Chapter 1
Pseudomonas aeruginosa Host Immune Evasion

Taylor S. Cohen, Dane Parker and Alice Prince

Abstract The opportunistic pathogen *Pseudomonas aeruginosa* is a common cause of pneumonia in immunocompromised and mechanically ventilated patients. By virtue of its large genome and genetic flexibility, *P. aeruginosa* is able to adapt to its environment regulating the expression of a large repertoire of virulence factors. It is capable of forming biofilms that favor persistence and evasion of phagocytic clearance. Within a biofilm the bacteria communicate via soluble quorum sensors regulating production of elastases and proteases. *P. aeruginosa* is able to modify expression of pathogen associated molecular patterns such that recognition by host immune receptors is minimized. The bacteria are also able to subvert the innate immune system through direct interaction with host cells and host immune cytokines. These strategies of immune evasion and the genetic pathways that regulate them have contributed to the unusual success of *P. aeruginosa* as on opportunistic pathogen.

Keywords *P. aeruginosa* · innate immunity · biofilm

Pseudomonas aeruginosa is an opportunistic human pathogen especially common in health care associated facilities. Aspiration or contamination of the airways with *P. aeruginosa* is an infrequent cause of pneumonia in a normal host, but is a common pathogen in immunocompromised and mechanically ventilated patients (Richards et al. 1999; Rodríguez-Rojas et al. 2012; Lynch 2001; Craven and Hjalmarson 2010). Much of what we have come to understand about the ability of *P. aeruginosa* to evade immune clearance has come from the study of cystic fibrosis, a genetic disorder often associated with respiratory colonization with *P. aeruginosa*. This wealth of clinical and basic science information has been important in the understanding of host-pathogen interactions in the airway.

A. Prince (✉) · T. S. Cohen · D. Parker
Department of Pediatrics, Columbia University, 650 W 168th St., 10032 New York, NY, USA
e-mail: asp7@columbia.edu

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P. aeruginosa utilize numerous strategies to avoid eradication by the immune system. Its large genome and genetic flexibility, allows *P. aeruginosa* to rapidly adapts to the milieu of the airway, and formation of biofilms promotes persistence and evasion of phagocytic clearance (Richards et al. 1999; Lynch 2001; Craven and Hjalmarson 2010; Hoboth et al. 2009; Huse et al. 2010; Smith et al. 2006). Once within the airways, organisms alter expression of pathogen associated molecular patterns (PAMPs) such that they limit detection by the host, and directly interact with host signaling through injection of effector proteins or release of vesicles. This chapter will review our current understanding of these methods of persistence and the interaction between *P. aeruginosa* and innate immune cells within the host.

P. aeruginosa Rapid Genetic Diversification

The *P. aeruginosa* genome consists of a conserved core set of genes and a number of accessory genes that allow for rapid adaptation within the host (Richards et al. 1999; Lynch 2001; Craven and Hjalmarson 2010; Wiehlmann et al. 2007). *P. aeruginosa* exhibits a high mutation rate during infection allowing the bacteria to respond to selective pressure in the host environment through diversification of the population's collective gene pool (Richards et al. 1999; Rodríguez-Rojas et al. 2012; Lynch 2001; Craven and Hjalmarson 2010; Hoboth et al. 2009; Huse et al. 2010; Smith et al. 2006). The mismatch repair system, essential to limiting mutation, can be turned off through down-regulation of the genes *mutS*, *mutL*, and *uvrD*. Loss of this system promotes increased mutation rates and genetic diversification, ultimately altering expression of other global regulators such as Vfr and OprF that influence expression of numerous virulence factors (Richards et al. 1999; Lynch 2001; Craven and Hjalmarson 2010; Fito-Boncompte et al. 2011; Fuchs et al. 2010; Oliver et al. 2002; Oliver et al. 2000). In addition to diversification of virulence expression, increased mutation allows members of the bacterial community to utilize difference sources of nutrition, survive in aerobic and anerobic conditions, and withstand antimicrobial peptides produced by the host (Richards et al. 1999; Lynch 2001; Craven and Hjalmarson 2010; Hoboth et al. 2009; Huse et al. 2010; Smith et al. 2006; Williamson et al. 2012; Zhao et al. 2012). Therefore while an individual bacterium might not be able to survive within the host, the population persists as subpopulations are resistant to various host pressures.

P. aeruginosa contains numerous two component systems that enable the organisms to respond to the environment or specifically to conditions encountered within a eukaryotic host. One of these regulons involves the two-component signal transduction system PmrAB that can be activated by host DNA. This two-component system regulates a variety of functions including: adding arabinose to lipid A of LPS altering its structural providing resistance to polymyxin (Moskowitz et al. 2004; Mulcahy et al. 2008). PmrAB also controls a group of genes with similarity to those involved in spermadine synthesis and provides a surface layer containing putrescine and spermidine that protects the organism from antimicrobial peptides

as well as polymyxin and gentamicin (Johnson et al. 2012). Consistent with its role in protection from host antimicrobial compounds, PmrAB is also induced in the presence of several cationic peptides including LL-37 as well (McPhee et al. 2003). Thus the organism has the genetic flexibility to adapt to conditions that would eliminate other microbes.

P. aeruginosa Communities: Biofilms

Within the host, *P. aeruginosa* forms biofilms, which protect the bacteria from innate immune clearance (Rodríguez-Rojas et al. 2012; Hoboth et al. 2009; Davies et al. 1997; Singh et al. 2000). Biofilms develop in a three step process involving bacterial attachment to the epithelial surface, microcolony formation, and formation of the mature biofilm (Fig. 1.1). Individual bacterial cells released from the mature

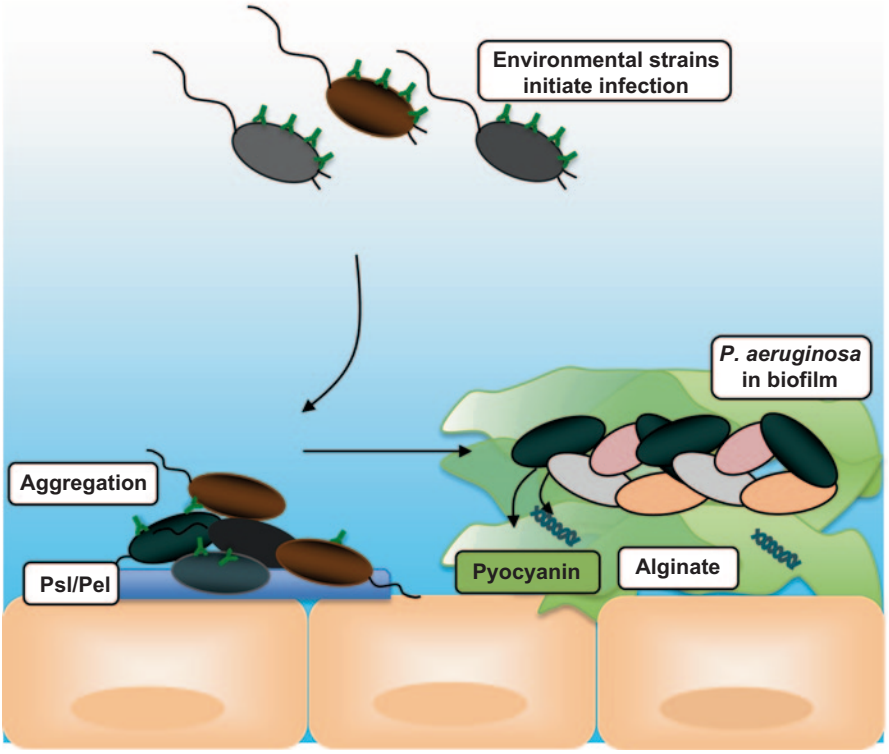

Fig. 1.1 Biofilm formation. Environmental isolates of *P. aeruginosa* aggregate within the host upon infection suppress expression of immunostimulatory virulence factors and initiate alginate production. Within the bacterial population gene expression in individual bacterium are diversified, allowing the population to adapt to the host's environment

biofilm known as planktonic cells facilitate dispersal of the infection (Stoodley et al. 2002). The biofilm matrix is composed of exopolysaccharides, nucleic acids and proteins. Initial formation is dependent on the *pel* and *psl* regulated exopolysaccharide production which firmly attaches bacteria to the epithelium (Colvin et al. 2011a, b; Ma et al. 2009; Jackson et al. 2004; Matsukawa and Greenberg 2004). Individual bacteria deposit Psl exopolysaccharide that attracts other bacteria and acts as the foundation of the biofilm (Zhao et al. 2013). Psl upregulates c-di-GMP levels in neighboring bacteria resulting in increased Psl production (Irie et al. 2012). Bacterial density and quorum sensing can suppress c-di-GMP and biofilm formation by repressing expression of genes within the *pel* locus (Ueda and Wood 2009). Upon initiation of the biofilm *P. aeruginosa* increases production of alginate, required for structural development of the bacterial community (Hentzer et al. 2001; Davies et al. 1998; Nivens et al. 2001). Production of this exopolysaccharide is regulated by *mucA*, and during the transition to a biofilm growth mode spontaneous selection of *mucA* mutants that promote alginate production allow the organisms to elude phagocytosis (Silo-Suh et al. 2002; Martin et al. 1993; Bjarnsholt et al. 2009). Host factors such as estrogen and mucus in the CF airway induce MucA mutations promoting colonization (Cattoir et al. 2012; Chotirmall et al. 2012). Alginate itself is immunogenic and elicits antibody production, which amplifies the host inflammatory immune contributing to pathology without resulting in clearance of the organisms (Pedersen 1992; Schiøtz et al. 1978).

The switch to the biofilm phenotype is highly correlated with persistent infection, however, it does not necessarily impair the ability of the bacteria to induce signaling in the host (Sanchez et al. 2013). Isolates from chronically colonized CF patients with altered immune phenotypes, loss of motility and increased mucosity, were not as lethal in a murine model as early isolates but were equally capable of inducing excessive lung inflammation and establishing a chronic infection (Bragonzi et al. 2009). A major component of the biofilm matrix is extracellular DNA, which can assist in initial biofilm development by attaching to F-actin released from neutrophils at the onset of infection (Parks et al. 2009; Whitchurch et al. 2002; Nemoto et al. 2003). Host pattern recognition receptors respond to the increase in DNA by upregulating inflammatory signaling, recruiting inflammatory monocytes to the lung, but not necessarily resulting in bacterial clearance (Fuxman Bass et al. 2010). Therefore, incorporated DNA from lysed bacteria into the biofilm structure maintains inflammatory signaling while preventing innate immune clearance.

Biofilm formation also facilitates the coordinate expression of numerous genes throughout the microbial population through secretion of highly soluble quorum sensors which have been identified as biomarkers of advanced infection (Struss et al. 2013). In *Pseudomonas* biofilms, homoserine lactones and quinolones act in concert with specific transcriptional activators to regulate gene expression (Guina et al. 2003; Wade et al. 2005). The Las system produces the homoserine lactone N-3-oxo-dodecanoyl that activates the transcriptional regulator LasR, influencing expression of elastases and proteases (Passador et al. 1993; Pearson et al. 1994). A second lactone, N-butanoyl, activates an alternate transcriptional regulator RhlR, which represses expression of the proteins that compose a major virulence factor,

the type III secretion system (TTSS). These signaling pathways regulate each other as LasR upregulates expression of RhlR and RhlR can control expression of LasR dependent factors in *lasR* mutant bacteria (Dekimpe and Déziel 2009). The *Pseudomonas* quinolone signal (PQS) activates its receptor PqsR which increases production of PQS, phenazine, and pyocyanin (Fito-Boncompte et al. 2011). PQS signaling is regulated by homoserine lactones, though through distinct mechanisms as LasR positively regulates and RhlR negatively regulates PQS production. Importantly, PQS correlates with bacterial virulence in animal models, potentially due to a relationship between PQS production and expression of inflammatory lipopolysaccharide (Guina et al. 2003).

Pyocyanin, regulated by transcriptional regulators LasR and OxyR, is produced by all *P. aeruginosa* strains, but at increased levels in biofilms (Vinckx et al. 2010; Schaber et al. 2004). It can directly interact with host cells inducing inflammatory signaling and reducing the function of antioxidants GSH and *N*-acetylcysteine by blocking the dual oxidase-based antimicrobial system (Rada et al. 2008; Look et al. 2005; Rada and Leto 2012). In the epithelium, the pyocyanin inhibits V-ATPase activity preventing endosomal acidification and perhaps inhibiting degradation of endocytosed bacteria (Ran et al. 2003). Pyocyanin may also negatively affect the function of the chloride channel CFTR, perhaps enhancing disease progression in cystic fibrosis (Schwarzer et al. 2008). Levels of pyocyanin have been correlated with decreased lung function in clinical studies, and in vivo experiments have documented decreased virulence of *P. aeruginosa* pyocyanin mutant strains (Fito-Boncompte et al. 2011; Lau et al. 2004; Hunter et al. 2012; Recinos et al. 2012). Therefore while cell-cell communication is especially important for formation and regulation of bacterial communities within the biofilm, it also results in the production of factors that interact directly with the host, inducing inflammatory signaling and interrupting host processes.

P. aeruginosa Altered PAMP Expression

P. aeruginosa presents an array of pathogen associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS), lipoproteins, and flagella that initiate host signaling through an numerous of receptor complexes. Toll like receptors (TLRs) such as TLR2 (bacterial lipoproteins) and TLR5 (flagellin) are expressed primarily on the cell surface and signal through the adapter protein MyD88 to activate innate immune signaling. TLR4 (LPS) is expressed both at the cell surface and within the endosome where it signals through the adapter protein Trif. Additional TLRs such as TLR3 (double stranded RNA), TLR7 (single stranded RNA), and TLR9 (CpG DNA) are intracellular receptors and activate similar signaling pathways. Host cells also express NOD-like receptors (NLRs) that activate caspase activation, cytokine release, and a form of inflammatory cell death called pyroptosis following recognition of bacterial peptidoglycans, flagellin, nucleic acids, and components of the type III secretion system (Akira et al. 2006). *P. aeruginosa* is a successful opportunist not

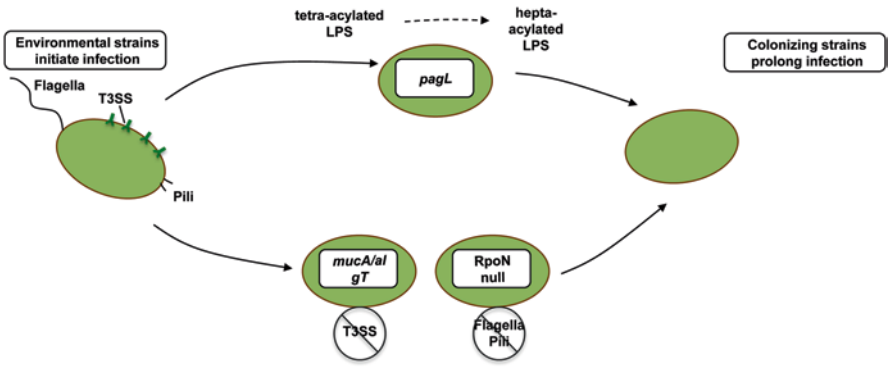

Fig. 1.2 Modification of virulence expression. Rapid genetic mutations upon infection result in alterations in *P. aeruginosa* virulence factor expression

only due to its many virulence factors but also due to its ability to adapt to the host environment minimizing activation of these host receptors and downstream signaling pathways (Fig. 1.2) (Bianconi et al. 2011).

Lipopolysaccharide (LPS), a major cell wall component all Gram-negative organisms is shed from the surface of bacteria into the airway and is recognized by TLR4 on host cells (Poltorak et al. 1998). The LPS-TLR4 interaction, mediated by adaptor proteins MD2 and CD14, results in activation of MyD88 (cell surface) and Trif (endosome) dependent signaling pathways (Kagan et al. 2008; Rowe et al. 2006; Visintin et al. 2001). The ability of LPS to activate host signaling pathways is influenced by the acylation state of side chains attached to the core signaling component of LPS, Lipid A, allowing for variation between the immunostimulatory capacity of LPS from various Gram-negative bacteria (Zughaier et al. 1999; Ganglloff et al. 1999). In fact mimics of triacylated LPS have been suggested as promising anti-inflammatories due to their inability to induce signaling while binding to TLR4 (Dunn-Siegrist et al. 2012). Lipid A alterations are controlled via transcriptional regulation (PhoP and PhoQ) of *pagP*, *pagL*, and *lpxO* genes that are responsible for acylation, deacylation, and hydroxylation of lipid A, respectively (Kawasaki et al. 2004; Geurtsen et al. 2006). In vivo selection of modified LPS, presumably in response to innate immune pressure, has been extensively studied. The affect of LPS structure on both acute and chronic infection has been clarified by analysis of isolates from newly infected infants with cystic fibrosis, and sequential isolates from colonized patients. Comparison of the LPS produced by *P. aeruginosa* isolates from infants with cystic fibrosis or non-CF patients with sepsis or bronchiectasis demonstrated that bacteria initially infecting the lungs of CF patients predominantly had a penta-acylated LPS lipid A with additional palmitate and aminoarabinose incorporated into the side chains (Ernst et al. 1999). These additions confer protection against cationic antimicrobial peptides (CAMPs) giving these organisms a significant survival advantage (Guo et al. 1998). The lipid A from CF isolates was more immunostimulatory when applied to human endothelial cells, resulting in more of

an interleukin-8 response than that of non-CF isolates. The differences in signaling were also attributed to palmitate additions to the side chains. Growth of the laboratory strain PAK in low Mg^{2+} media resulted in similar modifications to the side chain, indicating that these modifications could be in response to specific environmental stimuli within the CF lung (Ernst et al. 1999).

P. aeruginosa lipid A structure not only has an affect on the acute infection, but once in the CF lung further adaptations in side chain composition are observed. Analysis of sequential isolates from CF, confirmed to be clonal variants by pulsed-field gel electrophoresis and single nucleotide polymorphisms, demonstrated an enhanced ability of the later isolates to persist within the mouse lung (Lorè et al. 2012; Hajjar et al. 2002). While the murine data associated with these studies is controversial considering reports that murine TLR4 is unable to recognize alterations in lipid A, activation of NF-κB signaling in culture human epithelial cells was reduced in groups stimulated with later isolates. Analysis of the lipid A structure showed a blend of tetra- and penta- acylated lipid A in the early and mid isolates. Some samples from intermediate isolates also contained hexa-acylated lipid A, while the later isolates contained primarily hepta-acylated lipid A. These modifications persisted through serial passaging. Altered lipid A in the late isolate correlated with a mutation in the *pagL* gene that is responsible for deacylation, which was also observed in an independently published study of early and chronic infection in cystic fibrosis (Ernst et al. 2007; Cigana et al. 2009). These studies demonstrate that dynamic modifications to LPS in addition to other virulence adaptations enhance the ability of colonizing bacteria to persist within the airways.

In addition to changes in LPS structure, *P. aeruginosa* modifies expression of other virulence factors over the course of infection. The *mucA* mutant is associated with biofim formation and increased alginate production. Alginate and specifically the gene *algT* negatively regulate flagella production through inhibition of *fleQ* expression (Tart et al. 2005; Garrett et al. 1999; Cobb et al. 2004). The result is non-motile *P. aeruginosa* that lack flagella, but are also better able to evade immune clearance. Strains of *P. aeruginosa* lacking flagella have reduced virulence as they cannot activate TLR5 or NLRC4 signaling pathways, have an impaired ability to bind and colonize epithelial cell surfaces, and are resistant to macrophage engulfment (Hazlett et al. 1991; Saiman and Prince 1993; Saiman et al. 1990; Miao et al. 2008; Morris et al. 2009). Alternate regulation of flagella is also downregulated in colonizing infection (Mahenthiralingam et al. 1994). Flagellin and pilin synthesis are regulated by the alternative sigma factor σ54/RpoN and the majority of clinical isolates from long colonized patients are *rpoN* negative. Complementation of *rpoN* on a plasmid partially restores swimming ability. Therefore it seems that the presence of flagella, a highly inflammatory virulence factor, is not required for long term survival within the host, and a selective advantage exists for bacteria that lose flagellin expression.

Another group of virulence factors associated with strains of *P. aeruginosa* isolated from acute infections are those delivered by the type III secretion system (TTSS), which directly injects effector proteins into host cells and interrupts host signaling processes. The TTSS needle is composed of numerous proteins including

PscC, capable of activating the NLRC4 inflammasome independent of effector protein secretion (Miao et al. 2010). For pore formation in the host cell membrane, *P. aeruginosa* pilli must bind asialoGM1 on the host cell membrane (Saiman and Prince 1993; Sundin et al. 2002; Kang et al. 1997). Once the pore has formed, the bacteria inject a cocktail of proteins made up of a combination of ExoU, ExoY, ExoS, and ExoT. Individual bacterial isolates do not necessarily express each effector but they have major effects on the host.

While important for bacterial dissemination during acute infections, expression of TTSS components are reduced in colonizing strains of *P. aeruginosa* (Jain et al. 2004). The bacterium is able to sense the host and regulate expression and activation of the TTSS through the RsmAYZ regulatory cascade (O'Callaghan et al. 2012). Furthermore, *mucA* is part of the regulatory pathway upstream of TTSS expression, and selection of *mucA* mutants also selects for bacteria lacking TTSS expression (Wu et al. 2004). Therefore, as with other virulence factors such as flagella, expression of TTSS needle and effector proteins is repressed facilitating the selection of mutants that evade detection by the innate immune system and eradication by the host.

Evasion from Host Cells

The large array of virulence factors expressed by *P. aeruginosa* are also utilized to interact directly with host immune cells to avoid detection and clearance by the immune system (Table 1.1). *P. aeruginosa* can directly interact with host structural proteins and signaling pathways to promote colonization and dissemination from the airways. *P. aeruginosa* secretes several virulence factors that suppress the immune response. The pigment pyocyanin is able to suppress the production of the cytokines RANTES and MCP-1 as well as leukotriene B_4 and 5-hydroxyeicosateraenoic acid, both proinflammatory mediators (Müller and Sorrell 1991; Denning et al. 2003). The alkaline protease and elastase can degrade RANTES and MCP-1 as well as the neutrophil-activating protein-78 (ENA-78) (Leidal et al. 2003). The quorum sensing molecules *Pseudomonas* quinolone signal (PQS) and *N*-3-oxododecanoyl reduce IL-12 (Skindersoe et al. 2009). *N*-3-oxododecanoyl has significant immunomodulatory activity able to also inhibit TNF and high concentrations have inhibited antibody production in spleen cells (Telford et al. 1998; Hooi et al. 2004). Rhamnolipids have been shown to prevent induction of human beta defensin 2 (hBD-2), an important antimicrobial peptide, in response to flagellin of *P. aeruginosa* as well as in response to host cytokine IL-1β (Dössel et al. 2012). Outer membrane vesicles (OMV) of *P. aeruginosa* containing immunostimulatory bacterial components are released during infection and can directly interact with the host (Wessel et al. 2012; Bomberger et al. 2009). One protein found in OMVs, Cif, was shown to redirect cystic fibrosis transmembrane conductance regulator (CFTR) from recycling endosomes to the lysosome where it is degraded (Bomberger et al. 2011). Exotoxin A can inhibit interferon gamma synthesis, elastase can degrade interferon gamma

Table 1.1 *P. aeruginosa* virulence factors and their host targets

P. aeruginosa	Host Target	Reference
Rhamnolipid (alginate)	hBD-2	Dössel et al. 2012; Pedersen et al. 1990; Song et al. 2003; Mai et al. 1993; McClure and Schiller 1996; Krieg et al. 1988
	Phagocytosis	
	Neutrophil chemotaxis	
Elastase	Rantes	Leidal et al. 2003; Hong and Ghebrehiwet 1992; Theander et al. 1988; Mariencheck et al. 2003
	Complement	
	Phagocytosis	
Pyocyanin	VATPase	Müller and Sorrell 1991; Denning et al. 2003; Usher et al. 2002; Prince et al. 2008; Bruno et al. 1998; Rada and Rada 2013; O'Malley et al. 2003; Gloyne et al. 2011; Wilson et al. 1987
	Cytotoxic	
	CFTR	
	T-Cell proliferation	
	Ciliary beat	
	Cytokine production	
Tuf/Lpd	Complement	Kunert et al. 2007; Hallstrom et al. 2012
Outer membrane vesicles	CFTR	Wessel et al. 2012; Bomberger et al. 2009; Bomberger et al. 2011
Protease	Complement	Schmidtchen et al. 2003; Hong and Ghebrehiwet 1992
Alkaline phosphatase	Complement	Leidal et al. 2003; Schmidtchen et al. 2003; Kharazmi et al. 1986; Kharazmi et al. 1984
	Cytokine production	
	Myeloperoxidase	
ExoU/ExoT/ExoS	Actin polymerization	Parmely et al. 1990; Chung et al. 2009; Rieber et al. 2013; Bruno et al. 1998; Geiser et al. 2001; Soong et al. 2008; McMorran et al. 2003; Diaz and Hauser 2010; Cuzick et al. 2006; Galle et al. 2008; Jia et al. 2003
	Cytotoxic	
	T-Cell proliferation	
	Rho GTPases	
	Inflammasome	
Exotoxin A	Cytotoxic	Schmidtchen et al. 2003; Parmely et al. 1990; Michalkiewicz et al. 1999; Puri et al. 1994
	T-Cell proliferation	
	Cytokine production	
N-3-oxododecanoyl/PQS	T-Cell proliferation	Skindersoe et al. 2009; Telford et al. 1998; Hooi et al. 2004; Ritchie et al. 2005
	Cytokine production	

and the antimicrobial peptide LL-37 (Schmidtchen et al. 2003; Parmely et al. 1990; Michalkiewicz et al. 1999). Exotoxin A is a tremendously potent cytotoxin, and has been adapted for use in cancer therapy by fusing the toxin to cancer cell specific antibiodies (Kreitman et al. 2012; Mazor et al. 2012).

Another mechanism whereby *P. aeruginosa* can maintain a presence in the host is to prevent complement binding and activation. Particularly in the absence of *P. aeruginosa* specific IgG, complement deposition becomes important in the

opsonization and clearance of the bacteria. On the bacterial surface the elongation factor Tuf is able to bind the human complement regulator Factor H as well as plasminogen, preventing complement activity (Kunert et al. 2007). The enzyme, dihydrolipoamide dehydrogenase (Lpd) is also able to bind Factor H and plasminogen as well as Factor H-like protein-1 and complement Factor H-related protein 1 and (Hallstrom et al. 2012). Lpd, like Tuf is surface exposed and has been shown to promote survival in serum. Alkaline protease and to a lesser extent, elastase, are both able to degrade hemolytic complement (Schmidtchen et al. 2003; Hong and Ghebrehiwet 1992). Alkaline protease is able to inhibit opsonization by cleaving C2, which blocks deposition of C3b via both the classical and lectin pathways as well as the formation of C5a (Laarman et al. 2012). Each type of host cell (neutrophils, macrophages, dendritic cells, natural killer cells, lymphocytes, and epithelial cells) has a specific role in the innate immune response to *P. aeruginosa*. How each is affected differently by secreted and injected virulence factors is reviewed below.

Neutrophils

Neutrophils are thought to be essential to the clearance of *P. aeruginosa* and numerous virulence factors directly target neutrophil function. The mechanisms utilized by *P. aeruginosa* target three general functions of the neutrophil, their abilities to sense, move and kill pathogens. Direct physical interaction of *P. aeruginosa* with the neutrophil allows *P. aeruginosa* to absorb host sialic acids and bind to siglec-9 on the surface of neutrophils (siglecs are sialic acid recognizing immunoglobulin superfamily type I transmembrane lectins) (Khatua et al. 2012). The engagement of *P. aeruginosa* with siglec-9 inhibits neutrophil engagement, decreasing elastase and ROS production and reducing neutrophil NET (neutrophil extracellular trap) production.

The secreted virulence factors, alkaline phosphatase (AP) and elastase act upon neutrophils. Although the mechanism is largely unknown they are both able to inhibit chemotaxis (Theander et al. 1988). The surface protein alginate is also able to inhibit chemotaxis and prevent clearance of *P. aeruginosa* (Pedersen et al. 1990; Song et al. 2003; Mai et al. 1993). AP and elastase also inhibit myeloperoxidase-based killing, influencing oxidative burst and superoxide production (Kharazmi et al. 1986; Kharazmi et al. 1984). Both proteins at physiological concentrations inhibit phagocytosis, possibly by cleaving surface receptors required for uptake (Kharazmi et al. 1986). The secreted type III toxins ExoS, ExoT and ExoU through their ADP ribosyltransferase activity leads to neutrophil apoptosis (Diaz et al. 2008; Sun et al. 2012). Apoptosis has also shown to be induced by the phenazine, pyocyanin. Mediated by oxidative stress, pyocyanin causes early lysozyme dysfunction, change in pH leading to loss of acidification, membrane permeabilization and caspase-3 cleavage (Usher et al. 2002; Prince et al. 2008).

Macrophages and Dendritic Cells

Macrophages and dendritic cells are also targets of *P. aeruginosa* factors that reduce their ability to clear bacteria both directly and indirectly by affecting opsonization. The initial step in clearance by macrophages is the phagocytosis of invading pathogens. Rhamnolipids produced by *P. aeruginosa* inhibit this process as demonstrated in vivo by decreased uptake of zymosan particles by macrophages in the presence of exogenous rhamnolipid (McClure and Schiller 1996; Krieg et al. 1988). *P. aeruginosa* elastase degrade lung surfactant proteins, allowing the organism to avoid opsonization and phagocytosis (Mariencheck et al. 2003). The type III toxins ExoS and ExoT inhibit bacterial uptake by macrophages by interfering with actin polymerization. These toxins also kill macrophages by inducing apoptosis, as does exotoxin A, the quorum sensing molecule *N*-3-oxododecanoyl and the secreted type III toxin ExoU (Garrity-Ryan et al. 2000; Shaver and Hauser 2004; Ritchie et al. 2005; Diaz et al. 2008). Exotoxin A inhibits interferon gamma induced activation of the co-stimulatory molecules CD80 and CD86 while ExoU, once injected into macrophages by the type III secretion machine, inhibits inflammasome activation and causes cell death in the majority of immune cell types encountered by *P. aeruginosa* (Michalkiewicz et al. 1999; Sutterwala et al. 2007; Diaz et al. 2010).

Dendritic cells act as a link to the adaptive immune response. In vivo models have shown that chronic infection with *P. aeruginosa* leads to decreased dendritic cell expression of the co-stimulation molecules CD40, CD80, and CD86 (Mukae et al. 2010). Ex vivo analysis of dendritic cells from infected animals demonstrated enhanced apoptosis of dendritic cells and reduced capacity to stimulate and recruit T cells (Boontham et al. 2008). The quorum signaling molecules *Pseudomonas* quinolone signal (PQS) and *N*-3-oxododecanoyl have been shown to reduce DC induced T cell proliferation in vivo (Skindersoe et al. 2009). T cells are required for clearance of *P. aeruginosa*, therefore inhibition of T cell activation and recruitment could provide this opportunist the chance to colonize (Cohen and Prince 2013).

Natural Killer Cells

Natural killer (NK) cells are activated by interferons and macrophage derived cytokines to tackle both intracellular and extracellular infections (Small et al. 2008). They are critical in inflammation and their role in *P. aeruginosa* infection varies depending upon the infection model (Hazlett et al. 2007; Newton et al. 1992; Laghvani et al. 2005). Although these lymphoid cells are less studied some *P. aeruginosa* virulence factors have been shown to influence their function. Inhibition of NK cell function is thought to occur by cleavage of surface receptors necessary for binding of NK cell to their targets (Pedersen and Kharazmi 1987). Exotoxin A, already implicated in bacterial killing of macrophages can also inhibit NK cell cytotoxic activity involved in oncogenesis (Michalkiewicz et al. 1999). Exotoxin A exposure leads to apoptosis due to the generation of reactive oxygen species resulting from

PI3K, caspase-9 and MAPK (Chung et al. 2009). This process is thought to offer an explanation for disease relapse in immunocompromised individuals as studies have shown *P. aeruginosa* to enhance tumor metastasis.

T Cells

T lymphocytes act as a connection between the innate and adaptive immune systems. Interference with T cell cytokine production prevents bacterial clearance and blocks the hosts ability to mount an adaptive response (Cohen and Prince 2013). Several *P. aeruginosa* virulence factors affect the ability of T cells to proliferate in the response to IL-2. Shed flagella recruit myeloid-derived suppressor cells which restrict T-cell function (Rieber et al. 2013). Alkaline protease, elastase, exotoxin A, ExoS, pyocyanin and quorum sensing molecules all have been shown to inhibit T cell proliferation (Hooi et al. 2004; Ritchie et al. 2005; Bruno et al. 1998; Puri et al. 1994; Nutman et al. 1987; Ulmer et al. 1990; Gupta et al. 2011; Smith et al. 2002). Alkaline protease and elastase inhibit IL-2 by binding to its cognate sensor (Theander et al. 1988). Exotoxin A is toxic to T cells and has shown promise in the potential removal of activated lymphocytes in allograft rejection by killing $CD8^+$ cells when conjugated to IL-4 (Puri et al. 1994). The two quorum sensing molecules *N*-3-oxododecanoyl and PQS also inhibit proliferation (Hooi et al. 2004; Gupta et al. 2011). *N*-3-oxododecanoyl causes apoptosis in CD4+ cells and T cell lines by changing mitochondrial transmembrane potential (Ritchie et al. 2005; Jacobi et al. 2009).

Epithelial Cells

Epithelial cells in many cases are the first cell type to come into contact with pathogenic organisms. It is not surprising then that the virulence factors of *P. aerguinosa* influence their function in a variety of ways to persist within the host and avoid immune clearance.

One mechanism of avoiding host detection is co-opting cells to hide. *P. aeruginosa* is able to invade and survive, avoiding immune detection in epithelial cells. Inside epithelial cells it forms pod-like clusters akin to those observed with uropathogenic *E. coli* (Garcia-Medina et al. 2005; Anderson et al. 2003; Fleiszig et al. 1994). This intracellular niche also allows *P. aeruginosa* to avoid antibotics, even those capable of permeating cells. The invasion of *P. aeruginosa* into epithelial cells requires src kinases and lipid rafts. Mice lacking caveolin-1, an important component of lipid rafts, are resistant to infection (Zaas et al. 2009; Zaas et al. 2005; Esen et al. 2001). The decision to invade cells is also dependent on the availability of nutrients. When iron is low *P. aeruginosa* invades and when high, such as in the case of cystic fibrosis sputum it forms biofilms, which are also recalcitrant to clearance (Berlutti et al. 2005).

More commonly expressed effectors such as ExoS or ExoT interfere with activation of the Rho GTPases preventing host cells from internalizing bacteria, promoting reorganization of key tight junction proteins, and allowing invasion of the bacteria across epithelial barriers (Geiser et al. 2001). The GTPase-activating protein domain of ExoS in addition to targeting the Rho pathway also prevents ERM protein phosphorylation preventing their interaction with the actin cytoskeleton, and disrupting epithelial barrier function (Soong et al. 2008). ExoU, a phospholipase, is only found in a small fraction of isolated bacteria, but is the most potent of the effector proteins and is commonly associated with strains causing ventilator associated pneumonia (Schulert et al. 2003). Following injection into host cells it binds ubiquitin and is activated (Anderson et al. 2011). ExoU can activate signaling through MAPK signaling cascades and activates transcription factor AP-1 (McMorran et al. 2003; Diaz and Hauser 2010; Cuzick et al. 2006). Furthermore, ExoU and ExoS can inhibit activation of caspase-1, limiting production of inflammasome dependent cytokines and cell death pathways (Sutterwala et al. 2007; Galle et al. 2008). ExoS is also able to induce apoptosis in epithelial as well as other cells types via caspase-3 and 9 activation (Jia et al. 2003). Pyocyanin is also toxic to epithelial cells. It induces superoxide production in cells while inhibiting catalase (Rada and Rada 2013; O'Malley et al. 2003; Gloyne et al. 2011). Pyocyanin has also been shown to inhibit epithelial ciliary beat frequency, an important component of mucociliary clearance (Wilson et al. 1987).

Infection of polarized epithelial monolayers is thought to require bacterial access to components of the epithelial basal membrane. During infections in which epithelial integrity is not compromised, *P. aeruginosa* recruits components of the basal membrane to the apical surface of the cell (Kierbel et al. 2007). Rapidly following bacterial interaction with confluent epithelial monolayers, PI3K is recruited to the apical surface, resulting in local PIP3 production and localized increases in actin. Through a proposed vesicle transport mechanism components of the basal membrane are then transported to the apical surface without disrupting tight junction integrity. *P. aeruginosa* are then able to bind and invade epithelial monolayers at these sites. It is currently unclear how the bacterium is able to induce this localized depolarization.

Conclusion

It is becoming increasingly clear that co-evolution with eukaryotic hosts has enabled successful opportunistic pathogens such as *P. aeruginosa* to develop sophisticated adaptations to host innate and adaptive immune responses. This allows the pathogen to evade immune detection partially though resistance to antimicrobial agents. *P. aeruginosa* is tremendously adept at adapting to the multiple innate immune effectors encountered in the airway, and tremendous genetic and metabolic flexibility facilitate their ability to colonize, persist, and occasionally cause invasive infection and sepsis. Modifications of virulence expression impede recognition by

host immune receptors and direct interactions with innate immune cell function prevent efficient phagocytosis and killing. Preventative strategies must take into account these complex interactions with the host and the involvement of epithelial and endothelial cells as well as the phagocytes and immune cells that target these organisms.

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