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Minireview

MINIREVIEW – Pathogens & Pathogenicity

The contribution of *Pseudomonas aeruginosa* virulence factors and host factors in the establishment of urinary tract infections

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One sentence summary: *Pseudomonas aeruginosa* causes highly resistant urinary tract infections. It produces an arsenal of virulence factors that aid the infection process. Understanding these is key to the development of novel therapeutics.
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ABSTRACT

Pseudomonas aeruginosa can cause complicated urinary tract infections, particularly in people with catheters, which can lead to pyelonephritis. Whilst some subgroups appear more susceptible to infection, such as the elderly and women, the contribution of other host factors and bacterial virulence factors to successful infection remains relatively understudied. In this review, we explore the potential role of *P. aeruginosa* virulence factors including phenazines, quorum sensing, biofilm formation and siderophores along with host factors such as Tamm-Horsfall protein, osmotic stress and iron specifically on establishment of successful infection in the urinary niche. *P. aeruginosa* urinary tract infections are highly antibiotic resistant and require costly and intensive treatment. By understanding the infection dynamics of this organism within this specific niche, we may be able to identify novel therapeutic strategies to enhance the use of existing antibiotics.

Keywords: *Pseudomonas aeruginosa*; urinary tract infections; virulence factors; biofilm; antibiotic resistance; phenazines

INTRODUCTION

Urinary tract infections (UTI) are the most common healthcare acquired infections and account for over 30% of all nosocomial infections (Klevens *et al.* 2007). UTIs can be classified as uncomplicated or complicated. Uncomplicated UTIs occur in patients with normal, healthy urinary tracts. Complicated UTIs occur in patients with structurally or functionally compromised urinary tracts, as seen in catheterised patients and patients suffering from pyelonephritis—bacterial ascension of the kidney. In uncomplicated UTI, *Escherichia coli* is the primary causative agent responsible for up to 80% of cases with other Gram-negative microbes such as *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* being less frequently detected (7%–15%). However, complicated UTIs are more frequently caused by uropathogenic (based on site of isolation) *P. aeruginosa*, which shows a higher prevalence of antimicrobial resistance and greater propensity to form biofilms on medical devices than *E. coli* or *K. pneumoniae* (Nicolle *et al.*, 2005). *P. aeruginosa* urine sample isolates from UTI patients in England are more likely to be resistant to carbapenems than either *E. coli or K. pneumoniae* (Ironmonger *et al.* 2015).

Approximately 13% of *P. aeruginosa* infections are caused by multidrug-resistant strains (CDC 2013) and nosocomial *P. aeruginosa* infections have been identified as a worldwide healthcare

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issue (Rosenthal *et al*. 2016). The World Health Organization has recently named *P. aeruginosa* as a target of the highest priority for the development of new antibiotics (WHO 2017). Infection by multidrug-resistant *P. aeruginosa* was associated with a 70% increase in cost per patient when compared to non-resistant infection (Morales *et al*. 2012), and catheter-associated UTIs (CAUTI) cause an estimated 900 000 extra hospital days per year in the USA (Warren 2001). Elderly populations are particularly prone to CAUTI in long-term care facilities, while women are more susceptible to UTIs in general (Nicolle, Strausbaugh and Garibaldi 1996; Foxman 2014). *P. aeruginosa* is often highly resistant to antibiotics. A study by Rizvi *et al*. (2011) showed that *P. aeruginosa* isolates from UTIs in India were highly resistant to fluoroquinolones, (ciprofloxacin 85.8% resistant and ofloxacin 80.0% resistant); however, no resistance to antibiotics such as imipenem was detected. High resistance to fluoroquinolones has also been reported in Poland, along with increased resistance to carbapenems (Pobiega *et al*. 2016). Many factors are responsible for the inherent resistance of *P. aeruginosa* to antimicrobials: a cell wall with low permeability, a large and adaptable genome, mobile genetic elements and the formation of biofilms (Lambert 2002). Some antibiotics manage to permeate the cell wall through porins e.g. carbapenems access through OprD porins. Loss of OprD porins can result in resistance to carbapenems (Livermore 2001; Lister, Wolter and Hanson 2009). Even antibiotics that manage to permeate the cell well of *P. aeruginosa* face being exported by one of the many efflux pumps present. Several antibiotic efflux systems have been described including MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexXY-OprM (Poole 2001). Mutations in *gyrA* change the DNA gyrase targeted by quinolones resulting in resistance (Akasaka *et al*. 2001). Of the estimated 51 000 healthcare-associated *P. aeruginosa* infections that occur in the USA every year, ∼13% of them are multidrug resistant (CDC 2013). A test of 32 *P. aeruginosa* isolates from UTIs found that 19% of the strains were multidrug resistant and growth in artificial urine media (AUM) enhanced antibiotic tolerance up to 6000-fold (Narten *et al*. 2012). Therefore, current clinical practices of evaluating antibiotic resistance may underestimate enhanced resistance conferred by the biofilm lifestyle and adaptation to conditions in the urinary tract.

The pathogenesis of uropathogenic *E. coli* (UPEC)-mediated UTI has been extensively characterised, but very little is known about the role of *P. aeruginosa* virulence factors and the interplay between bacteria and host factors found in the urinary tract.

P. aeruginosa is widely recognised as having an arsenal of virulence factors that help to facilitate successful infection and colonisation across a wide range of environments. For many bacteria, the urinary tract represents a harsh, nutrient-limited environment; however, the versatility and size of *P. aeruginosa* genomes result in an ability to exploit this niche. This review will summarise the importance of *P. aeruginosa* virulence characteristics in the context of UTIs and the potential role of some of these virulence factors is described in Table 1.

SIDEROPHORES

Siderophores are iron chelating compounds that are secreted by bacteria and aid survival, especially in iron-limited environments. Pyoverdine and pyochelin are siderophores produced by *P. aeruginosa* (Table 1). Pyoverdine is a group of green fluorescent compounds that represents the primary iron-uptake system in *P. aeruginosa*. Pyochelin has a lower affinity for iron compared to pyoverdine and has been proposed to be associated with sustained inflammatory responses identified in chronic infections (Cornelis and Dingemans 2013). Siderophores, particularly pyoverdine, are essential for virulence in many models of infection including lung infection and burn models (Visca, Imperi and Lamont 2007); however, their role in UTIs is relatively unclear. A study by Tielen *et al*. (2011) revealed that pyoverdine could be detected in all 30 isolates from UTIs and CAUTIs that were investigated and iron-limited conditions have been suggested to be important in UTIs (Mittal *et al*. 2008a). Furthermore, microarray and qRT-PCR studies of other Gram-negative microbes causing UTIs have shown dramatic upregulation of siderophore-related genes *in vivo* in humans and mice compared with *in vitro* growth in LB, suggesting that iron acquisition may also be essential in this niche (Snyder *et al*. 2004; Hagan and Mobley 2009; Hagan *et al*. 2010). However, clinical isolates from the respiratory tract have also been found to produce pyoverdine and therefore this is not a niche-specific trait (Huston *et al*. 2004).

TOXINS

P. aeruginosa can produce a variety of toxins, including four type III toxins: Exoenzyme (Exo) S, ExoU, ExoT and ExoY (Fig. 1). *In vitro* expression of these toxins from isolates has been identified in *P. aeruginosa* isolates from various infection settings, particularly from acute infections (Hauser *et al*. 2002). Exo S is an effector protein of the type III secretion system and functions as an ADP-ribosylating enzyme (Iglewski *et al*. 1978). Levels of Exo S were significantly higher *in vitro* in *P. aeruginosa* isolates from wound and UTIs when compared to tracheal isolates (Hamood, Griswold and Duhan 1996) and increased with persistent infection. Infection isolates isolated longitudinally produced higher levels of Exo S, regardless of the site of infection (Hamood, Griswold and Duhan 1996; Rumbaugh, Griswold and Hamood 1999) suggesting a role for this enzyme in persistence. ExoU is a cytotoxin secreted by the type III secretion system. ExoU has phospholipase A2 activity and also impairs the recruitment of phagocytes (Diaz *et al*. 2008). The presence of ExoU has been identified in isolates from the urinary tract (Pobiega *et al*. 2016); however, nothing is known about the potential role of ExoU in UTIs and the roles of ExoT and ExoY are shown in Table 1.

EXOPOLYSACCHARIDES AND BIOFILM FORMATION

The ability of *P. aeruginosa* to form biofilms is an advantage in many infection situations and greatly enhances its ability to resist antibiotics and harsh environmental conditions. Exopolysaccharides, extracellular DNA (eDNA), pyocyanin, rhamnolipids and functional proteins are all factors that contribute to the formation of *P. aeruginosa* biofilms. Alginate, Pel and Psl are three polysaccharides produced by *P. aeruginosa*. High levels of alginate are commonly seen in cystic fibrosis isolates and these alginate overproducing strains are classified as mucoid. However, isolates from UTIs produce significantly lower levels of alginate *in vitro* when compared to isolates from various body sites (Ciragil and Söyletir 2004; Tielen *et al*. 2011; Rawat and Prasad 2015).

In non-mucoid strains, there is a greater reliance on Pel and Psl. Psl is important in surface attachment of biofilms *in vitro* but there is functional redundancy between Pel and Psl (Colvin *et al*. 2012). Biofilms formed by *P. aeruginosa* in a murine model of CAUTI did not require exopolysaccharides (Cole *et al*. 2014). It has been proposed that another secreted virulence

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factor, pyocyanin, binds to, and intercalates with, eDNA thereby increasing the viscosity of DNA solutions (Das *et al.* 2015). This may promote biofilm formation via this route in the urinary tract (Fig. 2). Pyocyanin can lead to the production of reactive oxygen species (ROS) and impaired wound healing (Fig. 1). 5-Me-PCA, a precursor to pyocyanin, has even greater redox potential than pyocyanin and could be even more helpful to cells through theoretically supporting ATP generation in combination with electron donors such as succinate in the anoxic locations of the biofilm (Sakhtah *et al.* 2016).

Rhamnolipids promote microcolony formation in the early development of biofilms and in the late stages aid structural

Table 1. Potential role of *P. aeruginosa* virulence factors in UTIs.

Virulence factor	Characteristics	Potential Role in UTIs	References
LasA	Staphylolytic zinc metallopeptidase of the M23A family; has reduced elastolytic activity compared to LasB; enhances elastolytic activity of LasB	Aid breakdown of host tissues (including elastin in the urinary tract) which could facilitate invasion and/or amino acid metabolism	Spencer *et al.* (2010) Cowell *et al.* (2003)
LasB	Zinc metalloprotease with the foremost elastolytic activity; necessary for activation of LasA	Biofilm formation; immunomodulation; Aid breakdown of host tissues (including elastin) and which could facilitate invasion and/or amino acid metabolism	Cathcart *et al.* (2011) Yu *et al.* (2014) van der Plas *et al.* (2016) Cowell *et al.* (2003) Golovkine *et al.* (2014)
Phospholipase A	Has activity that releases fatty acids from phospholipid substrate whereas phospholipase C releases phosphate esters; found commonly in UTI isolates	Could be implicated in apoptosis of host cells; possible generation of ROS	Steinbrueckner *et al.* (1995) Tielen *et al.* (2011) Kirschnek and Gulbins (2006)
Phospholipase C	Haemolytic (plcH) and non-haemeolytic (plcN) versions; Both hydrolyze phosphatidylcholine; plcH hydrolyzes sphingomyelin and phosphatidylcholine; plcN hydrolyzes phosphatidylserine and phosphatidylcholine	Haemolytic activity could aid iron availability in the iron scarce urinary tract	Ostroff, Vasil and Vasil (1990)
Phospholipase D	Secreted by H2 Type VI system; implicated in bacterial competition, chronic infection and eukaryotic cell invasion	Could aid persistence and/or invasion in the urinary tract	Russell *et al.* (2013) Wilderman *et al.* (2001) Jiang *et al.* (2014)
ExoS	Bifunctional type-III cytotoxin; almost never found in strains expressing ExoU; disrupts actin cytoskeleton; ADP-ribosylates broader range of host proteins than ExoT	Levels increase over time in UTIs, could aid persistence and immune evasion	Barbieri and Sun (2004) Engel (2003) Engel and Balachandran (2009)
ExoT	Bi-functional type-III cytotoxin; induces mitochondrial apoptosis in host cells; disrupts actin cytoskeleton	Could aid immune evasion	Barbieri and Sun (2004) Wood *et al.* (2015) Engel and Balachandran (2009)
ExoU	Type-III toxin; extremely cytotoxic phospholipase; almost never found in strains expressing ExoS	Some UTI isolates found with ExoU and low cytotoxicity; ExoU may serve other function or be a hindrance	Engel (2003) Tielen *et al.* (2011) Engel and Balachandran (2009)
ExoY	Type-III toxin with adenylate cyclase disrupting actin cytoskeleton; enhances production of the second messengers cGMP and cUMP in host cells	Unclear	Engel and Balachandran (2009) Beckert *et al.* (2014) Yahr *et al.* (1998)
Exotoxin A	Toxin Inhibits eukaryotic protein synthesis via ADP ribosylation of elongation factor 2 which can lead to cell lysis; stimulates inflammation and hepatotoxicity in animals; positively regulated by iron starvation and ToxR	Could aid immune evasion; stimulation of inflammation in the kidney could aid persistence	Pastrana *et al.* (2005) Morimoto and Bonavida (1992) Chiu *et al.* (2009) Walker *et al.* (1994) Gaines *et al.* (2007)
Alkaline protease	Type I secreted zinc metalloprotease; degrades host immune complements C1q, C2, C3 and cytokines IFN-γ and TNF-α	Increase iron availability via breakdown of transferrin; enhance amino acid metabolism via protease activity; aid immune evasion	Laarman *et al.* (2012) Shigematsu *et al.* (2001) Kim *et al.* (2006)
Pyoverdine and pyochelin (siderophores)	Main mechanisms for iron uptake and therefore survival in many environments. Pyoverdine displays high levels of diversity and the highest affinity for iron. Pyochelin has lower affinity but has been implicated in chronic infection and is association with pyocyanin	Urine, particularly in the bladder, is a low iron environment and siderophores would facilitate bacterial growth	Cornelis and Dingemans (2013)

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Table 1 *Continued*

Virulence factor	Characteristics	Potential Role in UTIs	References
Alginate	An *O*-acetylated linear polymer of D-mannuronate and L- guluronate residues (Evans and Linker 1973). Alginate overproduction (mucoidy) has been associated with chronic infection isolates from the CF lung. Alginate contributes to biofilm architecture but is not essential for biofilm formation (Stapper *et al.* 2004)	Although alginate plays a role in biofilm formation, the contribution of alginate in the urinary tract is thought to be minimal	Evans and Linker (1973) Stapper *et al.* (2004)
Pyocyanin	Type-II secreted, redox-active zwitterion; cytotoxic; blue at physiological pH	May impair ability of urothelial cells to repair and cause pain and urinary urgency in infection; induce inflammation	Hall *et al.* (2016) McDermott *et al.* (2012) McDermott *et al.* (2013)

development that depends on cell migration (Pamp and Tolker-Nielsen 2007). Rhamnolipids rather than affecting motility are responsible for the initiation of migration—termed seeding dispersal (Wang *et al.* 2013). Rhamnolipid expression is upregulated under iron-limited environments and correlates with increased surface motility and the formation of flat, unstructured biofilms (Glick *et al.* 2010). Furthermore, rhamnolipid-deficient *P. aeruginosa* cannot maintain the fluid-filled channels which are purported to aid nutrient diffusion through densely populated mature biofilms (Davey, Caiazza and O'Toole 2003). Since iron is thought to be limited in the urinary tract during infection, these qualities suggest that rhamnolipids could aid persistence of uropathogenic *P. aeruginosa* and enable ascension of the urinary tract. Rhamnolipid production could therefore be a key mediator of bacterial persistence in UTIs caused by *P. aeruginosa* (Fig. 2).

An operon of six genes (*fapABCDEF*) which encodes amyloid-like fimbriae (ALF) was found in *P. aeruginosa* and other pseudomonads including *P. fluorescens* (Dueholm *et al.* 2010). While the ALF in this strain were structurally similar to curli fimbriae purified from *E. coli*, the repeating 37 amino acid motifs found in FapC, the major subunit of the Fap fibril, were found to be distinct from those of *E. coli* curli. When the *fap* operon was expressed in *E. coli*, it resulted in an aggregative phenotype, whereas the control strain of *E. coli* remained planktonic. Orthologues to the genes of the fap operon were found in *P. aeruginosa* (Dueholm *et al.* 2010) and overexpression of this operon resulted in increased biofilm formation (Dueholm *et al.* 2013). The amyloid formed by the fap operon makes individual cells more resistant to drying, more hydrophobic and increases biofilm stiffness (Zeng *et al.* 2015). The hydrophobicity of amyloids enables binding of pyocyanin and the quorum-sensing molecules, 2-heptyl-3-hydroxy-4(1H)-quinolone and N-(3-oxododecanoyl)-l-homoserine lactone (Seviour *et al.* 2015). Fap proteins have yet to be studied in relation to uropathogenic *P. aeruginosa*, but may play an important role—especially considering the diminished role of exopolysaccharides in UTI isolates.

OTHER SECRETED ENZYMES

The vast majority of *P. aeruginosa* isolates are proteolytic and elastolytic (Nicas and Iglewski 1986). Proteolytic and elastolytic proteins are believed to contribute to the virulence and pathogenicity of *P. aeruginosa* (Fig. 1). The elastolysis-deficient mutant PAO-E64 demonstrated lower virulence than its PAO1 parent strain in a burned-mouse infection model (Nicas and Iglewski 1986) and rat-agar bead model of lung infection (Woods *et al.* 1982). From PAO-E64, the genes *lasA*, *lasB* and *aprA*, coding for LasA, elastase and alkaline protease, respectively, were implicated in proteolysis and elastolysis. LasA, like elastase and alkaline protease, has been shown to have its own elastolytic activity (Toder *et al.* 1994). Both *lasA* and *lasB* are transcriptionally regulated by *lasR* (Toder, Gambello and Iglewski 1991). In a study of tracheal, urinary tract and wound infections, high levels of elastase were seen in isolates studied *in vitro* from every site (Hamood, Griswold and Duhan 1996) suggesting that production is also beneficial during UTIs. The inability of a mutant PAO1 strain to colonise renal tissue in a mouse model was attributed to the mutant's inability to produce protease, elastase and rhamnolipid (Gupta, Gupta and Harjai 2013).

P. aeruginosa has a haemolytic and non-haemolytic version of phospholipase C (PLC)—PlcH and PlcN, respectively (Ostroff, Vasil and Vasil 1990). Elevated levels of PlcH were detected in 100% of CF patients with chronic *P. aeruginosa* infections (Hollsing *et al.* 1987). PLC levels *in vitro* were higher in *P. aeruginosa* isolates from UTIs, when compared to isolates from burns, wounds, CF sputum, pneumonia sputum and blood (Woods *et al.* 1986). However, in a study limited to isolates from tracheal, urinary tract and wound infections, PLC was highly produced at every site (Hamood, Griswold and Duhan 1996) and a more recent study rarely observed PLC (Tielen *et al.* 2011). While it is unclear whether there is a definitive role for PLC during UTI, it seems plausible that PLC-mediated red blood cell lysis and liberation of haem may provide a route for increased iron acquisition by bacteria, though further study is needed.

QUORUM SENSING

P. aeruginosa QS has been studied extensively and is currently proposed to consist of four interlinked QS systems: *las*, *rhl*, *pqs* and *iqs* (Lee and Zhang 2015). This has been reviewed recently (Lee and Zhang 2015). QS signal molecules (acyl-homoserine lactones, AHL) are expressed constitutively. Once a threshold level of signal molecule, indicative of cell density, has been reached, coordinated gene expression is induced. In *P. aeruginosa*, ~10% of the genome is thought to be under the complex control of QS.

The *rhl* QS system is important in *P. aeruginosa* UTI pathogenesis with a PAO1 knockout of the *rhl* regulator, *rhlR*, showing significantly reduced virulence in a mouse model which could be restored through the addition of C4 homoserine lactone (Gupta, Harjai and Chhibber 2016). Bacteriological and histological assessment of mice infected with Δ*rhlR* PAO1 showed reduced

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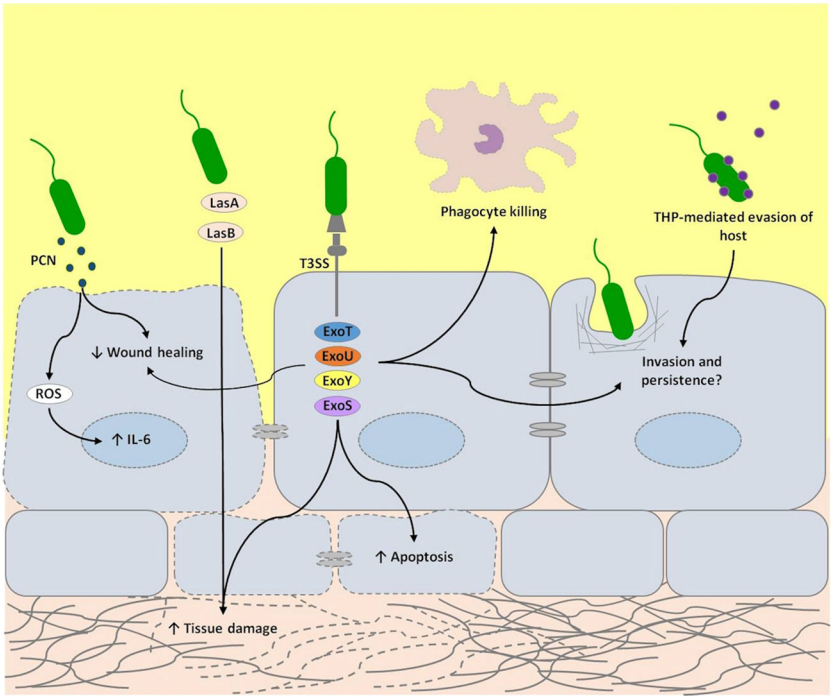

Figure 1. Schematic representation of urothelium–*P. aeruginosa* interactions and their effects on host cell physiology in urinary tract infections. *P. aeruginosa* possesses a broad array of virulence factors that facilitate colonisation and propagation of infection. *Pseudomonas aeruginosa* is known to express 4 type III secretion system (T3SS) effector proteins (ExoS, ExoU, ExoT and ExoY) which are delivered into host cells during infection. ExoU facilitates phagocyte killing and in conjunction with ExoT is known to activate pro-apoptotic pathways and delay wound healing in other epithelial cell types. ExoS may aid persistence and immune evasion via cytoskeletal disruption, enabling *P. aeruginosa* to act as an intracellular pathogen. While ExoY is also known to cause actin cytoskeletal disruption, its specific role in UTI is unclear. Other secreted virulence determinants such as proteolytic and elastolytic proteins, LasA and LasB, are known to cause tissue damage and facilitate persistence in chronic infections. *P. aeruginosa* produces several phenazines that enhance competitiveness in polymicrobial infections. One such example, pyocyanin (PCN), is found in higher levels in urinary isolates than other infections and reduces urothelial cell viability via a mechanism thought to be regulated by induction of oxidative stress via generation of ROS which increase transcription of IL-6. PCN causes urothelial cell senescence which may play a role in impaired wound healing in chronic infections. THPs are also implicated as a secreted host factor that enhances the virulence of *P. aeruginosa* in CAUTI, enhancing persistence and immune evasion.

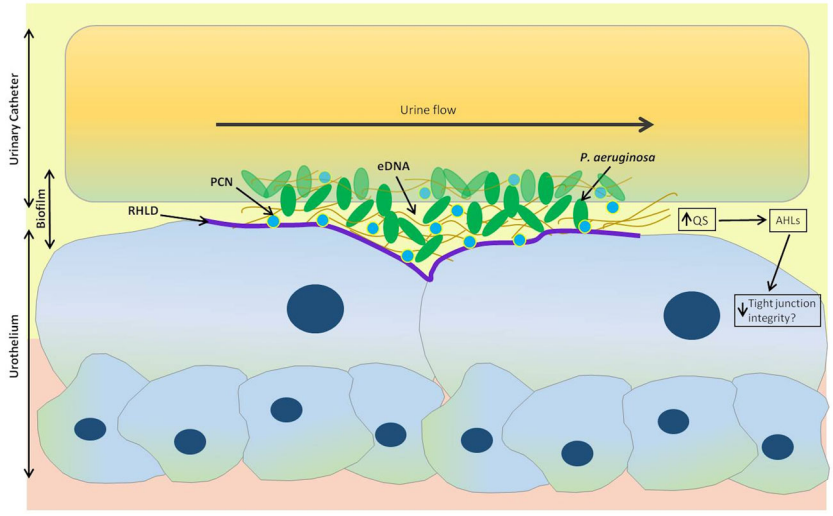

Figure 2. Proposed model for biofilm formation of *P. aeruginosa* capable of causing UTIs and CAUTIs. *P. aeruginosa* is capable of causing infection both in the presence and absence of a urinary catheter. Pyocyanin (PCN) is thought to facilitate cross-linking of eDNA leading to increased viscosity that facilitate initial biofilm formation. Secreted rhamnolipids (RHLD) promotes microcolony formation early in biofilm formation and aids structural integrity as development progresses. Within the biofilm, increased levels of QS occur and the signalling molecules, AHLs, may interact directly with the urothelium to decrease the integrity of tight junctions.

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bacterial load in the bladder and kidneys at days 1, 3 and 5 post-infection compared to PAO1-infected control mice. Furthermore, *in vitro* the *rhlR* mutant showed significantly reduced expression of elastase (55.17%), protease (12.72%) and rhamnolipid (12.67%), essential virulence factors in *P. aeruginosa* pathogenicity, compared to wild-type PAO1 (Pearson, Pesci and Iglewski 1997). In an acute pyelonephritis model of infection, a QS-deficient mutant (double *lasI rhlI* knockout) showed reduced inflammation and polymorphonuclear cell infiltration compared to the wild-type strain (Gupta *et al* 2013). QS activity was detected in all isolates from UTIs using a broad plate-based assay and may suggest the importance of a functioning QS system in UTIs (Tielen *et al*. 2011). QS mutants are frequently found in *P. aeruginosa* isolates from the respiratory tract (Heurlier *et al*. 2005). *Pseudomonas aeruginosa* with a defective QS system (double *lasI rhlI* knockout) has been shown to produce an altered, flatter biofilm (Davies *et al*. 1998) which may have an impact on the ability of the bacterium to form surface-attached CAUTI. *P. aeruginosa* biofilms from 14 out of 14 CAUTI isolates produced AHL when grown on catheters in a simple, physical model of a bladder and *in vitro* (Stickler *et al*. 1998).

In addition to the presence of an active QS system in *P. aeruginosa* isolates, QS signal molecules themselves induce renal tissue inflammation and cytokine response (Gupta, Chhibber and Harjai 2013) and affect the integrity of tight junctions in human epithelium (Vikström *et al*. 2009) although the effect on urothelium directly has not been studied (Fig. 2).

MOTILITY

Motility can be an important factor in allowing *P. aeruginosa* to colonise and exploit new niches. *P. aeruginosa* displays three types of motility: swimming, swarming and twitching. Swimming involves the rotation of a single polar flagellum. Swimming, through flagella movement, has been linked to triggering neutrophil extracellular traps (Floyd *et al*. 2016) and phagocytosis by neutrophils (Lovewell, Patankar and Berwin 2014), the first line of defence in human infections. Twitching motility involves the extension and retraction of type IV pili and therefore play a vital role in bacterial attachment and initial colonisation, particularly on mucosal cell surfaces (Hahn 1997). In addition, type IV pili can further contribute to virulence and bacterial adaptation through the mediation of pili-dependent phage infection (Davies *et al*. 2016). Swarming motility requires multicellular coordination of bacteria across semi-solid (viscous) surfaces, including mucosal sites. The complex behaviour has been linked to increased antibiotic resistance and large shifts in bacterial gene expression (Overhage *et al*. 2008). The role of motility and down-regulation of flagellin during chronic infection has been studied in isolates from CF lung infections (Mahenthiralingam, Campbell and Speert 1994); however, the role in UTIs is not known. Tielen *et al*. investigated swimming, twitching and swarming motility in *P. aeruginosa* isolates from UTIs. Over 90% of isolates displayed swimming activity and over 70% displayed twitching motility. The most variation in motility was seen in swarming. The ability to swarm was increased in isolates associated with UTIs compared to CAUTIs. Within the CAUTIS isolates, swarming was higher in acute CAUTIS compared to chronic CAUTIs. This may suggest that swarming motility is associated more with acute infection compared to chronic infection; however, the total number of isolates in this study was relatively limited (Tielen *et al*. 2011).

P. aeruginosa isolates from the urinary tract can produce a plethora of virulence factors; however, many of these studies have studied the isolates under *in vitro* conditions, and therefore the importance in vivo must be viewed with caution. In addition to known virulence factors, *P. aeruginosa* genomes carry an abundance of hypothetical proteins with unknown functions. It is possible that some of these may play important roles in infections and therefore there is much work to be performed to unlock this wealth of information.

HOST FACTORS

Certain patient groups display greater susceptibility to UTIs, and there is an increased risk associated with being female and either very young or elderly. Complicated UTIs are often associated with structural and functional abnormalities of the urinary tract. In addition to the risk factors above, genome wide association studies have identified several genes associated with increased risk to UTIs including mutations in DSTYK, HSPA1B, CXCR1, CXCR2, TLR2, TLR4 and TGFB1. However, these have been associated with UTIs in general and not *P. aeruginosa* specific.

In addition to host genetics, the urinary tract environment also plays a role in bacterial infections.

Tamm-Horsfall protein

Tamm-Horsfall protein (THP), also known as uromodulin, is a polymeric glycoprotein that can bind to a variety of surfaces by n-linked and o-linked glycans and is encoded by the *UMOD* gene (Pennica *et al*. 1987; Kumar and Muchmore 1990; Serafini-Cessi, Malagolini and Cavallone 2003). THP is produced in the thick ascending limb of the loop of Henle in the kidneys and is the most abundant protein in human urine. While most investigations of THP's potential role in preventing UTIs focus on *Escherichia coli*, these observations may also translate to other Gram-negative uropathogens such as uropathogenic *P. aeruginosa*, given their overlapping virulence profiles and proposed mechanisms of pathogenicity (Fig. 1). Studies suggest that bacterial clearance of *E. coli* from the urinary tract may be mediated by binding of type-1 fimbriae to THP mannose moieties, thus preventing fimbrial adhesion to mannose-rich uroplakin Ia and Ib glycoproteins found in the uroepithelium. However, the precise molecular interactions between THP and type 1 fimbriae are unclear. (Pak *et al*. 2001). These *in vitro* observations are further supported by *in vivo* studies of homozygous $THP^{-/-}$ knockout mice which are more vulnerable to bladder colonisation by type 1 fimbriated *E. coli* (Bates *et al*. 2004). While type 1 fimbriae are not found in *P. aeruginosa*, other adhesive filaments such as type IV pili, are known to be important regulators of *E. coli* colonisation in mouse models of UTI and thus their presence in *P. aeruginosa* may contribute to UTI pathogenesis (Subashchandrabose *et al*. 2013). The identity of the specific bacterial adhesins and host receptors that mediate adhesion of *P. aeruginosa* to epithelial cells in the urinary tract via type IV pili are unclear. However, *P. aeruginosa* pili are known to bind to glycolipids expressed in epithelial cells with specificity towards the Galβ1–3GlcNAc and Galβ1–4GlcNAc residues (Ramphal *et al*. 1991; Sheth *et al*. 1994; de Bentzmann *et al*. 1996; Comolli *et al*. 1999). THP has been shown to be an excellent ligand for Galβ1–4GlcNAc active lectins, suggesting that THP may bind directly to type-IV pili and prevent *P. aeruginosa* adhering to host cells in a similar manner to that shown for type 1 fimbriae, albeit via a different glycoprotein (Wu *et al*. 1995).

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While laboratory studies suggest that THP may have a protective role in preventing UTI, clinical findings in CAUTI patients may refute this evidence. Studies of both latex and silicone catheters removed from 20 patients had bound THP, with elevated levels detected on catheters colonised with bacteria compared to culture negative samples. Given that silicone and latex catheters with bound THP facilitated binding of *E. coli* and *P. aeruginosa* (Raffi *et al.* 2009), this raises the possibility that catheters prevent THP-mediated elution of uropathogens thereby promoting UTIs.

The presence of THP at 50 μg ml^{-1} *in vitro* resulted in increased *P. aeruginosa* virulence factor production (protease, elastase, PLC, alginate, pyoverdin, pyochelin) *in vitro* compared to a control grown in the absence of THP. However, a further increase from 50 to 70 μg ml^{-1} resulted in a significant fall in measured virulence factor production suggesting that the effect is concentration dependent (Mittal *et al.* 2006). An increased renal load of *P. aeruginosa* was detected when the bacteria were coated with THP along with decreased adherence and killing of the coated bacteria by murine peritoneal macrophages (Harjai *et al.* 2005). In the event that *P. aeruginosa* manages to colonise the urinary tract (or the kidney where THP is membrane bound), THP could aid *P. aeruginosa* in evading the host immune system and increasing virulence (Hawthorn, Bruce and Reid 1991).

The normal range of excreted THP is 9.3–35.0 mg day^{-1} in males and 9.0–36.3 mg day^{-1} in females. The mean amount of THP excreted was significantly less in females (15.2 $\pm$ 1.6 mg day^{-1}) than in males (21.3 $\pm$ 1.2 mg day^{-1}) (Glauser *et al.* 2000). In a large study of community-living elderly patients, those with the highest urinary THP concentrations (upper quartile) were significantly less likely to develop a UTI than those with the lowest urinary THP concentrations (lower quartile) (Garimella *et al.* 2016). Small molecular weight mannosides that act in a similar way to THP, by inhibiting the FimH adhesin of *E. coli* used for attachment in type 1 fimbriated *E. coli*, have shown promise at combatting UPEC in a murine UTI model (Cusumano *et al.* 2011). Future studies that characterise the molecular interactions that mediate type IV pilus binding to bladder cells and THP will lead the way for development of similar small molecule inhibitors that can disrupt binding of uropathogenic *P. aeruginosa* and thus prevent UTI.

Iron

Bacteria attempting to grow in the urinary tract experience conditions with very little iron (Shand *et al.* 1985). Growth of *P. aeruginosa* in iron-depleted medium decreased phagocytosis while enhancing adherence to uroepithelial cells and expression of all the major virulence factors compared to growth in iron-replete medium (Mittal *et al.* 2008a). Infection of mice with *P. aeruginosa* grown under iron-depleted conditions resulted in higher renal, bladder and urine bacterial counts as well as more extensive tissue damage compared to infection with *P. aeruginosa* grown under iron-replete conditions (Mittal *et al.* 2008a). To better understand how *P. aeruginosa* adapts to growth in the urinary tract, *P. aeruginosa* was grown anaerobically in AUM and analysed at a systems level using transcriptomics, proteomics, metabolomics and enzyme activity analyses (Tielen *et al.* 2013). A total of 86 genes related to iron acquisition were active and upregulated when grown anaerobically in AUM compared to growth in 10-fold diluted LB broth (Tielen *et al.* 2013).

Osmotic stress

The human urinary tract experiences highly variable osmotic concentrations. Increasing the osmolarity of a growth medium from 100 to 300 mOsmol L^{-1} resulted in enhanced growth and virulence while further increase of osmolarity to 350 mOsmol L^{-1} resulted in significant reduction in growth and virulence of *P. aeruginosa* (Mittal *et al.* 2009). Typical osmolarity of human urine ranges from 500 to 800 mOsmol L^{-1}. The aforementioned study suggests that there is a range of osmolarity that may predispose patients to UTI but further research is needed. Sequencing of a small colony variant of *P. aeruginosa* isolated from a chronic CAUTI highlighted the presence of several genes involved in osmotic stress protection such as a sodium/hydrogen ion antiporter (*nhaA*) and a compatible solute glycine betaine transporter (PAMH27_5169 to PAMH27_5171) (Tielen *et al.* 2014).

OUTLOOK

The rise in antimicrobial-resistant organisms is alarming, and therefore there is a need to develop treatment strategies that minimise the selective pressures applied. In the case of uropathogenic *P. aeruginosa*, this could involve a focus on clearing bacteria from the urinary tract rather than killing them outright. However, this would still select for bacteria that could avoid clearance and survive in the urinary tract niche. A more realistic but challenging strategy is the development of new therapeutic targets and the appropriate combination of existing therapies to reduce selection for bacterium resistant to individual therapies.

Sublethal doses of antibiotics have been shown to enhance the formation of *P. aeruginosa* biofilms (Bagge *et al.* 2004; Hoffman *et al.* 2005). The vast majority of antibiotics are designed with planktonic bacteria in mind. In the future, there needs to be a greater focus on targeting biofilms. Pyocyanin and eDNA make enticing therapeutic targets because of their aforementioned importance in promoting biofilm formation in UTI isolates of *P. aeruginosa*. Addition of 250 and 500 μM of the antioxidant glutathione decreased the intercalation of pyocyanin with DNA in a DNA–pyocyanin mixture (Das *et al.* 2015). Confocal microscopy showed that the biofilms of PA14 had significantly lower surface coverage when grown in the presence of DNase I (Das *et al.* 2015), and therefore combining these biofilm inhibitors with traditional antibiotics may enhance treatment outcomes.

Oxidative stress occurs when damaging free radicals outnumber antioxidants and can cause tissue damage. Pyocyanin has been shown to induce oxidative stress in human endothelial cells by the generation of hydrogen peroxide. This depletes levels of glutathione, an antioxidant, and increases oxidative stress (Muller 2002). Urethral infection of mice with *P. aeruginosa* resulted in increased levels of reactive nitrogen intermediates, ROS and tissue damage while reducing antioxidant capacity. Levels of oxidative stress, and associated biomarkers, were reduced when infected mice were treated with the antioxidant N-acetylcysteine (Mittal *et al.* 2008b). There are many other antioxidants that could be used to treat uropathogenic *P. aeruginosa* which have shown promise in treating other uropathogens (Allameh and Salamzadeh 2016).

Many other novel therapeutic strategies are under development including small molecule inhibitors, phytochemicals, antibody-based therapy, bacteriophage therapy, photodynamic therapy, antimicrobial peptides, enzyme-based therapy and nanoparticles (Sharma *et al.* 2014). Through understanding the factors that are important for virulence within the urinary tract,

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it may be possible to develop effective treatments aimed at reducing *P. aeruginosa* pathogenesis within this understudied niche.

CONTRIBUTIONS

JWN, RVF and JLF contributed equally to the conception, planning and writing of this manuscript.

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