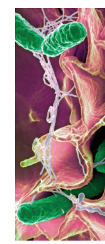

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MINIREVIEW

Pseudomonas aeruginosa: new insights into pathogenesis and host defenses

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This review about *Pseudomonas aeruginosa* acute and chronic virulence is timely and extremely well presented. It presents both the response of the host and the virulence factors produced by the bacterium.

Keywords
Pseudomonas; virulence; host defense; genome; Antimicrobial resistance; Regulatory systems.

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Abstract

Pseudomonas aeruginosa is a metabolically versatile bacterium that can cause a wide range of severe opportunistic infections in patients with serious underlying medical conditions. These infections are characterized by an intense neutrophilic response resulting in significant damage to host tissues and often exhibit resistance to antibiotics leading to mortality. Treatment of persistent infections is additionally hampered by adaptive resistance, due to the growth state of the bacterium in the patient including the microorganism's ability to grow as a biofilm. An array of *P. aeruginosa* virulence factors counteract host defences and can cause direct damage to host tissues or increase the bacterium's competitiveness. New prevention and treatment methods are urgently required to improve the outcome of patients with *P. aeruginosa* infections. This review describes the two main types of *P. aeruginosa* lung infections and provides an overview of the host response and how the genomic capacity of *P. aeruginosa* contributes to the pathogenesis and persistence of these infections.

Introduction

The Gram-negative bacterium *Pseudomonas aeruginosa* is an opportunistic pathogen that normally inhabits the soil and surfaces in aqueous environments. Its adaptability and high intrinsic antibiotic resistance enable it to survive in a wide range of other natural and artificial settings, including surfaces in medical facilities. Serious *P. aeruginosa* infections are often nosocomial, and nearly all are associated with compromised host defenses such as in neutropenia, severe burns, or cystic fibrosis (CF; Table 1; Lyczak *et al.*, 2000). Therapeutic options are increasingly limited due to the continued emergence and spread of antimicrobial resistant strains; as a result, *P. aeruginosa* infections demonstrate high morbidity and mortality. In the United States, *P. aeruginosa* is among the most common hospital pathogens and is the second most common pathogen isolated from patients with ventilator-associated pneumonia (VAP; Hidron *et al.*, 2008). Given the severity of *P. aeruginosa* infections and the limited antimicrobial arsenal with which to treat them, finding alternative prevention and treatment strategies is an urgent priority.

Airway infections of *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is one of the most common pathogens causing respiratory infections of hospitalized patients. Airway infections are often classified into two types, acute or chronic, and transmission can be either hospital- or community-acquired, although the latter is rare and almost always associated with an underlying defect in immunity (Arancibia *et al.*, 2002). Acute nosocomial pneumonias are typically the result of direct trauma, such as damage to the epithelium due to intubation or smoke inhalation. Chronic infections can result when a patient's underlying medical condition does not allow for an effective immune response, such as in the elderly or individuals with CF.

Acute lung infections

The high incidence of *P. aeruginosa* in healthcare institutions is contributed to by the poor health status of the patients, the high carriage rate of often multidrug-resistant strains in hospital wards, and prior use of broad spectrum

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Table 1 Common pseudomonal infections and risk factors

Infection	Major risk factors
Soft tissue	Burns, open wounds, postsurgery
Urinary tract	Use of urinary catheter
Bacteremia	Immunocompromised
Diabetic foot	Diabetes, impaired microvascular circulation
Respiratory/pneumonia	Old age, COPD, cystic fibrosis, mechanical ventilation
Otitis externa (swimmer's ear)	Tissue injury, water blockage in ear canal
Keratitis (corneal infection)	Extended contact lens wear, contaminated contact lens solution
Otitis media folliculitis (hot tub rash)	Improperly cleaned hot tubs

COPD, chronic obstructive pulmonary disease.

antibiotics (Otter *et al.*, 2011). Although rates vary between studies and institutions, VAP generally demonstrates the highest mortality, as great as 30% (Williams *et al.*, 2010). Patients with VAP often suffer from a breached epithelium induced by the insertion of the endotracheal tube, which can itself serve as a reservoir for *P. aeruginosa* growing as a biofilm on the plastic surface (Williams *et al.*, 2010). These biofilms are difficult to remove and treat as biofilm-associated bacteria exhibit highly increased resistance to antibiotics and disinfectants. This in part explains the relative success of antibiotic treatment regimens that are started prior to the formation of biofilms compared with the persistence of *P. aeruginosa* infections after a biofilm has developed.

Acute lung infections also occur in those who are unable to mount an appropriate host response. Underlying immune deficiencies that can predispose to *Pseudomonas* infection include old age, neutropenia due to cancer chemotherapy, or immunosuppression due to organ transplant. Thus, community-acquired pneumonia is more common in these patients than in patients who are otherwise healthy (Williams *et al.*, 2010). Nosocomial infections are also of high incidence because immune deficient patients are frequently hospitalized and therefore exposed to *Pseudomonas* reservoirs in the healthcare setting.

Chronic lung infections

If not eradicated during the acute infection phase, *P. aeruginosa* can adapt to the lung environment to grow as a biofilm resulting in a chronic infection. The best-known cases of chronic pseudomonal lung infections are those in patients with CF, most of whom develop a *Pseudomonas* lung infection by adolescence and can live with such an infection for 20 or more years. In individuals with CF, a mutation in the cystic fibrosis transmembrane regulator (CFTR), a cAMP-dependent chloride channel, results in a dehydrated and thickened airway surface liquid (ASL) that hinders mucociliary clearance from the conducting airways. Inhaled bacteria take up residence in the altered ASL and cause an initial acute infection and vigorous inflammatory response. The thickened ASL severely impairs the immune response, and the persistent immunological stimulation by the bacteria and/or the inability of the host to control inflammation results in chronic lung inflammation (Sadikot *et al.*, 2005; Williams *et al.*, 2010). In addition, there is some evidence that the CFTR mutation itself influences the ability of the host to control bacteria-induced inflammation (Blohmke *et al.*, 2012). The destruction of lung function due to the hyperactive inflammatory response, possibly exacerbated by bacterial toxins, causes the progressive deterioration of lung function and ultimately makes these lung infections fatal.

Several studies have followed the progression of *Pseudomonas* infections in patients with CF over the course of many years. The results of these studies demonstrated that phenotypic and genotypic changes occur in *P. aeruginosa* over time (Smith *et al.*, 2006; Hogardt *et al.*, 2007; Tingpej *et al.*, 2007; Mena *et al.*, 2008). Typically, the changes are such that the bacterium isolated from an established chronic infection is less inflammatory and less cytotoxic than the strain isolated years earlier from the same patient during the initial acute phase of the infection. In particular, these changes include a loss of flagellum and pili, necessary for adherence and motility, and by corollary for the injection of type 3 secreted toxins (as adherence is a prerequisite); the mutation of *mucA*, *mucB*, or *mucD*, causing the cells to form mucoid colonies that may protect them from the innate immune system (Mathee *et al.*, 1999); the evolution of highly antibiotic resistant small colony variants that are promoted by prolonged antibiotic therapy; changes in lipopolysaccharide, including altered lipid A (Ernst *et al.*, 2006, 2007), and loss of O-antigen (Hancock *et al.*, 1983); and alterations in quorum sensing (QS), such as inactivation of *lasR* (Winstanley & Fothergill, 2009). Such changes are promoted by the frequent emergence of mutator strains in the CF lung (Oliver, 2010). These altered strains are comparatively avirulent when used to infect mice in models of acute lung infection, but are unhampered in their ability to establish chronic infections (Bragonzi *et al.*, 2009).

Chronic pseudomonal lung infections are also associated with people who have chronic bronchiectasis and chronic obstructive pulmonary disease (COPD). Chronic bronchiectasis is the irreversible dilation of bronchial airways caused by the destruction of muscle and elastic tissue, usually the result of a severe childhood respiratory infection. The damage is usually restricted to the lobe in which the infection originated, and the subsequent infection does not spread. Unlike patients with CF, those with non-CF bronchiectasis do not generally have genetic abnormalities causing a defect in their immune systems, and thus, the disease is the result of impaired mechanical clearance resulting from the damage caused by the primary infection (Williams *et al.*, 2010). COPD is caused by chronic inflammation of lung tissues leading to the narrowing of airway passages resulting in a restriction of airflow. Cigarette smoking is considered the most significant risk factor for the development of COPD, whereby noxious chemicals in cigarette smoke dysregulate the normal responses of the innate immune system within the lung (Provinciali *et al.*,

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2011). Patients with COPD are frequently elderly, as the complex process of aging contributes to a general decline in lung function and the changes brought about by the cigarette smoke typically occur gradually over decades. The incidence of *Pseudomonas* infections in patients with COPD ranges from 4% to 15%, and the clinical manifestations of these infections blur the boundary between acute and chronic with both mild bronchitis and pneumonia with sepsis being common (Williams *et al.*, 2010). Many patients with COPD are able to clear the infection, but almost as many develop a persistent infection that is characterized by periodic exacerbations (Murphy *et al.*, 2008). The 1- and 2-year mortality rates after hospitalization due to an acute exacerbation of COPD are high, ranging from 22% to 49%. With the current global increase in smoking rates (largely in low-income countries), COPD is a leading cause of death that is increasing in prevalence (Hurd, 2000).

Host response to *Pseudomonas* airway infection

Humans can breathe in excess of 10 000 L per day (Flato *et al.*, 1996), and the inhaled air contains microorganisms and particulates from the environment. Despite this, the lungs of a healthy individual generally remain free from infection, reflecting the efficiency of innate immunity. In the conducting airways, the epithelium is the first line of defense against infectious agents, playing a broad range of roles in the innate response to infection. Several cell types play a role in the immunological defenses of the airways, including dendritic cells, T cells, macrophages, and neutrophils (Fig. 1). The prevention of colonization and clearance of *P. aeruginosa* from the airways therefore involves the coordinated effort of many cell types, and for this reason, persistence in the lung is no easy task for a microorganism. The symptoms and outcomes of *P. aeruginosa* infection depend on both the appropriate response of the host and bacterial virulence factors that largely act to counteract the host response. An overview of the host response to a *Pseudomonas* infection is given here.

Epithelial cells

Inhaled air starts its journey in the nasal and tracheal passages. From there, the conducting airways branch multiple times in the lung bronchi into increasingly smaller passages where they end in the gas-exchanging alveoli. The conducting passages are lined with a pseudostratified epithelium consisting of several morphologically distinct cell types that fulfill a number of critical functions. As the first site of contact for inhaled particles, including pathogens, the epithelial cells form a physical barrier to infection and act as sentinels to alert the innate and adaptive immune systems to infection (Whitsett, 2002).

Ciliated epithelial cells are the predominant cell type within the airway, comprising more than 50% of the epithelial surface. Each ciliated cell contains approximately 300 hair-like extensions of the cell membrane called cilia, which are powered by numerous mitochondria. These cells rhythmically beat their cilia in a unidirectional manner to push particles upwards and out of the lung. Thus, the primary role of these cells is the transport of mucous and mucous-

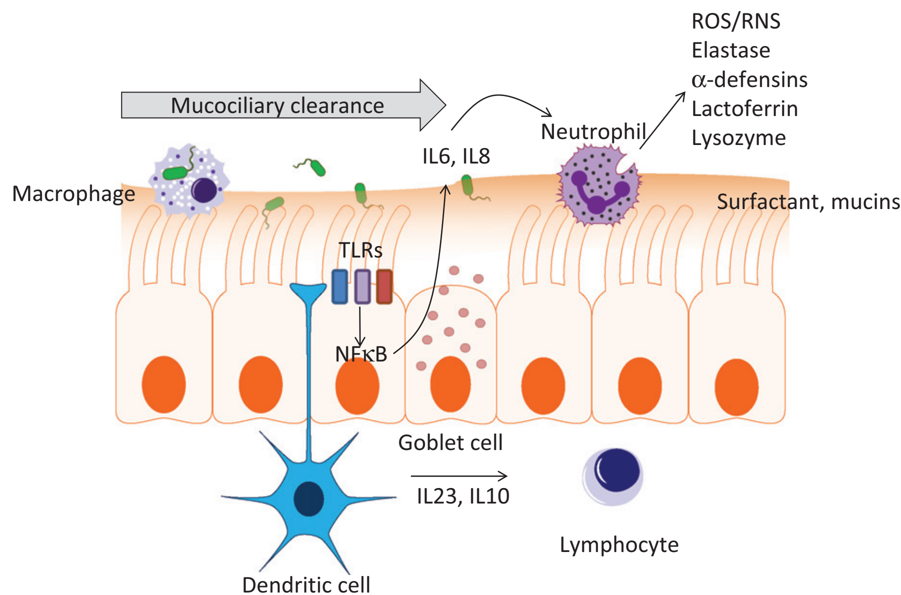

Fig. 1 Airway defenses render the lung an inhospitable environment to inhaled microorganisms. Bacteria become trapped in the viscous mucous layer, which is swept out of the lung by the rhythmic unidirectional beating of millions of cilia. Flagella, lipopolysaccharide, and type 4 pili of *Pseudomonas aeruginosa* are highly inflammatory and can be recognized by host pattern recognition receptors such as TLRs on various host cells to initiate an inflammatory response via the NFκB signaling pathway. Activated alveolar macrophages as well as neutrophils recruited by IL8 phagocytose and kill *P. aeruginosa*. Dendritic cells sample the lumen of the lung from the basal lamina and activate the adaptive response (B cells and T cells). The lumen of the lung is also made inhospitable for microorganisms by the presence of secreted antimicrobial peptides such as α-defensins, lactoferrin, and lysozyme.

encased particles, including microorganisms, from the lung to the throat (Knight & Holgate, 2003).

Secretory cells contain numerous granules for the production, storage, and secretion of mucin glycolipids (goblet cells) and bronchiolar surfactant (Clara cells and type 2 epithelial cells). Mucins are high molecular weight and highly glycosylated macromolecules that effectively bind and trap many foreign particles. The unfolding of the diverse carbohydrate chains of the mucus layer is dependent on the level of hydration, ion concentration, and pH (Knowles & Boucher, 2002). It has been proposed that one consequence of the CFTR mutation is the dehydration of the mucous layer, causing the carbohydrate side chains of the mucins to improperly unfold, hampering their ability to bind foreign particles, and making them more likely to bind to the cell-tethered mucins MUC1 and MUC4, thus effectively gluing the mucous layer to the epithelium and preventing mucociliary clearance (Knowles & Boucher, 2002). Clara cells in the lower bronchial passages and type 2 epithelial cells in the alveoli secrete pulmonary surfactant, a lipoprotein complex that lowers the surface tension at the air–liquid interface and thereby prevents alveolar collapse at the end of exhalation. Surfactant proteins have additional roles in binding and opsonizing microbial pathogens (Chroneos *et al*., 2010).

Epithelia also secrete many other molecules that may play roles in the defense of the lung. Complement proteins secreted by the epithelial cells act to bind infectious agents and promote phagocytosis. Cytokines and chemokines, particularly the powerful human neutrophil attractant IL-8, are also secreted by epithelial cells, upon activation of their toll-like receptors (TLRs), to enable recruitment and activation of cells of the innate and adaptive immune systems (Holt *et al*., 2008). Host defense (antimicrobial) cationic peptides, such as β-defensins and LL-37, and cationic proteins like lysozyme and lactoferrin are secreted into the lumen of the lung or deposited by degranulation of phagocytic cells, and are found in increased concentrations during infection or inflammation (Devine, 2003). However, the specific role of these peptides in the defense of the lung is a topic for discussion. The antimicrobial activity of these peptides has been shown to be sensitive to high salt concentrations, particularly to divalent cations such as Ca^{2+} and Mg^{2+} which exist in millimolar concentrations in most tissues. Furthermore, polysaccharides such as anionic glycosaminoglycans (e.g. heparin), and possibly mucins, bind to these cationic peptides and inhibit their action. Conversely, such peptides have profound immunomodulatory activities, which include activities that aid in the resolution of infection and inflammation such as cellular recruitment and anti-inflammatory activity in neutralizing microbial inflammatory stimuli like lipopolysaccharide (Afacan *et al*., 2012; Hancock *et al*., 2012).

Phagocytic cells

A hallmark of the inflammatory response to a *Pseudomonas* lung infection is the recruitment of neutrophils. This recruitment is dependent on the production of chemokines, particularly IL-8 (human) and KC (mouse), members of the CXC chemokine family. Mice that are administered anti-CXCR antibody demonstrate a 50% reduction in the number of neutrophils recruited to the lungs when subsequently challenged with *P. aeruginosa* and have much poorer survival rates (Tsai *et al*., 2000). Neutrophils phagocytose and kill bacteria in the lung through a number of highly effective microbicidal molecules including reactive oxygen and nitrogen species, and nonoxidative molecules such as defensin antimicrobial peptides, lysozyme, and neutrophil elastase. Although neutrophils are important in host defenses, when they are stimulated by inflammatory cytokines or bacterial molecules like lipopolysaccharide they become highly inflammatory and degranulate, causing considerable local damage (Williams & Parkos, 2007). Fortunately, their limited life span (< 24 h) and removal by noninflammatory apoptosis help to limit this damage. In chronic infections where the stimulation of the immune system by the bacteria is persistent, the neutrophilic response has greater potential to injure the surrounding host tissues (Williams *et al*., 2010). This appears to be the case for CF, although CF lung neutrophils also seem to be functionally defective, as they fail to clear the infection. The basis for this is not well understood and may reflect a particular state of the neutrophils or the regulatory influences of other cells in the lung. It has been suggested that the dehydration of the airway surface fluid in CF might trap neutrophils at localized sites and cause the induction of neutrophil necrosis rather than apoptosis, contributing to lung pathology (Downey *et al*., 2009; Hayes *et al*., 2011).

Alveolar macrophages also play an important role in the defense of the lung alveoli. These cells phagocytose particles, sequester antigens, and secrete small amounts of cytokines and chemokines in the steady state, but when activated during infection, these functions become enhanced. Although macrophages have phagocytic capabilities, the role they play in *Pseudomonas* infections is ambiguous. In some murine acute infection models, depletion of lung macrophages resulted in a lack of chemokine production, deficient neutrophil recruitment, and defective phagocytosis (Kooguchi *et al*., 1998; Fujimoto *et al*., 2002; Ojielo *et al*., 2003). Conversely, other studies demonstrated that macrophage depletion did not affect the severity of the infection (Morissette *et al*., 1996; Cheung *et al*., 2000). A variety of studies have implicated CFTR in the regulation of inflammation (with CFTR mutations promoting an elevated response to microbial agonists; Cohen & Prince, 2012). Thus, the altered cytokine environment caused by hyper-inflammation in the CF lung may impact on the efficiency of microbial phagocytosis and killing.

Pseudomonas aeruginosa pathogenesis and major virulence factors

As mentioned previously, analyses have revealed that *P. aeruginosa* isolated from acute infections differ substantially in phenotype from those isolated from chronic infections (Smith *et al*., 2006). Isolates from acute infections express a wealth of virulence factors, while in contrast, many isolates from chronic CF lung infections lack some of

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the most inflammatory bacterial features, such as flagella and pili, and downregulate other virulence mechanisms such as the type 3 secretion system (T3SS; Hogardt & Heesemann, 2010). Furthermore, isolates from chronic infections more readily form biofilms and overexpress the exopolysaccharide alginate, causing these strains to become mucoid (Sadikot *et al*., 2005; Kipnis *et al*., 2006). What follows is a description of key virulence factors known or suspected of contributing to respiratory pathogenesis. However, clinical data by nature are correlative and can be confounded by multiple mutations in a single isolate, or the presence of multiple isolates with differing genotypes and phenotypes, so the contribution of specific virulence factors to human disease has usually not been proven. Nevertheless, we have endeavored to describe the contribution of these virulence factors to human disease where data are available. A summary of virulence factors is depicted in Fig. 2.

Flagella and type 4 pili

Each *P. aeruginosa* cell possesses a single polar flagellum and several much shorter type 4 pili also localized at a cell pole. These proteinaceous appendages function both as adhesins and as major means of motility. Flagella and pili can also initiate an inflammatory response.

The whip-looking flagellum provides swimming motility through a rotating corkscrew motion in an aqueous environment and is an essential part of bacterial chemotaxis. Bursts of straight line swimming are interspersed with 'tumbles', wherein flagella rotation is transiently reversed and motility is halted in order for the bacterium to reorient itself. During an infection, the bacterium can adhere to host epithelial cells through the binding of its flagellum to the asialyated glycolipid asialoGM1 and can elicit a strong NFκB-mediated inflammatory response via signaling through TLR5 and a caspase-1-mediated response through the Nod-like receptor, Ipaf (Miao *et al*., 2007). Nonflagellated mutants are defective in models of acute infection (Brimer & Montie, 1998; Feldman *et al*., 1998), yet a large proportion of isolates from chronic infections demonstrate downregulation of flagella and/or flagella-mediated motility or are aflagellate (Wolfgang *et al*., 2004). As flagella are believed to be required for the establishment of infections, clinical vaccine trials have been undertaken to prevent initial infection and thereby the subsequent progression to a chronic infection; however, to date, these have not shown much success (Doring *et al*., 2007; Johansen *et al*., 2008).

Type 4 pili are arguably the most important adhesins of *P. aeruginosa* and are also involved in twitching motility and the formation of biofilms. Located at a cell pole, type 4 pili extend and retract like grappling hooks to pull the cell along

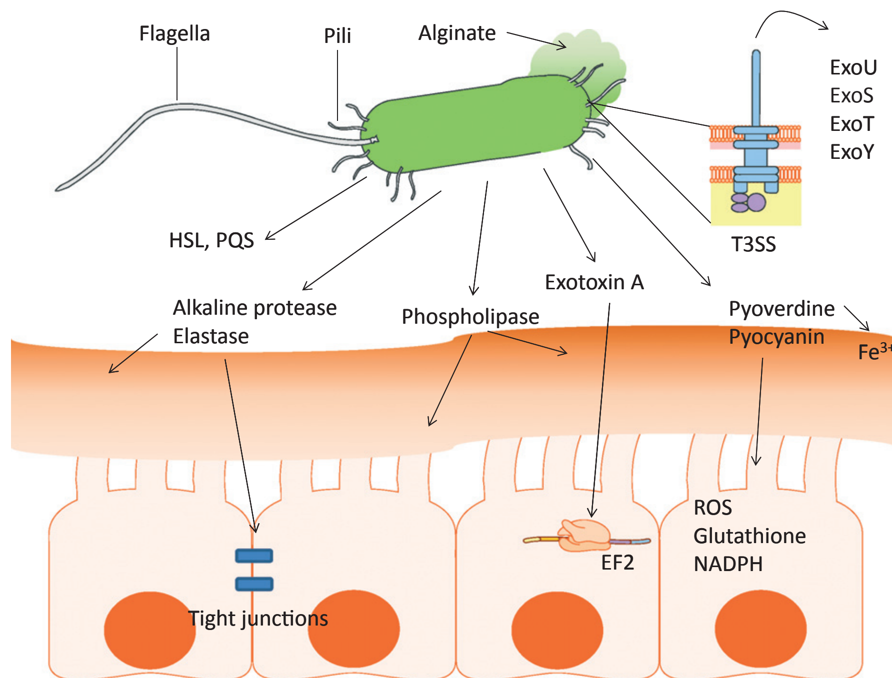

Fig. 2 A multitude of virulence factors are produced by *Pseudomonas aeruginosa*. Flagella and type 4 pili are the main adhesins, capable of binding to host epithelial gangliosides, asialoGM1 and asialoGM2. Along with lipopolysaccharide, these surface appendages are also highly inflammatory. Once contact with host epithelia has occurred, the T3SS can be activated, which is able to inject cytotoxins directly into the host cell. Several virulence factors are secreted by *P. aeruginosa* and have varying effects on the host. Several proteases are produced, which can degrade host complement factors, mucins, and disrupt tight junctions between epithelial leading to dissemination of the bacteria. Lipases and phospholipases can target lipids in the surfactant as well as host cell membranes. Pyocyanin, a blue-green pigment, can interfere with host cell electron transport pathways and redox cycling. Pyoverdine captures Fe^{3+} to allow for a competitive edge in an environment in which free iron is scarce.

solid surfaces by a process termed 'twitching motility' (Kipnis *et al.*, 2006). Together with flagella, pili also facilitate swarming motility, a highly coordinated form of motility on semi-solid surfaces (Kohler *et al.*, 2000; Yeung *et al.*, 2009). Pili can also lead to aggregation, causing the bacteria to form microcolonies on target tissues, effectively concentrating the bacteria in one location and potentially offering protection from the host immune system and from antibiotics (Craig *et al.*, 2004; Sriramulu *et al.*, 2005). Certainly, microcolonies of *P. aeruginosa* in the lung sputum of chronically infected patients with CF have been observed and resemble mucoid colonies grown in the laboratory (Bjarnsholt *et al.*, 2009). Conversely, pili are the major adhesin involved in nonopsonic phagocytosis of *Pseudomonas* (Kelly *et al.*, 1989). Pilin-deficient mutants or those impaired in twitching motility demonstrate reduced virulence in various models. Like flagella, pili are targets of antipseudomonal therapy, including immunization; however, these efforts are hampered by the antigenic variability of pili across *P. aeruginosa* strains (Kipnis *et al.*, 2006).

Type 3 secretion system

T3SS are shared among many pathogenic Gram-negative bacteria as a means of injecting toxins directly into host cells. As such, the *P. aeruginosa* T3SS is a major determinant of virulence, and its expression is frequently associated with acute invasive infections and has been linked to increased mortality in infected patients (Sadikot *et al.*, 2005; Hauser, 2009). The needle-like appendage of the T3SS, evolutionarily related to flagella, permits the translocation of effector proteins from the bacterium into the host cell through a pore formed in the host cell membrane. Only four effectors have been identified – ExoY, ExoS, ExoT, and ExoU – far fewer than many other well-characterized T3SS (e.g. *Salmonella enterica* SPI-1 has 13, *Shigella* sp. have 25; Hauser, 2009).

The T3SS of *P. aeruginosa* is encoded by 36 genes on five operons, with six other genes encoding the effector proteins and their chaperones scattered elsewhere in the chromosome (Hauser, 2009). The entire system is transcriptionally controlled by ExsA, a member of the AraC family of transcriptional activators (Yahr & Wolfgang, 2006). The four effector proteins of *P. aeruginosa* T3SS are expressed variably in different strains and isolates. Nearly all strains express one of the two major exotoxins *exoU* or *exoS* but very rarely both (Shaver & Hauser, 2004), while most strains express *exoY* and *exoT*¸ which have minor roles (Hauser, 2009). ExoS is bifunctional, including both N-terminal GTPase-activating protein activity and C-terminal ADP-ribosyltransferase (ADPRT) activity. Both activities have an effect on actin cytoskeletal organization, although the ADPRT activity is understood to play a larger part in pathogenesis. ExoU is a phospholipase and is estimated to be 100 times more potent a cytotoxin than ExoS and capable of causing rapid death of host eukaryotic cells due to loss of plasma membrane integrity consistent with necrosis (Kipnis *et al.*, 2006; Hauser, 2009). The exact contribution of each of the toxins to pathogenesis is unclear, but it is thought that the T3SS may allow *Pseudomonas* to exploit breaches in the epithelial barrier by antagonizing wound healing during colonization and to promote cell injury directly (i.e. via ExoU) and indirectly (i.e. recruitment and activation of neutrophils) leading to the symptoms of bacterial pneumonia (Hauser, 2009).

QS and biofilm formation

QS is a mechanism shared by many bacteria that allows for a coordinated adaptation of a bacterial population to environmental changes, including the adaptation to the lung environment. This adaptation is mediated by small membrane-diffusible molecules called autoinducers. These molecules are constitutively produced by each bacterium and act as cofactors of specific transcriptional regulators when they reach high enough threshold concentrations. The concentration of autoinducer molecules in the medium is proportional to the concentration of bacteria such that when the bacterial population increases to a critical mass (i.e. 'quorum'), and the concentration of autoinducers becomes sufficient to cause activation of specified downstream genes resulting in a coordinated response across the entire bacterial population. It is estimated that as many as 10% of genes in the genome and more than 20% of the expressed bacterial proteome are regulated by QS (Deep *et al.*, 2011).

Pseudomonas aeruginosa produces three autoinducers. Two of these autoinducers are acyl homoserine lactones (AHLs): 3-oxo-dodecanoyl homoserine lactone (3-oxo-C12 HSL) is produced by the LasI AHL synthase and acts on the LasR transcriptional activator, and butyryl homoserine lactone (C4 HSL) is produced by the RhlI AHL synthase, which acts on the RhlR transcriptional activator. The third autoinducer is a 2-heptyl-3-hydroxy-4-quinolone designated the *Pseudomonas* quinolone signal, which is synthesized by a complex multistep process involving two operons, *pqsABCDE* and *phnAB*, and three genes located outside these operons, *pqsR*, *pqsH*, and *pqsL* (Deep *et al.*, 2011; Heeb *et al.*, 2011). These QS systems act in a hierarchical manner, with the *las* system positively regulating both *rhl* and the production of quinolones (Heeb *et al.*, 2011). Cell survival, biofilm formation, and virulence are controlled by these systems; thus, strains deficient in any one of these systems demonstrate reduced pathogenicity (Pearson *et al.*, 2000; Sadikot *et al.*, 2005; Kipnis *et al.*, 2006).

Biofilms are highly organized, structured communities of bacteria attached to one another and to a surface, and their formation is intricately linked to QS (Bjarnsholt *et al.*, 2010). These communities are encased in extracellular polymeric substances (EPS) that can consist of polysaccharides, nucleic acids, lipids, and proteins. The EPS matrix makes up the majority (50–90%) of the volume of the biofilm and imparts both a physical and chemical robustness to the community by resisting mechanical forces (e.g. flowing water) and decreasing the penetration of toxic chemicals (e.g. antibiotics, host defense molecules; Hall-Stoodley & Stoodley, 2009; Lieleg *et al.*, 2011). Furthermore, the bacteria within the biofilm also differ substantially from their

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planktonic (free swimming) brethren in terms of their transcriptional profile (Waite *et al.*, 2006). Relative oxygen and nutrient limitation within the biofilm may contribute to the slow mode of growth observed by biofilm bacteria, as well as to an upregulation of the general stress response alternative sigma factor RpoS; all of these factors might lead to increased antibiotic resistance (Mah & O'Toole, 2001), which has also been proposed to be due to adaptive changes in gene expression, slow penetration, QS, and higher extracellular concentrations of antibiotic destroying enzymes. The antibiotic and disinfectant resistance of bacterial biofilms contributes tremendously to their resilience, and therefore, biofilms are a major medical problem. Biofilms can form on inserted medical equipment such as catheters and endotracheal tubes (Veesenmeyer *et al.*, 2009), and it has been proposed that *P. aeruginosa* can grow as a biofilm on host tissues/epithelial surfaces during chronic infections, particularly in the CF lung (Bjarnsholt *et al.*, 2010).

The transition of *P. aeruginosa* from the motile to sessile state in biofilms, and back again, manifests itself as a multitude of physiological changes. The first phase is initial contact followed by strong (effectively irreversible) attachment. This is mediated by type 4 pili, flagella, and the more recently discovered Cup fimbria (Mikkelsen *et al.*, 2011). What initiates this transition is partly dependent on cell-to-cell signaling via the Las and Rhl quorum-sensing systems and on environmental cues such as antibiotics, pigments, and siderophores (Lopez *et al.*, 2010). For example, the antibiotic imipenem has been shown to cause a thickening of biofilms due to the induced expression of alginate polysaccharide (Bagge *et al.*, 2004). After irreversible attachment, bacteria in the biofilm multiply as microcolonies and produce an EPS matrix. Three polysaccharides are produced for the *P. aeruginosa* EPS, with the importance and contributions of each varying according to the strain. Alginate is overproduced by mucoid strains that are often isolated from the lungs of patients with CF. It is widely considered to participate in the formation of biofilms in the CF lung where it is thought to protect the bacteria from the host response; however, evidence also suggests that alginate itself is not a requirement for biofilm formation *in vitro* (Wozniak *et al.*, 2003; Ryder *et al.*, 2007). The Pel polysaccharide is produced by most strains, while the Psl polysaccharide is not fully encoded in all strains (e.g. strain PA14 contains a partial deletion in the *psl* locus; Lopez *et al.*, 2010). Continued maturation of the biofilm leads to mushroom-shaped structures that are interspersed with fluid-filled channels allowing for the exchange of waste products and nutrients (Kaplan, 2010). Subsequently cells can detach from the biofilm and disperse through the environment, where they are able to adhere to another surface, renewing the cycle of biofilm formation.

The shift between motile and sessile states is influenced by several regulatory systems that appear to intersect at various nodes. The GacA/GacS two-component system has for many years been implicated in both biofilm formation and virulence. An activated GacA response regulator (RR) promotes the transcription of the two small regulatory RNAs, RsmY and RsmZ, which then bind and inactivate the translational repressor RsmA (Mikkelsen *et al.*, 2011). Sequestered RsmA causes the formation of biofilm through the increased production of Pel and Psl polysaccharides and the second messenger cyclic-di-GMP (Moscoso *et al.*, 2011) and the downregulation of several virulent extracellular products, such as pyocyanin, hydrogen cyanide, and elastase (Gooderham & Hancock, 2009). The hybrid sensor RetS influences this system by repressing GacA and by affecting cyclic-di-GMP production via the diguanyl cyclase and regulator, WspR (Moscoso *et al.*, 2011). Twenty-five other regulators have been shown, to a greater or lesser extent, to reciprocally regulate biofilm formation (reflecting the sessile state) and swarming motility (reflecting the motile state; Yeung *et al.*, 2009).

Proteases

Several proteases are secreted by *P. aeruginosa*. These proteases have established roles in ocular infections and in sepsis, where they can degrade immunoglobulins and fibrin, and disrupt epithelial tight junctions (Kipnis *et al.*, 2006). While their contribution to lung infections is less clear, proteases have been shown to contribute to tissue damage in respiratory infections, including the degradation of host lung surfactant (Fleiszig & Evans, 2002; Hobden, 2002; Kipnis *et al.*, 2006).

Alkaline protease is a type 1 secreted zinc metalloprotease that is known for its degradation of host complement proteins and host fibronectin (Laarman *et al.*, 2012). In a murine model of sepsis, alkaline protease in combination with pseudomonal exotoxin A was prepared and administered as an inactivated toxoid vaccine and demonstrated statistically significant protection against subsequent infection by *P. aeruginosa* (Matsumoto *et al.*, 1998). Moreover, alkaline protease has been shown to interfere with flagellin signaling through host TLR5 by degrading free flagellin monomers and thereby helping *P. aeruginosa* to avoid immune detection (Bardoel *et al.*, 2011).

Pseudomonas aeruginosa produces two elastases, LasA and LasB, which are regulated by the *lasI* quorum-sensing system and secreted via type 2 secretion systems (Toder *et al.*, 1994; de Kievit & Iglewski, 2000). Most *P. aeruginosa* investigations reserve the term 'elastase' for LasB and 'staphylolysin' for LasA. This is because LasA, a serine protease, is able to hydrolyze the penta-glycine bridge required for peptidoglycan stabilization in the cell wall of staphylococci, but has only a fraction of the elastolytic abilities of LasB and rather is thought to enhance the proteolytic activity of LasB (Toder *et al.*, 1994; Matsumoto, 2004) . LasB has been observed to degrade the opsonizing lung surfactant proteins A and D (Mariencheck *et al.*, 2003). As a result, Δ*lasB* mutants are more susceptible to phagocytosis and are attenuated for virulence (Kuang *et al.*, 2011).

Protease IV is a serine protease that can degrade complement proteins, immunoglobulins, and fibrinogen. Injections of protease IV onto the cornea in a rabbit model of ocular infection caused erosion of the corneal epithelium,

while infection of corneas with a protease IV deficient strain showed reduced virulence (Engel *et al.*, 1998). Furthermore, protease IV degradation of host surfactant proteins A and D has been shown to inhibit the association of *P. aeruginosa* with alveolar macrophages, demonstrating a role for this protease in *P. aeruginosa* survival during infection (Malloy *et al.*, 2005).

Lipopolysaccharide

Lipopolysaccharide is a complex glycolipid that forms the outer leaflet of the outer membrane and has roles in antigenicity, the inflammatory response, exclusion of external molecules, and in mediating interactions with antibiotics (King *et al.*, 2009). *P. aeruginosa* produces a three-domain lipopolysaccharide consisting of a membrane-anchored lipid A, polysaccharide core region, and a highly variable O-specific polysaccharide (O-antigen or O-polysaccharide). The importance of lipopolysaccharide to the bacterium and to host pathology and antibiotic resistance has subjected it to intense study, and a great deal is now known about its biosynthesis and the contributions of its structural domains to the above observations. Due to space limitations, we have limited our discussion of lipopolysaccharide to lipid A and O-polysaccharide, the two components that contribute the most to *Pseudomonas* infections. For two excellent reviews on *Pseudomonas* lipopolysaccharide, we refer the reader to King *et al.* (2009) and Lam *et al.* (2011).

Lipid A

Lipid A is an atypical glycolipid that anchors the lipopolysaccharide into the outer membrane. Like the lipid A from other Gram-negative bacteria, *P. aeruginosa* lipid A is composed of a diglucosamine biphosphate backbone with *O*- and *N*-linked primary and secondary fatty acids. Structurally, the number, position, and nature of the linked acyl groups and the type of substituent to the phosphate groups can vary between isolates and can also arise due to growth conditions (Lam *et al.*, 2011). As the 'business end' of lipopolysaccharide, lipid A can be sequentially bounded by host cell coreceptors MD2 and CD14 leading to activation of the TLR4 to NFκB signaling pathway and triggering the production of pro-inflammatory cytokines and chemokines, inflammation, and eventually endotoxic shock (Teghanemt *et al.*, 2005; Akira *et al.*, 2006).

Modifications to lipid A can alter the bacterium's susceptibility to polymyxins and cationic antimicrobial peptides as well as change its inflammatory properties. Laboratory-adapted *P. aeruginosa* strains grown in rich medium exhibit penta- or hexa-acylated lipid A forms, which differ by the presence of a decanoic acid at the three-position. Penta-acylated species are predominant (*c.* 75%) in laboratory strains and in isolates from acute infections (King *et al.*, 2009). Conversely, isolates from chronically infected CF lungs demonstrate hexa- and sometimes hepta-acylated species with increased inflammatory properties, and the extent of these modifications appears to increase with the severity of lung disease (Ernst *et al.*, 2007). The increased inflammatory potency of these hyperacylated lipid A species is thought to be due to an alteration in the binding of lipid A to MD2 (Teghanemt *et al.*, 2005), while the addition of aminoarabinose in constitutive *phoQ* mutant strains also leads to more inflammatory lipopolysaccharide (Gellatly *et al.*, 2012). Similarly both types of changes and especially aminoarabinose addition to either or both phosphates can contribute to resistance to cationic antimicrobial peptides such as polymyxins (Ernst *et al.*, 1999). Indeed, as inhaled colistin is routinely administered to the lungs of patients with CF, it is of no surprise that an altered lipid A promoting resistance has been isolated from *Pseudomonas* from these patients (Ernst *et al.*, 2007; Miller *et al.*, 2011). Many lipid A modifications are regulated and can be induced as a response to an environmental change; for example, the addition of aminoarabinose can be triggered by binding to epithelial surfaces, the presence of antimicrobial peptides acting through the ParRS or CpxRS two-component regulatory systems (TCSs), or limiting (nonphysiological) Mg^{2+} acting through the PmrAB or PhoPQ two-component systems.

O-polysaccharide

In wild-type strains, the lipid A domain is attached to a conserved nine or ten sugar, branched oligosaccharide core. This lipid A-core can be further substituted in approximately 15% of lipopolysaccharide molecules in *P. aeruginosa* by O-polysaccharide ('O-antigen'). Two types of O-antigen can exist simultaneously within a given *P. aeruginosa* cell, and they are distinct structurally and serologically. A-band ('common') polysaccharide is a homopolymer of D-rhamnose approximately 70 sugars long and which elicits a weak antibody response. In contrast, B-band ('O-specific') polysaccharide is a strain-variable heteropolymer both in chain length and in the nature of the sugars, and this lipopolysaccharide elicits a strong antibody response and is the chemical basis for serotyping (King *et al.*, 2009). Some strains of *P. aeruginosa* produce no O-polysaccharide at all ('rough' strains), while others substitute the lipid A and core with only one O-saccharide unit ('semi-rough'). Interestingly, many chronic *P. aeruginosa* isolates lose their expression of the B-band polysaccharide (Hancock *et al.*, 1983) with the A-band polysaccharide becoming the dominant antigen over time. This may be driven by selective pressure for the bacteria to evade host adaptive immune responses by suppressing the more antigenic O-specific polysaccharide (King *et al.*, 2009).

Other virulence factors

A number of other virulence factors are secreted by *P. aeruginosa* and can contribute to its pathogenicity. Exotoxin A is an ADPRT that inhibits host elongation factor 2 (EF2) thereby inhibiting protein synthesis and leading to cell death. This inhibition of protein synthesis also likely leads to the repression of the host immune response as demonstrated by the decrease in cytokines released from whole blood stimulated with heat-killed *P. aeruginosa* in the absence of exotoxin A (Schultz *et al.*, 2000). Exotoxin

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A-producing strains show a 20-fold increase in virulence in a murine model compared with exotoxin A-deficient mutants (Miyazaki *et al.*, 1995). The toxic properties of exotoxin A have also been shown to induce host cell death by apoptosis, and for that reason, exotoxin A has been investigated as an immunotoxin that targets tumor cells for anticancer therapy (Wolf & Elsässer-Beile, 2009; Du *et al.*, 2010).

Lipases and phospholipases break down surfactant lipids and the phospholipids of host cell membranes (Kipnis *et al.*, 2006). Phospholipases have been shown to degrade surfactant, 90% of which is lipid, causing an increase in surface tension (Holm *et al.*, 1991). Hemolytic phospholipases are able to directly lyse human and sheep erythrocytes (Ostroff *et al.*, 1990).

The blue-green pigment pyocyanin gives *P. aeruginosa* colonies their distinct color and causes oxidative stress to the host, disrupting host catalase, and mitochondrial electron transport (Lau *et al.*, 2004). Purified pyocyanin has been shown *in vitro* to induce apoptosis in neutrophils as well as inhibit the phagocytosis of apoptotic bodies by macrophages (Lau *et al.*, 2004; Bianchi *et al.*, 2008). It is also able to modulate the expression of the chemokines IL-8 and RANTES by airway epithelial cells (Denning *et al.*, 1998) and suppress cilia beating. Along with rhamnolipids, the production of pyocyanin has been shown to be partly controlled by the oxidative stress RR, OxyR, and is therefore thought to play a protective role against the reactive oxygen and nitrogen species produced by phagocytic cells during infection (Lau *et al.*, 2005; Vinckx *et al.*, 2010).

Iron chelation is a vital part of establishing infections and the progression to a chronic infection, as the host environment has little free iron due to its own sequestration molecules such as lactoferrin and transferrin. The siderophore, pyoverdine, is both able to sequester iron from host depots and to act as a signaling molecule. Iron-bound pyoverdine interacts with the *Pseudomonas* cell receptor FpvA, and this complex in turn interacts with the antisigma factor FpvR, causing the upregulation of exotoxin A, endoprotease, and of pyoverdine itself (Jimenez *et al.*, 2012). Several other iron siderophore transport systems exist, enabling uptake of iron complexed with endogenous siderophores (e.g. pyochelin), host heme, or the siderophores of other microorganisms (e.g. enterobactin; Cornelis, 2010).

Antimicrobial resistance

Infections by *P. aeruginosa* are notoriously difficult to treat due to its intrinsic ability to resist many classes of antibiotics as well as its ability to acquire resistance. All known mechanisms of antibiotic resistance can be displayed by this bacterium (intrinsic, acquired, and adaptive); sometimes all within the same isolate (Table 2). Resistance rates are on the rise despite the use of combination drug therapies (Moore & Flaws, 2011). As few new drugs are available to combat *P. aeruginosa* infections, there has been a return to the use of older drugs such as polymyxins that had previously fallen out of favor due to wide reports of toxic side effects (Livermore, 2002). Despite the reports of nephrotoxicity and neurotoxicity, for patients with CF suffering recurrent infections of multidrug-resistant bacteria, colistin (a polymyxin drug) has for the past 15 years been routinely administered via inhalation (Falagas & Kasiakou, 2006), demonstrating that the antibiotic resistance problem has been influencing therapeutic choices for many years.

Intrinsic resistance is encoded in the microorganism's chromosome. In the case of *P. aeruginosa,* intrinsic resistance is due to the low permeability of its outer membrane, the constitutive expression of membrane efflux (Mex) pumps, and the natural occurrence of an inducible chromosomal β-lactamase, AmpC (Strateva & Yordanov, 2009). The outer membrane is a semi-permeable barrier that restricts the uptake of small hydrophilic molecules such as β-lactam antibiotics to the channels of porin proteins embedded within the outer membrane. It is estimated that the *P. aeruginosa* outer membrane is 10- to 100-fold less permeable than that of *Escherichia coli,* having fewer large channel porins (formed by OprF) and a number of small channel porins (formed by proteins such as OprD and OprB; Breidenstein *et al.*, 2011). Six resistance–nodulation–division (RND) family efflux pumps have been described

Table 2 Example resistance mechanisms in *Pseudomonas aeruginosa*

Mechanism	Resistance class	Example(s)
Efflux pumps	Intrinsic	MexAB–OprM, MexCD–OprJ, MexEF–OprN, MexXY–OprM (cephalosporins, carbapenems, aminoglycosides, quinolones, ureidopenicillins)
Outer membrane impermeability	Intrinsic	OprF, OprD, OprB (carbapenems, aminoglycosides, quinolones)
β-lactamases	Intrinsic	AmpC (penicillins)
Targeted mutation	Acquired	DNA gyrase, DNA topoisomerase (quinolones) MexZ (quinolones, cefapimes, aminoglycosides)
Horizontal transfer	Acquired	Metallo-β-lactamases, ESBLs (penicillins, cephalosporins, carbapenems)
Membrane changes	Adaptive	Lipid A modification (aminoglycosides, polymyxins) AmpC upregulation (penicillins)

ESBL, extended spectrum β-lactamase.

for *P. aeruginosa,* although 12 have been identified genetically (Schweizer, 2003). These efflux pumps can eject a wide range of antibiotics; for example, MexAB–OprM and MexXY–OprM can collectively efflux β-lactams, chloramphenicol, fluoroquinolones, macrolides, novobiocin, sulfonamides, tetracycline, and trimethoprim and aminoglycosides (Livermore, 2002; Schweizer, 2003). The β-lactamase, AmpC, is located in the periplasm and can efficiently hydrolyze several β-lactam antibiotics such as penicillins and cephalosporins. It is expressed at low levels but can be induced by subinhibitory concentrations of certain β-lactams. The resistance imparted by efflux pumps and AmpC is intricately connected to restricted outer membrane permeability, because the concentration of β-lactams in the periplasm is dependent on the efficiency and rate by which they are transported through the porins of the outer membrane (Jacoby, 2009).

Acquired resistance can be the result of the genetic transfer and subsequent expression of a resistance cassette taken up by the bacterium or it may be the result of mutations in targets or the genes, including regulators, which stabilize or enhance intrinsic resistance mechanisms (Breidenstein *et al.*, 2011). DNA elements such as plasmids and transposons can be passed among bacteria via conjugation, transformation, or transduction and can impart resistance to one or more antibiotics in the otherwise susceptible recipient. These elements can also reinforce the intrinsic resistance of *P. aeruginosa;* for example, the transfer and expression of a second β-lactamase can increase resistance to particular β-lactam antibiotics and/or increase the range of β-lactams that can be resisted. Acquired resistance can also occur when a mutational event in a regulatory gene causes dysregulation of a preexisting resistance mechanism. For example, like the natural inducers, which include aminoglycosides and other antibiotics targeting ribosomes, a mutation in *mexZ*, which normally suppresses expression of *mexXY*, leads to the overexpression of the MexXY efflux pump (Matsuo *et al.*, 2004). Mutations that result in alterations of an antibiotic's target can also confer resistance, for example where a mutation in DNA gyrase reduces the binding affinity of the enzyme for fluoroquinolones leading to resistance (Schweizer, 2003; Breidenstein *et al.*, 2011).

Adaptive resistance occurs when environmental conditions such as various stresses including exposure to subinhibitory antibiotic concentrations, or growth states such as biofilm formation, swarming or surfing motility or association with epithelial surfaces lead to increased resistance. These conditions cause a change in gene expression resulting in an upregulation of genes that can confer resistance as mentioned above (Breidenstein *et al.*, 2011). A well-known adaptive resistance mechanism in *P. aeruginosa* causes resistance to cationic antimicrobial peptides. Under specific inducing conditions (limiting Mg^{2+}, exposure to peptides and polymyxins and epithelial cell interaction), a variety of sensor kinases (SKs) including PhoQ, PmrB, ParS, CprS, and CbrA independently upregulate the expression of the *arnBCADTEF-udg* operon, which causes the synthesis and addition of aminoarabinose to lipid A (McPhee *et al.*, 2003, 2006). This modification lessens interactions of these cationic peptides with the outer membrane by reducing the negative charge of lipopolysaccharide. This effect is transient because susceptibility returns when the specific inducing conditions are reversed.

Pseudomonas aeruginosa: genomic context

The success of *P. aeruginosa* as an opportunistic pathogen is due substantially to the versatility and adaptability encoded in its genome. As of September 2012, 36 strains of *P. aeruginosa* from both clinical and environmental sources had been fully or partly sequenced according to the NCBI Entrez database. Compared with most other bacteria that cause disease, *Pseudomonas* has a relatively large genome, ranging from 6.22 to 6.91 Mb (Silby *et al.*, 2011). The sequencing of multiple strains has revealed that the genome is arranged as an assortment of conserved regions interspersed by 'regions of genomic plasticity' that contain genes unique to each strain (Mathee *et al.*, 2008). This has led to *P. aeruginosa* being described as having a 'core' genome, containing a conserved set of genes common to the species and comprising as much as 90% of the genomic content, and an 'accessory' genome, containing genes that are generally found in only a few strains. A key facet of the *P. aeruginosa* genome is the large number of paralogous genes that have arisen by genetic duplication, because evolved independently to create families of gene products that overlap functionally but which have discrete properties or are regulated differently. When coupled with the increased metabolic and functional diversity displayed by *P. aeruginosa*, it seems likely that the evolution of the *P. aeruginosa* genome arose from selective pressure for environmental adaptability (Silby *et al.*, 2011).

Pseudomonas aeruginosa is famously metabolically versatile and has been isolated from numerous nutrient-poor settings, including surfaces in medical facilities. A familiar anecdote among *Pseudomonas* scientists is that for any real or imagined hydrocarbon, there is a species of *Pseudomonas* that can catabolize it given oxygen or nitrite and sufficient time. *Pseudomonas* has a well-known preference for growth on tricarboxylic acid (TCA) intermediates over sugars in the laboratory setting (mediated through CbrAB/Crc/CrcZ), and reflecting this, the sequencing of strain PAO1 (the first strain to be sequenced) revealed *c.* 300 cytoplasmic transport systems and a substantial number of genes encoding enzymes predicted to be involved in β-oxidation of various carbon compounds (Stover *et al.*, 2000). The vast majority of these transport systems are for the import of nutrients and other small molecules. Several mono-, di-, and tri-carboxylate transport systems were identified, yet very few sugar transporters were revealed when compared with the intensely scrutinized *E. coli*, the most closely related bacterium that had been fully sequenced at the time.

Perhaps more astonishing than its metabolic diversity is the sheer number of regulatory genes that *P. aeruginosa* encodes. The sequencing of PAO1 predicted 521 genes

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encoding regulatory proteins, nearly 10% of its genome, a far higher proportion than sequenced bacteria with smaller genomes (Stover *et al.*, 2000). Analyses of other bacterial genomes have demonstrated that bacteria that can survive in diverse environments have a larger proportion of their genomes dedicated to regulatory proteins than bacteria that are specialized to survive in a specific environment. Many of the identified regulatory genes in *P. aeruginosa* belonged to the two-component class of regulatory systems, which allow the bacterium to rapidly adapt to an environmental change.

Many other systems were identified in *P. aeruginosa*, which gave insights into the pathogenicity and persistence of this bacterium. These included numerous intrinsic drug resistance and efflux systems, protein secretion systems, and virulence factors (Stover *et al.*, 2000). Perhaps more telling, 45.8% of predicted open reading frames (ORFs) contained genes for which no function could be assigned or predicted. While many of these share sequence homology to predicted genes of unknown function in other sequenced bacteria, the majority did not show homology to any previously sequenced gene. Over a decade after PAO1 was sequenced, only 153 of these unknown genes had been functionally characterized (Winsor *et al.*, 2005) although nearly 700 are listed as conserved hypotheticals.

Two-component regulatory systems

TCSs are one of the main regulatory families that are used by a bacterium to rapidly adapt to changes in its environment. In terms of pathogenesis, the success of the adaptation of *P. aeruginosa* from its normal soil or aqueous habitat to the hostile environment of the host is a remarkable feat, and much of this is controlled by TCSs. *Pseudomonas aeruginosa* has more TCSs than any other known bacterial pathogen, and several of these have been implicated in virulence and/or antibiotic resistance. One example is the previously mentioned GacA/LadS/RetS regulatory circuit's involvement in biofilm formation and virulence.

TCSs constitute a primitive signal transduction system and generally consist of a membrane-bound SK that detects an extracellular stimulus and a cytoplasmic RR that acts to affect cellular change. In the classical scheme of two-component signal transduction, the SK detects an external signal (e.g. through ligand binding), which causes a conformational change and autophosphorylation at a conserved histidine residue. The SK then transfers the phosphate group to a conserved aspartate on the N-terminal of the RR, thereby activating the regulator's C-terminal output domain, frequently a helix-turn-helix DNA-binding domain (Galperin, 2006; Gooderham & Hancock, 2009). The activated RR proceeds to alter the expression of particular genes to cause a response to the stimulus. This process is reversible, and dephosphorylation of the RR serves to return the cell to its previous state.

TCSs are diverse, and structural and functional modifications of this classical system exist. Hybrid SKs can contain multiple phosphodonor and phosphoacceptor sites and can promote multistep phosphorelay schemes that can include small histidine relay proteins, while not all RRs have DNA-binding effector domains (Stock *et al.*, 2000). The effector domain of a RR may function as an enzyme, an intermediary in a phospho-transfer reaction, or through interaction with other proteins. Further, small molecules such as acetyl phosphate can serve as phospho-donors to RRs (Stock *et al.*, 2000). It is also possible for multiple SKs to phosphorylate the same RR or for a single SK to phosphorylate several RRs, as is the case for chemotaxis, in which a single SK, CheA, phosphorylates two RRs, CheB and CheY (Li *et al.*, 1995), or in the quorum-sensing cascade of *Vibrio harveyi* where the SKs LuxN, LuxQ, and CqsS can each transfer the phosphate to LuxU (Jung *et al.*, 2011).

In *P. aeruginosa*, there are a predicted 64 SKs and 72 RRs, and most of them (50 systems) are arranged as cognate pairs in an operon. The rest are not physically linked to any other two-component gene and are termed 'orphans'; this physical separation makes it difficult to predict cognate pairings (Gooderham & Hancock, 2009). Several TCSs in *P. aeruginosa* have been identified as contributing to virulence (e.g. GacA–GacS), biofilm formation (e.g. WspR–WspE), and antibiotic resistance (e.g. PhoP–PhoQ; Gooderham & Hancock, 2009).

Significance and conclusions

Although our understanding of *P. aeruginosa* has advanced considerably over the last few years, this bacterium remains a scourge in hospitals, causing virulent and persistent infections despite antibiotic treatment. Given its ubiquitous habitat, metabolic versatility, and complex regulatory controls, it is unlikely that *P. aeruginosa* will ever be completely eliminated from hospital settings; hence, tried and true methods of prevention and early intervention are likely to remain the most effective methods of treatment for at least the foreseeable future. Increased understanding of *Pseudomonas* regulatory systems and their effect on biofilm dynamics and QS may allow us to find and exploit weaknesses in this particularly resilient mode of growth or adapt current treatment regimens to prevent the formation of biofilms or of adaptive resistance. Indeed, investigations have commenced to target various TCSs as a means of therapeutics (Stephenson & Hoch, 2002). Vaccine development, for example targeting flagella or of type 4 pili as antigens, may also allow us to prevent infection in those who are most at risk.

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