factors).

The fact that *P. aeruginosa* can cross the corneal epithelium into the underlying stroma to cause disease in contact lens wearers, suggest a compromise in host defense. Here our research explored the role of antimicrobial peptides (AMPs) in limiting *P. aeruginosa* infectivity and traversal of the corneal epithelium. While epithelial-expressed antimicrobial peptides (AMPs) are a logical candidate defense factors (and have been shown important in clearing bacteria that have gained access to the corneal stroma), a role for AMPs in actual epithelial barrier function during health has only been assumed. The decrease in antimicrobial activity observed in cystic fibrosis (CF) patients (16) in the presence of high salt content (a major component of the tear fluid), brought into question the effectiveness of these peptides at the ocular surface.

The results of our study showed that AMPs, specifically human β-defensins (hBD-1, 2, and 3) and the human cathelicidin LL-37, contribute to the ability of human corneal epithelia to resist traversal by *P. aeruginosa in vitro*. They also show that murine β-defensin mBD-3 (the mouse ortholog of hBD-2) contributes to clearance of *P. aeruginosa* from the healthy mouse eye *in vivo,* and additionally limits *P. aeruginosa* adhesion to the corneal epithelial surface. These data suggest that in addition to their role in innate defense responses to ocular injury and infection (101, 197), AMPs are also involved in maintaining normal resistance to infection when the tissue is healthy. However, it is also now apparent that synergist effects between host defense proteins (i.e. lactoferrin and lysozyme) and antimicrobial peptides (i.e. hBD-2 and HBD-3) may help overcome the effect of salt concentration in the tear film to help eliminate microbes on the ocular surface.

In agreement with our overall objective, we also tested the hypothesis that prolong exposure to corneal epithelia increases *P. aeruginosa* capacity to adapt to and overcome epithelial barrier function that would otherwise limit traversal by using our *in vitro* traversal model. It maybe the pathogenesis of contact lenses involves providing a suitable niche for *P. aeruginosa* to persist to sub-lethal concentration of epithelial defense factors. Often biofilms are observed from contact lenses of infected eyes. Our results demonstrated bacteria that had traversed corneal epithelial cells (i.e. pre-

conditioned) acquired an enhanced capacity for subsequent traversal. Related, we therefore wanted to explore how epithelial impacts bacterial gene expression, as a first step towards identifying bacterial genes that contribute to penetration (and traversal) when the epithelial host defenses (i.e. antimicrobial peptides) are overcome.

Previous studies investigating mechanism of *P. aeruginosa* traversal have mostly been done using epithelial cell monolayers grown on permeable filters, e.g. MDCK cells (14, 15, 75), showing the involvement of elastase, exotoxin A (14) and the MexAB-OprM drug-efflux pump (76). While our microarray data showed previously implicated genes involved in traversal of other epithelia, e.g. *rsaL*, our results also showed novel roles for previous identified virulence genes. Importantly, we also identified a large amount of genes encoding hypothetical proteins involved in the traversal of multilayered human epithelia, e.g. PA4308. Our data suggest that traversal itself contributes to a complex set of events when *P. aeruginosa* attempt to cross the epithelial cell multilayers that ultimately determine the outcome. While further studies will be needed to elucidate the involvement of these genes, by investigating how epithelial invasion and traversal impacts *P. aeruginosa* gene expression, we can better understand the relevance of specific bacterial virulence traits on epithelial derived infections.

In summary, here we explored the role of the innate defenses of the ocular surface preventing infections against *P. aeruginosa*. Our attempt in understanding how the healthy cornea resists infection, and how this maybe compromised in contact lens wear lead us to investigate the early events (i.e. epithelial barrier) of infection. Understanding the complexity between virulence strategies of *P. aeruginosa* and ocular surface defenses we can hopefully accelerate efforts towards preventing infection of the eye (or other epithelial derived infection sites) and develop targets for novel therapies that interfere with disease initiation early on.

CHAPTER 6.

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CHAPTER 7.

SUPPLEMENTAL DATA.

Table S1. Real Time RT- qPCR primers and chromosomal deletion primers used in this study.

Gene	Sequence (5' to 3')[a]	Reference
hBD-1	F: GGAGGGCAATGTCTCATTCTG R: CTCTGTAACAGGTGCCTTGAA	(145)
hBD-2	F: TCCTCTTCTCGTTCCTCTTCA R: AGGGCAAAAGACTGGATGAC	(145)
hBD-3	F: CATTATCTTCTGTTTGCTTTGCTC R: CGATCTGTTCCTCCTTTGGA	(145)
LL-37	F: GAAGACCCAAAGGAATGGCC R: CAFAFCCCAGAAGCCTGAGC	(131)
β-actin	F: GATTACTGCTCTGGCTCCTAGC R: GACTCATCGTACTCCTGCTTGC	(131)
PA4308-Up	F: GAGCTCGGTGAACGCCTCGAACTCCTCGAT R: GGATCCGCGCTGAGCGCCAGGCCAT	This Study
PA4308-Down	F: GGATCCGCGACATCCATTCATCCTCGGG R: AAGCTTTTGCCAGCGGCCTTCACCG	This Study

[a] F, forward; R, reverse

Table S2. TER across human corneal epithelial cells before and after inoculation with 10^6 CFU of naïve PA01 or pre-conditioned PA01.

TER Readings (Ω/cm^2) Mean (± SD)	**HCE exposed to Naïve PA01**	**HCE exposed to Pre-Conditioned PA01**
Uninfected Cells	325 ± 4	320 ± 5
8 h Post-inoculation	321 ± 7	304 ± 4*

* $p < 0.05$ when compared to uninfected cells

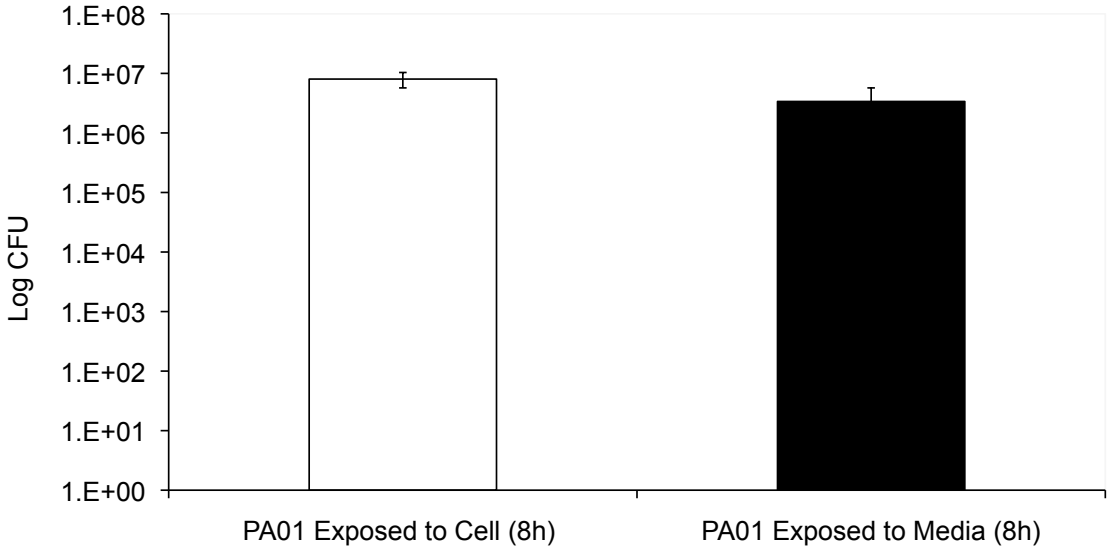

Figure S1. *P. aeruginosa in vitro* traversal assay using multilayered human corneal epithelia. PAO1 cultures were exposed to cells or KGM-2 basal media for 8 h prior to inoculation with 10^6 cfu added to the apical compartment and viable counts taken from basal compartment (i.e. traversed bacteria) 8 h post- inoculation.

Table S3. Complete gene list PAO1 naïve to traversal of multilayered human corneal epithelial cells relative to those grown in media only.

Gene ID	Up-Regulated	P-Value
PA0209_at	8.09482862	2.91E-05
PA4881_at	6.363597469	3.81E-05
PA0239_at	5.94504334	4.43E-05
PA2031_i_at	5.90207978	2.00E-05
PA4865_ureA_at	5.256483039	0.000181723
PA2297_at	5.166857965	0.000227599
PA0251_at	4.942779536	3.81E-05
PA0121_at	4.73923044	4.84E-05
PA2327_at	4.70080019	0.000161281
PA1217_at	4.504866874	4.43E-05
PA0135_at	4.381955516	0.000305414
PA1266_at	4.378396134	4.43E-05
PA0206_at	4.34929225	0.000401478
PA3377_at	4.214427731	0.000157135
PA0232_pcaC_at	4.086598861	0.000156254
PA0193_at	4.048160037	0.001265973
PA1281_cobV_at	4.037555742	0.001243417
PA2132_at	3.96647187	5.43E-05
PA0204_at	3.960189775	0.000129744
PA0205_at	3.831165561	0.000326042
PA0107_at	3.822023929	5.55E-05
PA1233_at	3.810262075	4.43E-05
PA4220_i_at	3.772536094	5.43E-05
PA0103_at	3.695736574	0.000202883
PA0151_at	3.679016406	0.000130323
PA1251_at	3.637526699	5.43E-05
PA4146_at	3.59941269	0.000176541
PA1342_at	3.598585411	0.000326042
PA4140_at	3.50584125	0.000303421
PA1219_at	3.490268991	0.000130323
PA0123_at	3.450025328	4.85E-05
PA0236_at	3.421243874	0.000282882
PA1237_at	3.394365231	0.000305414
PA0111_at	3.362106799	5.55E-05
PA0150_at	3.318692568	9.03E-05
PA0165_at	3.286496264	0.000284883
PA2368_i_at	3.266511675	0.00087798
PA0112_at	3.243808309	0.001932071
PA4111_i_at	3.220490973	0.00087798
PA0138_at	3.193394011	0.000157135
PA4106_at	3.137262594	0.000156254
PA0185_at	3.133337979	0.000157135
PA3441_at	3.084536857	0.001048338
PA2343_mtlY_at	3.065077712	0.001638523
PA0195_pntA_at	3.055962869	0.000674433
PA1262_at	3.053177761	0.000156254
PA3442_at	3.04481694	0.000160558
PA3891_at	3.026289648	0.000326042
PA2212_at	3.008527955	0.001565879
PA0230_pcaB_at	2.999434441	0.000236089
PA0281_cysW_at	2.998148575	0.000823212
PA0221_at	2.96888991	0.000314826
PA0160_at	2.959808053	0.003444762
PA0136_at	2.924919595	0.000202883
PA4150_at	2.924206868	0.000183473
PA5458_at	2.920607594	0.000483867
PA0192_at	2.870897178	0.001246754
PA4823_at	2.865282469	0.000192043
PA0207_at	2.831701972	0.000161281
PA3394_nosF_at	2.822230324	0.000157135
PA0470_at	2.813549908	0.000305414
PA0069_at	2.809421178	0.000187869
PA1355_at	2.804835464	0.003665024
PA2906_at	2.800200258	0.000202883
PA0218_at	2.797178629	0.002061007
PA2027_at	2.786713279	0.000466916
PA4822_at	2.776914016	0.000284883
PA4083_at	2.76130321	0.000773181
PA2292_at	2.755889157	0.001229213
PA2338_at	2.751758861	0.000135715
PA3343_at	2.734159552	0.000305414
PA4185_at	2.73054624	0.004904428
PA1848_at	2.720138258	0.000285959
PA5219_at	2.718294064	0.000374206
PA4219_at	2.678823266	0.000350905
PA0210_mdcC_at	2.675040692	0.000472645
PA0248_at	2.669049196	0.000202883
PA4218_at	2.66505347	0.000231826
PA4099_at	2.664357368	0.000350905

Gene ID	Up-Regulated	P-Value
PA2149_at	2.660571423	0.000314158
PA0203_at	2.659871593	0.001168995
PA3425_at	2.638605788	0.000350905
PA1268_at	2.629692326	0.000264442
PA0703_at	2.629574175	0.000202883
PA2180_at	2.628135879	0.000964187
PA5348_at	2.626887511	0.003962854
PA0097_at	2.617057346	0.000874789
PA3418_ldh_at	2.608022121	0.000177726
PA0334_at	2.602478822	0.006960641
PA4059_at	2.601375998	0.000183996
PA0215_at	2.600893051	0.005431461
PA4898_at	2.598432624	0.001168995
PA3938_at	2.598230179	0.000202883
PA0110_at	2.58698624	0.000327014
PA2204_at	2.584948368	0.000387743
PA5414_at	2.577730806	0.000706308
PA4163_at	2.568326376	0.000570763
PA0171_at	2.561472842	0.000202883
PA0148_at	2.556538667	0.002382964
PA3186_oprB_s_at	2.548618106	0.027913715
PA4860_at	2.546801485	0.002485032
PA0345_at	2.544892007	0.000243273
PA1232_at	2.522667927	0.000783652
PA4597_oprJ_at	2.514741566	0.000227599
PA0114_at	2.510103251	0.000374206
PA3909_at	2.507165559	0.001000011
PA0066_at	2.504613561	0.000350905
PA0174_at	2.496311746	0.003903091
PA0098_at	2.491464655	0.000274565
PA0079_at	2.487235642	0.00030852
PA1228_at	2.464376479	0.003537446
PA4116_at	2.460890239	0.000579919
PA1337_ansB_at	2.459781773	0.000264442
PA0325_at	2.457449734	0.00022037
PA2416_treA_at	2.456200218	0.00037923
PA1394_at	2.445518976	0.000407648
PA5465_at	2.437716781	0.000305414
PA0211_mdcD_at	2.427400015	0.001488952
PA1675_at	2.426314156	0.000716202
PA5525_at	2.417184726	0.000227599
PA1701_at	2.409636322	0.000593563
PA3881_at	2.409010132	0.000339728
PA3904_i_at	2.408064259	0.000485064
PA1888_at	2.406978123	0.000227599
PA2863_lipH_at	2.403836779	0.005616252
PA5415_glyA1_s_at	2.400552524	0.000282882
PA1879_at	2.399419735	0.000404803
PA3895_at	2.394189801	0.000583191
PA3371_at	2.392422303	0.000350905
PA0077_at	2.390949837	0.001761499
PA2366_at	2.386335025	0.001368503
PA2214_at	2.38152166	0.00113597
PA2916_at	2.374511349	0.000264713
PA1221_at	2.370566004	0.000350905
PA4125_hpcD_at	2.366800927	0.001062868
PA0055_at	2.363061982	0.000476231
PA3378_at	2.356470947	0.000783652
PA4193_at	2.351121978	0.000284883
PA4121_at	2.345044137	0.035240507
PA2339_at	2.341977988	0.000345655
PA4109_ampR_at	2.326407254	0.000502656
PA4776_at	2.319449268	0.001409531
PA3501_at	2.318160928	0.00133407
PA0213_at	2.315361869	0.000339728
PA4824_at	2.303242028	0.000760224
PA2134_at	2.301914765	0.002571333
PA0265_gabD_at	2.299955136	0.000350905
PA3383_at	2.298335084	0.003352109
PA0216_at	2.28995783	0.000468789
PA2274_at	2.289951335	0.000574064
PA1175_napD_at	2.288757515	0.021111558
PA3444_at	2.28844553	0.000279778
PA5196_at	2.285115577	0.007174821
PA4211_g_at	2.282421342	0.004366159
PA4058_at	2.27805738	0.001145686
PA1351_at	2.277188012	0.003044599
PA0116_at	2.274993926	0.003949322
PA5381_at	2.265781748	0.000480177

Note: Gene list from the microarray analysis was generated by comparing gene expression profile of PAO1 exposed to corneal epithelial cells that were naïve to traversal (Naïve PAO1) or those that traversed the multilayered epithelial cells (Pre-conditioned PAO1). Baseline reference for each condition was PAO1 grown in media only.

Table S3 continued. Complete gene list of PAO1 naïve to traversal of multilayered human corneal epithelial cells relative to those grown in media only

Gene ID	Up-Regulated	P-Value
PA0274_at	2.259722155	0.001463624
PA0113_at	2.257582694	0.000284883
PA1263_at	2.257084088	0.000773228
PA1317_cyoA_at	2.256879341	0.000964187
PA2881_at	2.250797722	0.002154809
PA5432_at	2.247427917	0.000305414
PA3412_at	2.245577329	0.002159079
PA3911_at	2.24420363	0.0010898
PA1230_at	2.243636797	0.001917488
PA0163_at	2.239111907	0.000472645
PA2862_lipA_at	2.230983369	0.001138978
PA4878_at	2.223156325	0.00081635
PA3126_ibpA_at	2.22181507	0.009569683
PA4741_rpsO_at	2.220789694	0.00601019
PA0147_at	2.218382434	0.002083131
PA2331_at	2.209305312	0.000472645
PA2514_antC_at	2.202477512	0.000823212
PA0149_at	2.198829809	0.002409448
PA1412_at	2.196367685	0.000650929
PA2178_at	2.191271764	0.001257482
PA1885_at	2.190600216	0.003292577
PA4107_at	2.189830528	0.008847299
PA2360_at	2.177623669	0.0015068
PA0198_exbB1_at	2.176263581	0.000783652
PA4182_at	2.176042013	0.006667608
PA5481_at	2.175090646	0.000502656
PA2270_at	2.169845245	0.001418488
PA1905_s_at	2.164627415	0.000515593
PA2919_at	2.163740376	0.001761499
PA4110_ampC_at	2.163727805	0.00249917
PA5460_at	2.163592362	0.000738762
PA3896_at	2.163430832	0.003326301
PA3463_at	2.153805501	0.012376185
PA5517_at	2.151746464	0.003048568
PA1226_at	2.151122245	0.000486606
PA2312_at	2.147872352	0.021509571
PA0180_at	2.137885759	0.004627737
PA4149_at	2.135259369	0.002675233
PA2381_at	2.1347715	0.000738762
PA1408_at	2.131366733	0.007556611
PA2003_bdhA_at	2.130735302	0.004332414
PA2315_at	2.130561115	0.001093647
PA2910_at	2.129300009	0.005260897
PA0078_at	2.125466422	0.003689534
PA2130_at	2.123864962	0.001076607
PA3934_at	2.121496544	0.00691379
PA4187_at	2.121119827	0.002009225
PA4830_at	2.119914218	0.001761499
PA0080_at	2.117296779	0.003252803
PA0467_at	2.114738649	0.010582621
PA1277_cobQ_at	2.114634474	0.008513528
PA4181_at	2.114353213	0.00618874
PA3937_at	2.114314367	0.00081635
PA4089_at	2.109897601	0.000783652
PA0152_pcaQ_at	2.109661095	0.000477355
PA0100_at	2.109458431	0.000871629
PA1909_at	2.109274737	0.002159079
PA0108_coIII_at	2.106478312	0.031485079
PA4135_at	2.106323963	0.0043156
PA4226_pchE_at	2.10610379	0.000570763
PA0132_at	2.101221699	0.044701064
PA4134_i_at	2.099784639	0.000999133
PA2184_at	2.097855739	0.001460744
PA4104_at	2.090944241	0.001650553
PA0074_ppkA_at	2.086963356	0.00272899
PA2914_at	2.085840414	0.000593563
PA3354_at	2.084948526	0.000706308
PA4879_at	2.081596167	0.001982444
PA4038_at	2.072270554	0.001014518
PA1259_at	2.070141826	0.005331381
PA0394_at	2.069450892	0.000587025
PA3424_at	2.065996336	0.001560539
PA4171_at	2.064819992	0.018785907
PA3411_r_at	2.063093615	0.004978206
PA0194_at	2.059443019	0.01970909
PA4873_at	2.05563334	0.002491726
PA0231_pcaD_at	2.054877006	0.002896475
PA3395_nosY_at	2.053354979	0.004384664
PA0115_at	2.05197521	0.001771244
PA0366_at	2.051723371	0.00213932
PA1274_at	2.050134306	0.0239003
PA4160_fepD_at	2.049454884	0.00136465
PA0522_r_at	2.043888493	0.00429483
PA3577_i_at	2.041575686	0.01327754
PA5500_znuC_at	2.039256265	0.00140953
PA0056_at	2.037687876	0.00206227
PA2243_at	2.037054438	0.0038317
PA2336_at	2.035835019	0.00690571
PA2296_at	2.034732042	0.00081635
PA0191_at	2.033313955	0.00191846
PA0076_at	2.029308862	0.00130427
PA4176_ppiC2_at	2.028352688	0.00126597
PA2086_at	2.02719597	0.00093878
PA0370_at	2.026360914	0.00139958
PA0810_at	2.025867648	0.00078365
PA3406_hasD_at	2.02450295	0.00233404
PA2055_at	2.022271701	0.00057406
PA0091_at	2.022103175	0.00367329
PA0095_at	2.021069307	0.00082321
PA2467_at	2.015969825	0.00124342
PA2135_at	2.015008432	0.00073876
PA4115_at	2.013303317	0.02947771
PA0226_at	2.010875201	0.00114569
PA1211_at	2.010483893	0.00095949
PA4153_at	2.006447293	0.0361065
PA2123_at	2.005628858	0.00073618

Note: Gene list from the microarray analysis was generated by comparing gene expression profile of PAO1 exposed to corneal epithelial cells that were naïve to traversal (Naïve PAO1) or those that traversed the multilayered epithelial cells (Pre-conditioned PAO1). Baseline reference for each condition was PAO1 grown in media only.

Table S3 continued. Complete gene list of PAO1 naïve to traversal of multilayered human corneal epithelial cells relative to those grown in media only

Gene ID	Down-Regulated	P-Value
PA1746_at	2.001903685	0.005539579
PA0497_at	2.002142068	0.006351809
PA5173_arcC_at	2.004302443	0.000610425
PA3163_cmk_at	2.014116959	0.000823212
PA3832_holC_at	2.016299661	0.001982444
PA2562_at	2.020582923	0.000783652
PA0480_at	2.031956016	0.004495976
PA1431_rsaL_at	2.032526227	0.001797616
PA5558_atpF_at	2.055626406	0.009781015
PA0646_at	2.056169249	0.002431486
PA3134_gltX_at	2.059538241	0.00231895
PA0664_at	2.074526141	0.000977782
PA2580_at	2.075956179	0.004684668
PA2765_at	2.086080439	0.000658663
PA3284_at	2.089058806	0.003326301
PA3148_wbpI_at	2.097380105	0.001243417
PA0981_at	2.099215446	0.001460744
PA4644_at	2.104029635	0.003038445
PA0668_tyrZ_at	2.105867015	0.000801881
PA1093_at	2.113176266	0.000783652
PA3146_wbpK_at	2.120247604	0.00249917
PA4236_katA_at	2.122706032	0.000658663
PA2981_lpxK_at	2.124403338	0.001334435
PA2987_at	2.127580948	0.001145686
PA0712_at	2.137406904	0.006390898
PA1530_at	2.144549312	0.000364448
PA2566_at	2.145229101	0.000476231
PA2516_xylZ_at	2.147379046	0.000570763
PA1539_at	2.147825496	0.000574064
PA1552_at	2.153528169	0.000574064
PA2740_pheS_at	2.163237489	0.000364448
PA0746_at	2.168138181	0.000783652
PA2973_at	2.177312473	0.000773181
PA4439_trpS_at	2.180582635	0.000479852
PA2697_at	2.195174358	0.001334435
PA4755_greA_at	2.199205069	0.002070173
PA2621_at	2.214835484	0.001138978
PA4672_at	2.222006002	0.000436779
PA3745_rpsP_at	2.229187777	0.004163686
PA0585_at	2.234393733	0.001673293
PA3656_rpsB_at	2.249916294	0.004756525
PA3744_rimM_at	2.293777649	0.001243417
PA3149_wbpH_at	2.296778365	0.002697839
PA3805_pilF_at	2.334903722	0.000350905
PA2768_at	2.34933245	0.003091275
PA5170_arcD_at	2.367950996	0.000350905
PA1515_alc_at	2.39570153	0.00037923
PA0582_folB_at	2.443166826	0.000550127
PA0938_at	2.443862652	0.000574064
PA1541_at	2.455392822	0.000345655
PA2775_at	2.470405319	0.004357798
PA3326_at	2.495116552	0.000303421
PA2967_fabG_at	2.495243273	0.000227599
PA4935_rpsF_at	2.51469807	0.003106323
PA4771_lldD_at	2.521744141	0.000233017
PA5282_at	2.521764705	0.000658663
PA3352_at	2.524775907	0.001243417
PA3006_at	2.526681604	0.001229213
Pae_orfD_at	2.538941664	0.000591236
PA0713_at	2.588395709	0.001181391
PA3407_hasAp_at	2.615800679	0.000874789
PA3572_at	2.617191691	0.000387743
PA3743_trmD_at	2.64388024	0.000202883
PA1656_at	2.684868115	0.000177894
PA0908_at	2.691865991	0.0031366
PA5232_at	2.827564384	0.000162146
PA5544_at	2.890848537	0.00248318
PA4745_nusA_at	2.938239978	0.000161281
PA2655_i_at	2.952381898	0.000156254
PA0894_at	3.006717271	0.000794384
PA2046_at	3.022126547	0.001761499
PA1001_phnA_at	3.066437876	9.03E-05
PA0954_at	3.075307152	0.011273709
PA3278_at	3.105725069	0.000806909
PA0996_at	3.110105311	0.000586203
PA2794_at	3.196588402	0.000502656
PA5357_at	3.206821425	0.001399584
PA0998_at	3.511285553	0.000303421
PA2501_at	3.525831184	0.000282882
PA4141_at	3.636763892	5.21E-05
PA4359_i_at	4.087425491	0.012395606
PA1000_at	4.190487422	4.43E-05
PA4498_at	4.206194611	7.34E-05
PA0999_fabH1_at	4.333959659	5.55E-05
PA0997_at	4.719652764	4.43E-05

Note: Gene list from the microarray analysis was generated by comparing gene expression profile of PAO1 exposed to corneal epithelial cells that were naïve to traversal (Naïve PAO1) or those that traversed the multilayered epithelial cells (Pre-conditioned PAO1). Baseline reference for each condition was PAO1 grown in media only.

Table S4. Complete gene list of PAO1 during traversal of multilayered human corneal epithelial cells relative to those grown in media only.

Gene ID	Up-Regulated	P-Value
PA4881_at	6.96862496	3.08E-05
PA3126_ibpA_at	6.479878381	0.000235137
PA4611_at	5.951863051	6.62E-05
PA1342_at	5.767837583	0.000142609
PA2031_i_at	5.105846078	2.78E-05
PA0209_at	4.773545589	0.000134756
PA2027_at	4.709412561	0.000135682
PA3229_at	4.682460223	0.000635936
PA5348_at	4.465658162	0.00064775
PA3819_at	4.294089858	0.001067649
PA4463_at	4.231651314	0.000135682
PA2297_at	4.05886761	0.00061003
PA3731_at	4.0234587	0.001239528
PA0251_at	3.991988284	8.73E-05
PA0232_pcaC_at	3.987490893	0.00022055
PA1198_at	3.963221359	0.002544314
PA4762_grpE_at	3.76715624	0.000440256
PA4865_ureA_at	3.744020997	0.000624254
PA0404_i_at	3.450615535	0.000155569
PA3031_at	3.445885136	0.007865732
PA5446_i_at	3.394323473	0.000174474
PA1217_at	3.392559793	0.00017079
PA2368_i_at	3.358414248	0.000938721
PA0265_gabD_at	3.309062242	0.000135682
PA3186_oprB_s_at	3.305676638	0.010549582
PA0160_at	3.304752329	0.00235943
PA2327_at	3.289181772	0.000624254
PA0236_at	3.21207635	0.000453275
PA5253_algP_at	3.208100865	0.001164715
PA2270_at	3.160826581	0.000297766
PA1266_at	3.144072436	0.000155569
PA1337_ansB_at	3.143193058	0.000144493
PA2952_etfB_at	3.061792561	0.000235137
PA0506_at	3.057950477	0.000135682
PA0239_at	3.042006794	0.000450983
PA0123_at	3.041877333	0.000135682
PA4751_ftsH_at	2.967873738	0.000661415
PA4922_azu_at	2.958851674	0.000296788
PA3441_at	2.94724549	0.00140674
PA3692_at	2.941734433	0.000255086
PA3904_i_at	2.906182154	0.00026263
PA4220_i_at	2.900414456	0.000235137
PA4140_at	2.891012921	0.000742403
PA1233_at	2.883363016	0.000155569
PA0165_at	2.880992484	0.000627005
PA5053_hslV_at	2.867250174	0.00138385
PA1592_i_at	2.862639823	0.003196206
PA3377_at	2.860850336	0.000674269
PA5054_hslU_at	2.843460387	0.001923299
PA0135_at	2.804148309	0.001822018
PA0107_at	2.798741716	0.000251091
PA4218_at	2.790295639	0.000235137
PA0206_at	2.778573953	0.002612053
PA1251_at	2.76772345	0.000235137
PA3385_at	2.767652738	0.003883184
PA0195_pntA_at	2.766079394	0.001172351
PA5255_algQ_at	2.758861964	0.00026263
PA2883_at	2.736721637	0.0017585
PA0376_rpoH_at	2.729533307	0.00054813
PA0121_at	2.72471473	0.00044183
PA4059_at	2.704139613	0.000233615
PA4776_at	2.702359214	0.000770533
PA5414_at	2.691168301	0.000674269
PA4099_at	2.668900946	0.000490323
PA3442_at	2.656793135	0.000324528
PA0329_at	2.647676362	0.003501949
PA1701_at	2.646785077	0.000526407
PA3691_at	2.64397079	0.000707617
PA0394_at	2.635601146	0.000235137
PA2132_at	2.613498905	0.000374893
PA4111_i_at	2.597856777	0.002614871
PA4115_at	2.597175384	0.008454793
PA2023_galU_at	2.567644814	0.000821489
PA2292_at	2.556715644	0.001950516
PA0150_at	2.552700855	0.000322108
PA1281_cobV_at	2.538057923	0.009213605
PA3343_at	2.5301742	0.000608956
PA2204_at	2.528763785	0.000624254
PA0114_at	2.509383337	0.000540355

Gene ID	Up-Regulated	P-Value
PA3577_i_at	2.505452437	0.004682688
PA4185_at	2.497875706	0.008352916
PA0110_at	2.496653896	0.000540355
PA1262_at	2.492708229	0.000422749
PA0204_at	2.488638738	0.000776139
PA4542_clpB_at	2.482607547	0.000540355
PA2338_at	2.472001914	0.000235923
PA2381_at	2.471057265	0.000440256
PA0171_at	2.468267598	0.000297766
PA0098_at	2.461169713	0.00036211
PA2853_oprI_at	2.450082633	0.000738164
PA0456_at	2.440821092	0.010901082
PA0230_pcaB_at	2.439514423	0.000707617
PA4116_at	2.43476627	0.000734117
PA1848_at	2.427859766	0.000636784
PA0103_at	2.425074984	0.001295379
PA5240_trxA_at	2.412687646	0.002537174
PA1041_at	2.402738155	0.001172266
PA3788_at	2.389564812	0.000775716
PA3911_at	2.385712313	0.000892578
PA0281_cysW_at	2.384368961	0.002858999
PA5119_glnA_at	2.378494438	0.001863343
PA3748_at	2.377446395	0.000450983
PA0079_at	2.362600616	0.00054813
PA1675_at	2.361803181	0.000938721
PA0836_at	2.360430507	0.000453275
PA1317_cyoA_at	2.357596137	0.000884514
PA0433_at	2.345977817	0.000661415
PA2366_at	2.339435013	0.001735926
PA3418_ldh_at	2.338942004	0.00036211
PA0151_at	2.330968555	0.000884514
PA0762_algU_at	2.329149936	0.011812857
PA3182_at	2.329025101	0.001448083
PA4153_at	2.324552559	0.017260099
PA0138_at	2.32295631	0.000674269
PA2180_at	2.320682071	0.002060565
PA4944_at	2.31848241	0.00173096
PA3891_at	2.318173155	0.001312407
PA5490_cc4_at	2.312500705	0.008148147
PA0185_at	2.305792011	0.000674269
PA5427_adhA_at	2.295036143	0.000941569
PA2339_at	2.288515027	0.000540355
PA4823_at	2.287430476	0.000665912
PA0149_at	2.287034801	0.002060565
PA4150_at	2.286639053	0.000674269
PA0111_at	2.281868407	0.000453426
PA0112_at	2.281358533	0.011140438
PA5458_at	2.276849556	0.001923299
PA4607_at	2.27542702	0.002451122
PA0078_at	2.27150689	0.002665975
PA3040_at	2.265207634	0.001003214
PA4106_at	2.259500675	0.000707617
PA3354_at	2.256403182	0.000587119
PA4181_at	2.255798516	0.004743831
PA3764_at	2.251212878	0.000661415
PA0139_ahpC_at	2.249750918	0.007107918
PA3458_at	2.248259377	0.000624254
PA2212_at	2.247578425	0.007518763
PA1676_at	2.24742944	0.00173096
PA0470_at	2.242351862	0.001110846
PA3306_at	2.240982628	0.000661415
PA4146_at	2.240045368	0.001429576
PA4125_hpcD_at	2.23736867	0.001599529
PA5196_at	2.237050428	0.008920056
PA5424_at	2.232275885	0.004513019
PA2467_at	2.228156272	0.000776139
PA3183_zwf_at	2.221550502	0.000793009
PA0218_at	2.219813505	0.007539676
PA2016_at	2.216848435	0.000707617
PA2248_bkdA2_at	2.210849386	0.001314709
PA0623_at	2.210583438	0.005156388
PA2906_at	2.209004053	0.000757169
PA4149_at	2.208334681	0.00235943
PA0193_at	2.199756045	0.019426583
PA4254_rpsQ_at	2.197198546	0.019426583
PA1232_at	2.193334746	0.001868643
PA1677_at	2.193099079	0.005841544
PA5463_at	2.19165534	0.015726227
PA3665_at	2.187012428	0.001025977

Note: Gene list from the microarray analysis was generated by comparing gene expression profile of PAO1 exposed to corneal epithelial cells that were naïve to traversal (Naïve PAO1) or those that traversed the multilayered epithelial cells (Pre-conditioned PAO1). Baseline reference for each condition was PAO1 grown in media only.

Table S4 continued. Complete gene list of PAO1 during traversal of multilayered human corneal epithelial cells relative to those grown in media only.

Gene ID	Up-Regulated	P-Value
PA0077_at	2.185982217	0.003265697
PA1219_at	2.178153128	0.00106172
PA0055_at	2.17755424	0.000892915
PA4870_at	2.177350818	0.00106172
PA1885_at	2.172863179	0.003724658
PA0116_at	2.172727055	0.005583971
PA0084_at	2.165919426	0.001946406
PA4223_at	2.164408618	0.000674269
PA2491_at	2.163101539	0.001405852
PA3885_at	2.163004784	0.000661415
PA0336_at	2.162983156	0.002537174
PA5171_arcA_at	2.161885278	0.001003143
PA1355_at	2.159731481	0.014703807
PA1263_at	2.158425607	0.001123014
PA5148_at	2.153952327	0.002060565
PA3909_at	2.152268852	0.002658475
PA1277_cobQ_at	2.150109005	0.008547588
PA4385_groEL_at	2.149454728	0.002063209
PA0703_at	2.147716146	0.000674269
PA5219_at	2.146168134	0.001523544
PA0108_coIII_at	2.143591005	0.029743366
PA4061_at	2.140655935	0.00106172
PA4182_at	2.139480198	0.008270729
PA0205_at	2.137843889	0.005341005
PA0203_at	2.134900204	0.004468225
PA3529_at	2.13276154	0.005383426
PA0943_at	2.132039525	0.002127505
PA0248_at	2.130216456	0.000714948
PA1237_at	2.128185953	0.003265697
PA3881_at	2.127073096	0.000802171
PA4219_at	2.125164747	0.001429576
PA2214_at	2.122843327	0.002464813
PA4067_oprG_at	2.121102148	0.0095871
PA4761_dnaK_at	2.117697878	0.004419257
PA0334_at	2.1169883	0.02177418
PA4465_at	2.115271561	0.00106172
PA5415_glyA1_s_at	2.112980044	0.000674269
PA3895_at	2.108382037	0.00138385
PA4042_xseB_at	2.100695799	0.003724658
PA0462_at	2.098833764	0.006220129
PA3371_at	2.08728166	0.000941569
PA4844_at	2.087026049	0.012493622
PA3976_thiE_at	2.086045862	0.012418411
PA3049_rmf_at	2.084597869	0.007835923
PA0097_at	2.083167576	0.003562341
PA1429_at	2.078848437	0.001633543
PA4058_at	2.077055809	0.00224991
PA0409_pilH_at	2.075202037	0.002442603
PA4920_nadE_at	2.071945043	0.001312407
PA2134_at	2.065924047	0.005386063
PA4523_at	2.063461649	0.003724658
PA0413_at	2.062651904	0.009080995
PA2320_gntR_at	2.062279064	0.002104087
PA1230_at	2.061032846	0.003562341
PA5432_at	2.049940558	0.000665912
PA1244_at	2.049443426	0.030829092
PA4822_at	2.048299207	0.001568683
PA5460_at	2.043395501	0.001172266
PA5312_at	2.042062828	0.011498743
PA0210_mdcC_at	2.04148442	0.002442603
PA0207_at	2.041046637	0.000938721
PA4578_at	2.038359131	0.004468225
PA3880_at	2.032476122	0.001295379
PA5436_at	2.025508663	0.012252166
PA5525_at	2.023075869	0.000707617
PA2140_at	2.017833021	0.000883298
PA5380_at	2.015694665	0.000738164
PA4163_at	2.014898854	0.002565412
PA3351_at	2.012792427	0.002979876
PA1333_r_at	2.011930307	0.043479919
PA3938_at	2.009732526	0.000938721
PA1596_htpG_at	2.008373271	0.001764177
PA0519_nirS_at	2.008230681	0.001752835
PA0100_at	2.005102876	0.001379458
PA4819_at	2.002756235	0.008900597
PA0211_mdcD_at	2.001070354	0.005386877

Gene ID	Down-Regulated	P-Value
PA4571_at	2.000946232	0.004933021
PA0864_at	2.00304222	0.002060565
PA4712_at	2.003704239	0.001194926
PA2987_at	2.015810738	0.001837578
PA3799_at	2.017255888	0.003130856
PA0663_at	2.017587962	0.004786242
PA5235_glpT_at	2.02201502	0.001197698
PA1530_at	2.040777103	0.000665912
PA2671_at	2.044266521	0.001429576
PA1668_at	2.046836488	0.006464339
PA2840_at	2.047115041	0.001316539
Pae_orfM_at	2.050085471	0.000674269
PA4745_nusA_at	2.050299065	0.00106172
PA1108_at	2.063509159	0.001389392
PA1519_at	2.071620266	0.002148876
PA3633_at	2.0795573	0.000744975
PA0746_at	2.079771903	0.001164715
PA3132_at	2.093497069	0.001123014
PA0483_at	2.095962073	0.000714948
PA5093_at	2.096042239	0.000775215
PA3121_leuC_at	2.10327695	0.000661415
PA2765_at	2.127515204	0.000705902
PA3832_holC_at	2.144992897	0.001443892
PA2621_at	2.145905887	0.001483436
PA1001_phnA_at	2.151989848	0.000624254
PA0998_at	2.172267109	0.003094737
PA2602_at	2.18183567	0.000624254
PA2998_nqrB_at	2.185062193	0.005156388
PA3513_at	2.213784398	0.017331118
PA2740_pheS_at	2.227533913	0.000440256
PA1656_at	2.229358055	0.00054813
PA2775_at	2.237559575	0.008148147
PA2981_lpxK_at	2.260435918	0.00106172
PA2580_at	2.261055622	0.003076618
PA0646_at	2.265281189	0.001429576
PA3149_wbpH_at	2.286814533	0.002992308
PA4755_greA_at	2.3263455	0.001633543
PA2947_i_at	2.342959251	0.003724658
PA3467_at	2.361732858	0.000374893
PA4718_at	2.391982196	0.000941569
PA1515_alc_at	2.395731171	0.000548004
PA1509_at	2.398485474	0.00155166
PA2566_at	2.435401864	0.000310803
PA2599_at	2.435482069	0.001598089
PA3743_trmD_at	2.457203193	0.00036211
PA4644_at	2.47639869	0.001295379
PA4498_at	2.484109077	0.000674269
PA4141_at	2.530301818	0.000288672
PA0585_at	2.613958788	0.000844056
PA1539_at	2.615281091	0.00026263
PA4672_at	2.649157968	0.000235137
PA3805_pilF_at	2.652208623	0.000251091
PA1541_at	2.671253582	0.000297766
PA0999_fabH1_at	2.701277363	0.00046397
PA0894_at	2.718956273	0.001402128
PA0986_i_at	2.719812138	0.000384583
PA4771_lldD_at	2.726046769	0.000235137
PA0712_at	2.749627077	0.001837578
PA4935_rpsF_at	2.820274749	0.001935324
PA5216_at	2.853009959	0.000233615
PA2516_xylZ_at	2.947876292	0.000202097
PA0908_at	2.951883688	0.002202756
PA0997_at	3.001259361	0.000235137
PA1000_at	3.009166855	0.000202097
PA0497_at	3.021587317	0.00081457
PA3399_at	3.022220186	0.005418618
PA2768_at	3.027522278	0.001023055
PA0996_at	3.099260574	0.000714948
PA0480_at	3.116656253	0.000632756
PA2697_at	3.37526898	0.000235137
PA0582_folB_at	3.408355246	0.00022055
PA0954_at	3.433993036	0.008148147
PA5282_at	3.530188699	0.000235137
PA5544_at	3.604616921	0.001123014
PA2046_at	3.771021978	0.000883298
PA5357_at	4.185647555	0.000674269
PA2794_at	4.336899918	0.000235137
PA2655_i_at	4.422223079	6.25E-05
PA4359_i_at	4.438550137	0.010514182

Note: Gene list from the microarray analysis was generated by comparing gene expression profile of PAO1 exposed to corneal epithelial cells that were naïve to traversal (Naïve PAO1) or those that traversed the multilayered epithelial cells (Pre-conditioned PAO1). Baseline reference for each condition was PAO1 grown in media only.

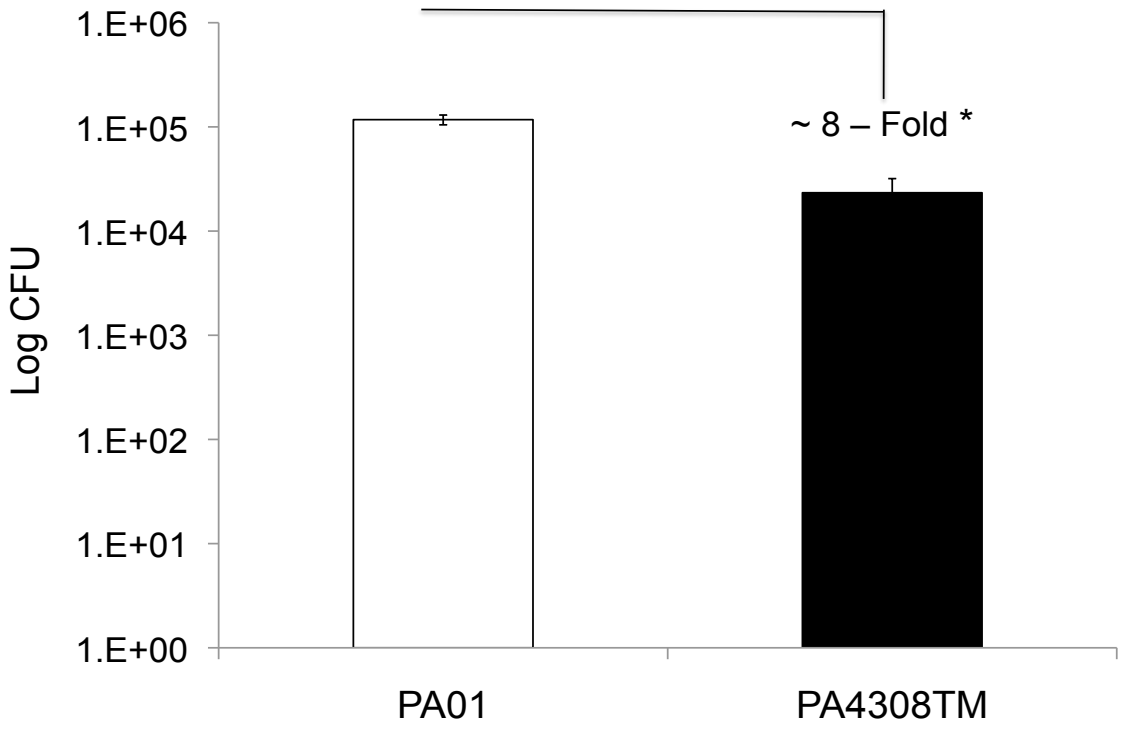

Figure S2. *P. aeruginosa* PAO1 and transposon mutant PA4308TM traversal of multilayered human corneal epithelia *in vitro* (A). PAO1 or PA4308TM (~10^6 cfu) were added to the apical compartment and viable counts taken from basal compartment (i.e. traversed bacteria) 8 h post- inoculation. * $p < 0.001$, student *t*-Test. One of two independent experiements is shown.