The University of Liverpool

Pseudomonas aeruginosa Pathogenesis in Urinary Tract Infections

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Abstract

Urinary tract infections (UTIs) cause high morbidity, mortality and economic burden. *Pseudomonas aeruginosa* can cause persistent UTIs and it has many virulence factors including quorum sensing systems and iron acquisition mechanisms. Furthermore, this pathogen is highly resistant to antibiotics and has been highlighted by the World Health Organisation as a pathogen for which there is critical need for new antimicrobials.

I have characterised *P. aeruginosa* UTI isolates from both the UK and Kuwait. The UTI isolates were variable in terms of their phenotypic characteristics and genomic composition. Multiple resistance genes linked to resistance to different classes of antibiotics including aminoglycosides, fluoroquinolones and β-lactams were identified from the sequence data using the CARD database, particularly in the isolates from Kuwait. These isolates were pan-resistance and multidrug resistance, with the ability to spread resistance via horizontal gene transfer.

Laboratory media are commonly used to study bacteria *in vitro*. Nutrient rich media does not accurately reflect the physiological environment, prompting the development of multiple media mimicking human body fluids such as Artificial Urine Medium (AUM). My results show that bacterial responses in LB and AUM were highly different. AUM and urine showed similar responses with regards to the increase in proteins associated with iron acquisition mechanisms and of the decrease in proteins associated with the Type III secretion system. However, differences were observed in other important pathways such as those involved in phenazines production. This suggests that AUM is a more reflective media than LB to study *P. aeruginosa* UTI isolates, particularly when the environment needs to be highly controlled/consistent, such conditions cannot be controlled in urine. The difference in the protein abundance profile in AUM suggest that further optimisation of the medium could be performed for better resemblance of urine.

Hormones are regulatory substances that are produced by the host and transported around the body. Sex-related hormones, oestrogen (oestradiol), progesterone and testosterone are examples of these. Previous studies have indicated that sex

hormones such as oestradiol may modulate *P. aeruginosa* mucoidy and alter innate immune responses in females. In this study, treatment with all three hormones decreased the abundance of *P. aeruginosa* proteins associated with iron acquisition mechanisms. Furthermore, individual hormones also showed specific bacterial changes such as increased protein abundance in pqs quorum sensing mechanism by oestradiol compared to testosterone and progesterone. This study suggests that *P. aeruginosa* pathogenesis may be variable in UTI infections based on the host hormonal profile. Understanding the individual role of host factors could allow us to fully understand the contribution of host components and the impact these may have on infection susceptibility and outcome. Therefore, further research is required into the role of host components, in particular sex hormones, in host-pathogen interactions.

Declaration

This thesis is the result of work performed mainly within two institutions: The Institute of Infection & Global Health under the supervision of Dr Jo Fothergill and the Institute of Translational Medicine under the supervision of Dr Rachel Floyd, who now resides at the Institute of Integrative Biology. All of these institutions are part of the University of Liverpool.

Some of the work presented in this dissertation was carried out in collaboration with others. Dr Christina Bronowski and John Newman assisted with the biofilm data in chapter 4. Sequencing data in chapter 3 was conducted by Dr Sam Haldenby in the Centre of Genomic Research at the University of Liverpool. Microscopy experiments was setup by the assistance of Dr Marco Marcello. Dr Dave Mason provided assistance with microscopy analysis and Jennifer Adcott with technical aspects of imaging at The Liverpool Centre for Cell Imaging. Dr Stuart Armstrong provided initial analysis of the proteomic data in chapter 4 and 5.

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List of abbreviations

°C	Celsius
PQS	2-heptyl-3-hydroxy-4-quinolone
3-oxo-C12HSL	3-oxo-C12HSL
A. baumannii	*Acinetobacter baumannii*
AAC	Aminoglycoside acetyltransferase
ABU	Asymptomatic bacteriuria
aEPEC	atypical enteropathogenic *Escherichia coli*
AHL	Acyl homoserine lactones
AMR	Antimicrobial resistance
ANT	Aminoglycoside nucleotidyltransferase
APH	Aminoglycoside phosphoryl transferase
AprA	Alkaline protease
AST	Antibiotic susceptibility testing
AUM	Artificial urine medium
BCG	Bacillus Calmette-Guerin
BHL	N-butyrylhomoserine lactone
C. elegans	*Caenorhabditis elegans*
CAD	Coronary artery disease
CAP	Cationic antimicrobial peptide
CARD	Comprehensive Antibiotic Resistance Database
CAUTI	Catheter Associated infections
C-di-GMP	Cyclic-di-GMP

CF	Cystic fibrosis
CG	Choriogonadotropin
CGR	Centre for Genomic Research
CHDL	Carbapenem-hydrolysing class D β-lactamases
CO	Carbon monoxide
COPD	Obstructive pulmonary disease
CRP	C-reactive protein
CV	Crystal violet
DARTS	Affinity Responsive Target Stability
DEHE	Hormone dehydroepiandrosterone
DGC	Diguanylate cyclase
DMPA	Depot medroxyprogesterone acetate
dNTPS	Deoxynucleotides
DPN	Diarylpropionitrile
dsDNA	Double stranded DNA
E. coli	*Escherichia coli*
ECF σ	Extracytoplasmic sigma factor
ECM	Extracellular matrix
eDNA	Extracellular DNA
ELISA	Enzyme-linked immunosorbent assay
EPS	Extracellular polymeric substances
ESBL	Extended-spectrum β-lactamases
ERα	Estrogen receptor α
ERβ	Estrogen receptor β

EUCAST	European Committee on Antimicrobial Susceptibility Testing
FIM	Florence imipenemase
FL	Fluoroquinolones
FN	Fibronectin
FSH	Follicle stimulating hormone
GCC	Gulf corporation council
GES	Guiana extended spectrum
GnRH	Gonadotropic releasing hormone
HCN	Hydrogen cyanide
HPI	High pathogenicity island
HRT	Hormone replacement therapy
IBC	Intracellular bacterial communities
IC	Interstitial cystitis
ICU	Intensive care unit
IL	Interleukin
IMC	Isothermal microcalorimetry
IMP	Imipenamases
IN	Inner membrane
KdoI and KdoII	3-deoxy-D-manno-octulosonic acid
KPC	*K. pneumoniae* carbapenemases
l-ARG	l-arginine
LB	Luria Bertani
L-Dab	L-diaminobutyrate
LESB	Liverpool Epidemic Strain

LH	Luteinising hormone
LOH	late-onset hypogonadism
LPS	Lipopolysaccharide
MAC	*Mycobacterium avium* complex
MBL	Metallo-beta-lactamases
MDCK	Madin-Darby Canine Kidney
MDR	Multidrug-Resistant
MIC	Minimum inhibitory concentration
MPP	Methyl-piperidino-pyrazole
MPS	Mucopolysaccharide
NDM	New Delhi metallo-β- lactamase
NET	Extracellular net traps
NGS	Next Generation Sequencing
NIH	National Institutes of Health
NK	Natural killer cells
OES	Other epidemic strains
P. aeruginosa	*Pseudomonas aeruginosa*
P. mirabilis	*Proteus mirabilis*
PAMP	Pathogen associated molecular pattern
PASP	*P. aeruginosa* small protease
PBP	Penicillin binding proteins
PBS	Phosphate buffered saline
PCA	Principle component analysis
P-CAD	Premature coronary artery disease

PDE	Phosphodiesterase
PES	Prairie Epidemic Strain
PHE	Public Health England
PMN	Polymorphonuclear leukocytes
PPT	Propyl-pyrazole-triol
PR-A	Progesterone receptors A
PR-B	Progesterone receptors B
QS	Quorum sensing
RGB	Regions of gene plasticity
RND	Resistance nodulation division
ROS	Reactive oxygen species
S. aureus	*Staphylococcus aureus*
S. saprophyticus	*Staphylococcus saprophyticus*
SA-β-gal	Senescence-associated β-galactosidase
SDC	Sodium deoxycholate
SERS	Surface-enhanced Raman scattering
SNP	Single nucleotide polymorphism
SPM	San Paulo metallo-β- lactamase
T3SS	Type III secretion system
TBDR	TonB- dependent receptor
TBDT	TonB-dependent transducers
TBE	Tris-borate-EDTA
TE	Thioesterase module
TFA	Trifluoroacetic acid

THP	Tamm-Horsfall protein
TLR	Toll-like receptor
TNF-α	Tumour necrosis factor α
UK	United Kingdom
UPEC	Uropathogenic *E. coli*
UPIa	Uroplakin Ia
USA	United States
UTI	Urinary tract infection
VIM	Verona integron-encoded metallo- β-lactamases
VUR	Vesico-uretric reflexes
WGS	Whole Genome Sequencing
WHO	World Health organisation
XDR	Extended-spectrum β-lactamases

Chapter 1

1 General introduction

1.1 Urinary tract: Anatomy and Function

The urinary tract is comprised of the urethra, bladder, two ureters and two kidneys. Urine is formed by the process of tubular filtration and secretion in the nephrons of the kidneys and then the diluted urine is transported to the bladder via two ureters [1]. In the bladder, urine is stored, and its composition maintained until expulsion from the body through the urethra. The bladder wall consists of three layers of smooth (involuntary) muscle tissue which promotes voluntary voiding of urine. Both inner and outer muscles are arranged longitudinally, however, the middle layer has a circular arrangement [2]. This muscular structure is covered by the peritoneum, a serosal membrane which covers the outermost surface [3]. Additionally, the bladder is a hollow structure lined with the uroepithelium which is considered the main protective barrier between the musculature, urine and causative agents of urinary tract infections (UTIs) [4]. The lamina propria divides the urothelium from the musculature and is comprised of an extracellular matrix containing multiple structures/cell types such as blood vessels, fibroblasts, adipocytes, and nerve endings [5]. The urothelium is known to play secretory roles by secreting compounds such as prostaglandins, nitric oxide, and acetylcholine [6]. The urothelium expresses purinergic receptors and discharges active chemicals such as ATP in response to chemical, mechanical and thermal stimuli [7].

1.2 Urinary tract infections

1.2.1 Prevalence, classification and diagnosis

UTIs are some of the most widespread infections in healthcare and community settings worldwide, with around 150 million people infected per annum [8], [9]. In the United States, UTI infections resulted in 10.5 million outpatient visits and an additional 2-3 million emergency department (ED) visits in 2007 [10]–[12]. In the period between 2006-2009, over 10.8 million ED visits were recorded with 16.7% amongst those admitted acute care hospitals in United States [13]. The economic burden is devastating, as 3.5 billion USD is spent on treating UTIs and related

complications. UTIs are the most common reason for prescribing antibiotics in Western Europe and the United States of America [14]. Children, older men and women of varying age groups are at higher risk of contracting UTIs [8]. The majority of UTIs are relatively easily treated. However, some infections can lead to complications such as bacteraemia, sepsis and death [8], [12].

UTIs are clinically classified into two groups: uncomplicated or complicated. The latter is used to describe UTI patients with structural or neurological abnormality [12]. This is caused by several factors that compromise the function of the urinary tract and the immune system [15]. These factors include the presence of indwelling catheters and renal calculi [8]. In addition, urinary obstruction, urinary retention, pregnancy and renal transplantation exacerbate complicated UTIs [12], [15]. In the absence of these complications the infection would be classified as uncomplicated [16], [17]. Infections may occur in the bladder, in the lower part of the urinary tract (cystitis) or in the upper part of the tract in one or both kidneys (pyelonephritis) [15], [16]. Cystitis symptoms may include some or all of the following symptoms; dysuria, haematuria and suprapubic tenderness, while flank pain and fever can be signs of acute pyelonephritis [18]. Risk factors include genetic susceptibility, female gender, obesity, previous UTI, vaginal infection, sexual engagement and diabetes [12], [15]. UTI infection may be resolved or can become recurrent due to either the persistent presence of the same uropathogen or re-infection with a different one [19].

Presence of bacteria in the bladder is known as bacteriuria, which is divided into asymptomatic (ABU) and symptomatic/significant [18], [20]. Historically, colony forming units above 10^5 per ml in two clean-catch samples of urine in women is considered clinically significant, with only one sample needed from men [18]. In catheter-associated urinary tract infection (CAUTI),a CFU $\geq 10^3$ derived from the catheter is considered positive [18]. Immune responses to UTI can be measured by the presence of an elevated count of polymorphonuclear leukocytes (PMNs) in urine (e.g. ≥10 PMNs per mm^3 of urine), clinically defined as pyuria [21]. Presence of symptomatic bacteriuria and pyuria is indicative of a UTI, without significant bacteriuria count, pyuria could be associated with kidney stones or malignancy (Table 1.1) [18].

Table 1.1 Common terminology used in clinical settings for UTI related diagnosis

Clinical classification	**Description**
Cystitis	Inflammation of the bladder
Urethritis	Inflammation of the urethra
Acute urethral syndrome	Symptoms of cystitis with significant Bacteriuria
Acute pyelonephritis	Kidney inflammation
Asymptomatic bacteriuria	Isolation of bacteria at significant counts without symptoms
Recurrent urinary tract infection	Persistence of infection or reinfection at least three times within 12 months
Pyuria	Presence of white blood cells in urine
Dysuria	Discomfort in urination, stinging and burning pain
Haematuria	Presence of excessive number red blood cells in urine

1.2.2 Studying uropathogenic bacteria

To study the events that lead to infection and progression in UTIs, cell culture-based assays have been used extensively [22]. These assays are comprised of immortalised, primary and cancer cell lines derived from the urothelium of the bladders or the proximal tubule epithelial cells of the kidneys [22]. The UTI mouse model has been involved heavily in the last two decades in understanding events leading up to invasion and colonisation of the bladder and kidneys during UTIs [23]. These models are considered as a good animal model to study host-pathogen interactions in humans. This is due, partly, to similar bladder structure and cellular composition between humans and mice [23]. Additionally, Type I pilus, a uropathogenic

Escherichia coli (UPEC) virulence factor can mediate attachment to the uroepithelium in both of humans and mice [23], [24]. A variety of Inbred mice can be utilised to reflect the genetic heterogeneity in humans that may result in individuals susceptible to the development of chronic infections [25]. Briefly, the process of developing a UTI in mice consists of multiple steps; female mice with different susceptibility to UTIs are anaesthetised followed by delivering the uropathogen of interest via a catheter into the mouse bladder. Collection of urine and blood samples from mice are utilised as a means of monitoring the infection. The mice are sacrificed at different time points depending on the experimental design. Bladders, kidneys, blood and urine are harvested or homogenised in preparation for analyses such as bacterial counts, measurement of leukocyte population, serum cytokines and microscopy [23]. One of the main microorganisms used in these studies is UPEC.

Several infectious agents are implicated in UTIs including fungi, viruses, Gram-positive and Gram-negative bacteria (Table 1.2). An overview of the key causative agents is given in the next page.

Table 1.2 Common aetiology of uncomplicated and complicated UTI infection, in descending order of prevalence in the United States [8]

Uncomplicated infection	Prevalence	Complicated infections	Prevalence
Escherichia coli	75%	*Escherichia coli*	65%
Klebsiella pneumoniae	6%	*Enterococcus spp*	11%
Staphylococcus saprophyticus	6%	*Klebsiella pneumoniae*	8%
Enterococcus spp	5%	*Candida* species	7%
Group B *Streptococcus*	5%	*Staphylococcus aureus*	3%
Proteus mirabilis	2%	*Proteus mirabilis*	2%
Pseudomonas aeruginosa	1%	*Pseudomonas aeruginosa*	2%
Staphylococcus aureus	1%	Group B *Streptococcus*	2%

1.2.3 UTI causing bacteria

1.2.3.1 *Escherichia coli*

Escherichia coli (E.coli) is a Gram-negative bacteria and the most common causative agent amongst uncomplicated and complicated UTIs, causing around 65 to 90% of infections (Table 1.2) [8], [12]. The bacterium resides as part of the gastrointestinal flora as a facultative anaerobe. The pathogen colonises the periurethral and vaginal areas prior to bladder invasion by using adhesive appendages or adhesins. By utilising an arsenal of virulence factors such as adhesins, toxins, flagella, polysaccharides and iron-acquisition mechanisms, it can also ascend to the kidneys, which can result in more complications [26]. Four phylogroups (A, B1, B2 and D) have been identified based on the their pathogenicity islands and virulence factors, most UPEC isolates belong to phylogenetic group B followed by group D [26], [27]. Multiple reference

UPEC strains are utilised to study a host-pathogen interactions such as UTI89 and CFT073 [28], [29].

Despite the increasing rates of resistance to antibiotics by UPEC [30], treatment is generally easier compared to other uropathogens such as *Pseudomonas aeruginosa,* since most UPEC are sensitive to a large number of classes of antibiotics which can clear the infection. These include the carbapenem, imipenem (up to 100% clearance success rate), ertapenem (99.98%), the aminoglycoside amikacin (99.94%), and the nitrofuran nitrofurantoin (99.91%) [26].

1.2.3.2 Klebsiella pneumoniae

Klebsiella pneumoniae is a Gram-negative encapsulated bacterium that is generally found in the flora of the skin, mouth and intestines [31]. It is involved in a range of infections such as UTIs, soft tissue infections, pneumonia and bacteraemia in susceptible individuals [32]. In UTIs, *K. pneumoniae* is the second most common causative pathogen of invasive bloodstream infections due, in part, to the action of several key virulence factors [33]. *K. Pneumoniae* produces a polysaccharide capsule that surrounds the outer membrane with 70 known different antigenic types which facilitate immune evasion. The key function of the capsule is to neutralise phagocytosis in the host [34]. The importance of other virulence factors such as fimbriae, liposaccharides, urease, siderophores and efflux pumps has been demonstrated in a variety of infections [35]. Carbapenem-resistant *K. pneumoniae* have been listed as a pathogen with critical priority in the development of new antimicrobials by the World Health organisation (WHO) [36].

1.2.3.3 *Proteus mirabilis*

Proteus Mirabilis is a member of Enterobacteriaceae family and is a Gram-negative, facultative anaerobe [37]. The bacterium contributes to 1%-2% of uncomplicated UTIs and 5% of complicated infections following catheterisation [38][39]. This uropathogen is known for its ability to utilise swarming motility and hyper-flagellar movement as a prominent virulence factor [40]. CAUTI biofilm formation is enhanced by potent urease activity, which leads to crystalline biofilm formation and could act as a protective armour [41], [42]. Upon bladder invasion, the pathogen can form

stones and consequently ascend to the kidneys and develop kidney stones followed by bloodstream invasion [43].

1.2.3.4 *Enterococc*i Spp

Enterococci are Gram-positive, catalase negative, facultative anaerobes [44]. Enterococci are members of the gut flora, they can cause nosocomial infections in the urinary tract, wounds and the bloodstream [45]. About 5% of uncomplicated UTIs and 11% in complicated infections are caused by enterococci, ranked second only to *E. coli* [8], [18]. *Enterococcus faecalis* and *Enterococcus faecium* are the two common causative agents in UTI and amongst all enterococcal associated infections [46]. The latter is intrinsically resistant to penicillins and can be vancomycin-resistant, prompting the WHO to classify it as a ''High priority'' bacteria in need of urgent development of new treatment options [36].

1.2.3.5 *Pseudomonas aeruginosa* in UTIs

P. aeruginosa are Gram-negative motile rods that have one polar flagellum [47]. This bacterium is an opportunistic pathogen that infects immunocompromised people or those with a breach in normal bodily defences. *P. aeruginosa* is a versatile pathogen that can infect all tissues and systems of humans such as the urinary tract, respiratory tract, bone, joint, soft tissue (burns) and dermatitis [48]. The bacteria also infect susceptible patients suffering from conditions such as cystic fibrosis (CF), cancer and acquired immunodeficiency patients (AIDS) [49]–[51]. The ability to exist in nosocomial settings poses a major challenge to healthcare systems around the world. The WHO has declared *P. aeruginosa* as one of the most critical pathogens in need of urgent new treatments such as antibiotics [36]. *P. aeruginosa* is ubiquitous in soil and water and can be found in biofilms on surfaces and substrates or as a swimming planktonic cell [48]. At 37°C, the bacteria can grow optimally but can also grow at a variety of temperatures including up to 42°C [52]. The bacterium is intrinsically resistant to many antibiotics, can resist weak antiseptics and high concentrations of salts and dyes. *P. aeruginosa* has been found growing in distilled water demonstrating its ability to grow with minimal growth requirements [52].

P. aeruginosa is not found in significant numbers in the gut or vaginal microbiomes in healthy individuals [18]. Thus, the primary route to the urinary tract occurs in

nosocomial settings, UTIs account for 40% of hospital acquired infections [47]. The percentage of UTIs attributed to *P. aeruginosa* is estimated at 12.0%, indicating that the pathogen is proficient at causing these infections [47]. The presence of patients in hospitals for more than 30 days increases the risk of acquiring UTIs by almost 100% [53]. Infections caused by CAUTI *P. aeruginosa* are usually known to be severe, persistent and antibiotic resistant [54]. *P. aeruginosa* isolates are more likely to be carbapenem resistant than any other uropathogens according to recent studies in England [55]. Insertion of catheters pave the way for progression of uropathogens by comprising defences of the bladder due to fibrinogen accumulation on the catheter [8], [56]. Consequently, *P. aeruginosa* invades the periurethral space in order to initiate infection. They then ascend to the urethra and subsequently to the bladder where they can further ascend to the kidneys via the ureter to colonize it [57], [58]. Without medical intervention, the uropathogen can cross the tubular epithelial cell barrier and cause bacteraemia and potentially sepsis [8]

1.3 Initiation of *P. aeruginosa* UTI

1.3.1 Adhesion

Adherence is a major pathological step in invasion and colonisation of the host. The epithelial layers of the human host are protected by fibronectin (Fn), a large extracellular matrix, dimeric adhesive glycoprotein [59]. Fn is present during pathological changes and wound healing of the epithelium [60]. The protective coating can induce phagocytosis, though it can be lost during stress and illness [61]. *P. aeruginosa* is also capable of adhering to mucin, which is a glycoprotein that provides protection on mucous membranes [62]. The mucin layer acts as a primary defensive barrier. Its role is to resist adherence of uropathogens and harmful elements in urine [63]. The bladder contains a mucopolysaccharide (MPS) layer with anti-adherence bacterial properties [64]. Epithelial damage is thought to occur after extracellular bacterial multiplication and biofilm formation [8]. The main adhesins in *P. aeruginosa* are the flagella and type IV pili, the pili are considered the most important adhesin [48], both structures will be discussed in detail in section 1.3.3 and 1.3.4, respectively.

1.3.2 Motility

P. aeruginosa possess at least three types of motility mechanisms, reflecting its ability to survive in different niches. It utilises a single polar flagellum to swim in aqueous environments and in low-agar (<0.4%) medium, cellular movements respond to repellents and attracts via chemotaxis [65]. Movement by extension and retraction is known as twitching motility, in which type IV pili are required [66]. Both types of movements can be used to move across abiotic surfaces [67]. Swarming motility is the third distinctive type, *P. aeruginosa* propagates across semisolid surfaces in a multi-factorial process depending on flagella, pili, rhamnolipids and amino acids [68]. In 2008, a fourth type of motility was discovered in PAO1 flagella- type IV pili double mutants and was given the name sliding motility. The movement is similar to swarming since it responds to the same environmental conditions and regulatory proteins [69]. Further discussion of the role of motility will take place in chapter 3.

1.3.3 Flagella

P. aeruginosa tend to have one polar flagellum that aids in motility and chemotaxis functions; mainly enabling swimming in liquids and some surfaces [70]. The flagella consist of three components; the basal body embedded into the cell surface, a curved short hook and a long external filament [71]. This organelle is anchored in the cell by several other proteins that help stabilize it and consequently, improve its chances of nutrient acquisition [71]. 50 genes are involved in synthesis, preserving and executing these functions, highlighting its significance to the bacterium for adaptation and survival. These genes are often highly conserved across bacterial species [72]. Genes such as *fleQ, fleS, fleR, flgM, fliA* and *fleN* are clustered in three separate regions on the chromosome. These encode multiple proteins that are involved in control and regulation of flagella [65] . Changes in flagellar motility are essential for the lifestyle shift from planktonic cells to sessile forms that could ultimately lead to biofilm formation [73]. Multiple flagellar genes (excluding *fliC)* were shown to facilitate binding to respiratory mucin [74]. Cyclic-di-GMP (C-di-GMP) is a secondary messenger that attaches to FleQ, a master regulator, resulting in suppression of flagella genes and promoting the expression of exopolysaccharides Psl and Pel [75], [76]. Thus, C-di-GMP is inversely correlated with flagellar motility. The switch from

flagellar movement to biofilm formation is also observed in CAUTI as reported by Cole *et al* (2016) upon investigating the role of C-di-GMP in biofilm formation [77]. Briefly, the study utilised a murine CAUTI model, which was infected by strains overexpressing either diguanylate cyclases (DGCs) (generators of cellular C-di-GMP), or by phosphodiesterases (PDEs) that remove C-di-GMP from the cell. The strains which overexpressed the DGCs were more capable of infecting the bladder and disseminating to the kidneys compared to wild-type strains. In contrast, strains overexpressing PDEs exhibited attuned ability to infect the bladder and progress to the kidneys compared to *P. aeruginosa* wildtype. These results indicate that C-di-GMP is key in the switch to a sessile life-style typical in CAUTIs [77].

Flagella have been demonstrated as a key virulence factor. Experiments in a burned mouse model demonstrated that strains lacking flagella were less virulent than their flagellated counterparts [78]. Global deficiency in flagella and pili was attributed to loss of *rpoN* gene which led to attenuated mutants unable to colonise and persist in a rat mucosal model [79]. *P. aeruginosa* flagella are a virulence factor and also highly immunogenic. It has therefore been investigated as a vaccine target [80]. Flagellin subunits within the flagellar complex are classified as pathogen associated molecular pattern (PAMP) which activate innate immunity via toll-like receptor 5 (TLR5) in the lung [81], [82]. The resulting inflammatory response contributes to *P. aeruginosa* eradication and may lead to induction of extracellular net traps (NETs) [83]. Isolates from CF patients are often flagella- deficient suggesting that loss may aid evasion of the immune system to gain a survival advantage in chronic respiratory infections [84].

1.3.4 Pili

Pili are filamentous appendages that allow bacterial cells to adhere to cell surfaces [85].The appendages are comprised of pilin, a 15 kDa protein encoded by the *pilA* gene [86]. Five distinct groups of *pilA* have been identified so far in *P. aeruginosa* strains [85]. Pilins are regulated by PilS and PilR proteins, a two component regulatory system [86]. The number of mapped genes involved in regulating pilin activity is 40. Similar to flagella, PilA has been investigated as a vaccine target. Hertle *et al* (2001) showed that an immune response was mounted against the variable C-termini of the

protein via asialo-GM1 receptor on epithelial cells [87]. *P. aeruginosa* isolates with mutated *pilA* may have a selective advantage based on a reduced immune response.

Type IV pili are the most significant and extensively studied type in *P. aeruginosa* and can promote biofilm formation and twitching motility [88]. The latter is a distinctive type of motility in which extension, tethering and retraction of the pilus occurs [89]. Type IV pili associated motility enhanced the virulence of *P. aeruginosa* in keratitis isolates in a mouse model. In UTI isolates, a study by Tielen *et al* (2011) showed that 70% of isolates can perform twitching motility, suggesting a potential role in infection and biofilm formation [90].

1.3.5 Outer membrane proteins

The outer membrane plays a crucial role in survival and pathogenicity of *P. aeruginosa.* It acts as a selective permeability barrier [91]. The outer membrane proteins contribute to many facets of growth and development of the cell, such as maintaining the integrity of the cell and affecting the passage of nutrients across it [92]. *P. aeruginosa* produces as many as 100 outer membrane proteins with variable structures and functions such as OprF, OprD and TonB-dependent receptor [93]. This is exemplified by porins which were defined by Henderson *et al* (2016) as 'the outer membrane β-barrel proteins which allows the passage of solutes or contribute to the envelope stability' [94].

Porins also contribute to antimicrobial resistance, loss of *oprD* was found to be the main mechanism of resistance to imipenem in *P. aeruginosa* isolates obtained from sputum, urine, blood and tissue [95]. OprF porin has been studied extensively. It is involved in biofilm formation, maintenance of the integrity of the cell membrane, adhesion to mammalian proteins, and outer membrane vesicle biogenesis [92]. To assess the impact of *oprF* deletion on full virulence, Fito-Boncompte *et al,* (2011) conducted a comparative phenotypic analysis between PAO1, an isogenic *oprF* mutant, and an *oprF*-complemented strain. These strains were used to infect four infection models: the plant *Cichorium intybus,* the worm *Caenorhabditis elegans,* glial cells of rats and human Caco-2/TC7. The absence of *oprF* impacted the function of several virulence factors. Namely, impaired adhesion to animal cells, attenuated

production of ExoS and ExoT, and impaired quorum sensing dependent virulence factors such as exotoxin A, pyocyanin, lectin PA-1L and elastase [96].

1.3.6 Lipopolysaccharides

The cell wall of Gram-negative bacteria contains an endotoxin known as lipopolysaccharide (LPS) which is a major cell wall component [97]. LPS is present in the outer membrane of the cell wall, which is part of the cell wall along with inner membrane (IN) and peptidoglycan [98]. The structure of LPS is comprised of three distinct regions; a hydrophobic lipid region, a core oligosaccharide, and a repeating O-linked oligosaccharide. The molecular weight of LPS is over 10,000 D [98]. *P. aeruginosa* LPS contains a basic lipid A structure which is comprised of an N- and O-acylated diglucosamine bisphosphate backbone [4-P-β-D-GlcpNII-(1→6)-α-D-GlcpNI-(1→P], the primary acyl groups can be variable chemically [99]. Most strains of *P. aeruginosa* utilised in laboratory settings produce a penta-acylated LPS (75%) and smaller subset of strains (25%) produce hexa-acylated LPS [99]. The difference in both isoforms is that penta-acylated LPS does not possess an O-linked 3-hydroxy decanoic acid (10:0(3-OH)) group at position 3 of the first glucosamine [99].

The second LPS domain is called the core oligosaccharide, which is classified into the inner and outer core[100]. The inner core is comprised of sugar components, which in turn, is comprised of two residues of 3-deoxy-D-manno-octulosonic acid (KdoI and KdoII) and two residues of L-glycero-D-manno-heptose (HepI and HepII) [101]. The outer core, on the other hand, is comprised of one D-galactosamine (GalN), one L-Rha, and three or four D-glucose (GlcI–GlcIV) residues [102].

The third major component of LPS is the O-antigen, on which the serotype of *P. aeruginosa* is based upon [102]. Structurally, the O-antigen consists of N-acyl derivatives of rhamnose and different amino sugars to make up a compound known as monosaccharides [103].

The interaction between the *P. aeruginosa* LPS and the CF lung has been an area of interest. CF isolates express either smooth O-side chains (long) or rough side chains (few or none). Alteration from smooth to rough variants occurs in the CF lung and is linked to switching from serum sensitivity to serum resistance [104]. Ernst *et al,*

(2003) reported that during CF infection, *P. aeruginosa* modify its lipid A structure in response to different environmental conditions. As an example, a novel hepta-acylated variant was found in a subset of *P. aeruginosa* clinical isolates obtained from CF patients with severe pulmonary disease [105].

As in other gram-negative bacteria, the lipid A region of the LPS is targeted by TLR4 upon recognition by PAMPs, these are part of the innate immune defences of the host [106]. *P. aeruginosa* alteration of its lipid A structure modulates a strain dependent host inflammatory response [105].

1.4 Virulence Factors

1.4.1 Quorum sensing associated virulence factors

1.4.1.1 Quorum sensing

Many bacterial species can form interactive communities capable of communicating via chemical signals. This process is known as quorum sensing (QS) [107]. This is achieved by gene regulatory mechanisms that are highly dependent on the density of bacterial cell populations [108]. The regulatory mechanism is controlled by secreted signalling molecules which are low molecular weight compounds. These compounds are referred to as auto-inducers and act as activators depending on the concentrations of the signal [109]. Crucially, the QS system contains 3 major elements known as QS synthase, the signal molecule and the signal receptor [110]. When cell density is low, the signalling molecules are at a low level and generally diffuse away without reaching a critical threshold level. At higher cell densities, the signalling molecules reach the threshold level thus activating downstream gene expression [109]. This process of QS allows the co-ordination of bacterial communities which can adapt/respond to environmental changes. It has been shown that in *P. aeruginosa*, QS can control expression of about 10% of the entire genome [111]. QS systems are extremely important for bacteria since they control aspects such as virulence factors, swarming motility as well as efflux pump activity that can promote antibiotic resistance [112].

P. aeruginosa contains a sophisticated QS system where four interconnecting signalling systems (las, rhl, pqs and iqs) are involved in orchestrating bacterial

responses to environmental changes (Figure 1.1) [109]. In the las system, lasI encodes the synthase enzyme which produces an auto inducer signal, 3-oxo-dodecanoyl homoserine lactone (3-oxo-C12HSL). The 3-oxo-C12HSL binds and activates LasR. The discovery of the las system was unravelled by the identification of LasR as a key regulator of *lasB* gene, which encodes the metalloprotease elastase [113]. Further discoveries revealed that LasR is essential in the transcription of other virulence factor genes such as *lasA and toxA* expression [113]–[115]. In the rhl system, the signal molecule is butyryl homoserine lactone (C4-HSL) [116]. C4-HSL is produced by the *rhlI* synthase which acts on the *rhlR* transcriptional activator [109], [117]. QS signal molecules are thought to directly impact on host cells where they were shown to provoke inflammatory response in renal tissues [118]. Furthermore, 3-oxo-C12HSL can disrupt adherent junctions in epithelial tissues [119].

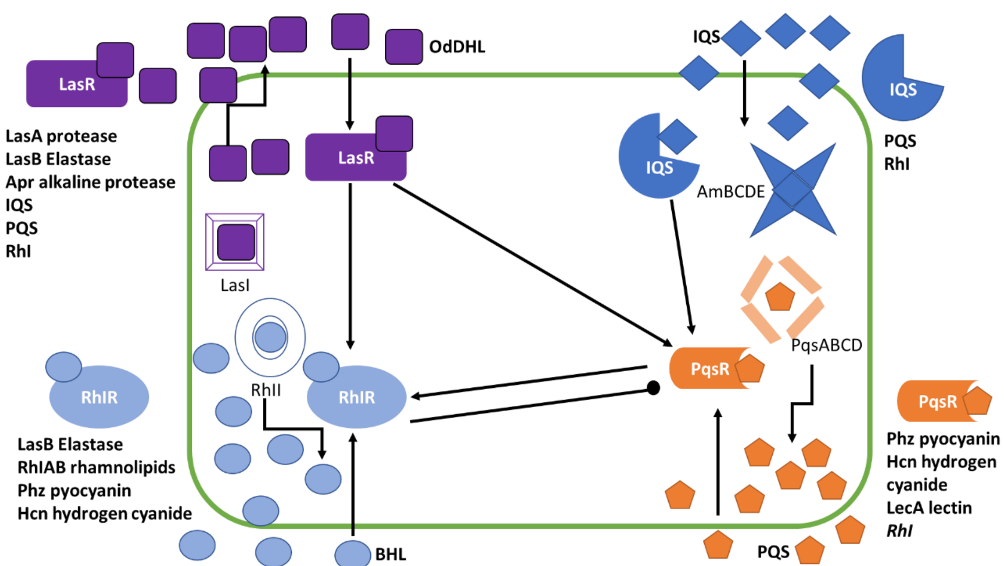


Figure 1.1 An outline of *P. aeruginosa* QS hierarchy and the regulons involved in conducting and release of associated virulence factors. Stimulatory effect represented by arrows, while inhibitory are indicated by perpendicular line [109]

A third QS system signalling molecule was discovered in 1999 known as PQS [120]. The structure of the molecule is 2-heptyl-3-hydroxy-4-quinolone and the regulator, is PqsR [121]. The regulator binds to the promoter region of *pqsABCD* and regulates the expression of the operon. This QS system is linked to *P. aeruginosa* virulence [122]–[124]. Reduced biofilm formation was observed and down-regulation of virulence factors pyocyanin, rhamnolipids, and elastase occurred due to a mutation

in the PQS system [125], [126]. In multiple model organisms; plants and nematode, PQS is necessary for complete virulence [125], [127], [128]. Bala *et al,* (2014) investigated the impact of the PQS system to assess its impact in mouse model of acute UTI pathogenesis. Upon deletion of *pqsA* and *pqsH,* attenuated virulence was observed, and lower pro-inflammatory cytokines produced against the mutants compared to PAO1 wild type [129].

The most recently identified QS system is IQS. The IQS signal molecule is 2-(2-hydroxyphenyl)-thiazole-4-carbaldehyde and produced by the autoinducer synthase AmbBCDE [130]. Disruption of the auto-inducer affects the Rhl and PQS systems negatively and consequently, reduces the expression of pyocyanin, elastase, and rhamnolipids. These phenotypes, however, can be re-established with the addition of 10 nmol/L IQS [131]. When *P. aeruginosa* encounters environments in which phosphate is scarce, the IQS system is thought to partially assume the role of the las system [131].

1.4.1.2 Biofilm formation

Biofilms contain a group of organized microorganisms sharing polymeric substances within a protective matrix [132]. Biofilms are ubiquitous in almost every environment and can be formed on biotic and abiotic surfaces [133]. They may be single species or polymicrobial and occur in different chronic infections. In short term CAUTI, infections are caused by a single species, whereas, long-term CAUTI tend to be polymicrobial with Gram-negative microorganism such as *E. coli*, *P. aeruginosa* and *K. pneumoniae* [134]–[136]. These communities are attached to a matrix made primarily of polysaccharides along with lipids, proteins and nucleic acids to form extracellular polymeric substances (EPS) [137]. Other macromolecules such as glycopeptides, lipids and lipopolysaccharides are involved in constructing a scaffold which protects the structure of biofilms [138].

The significance of the role of biofilms in infection is highlighted by their prevalence in almost 80% of infections [139]. Evidence of their role in pathogenicity and persistence can be witnessed in a range of infections such as *P. aeruginosa* ventilator associated pneumonia and diabetic foot infections by *Staphylococcus aureus* [140], [141]. Biofilms can form on medical devices such as catheters or implants [142].

Microbial communities within biofilms utilise gene transfer to exchange DNA including resistance genes and virulence factors [143]. Furthermore, the EPS in the matrix acts as shield to prevent diffusion of antibiotics across the biofilm [144]. Biofilm formation *in vitro* leads to the formation of mushroom-like structures [145]. Five stages of biofilm formation take place; 1) *P. aeruginosa* cells attach to the surface 2) EPS are produced to solidify the attachment. 3) The architecture of the biofilm starts developing followed by 4) Maturation of the architecture of the biofilms, and eventually 5) Dispersal of planktonic cells occurs [146].

Several virulence factors and cell components contribute to biofilm formation including rhamnolipids, exopolysaccharides, extracellular DNA and, type IV pili [130]. *P. aeruginosa* utilises exopolysaccharides such as alginate, pel and psl during biofilm formation [137]. Alginate is the first and the best characterised owing to its presence in the lungs of CF patients where it gains mucoidy phenotype in over-producing clinical isolates [147]. In other non-mucoid clinical and environmental strains, either pel or psl is expressed [147]. In UTI isolates, expression of alginate was observed in lesser amounts than isolates derived from other types of infection [90], [148]. *In vivo* murine model of CAUTI revealed that alginate is not needed to form biofilm [56].

Several complex processes involving numerous genes and environmental factors contribute to biofilm formation such as C-di-GMP, small RNAs, and QS systems [149] however the roles are not fully understood [150]. The role of the QS systems in *P. aeruginosa* in biofilm formation is complex. There is little evidence that QS contributes to biofilm attachment and the early stages of biofilm growth, however there may be a role in biofilm maturation [151]. QS controls the production of rhamnolipids which contribute to the channels involved in the distribution of nutrients and waste removal in biofilms [152], [153].

1.4.1.3 Pyocyanin

Pyocyanin is a phenazine pigment and a secondary metabolite produced by *P. aeruginosa* as a secreted, potent virulence factor [154]. Pyocyanin is a product of several phenazine encoding genes, some of which are located on two operons known as *phzABCDEFG* and other genes such as *phzM*, *phzH* and *phzS* (Figure 1.2) [155]. Structurally, pyocyanin contains a nitrogen ring which belongs to aromatic tricyclic

phenazines [156]. It is considered as a weak acid (pKa= 4.9) since it harbours a phenol group [156], [157]. At physiological pH level of 7.4, pyocyanin appears as a blue colour that could turn to red if pH drops to acidic levels [156], [158], [159]. As a zwitterion with low molecular weight, pyocyanin can penetrate host cell membranes easily [154], [156], [158].

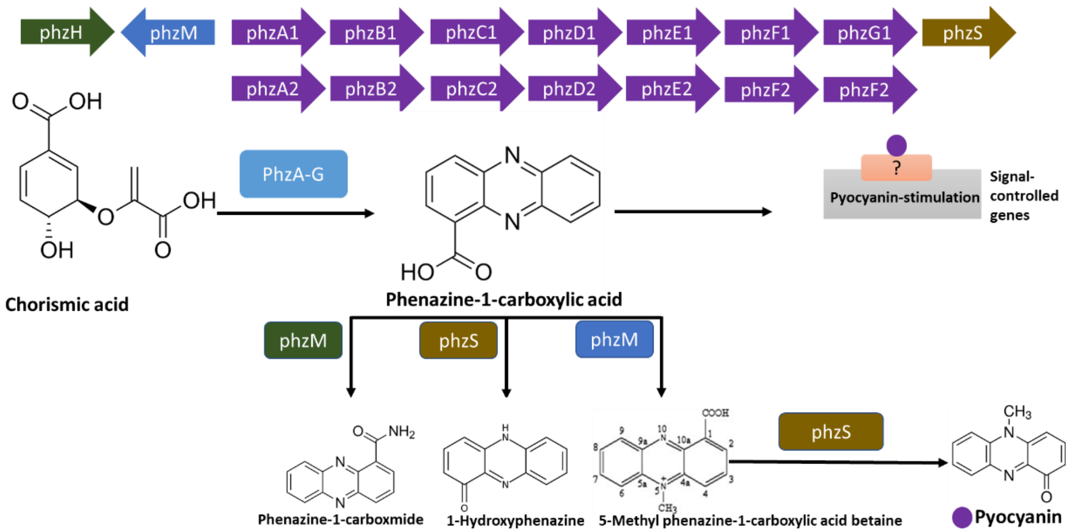


Figure 1.2 The signalling system and biosynthesis of pyocyanin production. Chorismic acid is converted via the PhzA to -G protein pathway into phenazine-1-carboxylic acid, which, in turn, is converted into different phenazines by the action of enzymes PhzS, PhzM and PhzH. The product of phzM, 5-Methyl phenazine-1-carboxylic acid betaine is converted to pyocyanin by phzS. Adapted from [160]

Pyocyanin is controlled by QS mechanisms [161]. Once the threshold of cell density is reached, LasR and RhlR activate genes contributing to pyocyanin biosynthesis [154]. The PQS quorum sensing via *pqsR* regulates pyocyanin positively by acting on *rhlR* and to a lesser extent *lasR*. GacS/GacA is a global signal transduction two-component system in Gram-negative bacteria, which has been implicated in swarming motility, biofilm formation, antimicrobial resistance and virulence of type III and VI secretion systems [162]. Virulence factor regulator (vfr) is another transcriptional regulator which is controlled by cyclic AMP. This promotes the Type III secretion system, ToxA, type IV pili and las QS system both directly and indirectly [163]. Both systems (GacS/GacA and VfR) have a role in pyocyanin production as mutants in both systems produced less pyocyanin [161].

Pyocyanin can cause cell cytotoxicity by activating intracellular reactive oxygen species (ROS) such as superoxide and hydrogen peroxide [164]–[166] . This activation event can have deleterious impact on host cells by disrupting the cell cycle and damaging DNA [164], [167]. Strains that discharge higher amounts of pyocyanin cause greater levels of oxidative stress, leading to significant cell lysis and subsequent release of extracellular DNA (eDNA) [168]. Pyocyanin intercalates with eDNA to form biofilms in UTIs by promoting cell to cell interactions and exerting influence on cellular surface properties [168]. Recent experimental evidence suggests that this is the route by which biofilm formation occurs in UTIs.

Pyocyanin has been detected in sputum samples of CF patients with concentrations reaching 130 µM [165]. Reports of existing pyocyanin in the bladder or elsewhere in the urinary tract are currently lacking [134]. However, *in vitro* detection of UTI isolates demonstrated that the concentration of pyocyanin could reach up to 39 µM [169]. UTI isolates appear to produce more pyocyanin compared to other *P. aeruginosa* isolates in other types of infection according to a report by Al-Ani *et al,* 2016 [169]. Urothelial cells can be susceptible to damage by pyocyanin. Mcdermott *et al* (2012) showed that treating urothelial RT4 cells with 25 µM or greater reduced cell viability [170]. Pyocyanin induction of 25-50 µM accelerated RT4 cellular senescence as witnessed by the increased production of senescence-associated β-galactosidase (SA-β-gal). This impact may inhibit cell repair processes by the host, resulting in recurrent UTI [171].

1.4.1.3 Elastase and LasA

Elastin is a major component of the human extracellular matrix (ECM) that is vital in many mammalian tissues such as the bladder, lungs, arteries, ligaments, tendons [172], [173]. Elastolytic activity can be observed in nosocomial UTI pathogens such as *S. aureus* and *P. aeruginosa* [174], [175]. The vast majority of *P. aeruginosa* isolates possess the ability to be proteolytic and elastolytic [176]. LasA is a serine protease that acts synergistically with elastase and alkaline proteinase to impose proteolytic activity on elastin and sensitise it to degradation to other proteolytic enzymes, including elastase [177]. Expression of both proteins are controlled by LasR [113], [178].

LasA protease, which is also known as staphylolysin, has been implicated in several types of *P. aeruginosa* infections; including keratitis (where a functional LasA,LasB and LasR is required for full virulence) [179], tracheal, wound, UTI [180] and chronic infection in CF lungs [181]. In the latter, high levels of *lasA* mRNA transcripts were expressed in the CF isolate LES431 [181]. However, it is unknown whether elastase has a prominent role in UTI [175]. In a mouse UTI model, an isogenic mutant to PAO1, failed to colonise the renal tissue due to attenuated virulence in proteases, rhamnolipids and elastases [118].

1.4.1.4 Alkaline protease

Alkaline protease (AprA) is one of the earliest discovered virulence factors with proteolytic activity in *P. aeruginosa* [182]. This metalloprotease is secreted via a type I secretion system The pathway depends on three major components; AprD (ATP binding Cassette), AprE (membrane fusion) and (AprF) outer membrane [183]. The exact role of alkaline protease in invasion and colonisation of CF lungs has not been fully identified. However, production of antibodies directed at this protease *in vivo* suggests it is involved in CF lung pathogenesis [184]. Alkaline protease can inhibit cytokine TNF-α induced signalling in A549 epithelial cells and therefore this process may alter immune signalling in the lungs of infected CF patients by inhibiting pro-inflammatory responses [185]. Although the exact role of alkaline protease in keratitis may not be clear, in isolates from keratitis infection there was a correlation between alkaline protease production and enhanced virulence [186]. A recent study examined 93 *P. aeruginosa* isolates from acute and chronic infections from blood, CF, urine, tracheal, rectum, sputum and venous catheters samples, by utilising *in vitro* zymography, elastase, and gelatinase assays. The authors found high levels and heterogenous production of alkaline protease, with the highest levels detected in the tracheal isolates [187].

1.4.1.5 Phospholipase C

Phospholipases are a group of diverse enzymes that are capable of hydrolysis of one or more ester linkages in glycerophospholipids [188]. Phospholipase C (PLC) targets and hydrolyses the phosphodiester bond in the phospholipid backbone and is therefore a significant virulence factor which suppresses the neutrophil respiratory

burst [189][188]. PLC is found in multiple bacteria such as *Clostridia, Bacillus, Listeria* (Gram-positive) and *P. aeruginosa* [190]. PLC is classified into haemolytic (PLC-H), and non-haemolytic (PLC-N) [191]. PLC-H and PLC-N are encoded by a set of genes that share DNA homology. Though they possess different substrate specificities [192], both can hydrolyse phosphatidylcholine. PLC-H can also target sphingomyelin while PLC-N exerts hydrolytic activity on phosphatidylcholine [192].

Lema *et al,* (2000) provided evidence of PLC-H hydrolysis of cow lung surfactant extract (CLSE) which is similar in composition with human surfactant [193]. Comparative analysis between mucoid and non-mucoid CF isolates revealed, that non-mucoid strains produced greater amounts of PLC-H [193], [194]. Although PLC-H appears to contribute to bacterial virulence at all stages of CF lung infection, it is more apparent in the final stages where lung deterioration and collapse occurs [195]. The exact role of PLC has not been elucidated yet in UTI pathogenies.

1.4.1.6 Rhamnolipids

Rhamnolipids are amphipathic glycolipids that contain L-rhamnose and β-hydroxy fatty acids moieties, and as a surfactant, rhamnolipids perform multiple functions in pathogenicity and are under the regulation of the QS system [196]. The Rhl QS system regulates production of 3 enzymes; RhlA, RhlB and RhlC, which, in turn, synthesize rhamnolipid and its associated precursors 3-(3-hydroxyalkanoyloxy) alkanioc acid (HAA) [196], [197] [198]. Rhamnolipids were detected in the CF lung and their role is thought to be in dissolving surfactants in acute and chronic lung infection [118],[199]. Several studies have suggested a role for rhamnolipid in swarming motility through reducing surface tension, thereby promoting flagellum movement along semifluid surfaces [200], [201]. This may be of benefit in the urinary tract as rhamnolipids, could promotes swarming motility on urinary catheters. *In vitro* and *in vivo* UTI analysis showed that absence of *rhl* QS (and therefore absence of rhamnolipid) reduced bacterial virulence [118].

1.4.1.7 Hydrogen Cyanide

P. aeruginosa is one of the few bacteria that produce and secrete hydrogen cyanide (HCN), a highly toxic virulence factor that inhibits multiple biochemical cellular processes in host cells (Table 1.3) [202]. The Rhl QS system controls HCN [203]. A HCN

concentration of 40 µM in host blood has been shown to be lethal [204]. Ryall *et al,* (2008) found a high level of HCN (mean average 72 µM) in the sputum of CF and bronchitis patients and absence of *P. aeruginosa* correlated with absence of HCN [205]. A clinical study on 167 CF patients observed that 74% of *P. aeruginosa* isolates produced HCN, and up to 83% of patients were infected with at least one strain that could produce HCN [206]. Reduction of lung function was observed in CF patients due to HCN production, which may have a role in *P. aeruginosa* persisting in the lung by affecting the host ability to resist and clear the infection [205]. The role of HCN in *P. aeruginosa* UTI has not been studied yet.

1.4.2 Virulence factors not controlled by QS

1.4.2.1 Type III secretion system

T3SS system has been implicated in *P. aeruginosa* infections such as CF and keratitis, however, the role in UTI pathogenesis remains unclear. In addition, the possible involvement of the T3SS systems in UTI pathogenesis of other Gram-negative bacteria is unclear. For example, a study on the contribution of T3SS to the pathogenesis of *P. mirabilis* in an acute ascending UTI mouse model revealed no involvement, despite the presence of a fully functional T3SS system [207]. Miyazaki *et al,* 2002 identified 3 strains out of 100 UPEC isolates which contain T3SS system [208]. A study on atypical enteropathogenic *Escherichia coli* (aEPEC) found an association between adherence to HeLa epithelial cells and T3SS [209]. Shulman *et al,* (2018) examined an *E. coli* strain (O2) known to cause UPEC and sepsis, the strain contains a pathogenicity island ETT2 that includes predicted genes of T3SS. However, no evidence was found of T3SS effectors in the secretome [210].

1.4.2.2 Structure of the Type III secretion system (T3SS)

The T3SS is a macromolecular structure that is evolutionarily related to the flagellar complex in Gram-negative bacteria [211]. The structure spans multiple layers of the bacterial and host membranes including the inner membrane, the periplasmic space, the peptidoglycan, the outer membrane and then into the cytosol of the host cell [212]. The T3SS machinery can be categorised into; the secretion apparatus, the translocation or the targeting apparatus, the secreted toxin (effectors) and the cognate chaperones (Figure 4.1) [213].

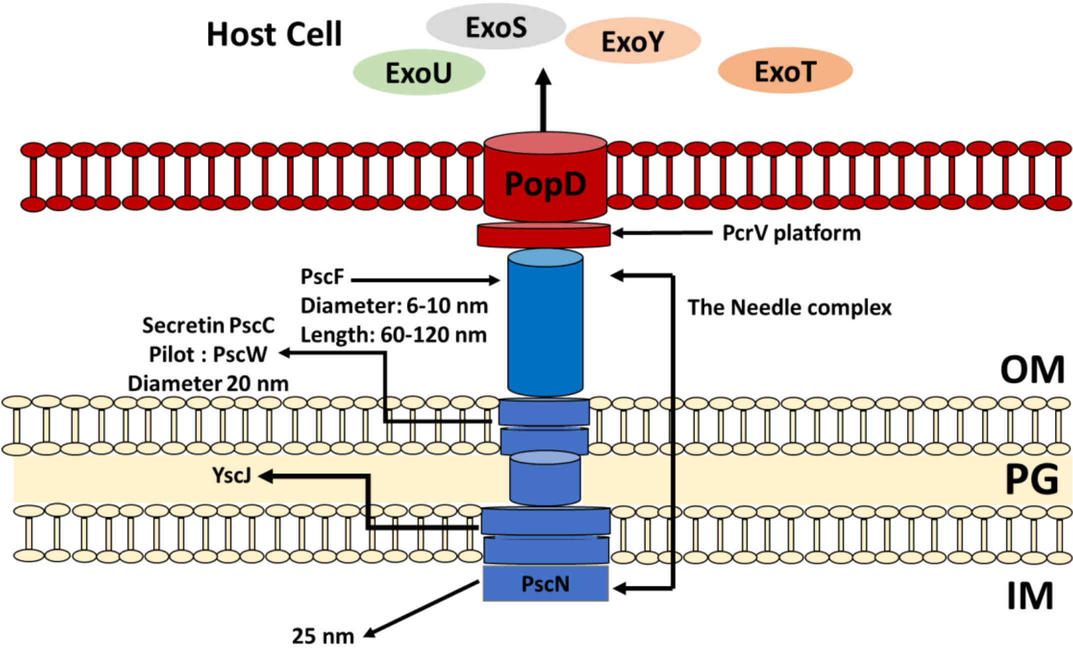


Figure 1.3 A schematic diagram showing the T3SS secretion apparatus. The system consists of the needle complex, the translocation apparatus, effector proteins and chaperones. Modified from [213]

1.4.2.3 Regulation of the T3SS proteins

Regulation of the T3SS injectosome and the effectors are strictly controlled. The system comprises of 36 genes on five clustered operons, with an additional six genes located around the chromosome, all contributing to the biogenesis of the T3SS [212].

Expression of the T3SS is tightly controlled and is upregulated in response to environmental cues, such as low concentrations of extracellular Ca^{+2}, casein and albumin and contact with the host cell [214]–[216]. The transcriptional activator ExsA binds to its own promoter and a consensus element on the promoter of T3SS genes [217]. The secretion activity of the T3SS apparatus is dependent on the coupling of EsxA with three proteins; ExsD, ExsC and ExsE [218]. ExaA, the transcriptional activator, is inhibited by ExsD when the needle is not in contact with the host plasma membrane [219]. Upon initiating injection, however, ExsE is released from its bound state with ExsC to inactivate ExsD. This, in turn, releases ExsA and allows it to bind the promoter region of T3SS (Figure 4.2) [220], [221]. For a detailed analysis of the transcriptional activity see Hauser *et al,* 2009 [212].

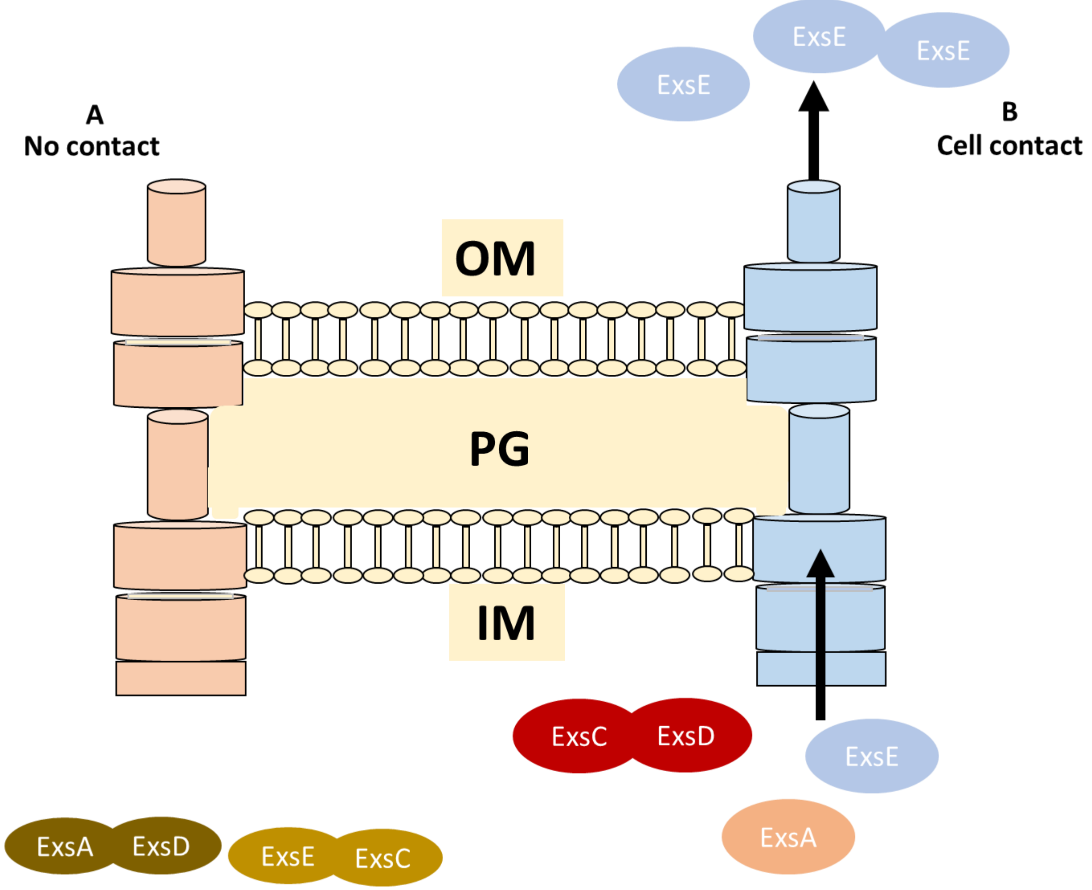


Figure 1.4 Proteins involved in the control of the T3SS. ExsA is the transcriptional regulator which activates the T3SS machinery depending the coupling of the protein with ExsD or ExsE. A): prior to cell interaction with the host cell, ExsA is coupled with ExdD. B): ExsD is replaced with ExsE which turns on the T3SS secretion system [213]

1.4.2.4 The needle complex

The needle complex consists of the basal body and a needle-like structure called the injectosome [212]. The basal body is an intracellular component which spans the inner membrane, the peptidoglycan, and the outer membrane [222]. This structure acts as the base for the injectosome which is anchored within [213]. Multiple proteins contribute to the functionality of the basal body, such as PscJ, PscC, PscN and PscW [212]. The protein PscJ is predicted to be a component of the inner membrane segment of the basal body, while PscC functions as an outer membrane component that belongs to the secretin family of outer membrane proteins [213]. Secretins produce large pores dependent on the formation of 12-14 subunits forming a homomultimeic ring that covers the length of the outer membrane and protrudes into the cytoplasm [223]. PscC forms a channel in the outer membrane by

oligomerisation with PscW and proteins PscJ (lipoprotein) and PscN (ATPase) to form part of the basal body [224]–[226]. The injectosome assembly is dependent on the helical polymerisation of PscF proteins [227]. The aim of the needle is to deliver the effectors from the bacterial cell, through the basal body to the targeting apparatus [228].

1.4.2.5 The Translocation apparatus

The function of the translocation apparatus (also known as the targeting apparatus), is to transport the effector toxins from the needle across the plasma membrane and eventually into the cytosol of the targeted host cell [212], [213]. Contact with the host cell initiates the formation of the injectosome, which in turn leads to the activation of the translocational apparatus with subsequent insertion into the plasma membrane of the host cell and formation of a fully functional translocation pore [212]. Three proteins are required to form the targeting apparatus: PopB, PopD and PcrV, known as the translocators. The interaction of those proteins as a collective unit forms the translocation pore [229]. PopB and PopD are comprised of hydrophobic domains which oligomerise in order to form the pore [230]. PcrV is hydrophilic and is required for a functional pore [231].

1.4.2.6 Effector proteins

Four effector proteins are currently known to be injected into the cytosol of the host cell, ExoS, ExoT, ExoU and ExoY [212]. Experimental evidence based on bacterial persistence in the mouse lung model of acute pneumonia suggest that *P. aeruginosa* producing ExoU causes more severe infections followed by ExoS [232].

ExoS and ExoT contain two domains with differing functions; N-terminal GTPase-activating protein (GAP) and ADP-ribosyltransferase (ADPRT) [213]. The GAP domain is biochemically identical in both effector proteins. GAP target GTP proteins in the cell and causes alterations to the inactive GDP-bound state [233]. Targeting of GTPases such as Rho and Cdc42 leads to the actin cytoskeletal rearrangement and consequently cell rounding, detachment and inhibition of phagocytosis. The ExoS Gap domain causes internal apoptosis to host cells [234]. Evidence of T3SS ExoT-dependent inhibition of cytokinesis in mammalians cells has emerged. Such inhibition leads to impaired host cell division which plays a partial role in wound healing [235].

ADPRT functions and targets differ between ExoS and ExoT. ADPRT in ExoS acts as a modulator of cytotoxicity in contrast to ExoT, in which it interferes with phagocytosis of the host cell [212]. The ADPRT domain of ExoT acts synergistically with the GAP domain to disrupt cytoskeletal formation and is involved in delayed wound healing, allowing *P. aeruginosa* to invade through the compromised mucosal barriers [236]. The ADPRT domain is also responsible for causing cell apoptosis by probable inhibition of Crk [237]. ExoS is known to cause apoptosis, albeit at a later stage of the infection. ExoS ADP ribosylates an extensive range of host proteins, including members of the Ras superfamily, the intermediate-filament protein vimentin, and the cellular mediator cyclophlin [213], [238]. ADP ribosylates radixin, ezrin and moesin, interfering with the cytoskeletal rearrangements [239], [240]. Thus, the impact of ADP ribosylation of host proteins leads to apoptotic-like cell death, cytoskeletal rearrangements, inhibition of DNA synthesis, endocytosis and movement of cellular vesicles[212], [213]. ExoS also can promote bacterial survival, escape and killing of macrophages once internalised within the phagosome by increasing the expression of bacterial *mgtC* and *oprF* [241]. Mgtc promotes growth within macrophages while OprF is an outer membrane protein which is implicated in survival inside macrophages [92], [96], [242].

ExoU has a severe debilitating impact on host cells owing to its cytotoxicity and broad phospholipase A2 activity [243]. The toxin targets and kills innate immune components such as macrophages and neutrophils. Depletion of the latter leads to localised immunosuppression and increased susceptibility to secondary infections [244], [245]. ExoU inhibits pro-inflammatory cytokine production in macrophages and is correlated with acute cytotoxicity [246]. Modulatory effects on host immunity by ExoU and extensive epithelial tissue damage contribute to pathogenicity and extensive damage to the host [213].

ExoY is the latest discovered effector and its exact function is yet to be fully elucidated [212]. ExoY is an adenylate cyclase that increases intracellular concentration of cyclic AMP resulting in alteration of the actin cytoskeleton of the host cell [247]. ExoY functions through the target human effector, the filamentous form actin [248]. Kloth *et al* (2018) demonstrated that ExoY contributes to *P. aeruginosa* acute

pathogenicity. The authors reached this conclusion upon observing pathological changes such as a decrease in lung barrier integrity, increased apoptosis and differential cytokine secretion [249]. ExoY is toxic to the yeast *Saccharomyces cerevisiae* and induces cell rounding in eukaryotic immune cells [250], [251]. The role of these effectors in UTIs has not been widely studied.

1.4.2.7 Chaperones

Chaperones are cytosolic, small proteins which assist the assembly and the operation of T3SS secretion system [252]. Chaperones are classified into three classes dependent on the type of the protein it is associated with. Class I chaperones contribute to the formation of pores, class II assist in forming needle-like structures and class III chaperones are associated with effector molecules or the toxins [213]. The unifying role of the chaperone proteins are to ensure a functional system; however, this is achieved by different mechanisms [253]. For instance, Class I chaperones maintain the helical components of the needle by masking the binding domains which subsequently polymerise and further deliver proteins to the export channel [254]. Class II chaperones inactivate the toxicity of hydrophobic translocators, this prevents premature signalling within the bacterium when the pore is formed and ultimately, the injectosome is formed [254], [255]. Prior to the formation of the ring structure of the translocon, both translocator proteins PopB and PopD are chaperoned by PcrH [256]. Effector proteins are associated with type I chaperones, which interact with their cognate partner via the chaperone binding domain. For instance, chaperone SpcS folds ExoS and ExoT to achieve the full functional capacity leading to full secretion[257]. ExoU is chaperoned by SpcU but no chaperone has been identified for ExoY [253].

1.4.2.8 T3SS and impact on disease

T3SS is not essential for *P. aeruginosa* to establish an infection, however, its role in pathogenicity of *P. aeruginosa* has been well characterised in acute and chronic *in vivo* animal models [258]. Expression of the T3SS has been associated with a poorer prognosis, greater disease severity, and higher rates of mortality [213], [259], [260]. In a clinical study involving 108 patients, respiratory tract and blood *P. aeruginosa* isolates were examined by immunoblot analysis to detect T3SS proteins. Expression

of ExoS, ExoT, ExoU and PcrV was associated with a relative risk of 6-fold greater mortality [261]. A recent study analysing the association of virulence and T3SS expression observed that most virulent *P. aeruginosa* isolates possessed an *exoU*+ phenotype in a *C. elegans* bloodstream infection model [262]. Interestingly, clinical isolates expressing T3SS are predominantly found in acute infections rather than the chronic infections such as CF, indicating that T3SS virulence may play a bigger role in acute infections [213].

The impact of T3SS on the pathophysiology of infection has several knock-on effects in the host, largely dependent on the site of infection. In respiratory tract infections, disruption of the host epithelial barrier increases susceptibility to infection due to the increased amount of asialoGM1 on the basolateral surface of epithelial cells [263]. Bacteria containing TS33 proteins bind to asialoGM1 and inject the effector proteins into the cytosol of the host cells which leads to tight junction disruption and further infiltration by invading *P. aeruginosa* cells [264]. Disruption of the epithelial and endothelial barriers allows *P. aeruginosa* dissemination into the bloodstream accompanied by secretion of pro-inflammatory mediators, resulting in eventual septic shock [264], [265]. In wound infections, ExoT causes delays in the healing process, potentially leading to invasion and dissemination into the blood stream [236].

The impact of T3SS on the immune function of the host is profound. Pro-inflammatory responses can be enhanced by T3SS due to the stimulation of IL-1β maturation leading to acute lung injury [244]. Neutralisation of alveolar macrophages and neutrophils in the lung by T3SS effectors also results in impaired bacterial clearance from the lung which further exacerbates the infection [213]. Failure to clear *P. aeruginosa* from the lung can lead to further secretion of effector proteins into the host cells and therefore a possible rise of pneumonia-like symptoms [266]. Hence, *P. aeruginosa* can self-promote this cycle by which pro-inflammatory responses are enhanced and innate response are neutralised, leading to further damage in the lung, bad prognosis and eventual mortality [213], [266].

Knowledge of UTI *P. aeruginosa* isolates ability to utilise T3SS is scarce. Schaber *et al,* 2004 found that the QS-deficient UTI isolate CI-5 did not possess either of ExoS or

ExoT [267]. In a recent study by Boulant *et al*, 2018, 339 isolates were obtained from invasive pulmonary infections, chronic CF infections, septicaemia, UTI, and isolates derived from environmental origins. PCR detection revealed that the prevalence of *exoU* was only 18.2% in 32 UTI isolates [268]. Tielen *et al,* (2011) detected 9 out of 30 isolates (28%) which were *exoU* positive. Interestingly, 4 ExoU positive isolates were low in cytotoxic activity, potentially due to a loss of function mutation [90]. Currently, knowledge regarding the potential role for ExoU remains elusive [269]. As for ExoS, the levels of this effectors could increase over time in UTI setting and it could aid persistence and immune evasion [270]–[272]. ExoT could potentially aid immune evasion during invasion and colonisation of the urinary tract [271]–[273]. Currently, no clear evidence has been shown of the involvement of T3SS other Gram-negative uropathogens such as UPEC and *P. mirabilis* [207]–[210]

1.4.2.9 Iron acquisition mechanisms

Iron acquisition mechanisms are crucial for *P. aeruginosa* for invasion and colonisation of the urinary tract [8]. *P. aeruginosa* utilises mechanisms to ensure its survival in different niches. Production of iron chelating molecules such as pyoverdine and pyochelin enables the bacterium to intake ferrous (Fe^{+3}) from the host, particularly from transferrins and lactoferrins [274], [275]. *P. aeruginosa* can also hijack siderophores produced by other microorganisms such as the siderophore enterobactin from *E. coli* [276], [277]. *P. aeruginosa* produces other compounds that have iron chelating activity. This includes pyocyanin which can chelate ferrous iron (Fe^{+2}) from the host (taken up by the Feo system) [278]. In addition, host haemoglobin can be targeted by haem proteins which are then transported through the Has and Phu iron uptake systems [279]. This will be discussed in detail in chapter 5.

Table 1.3 A summary of the key virulence factors utilised by *P. aeruginosa*

Virulence factor	Structure and/or function	Possible role in UTI pathogenesis
Pilus and Non-Pilus Adhesins. E.g. type IV pili (Tfp) and flagella.	Cell Surface attachments: Pili play a role in attachment and invasion of host cells. Non-pilus adhesins binds to mucin. asthma [62], [63].	Involved in attachment to catheter surfaces and uroepithelial cells [280].
Pyochelin/ pyoverdine siderophores	Extracellular Fe^{3+} chelating molecules scavenging scarcely available iron from host cells to ensure adaptability and survival [275].	May become more virulent in iron-limited environments such as the bladder [280].
Pseudomonas exotoxin A	Disrupts the function of protein synthesis by ADP-riboslation of Ef-2 elongation factor [281].	Causes multiple organ injury including the kidney [282].
Haemolysin	Produced by multiple bacterial species, causes lysis of host red blood cells [283].	Implicated in renal colonization and tissue damage [280].
ExoU	Type III cytotoxic phospholipase, Correlation exists between ExoU and acute cytotoxicity in the epithelium and macrophages [284].	Causes rapid necrotic cell death [270].
ExoT	Bi-functional type III cytoxin with rhoGAP domain. Encoded by almost all strains, causes capsase cell dependent in strains producing ExoS with ExoT [49].	May protect against needle-mediated injury upon during its insertion in the host cell. Not proven in UTI isolates. Possible role in immune evasion.
ExoS	Bi-functional type III cytoxin with rhoGAP domain and. Responsible for disruption of cytoskeletal arrangements [285].	A potential role in macrophage evasion [286].
ExoY	An adenylate cyclase that could disrupt actin cytoskeletons [270].	Function in UTI infection has not been established.
Alginate	An O- acetylated linear polymer composed of β-mannurate and L-guluronate residues. Aids in biofilm adherence [287].	No fundamental role in biofilm formation in UTIs [288],[289].
Pyocyanin	Nitrogen containing aromatic compound, a zwitterion that produces blue colour in neutral pH environments[134] .	Can cause reduction in urothelial cell viability [168].
Hydrogen Cyanide	Neutralisation of immune response and contributing to colonisation and virulence [204], [205].	Urothelial damage

1.5 Antimicrobial resistance

1.5.1 Antimicrobial resistance: Overview

Antimicrobial resistance (AMR) is major health-threat throughout the world and a great concern to health authorities and patients alike [290]. Antibiotics have been at the forefront of the success of modern medicine since Alexander Fleming discovered penicillin activity against *S. aureus* in 1928 [291]. With the dwindling efficacy and high rates of resistance in nosocomial settings, significant life-saving procedures such as surgery, transplantation and treatments of the immunocompromised are being compromised [292]. *P. aeruginosa* is a major cause of hospital-acquired infections. It is frequently found in burns, respiratory, urinary tract, bloodstream and device-related infections [293]. The bacterium is resistant to multiple antibiotics, with a notable rise in resistance against β-lactams, cephalosporins and carbapenems [294].

The majority of antibiotics need to penetrate the cell membrane of *P. aeruginosa* to be effective [295]. β-lactam antibiotics such as penicillins, cephalosporins, carbapenems and monobactams are comprised of a β-lactam ring (Figure 1.3) [296]. The antibiotics are designed to target and bind penicillin-binding proteins (PBP) produced by bacteria, and interfere with the synthesis of peptidoglycan [297]. Aminoglycosides used against *P. aeruginosa* include gentamicin, amikacin and tobramycin which inhibit protein synthesis upon binding to ribosomal 30S subunits [298]. Colistin, is a last resort antibiotic utilised to treat Gram-negative (including *P. aeruginosa)* MDR infections and belongs to the antibiotics class of polymyxins [299]. This class of antibiotics is comprised of polypeptides and targets bacterial LPS, resulting in increased outer membrane permeability [300]. Interference with DNA replication by targeting DNA gyrase and topoisomerase is another antibiotic action and this is utilised by quinolones such as ciprofloxacin and levofloxacin (Table 1.4) [301].

Aminoglycoside
e.g. tobramycin

Fluoroquinolone
e.g. ciprofloxacin

β-lactams
e.g. carbenicillin

Polymyxins
e.g. colistin

Figure 1.5 General chemical structure of antibiotics used for *P. aeruginosa* treatment, adapted from [302]

Table 1.4 A summary of major antibiotics used to treat *P. aeruginosa* infections, adapted from [303]

Class	Examples	Resistance	
		Chromosomal	Acquired resistance
Aminoglycosides	amikacin, tobramycin gentamicin	• Increases efflux pumps such as MexXY-OprM • Causes reduction of transportation across the cytoplasmic membrane	• Aminoglycoside-modifying enzymes (AMEs). • 16S rRNA methylases (extremely rare).
Fluoroquinolones	ciprofloxacin, levofloxacin	• Induction of mutations in quinolone resistance determining region (QRDR) of gyrA, gyrB, parC, parE genes. • Increases efflux of MexAB-OprM, MexCD-OprJ, and MexXY-OprM pumps)	• Aminoglycoside acetyltransferase variant aac(6')-Ib-cr which promotes reduction to ciprofloxacin susceptibility.
β-Lactams Carboxypenicillins Ureidopenicillins 3rd generation Cephalosporins 4th generation Cephalosporins Monobactams Carbapenems	carbenicillin ticarcillin piperacillin ceftazidime cefepime cefpirome aztreonam meropenem imipenem doripenem	• Increases efflux pumps, specifically the MexAB-OprM pump which promotes resistance to all β-lactams except imipenem. • Induces intrinsic overexpression of AmpC β-lactamase and promotes resistance to all β-lactams except carbapenems and carbenicillin. • Promotes lower outer membrane permeability via the loss of OprD porin, this results in resistance only to imipenem and reduced susceptibility to meropenem.	• Acquired penicillinases e.g. TEM-1, PSE-1 (confers resistance only to penicillins). • Extended spectrum β-lactamases e.g. PER-1, VEB-1 (confers resistance to all β-lactams except carbapenems). • Carbapenemases (resistance to most β-lactams including carbapenems. – Class A (e.g. GES-5) – Class B /MBLs (e.g. VIM, GIM NDM; enzymes not involved in resistance to aztreonam. – Class D (e.g. OXA-40-like; enzymes involved in carbapenem resistance.
Polymyxins	Colistin, polymyxin B	Promotion of LPS modification via two component systems such as PhoQP and PmrAB	

1.5.2 Pseudomonas uropathogenesis and antimicrobial resistance

The WHO has declared carbapenem resistant *P. aeruginosa* as a major and critical public health issue in need of developing therapeutic intervention [36]. The current new agents/combinations such as ceftolozane/tazobactam and ceftazidime/avibactam have increased the options with which the opportunistic

pathogen is treated [304] However, both treatments have already encountered *in vitro* and *in vivo* resistance [304], [305]. Despite the emergence of potential new treatments in phase III clinical trials such as meropenem-vaborbactam, mipenem-relebactam murepavadin and cefepime-zidebactam, the efficacy will not be assessed until prolonged period of consistent clinical use [306]–[308]. Thus, the current last resort for UTI treatment is colistin [309].

1.5.2.1 Aminoglycosides

Aminoglycosides are bactericidal agents used to treat *P. aeruginosa* infections by disrupting protein synthesis via binding 30S subunits [310]. Antibiotics such as streptomycin, gentamicin, amikacin and tobramycin are usually deployed with other β-lactams to increase the impact of bactericidal activity [311]. Multiple resistance mechanisms are involved in decreasing *P. aeruginosa* susceptibility such as decreased outer membrane permeability, efflux pumps and amino acid substitutions in ribosomal proteins [312].

1.5.2.2 Fluoroquinolones

Fluoroquinolones target DNA synthesis in bacteria by interacting with the enzymes DNA gyrase and topoisomerase IV [313]. Two members of this class of antibiotics are used to treat *P. aeruginosa* infections, ciprofloxacin and levofloxacin [314]. *P. aeruginosa* can develop resistance to this class of antibiotics by developing mutations on quinolone resistance genes in the (QRDR) region which encodes DNA gyrase *(gyrA or gyrB)* or topoisomerase IV (*parC or parE*) enzymes [315]. *P. aeruginosa* has also been identified with a transferable quinolone resistance determinant called *aac(6')-Ib-cr*. This encodes the enzyme AAC(6')-lb-cr. In comparison to AAC(6')-Ib, AAC(6')-lb-cr contains two amino acid substations which enables it to acetylate ciprofloxacin (not levofloxacin) and aminoglycosides [316], [317].

1.5.2.3 β-Lactams

β-Lactams are the largest class of antibiotics and these inhibit cross-linking of the peptidoglycan, a major component of the bacterial cell wall [318]. Inhibition mechanisms take place by targeting bacterial D-alanyl-D-alanine transpeptidase enzymes (PBPs) [319]. Many β-Lactams are ineffective against *P. aeruginosa* due to

the low membrane permeability, chromosomally encoded AmpC-type β-Lactamase and constitutive expression of efflux pumps [320]. See section 3.4.3 for detailed information on β-Lactamases classes.

Effective antibiotics in this class which often neutralise *P. aeruginosa* include carboxypenicillins (e.g. carbenicillin), ureidopenicillins (e.g. piperacillin), some third-generation (e.g. ceftazidime) and fourth generation cephalosporins (e.g. cefepime), monobactams (e.g. aztreonam) and carbapenems (e.g. imipenem, meropenem and doripenem only) [312]. For detailed information on resistance see section 3.4.

1.5.2.4 Polymyxins

The polymyxin class of antibiotics, consisting of colistin (polymyxin E) and polymyxin B, interfere with bacterial outer membrane binding by attaching to LPS [321]. The use of polymyxins dates to the 1940s, however their widespread use was unpopular due to nephrotoxic and neurotoxic side effects and the emergence of aminoglycosides and carbenicillin in the 1960s [322]. The rise in resistance, however, has meant that these antibiotics are being revisited and are particularly attractive due to the unusual mode of action. Delivery of colistin can occur two ways; intravenously and as nebulised drug for CF patients. The nebulised form is particularly attractive as safety issues are lessened due to the lower rates of absorption in other tissues [323], [324]. The vast majority of *P. aeruginosa* isolates are susceptible to colistin. However, resistance can occur due to LPS mutations and modified lipid A. In complicated UTI patients infected by XDR *P. aeruginosa,* colistin is used as a last resort antibiotic [309]. The commercially available form of this antibiotic for intravenous use is a prodrog of colistin called colistimethate sodium [325], which is eventually converted into colistin in the patient upon administration with 60%-70% immediate clearance in urine [326], [327].

1.6 Hormones

In addition to the wide spectrum of bacterial virulence factors that can affect the pathogenesis of infection, there are also important roles played by host factors. Host factors include many different components. However, in this thesis, I will be focusing on the impact of sex hormones.

1.6.1 Hormone classification

Hormones are chemical mediators produced by humans, these signals are released by the hypothalamus-pituitary-gonads axis into the peripheral circulation [328]. Hormones perform several vital functions and aim to control homeostasis, development, reproduction, maintenance, energy use and growth [329]. Despite the small concentrations of hormones released into the blood, multiple tissues and cells in the body contain specific receptors which allow recognition and response [330]. The impact of hormones varies amongst different tissues and cells, several examples of the impact of hormones on health and disease will be discussed further in this section.

Three major classes of hormones have been recognized; Protein/peptide hormones, terpenes and amino acid derivatives hormones as shown in (Table 1.5). Sex hormones and steroids belong to the second class [331].

Table 1.5 Classes of hormones and examples of each class. Sex hormones (steroids) are made by stimulation of hormones belonging to class one such as luteinising hormone (LH) and follicle stimulating hormone (FSH)

Hormone class	Hormone example
Protein/peptide	Regulating production (gonadotropins), Energy balance (insulin) and blood pressure regulation
Terpenes; Juvenile & Steroid hormones	Sex steroids such as testosterone and oestrogen
Amino Acid derivatives	Serotonin and melatonin

1.6.2 Sex hormones

Sex hormones are a group of hormones produced by male testes and female ovaries. In males, these hormones are known as androgens and testosterone is the main androgen. In females, two principal hormones are involved in promoting secondary female characteristics and reproductive functions. Each hormone will be discussed here and in further detail in chapter 5.

1.6.2.1 The impact of luteinizing hormone and follicle stimulating hormone synthesis

Human gonadotrophin hormones are comprised of three hormones: luteinizing hormone (LH); follicle stimulating hormone (FSH) and choriogonadotropin (CG) [331]. The latter is released by the placenta to aid pregnancy [332]. LH and FSH are released by cells in the anterior pituitary known as gonadotrophs, following exposure to gonadotropic releasing hormone (GnRH) from the hypothalamus [333]. Both hormones stimulate the gonads in male and female reproductive systems, specifically, the testes and the ovaries [334], [335]. The structure of both hormones comprises of alpha and beta chain subunits [331]. The alpha chains are identical, the beta chain is variable, and therefor determines the biological specificity of the hormones [336], [337]. In human males, the LH binds to receptors on Leydig cells in the testes to stimulate the production of testosterone [338], in females, testosterone is also produced in theca cells of the ovary prior to conversion to oestradiol by granulosa cells [339].

1.6.2.2 Testosterone

Testosterone is structurally known as 17-beta-hydroxy-4-androstene-3-on [340]. The testes produce almost all testosterone in men from cholesterol and acetate. Smaller amounts are secreted by the zona reticularis of the adrenal gland [341]. Testosterone binds receptors in cells/tissue resulting in the promotion of protein synthesis [331]. Androgen receptors (AR) are the receptors that mediate the functions of testosterone [342]. Testosterone increases muscle strength and mass, bone density and stimulates male secondary characteristics such as growth of body hair and larynx enlargement. When males reach adulthood, major synthesis of testosterone takes place in gonads. Leydig cells produce testosterone in males while theca cells synthesise testosterone in the female ovarian follicle. In the latter, testosterone is converted into oestradiol by aromatase activity [343]. The placenta can also release testosterone [343].

The synthesis of androgens in the gonads of both genders is regulated tightly by the secretion of GnRH [344]. This hormone (GnrH) is released from the hypothalamus and this leads to the stimulation of LH and FSH from the pituitary gland. LH then acts

by stimulating the synthesis of testosterone in the testicular male and ovarian female tissue. Testosterone inhibits GnrRH and LH release from the hypothalamus and the pituitary, respectively (Figure 1.4) [345].

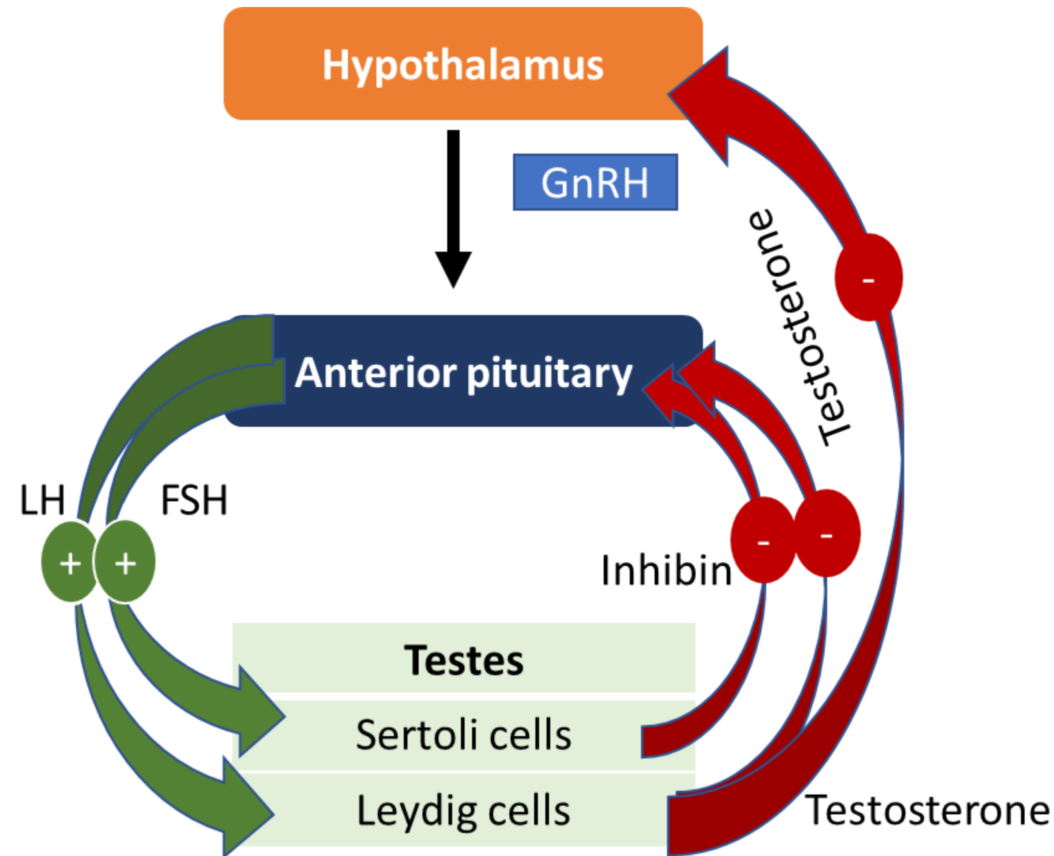


Figure 1.6 Testosterone production in humans. Negative feedback loop clarified as production of testosterone and inhibin by the testes inhibits the synthesis of LH, FSH and GnRH hormones. The process starts by secretion of GnRH from the hypothalamus. GnRH stimulates LH and FSH in anterior pituitary, which in turn, stimulate the production of testosterone

1.6.2.3 Oestradiol

Oestradiol is a central member of estranges family with oestriol and oestrone. Structurally, oestradiol contains two (OH) groups while oestrone and oestriol possess one and three (OH) groups, respectively [346]. The oestrogen derivatives fluctuate during the female lifespan as oestradiol is produced in higher quantities and is the principal oestrogen in the reproductive years [347]. All of these derivatives originate from endogenous and exogenous sources. The ovary, as an endogenous source and the principal producer of oestrogens, directly secretes oestrogens during the

reproductive cycle, and the adrenal secretory activity elevates conversion of androgens to oestrogen in the peripheral tissues [348]. Extragonadal sources such as the brain, liver, muscle and mesenchymal cells of adipose connective tissues, discharge small amounts in comparison to ovary [349], [350]. Upon progression to menopause, oestradiol starts to diminish [351]. Oestradiol promotes and maintains female secondary characteristic post-puberty such as breast growth, fat and bone deposition, and alterations in body shape. Oestradiol is discharged from the ovaries into the blood where coupling with sex hormone binding globulin occur, followed by cell entry to the nucleus to bind with DNA [331]. Oestrogen acts via intracellular receptors such as oestrogen-binding proteins ERα and ERβ [352]. The role of oestradiol in pregnancy is to maintain a balanced environment for the oocytes to thrive [353]. Oestradiol's impact is not restricted to growth and reproduction only, it plays a major role in homeostatic feedback mechanisms. Indeed, oestradiol production inhibits GnRh/LH release in a process called a negative feedback loop in males and females. In the latter, however, oestradiol switches to positive feedback to generate a surge of GnRh/LH pre-ovulation (Figure 1.5) [354].

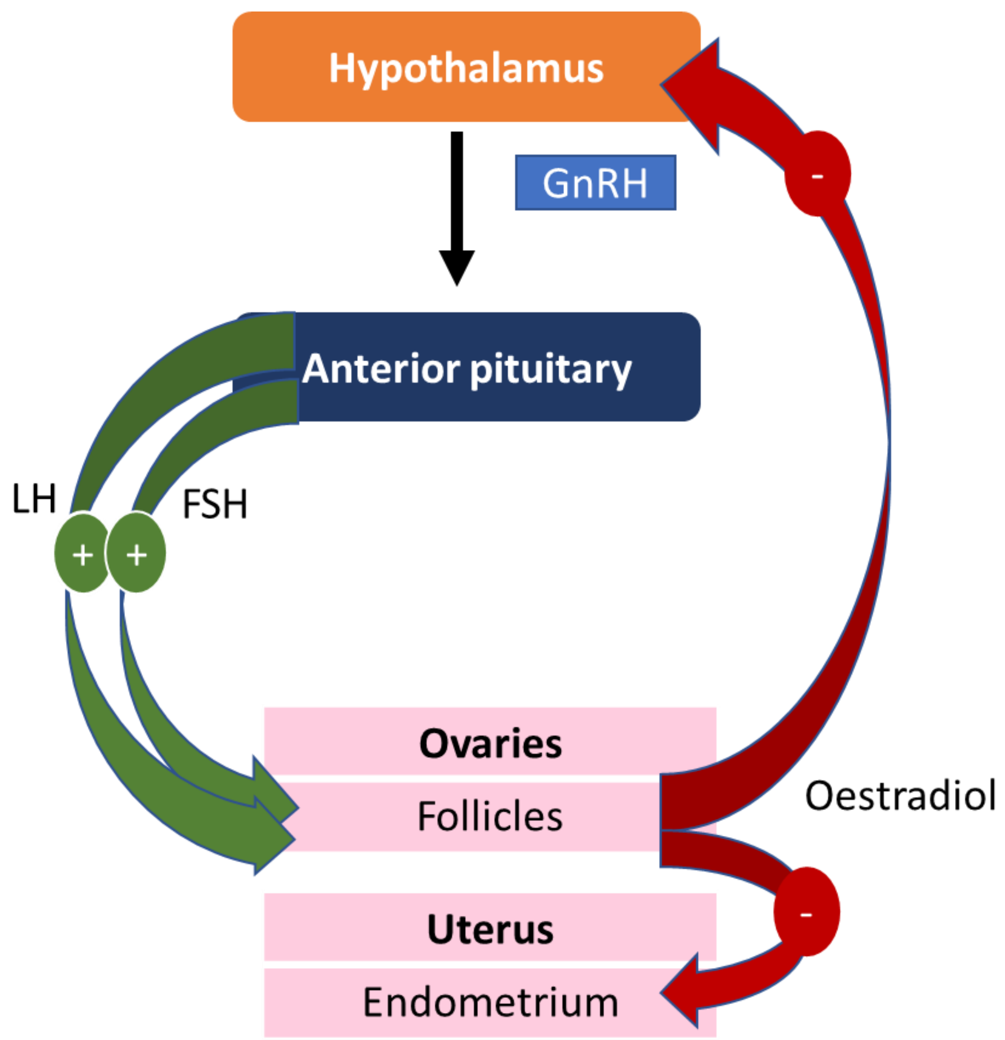


Figure 1.7 The process of oestradiol production During the follicular phase, GnRH stimulates the production of FSH and LH from the pituitary. Both hormones lead to the stimulation of oestradiol from the production oestradiol from the ovaries. Oestradiol inhibits the production of GnRH from the hypothalamus and keeping the levels of LH and FSH low. Oestradiol acts on the endometrium and causes the endometrial arteries to constrict leading to menstruation

1.6.2.4 Progesterone

Production of progesterone occurs in the gonads and the adrenal cortex in males and females [355]. Progesterone is involved in pregnancy and fluctuates during the menstrual cycle in reproductive age women [356]. Structurally, progesterone is known as pregn-4-ene-3,20-dione [357] and it binds to progesterone receptors (PR). These receptors are expressed in the granulosa cells of preovulatory follicles of the ovary [358]. Low levels of progesterone are observed pre-pubescence and post-

menopause. Physiologically, progesterone slows-down the pulsatile GnRH secretion by acting on the hypothalamus resulting in oestradiol priming [359]. In men, production of progesterone occurs in the testicles and the adrenal glands, and in contrast to women, serum levels of progesterone are not altered periodically or by age [360]–[362]

1.6.3 Sex Hormones in disease

Sex steroids impact on disease in some cases can be a clear, direct consequence of alteration in circulating hormones. This is exemplified by the protective effect of oestradiol to infections caused by *Mycobacterium spp* [363], [364]. Women are more susceptible to UTI and recurrent infections post-menopause [18]. In other diseases the link is less clear and is associated with increased risk but is not necessarily causal This will be discussed in detail in chapter 5, examples of sexual dimorphism and an overview of some of the roles those hormones plays are provided below.

1.6.3.1 Oestradiol in disease

Oestradiol has been linked to many disease conditions (Figure 1.6). Lack of oestrogen production in females is caused by conditions such as Turner syndrome [365]. Genetic defects in the adrenal and gonadal secretions impair fertility [366]. Oestradiol decreases the biomechanical stiffness of the cornea post-surgery [367]. In pulmonary disease, oestrogens including oestradiol are thought to predispose female patients to higher rates of morbidity and mortality, though the exact underlying mechanisms are not fully elucidated yet [368].

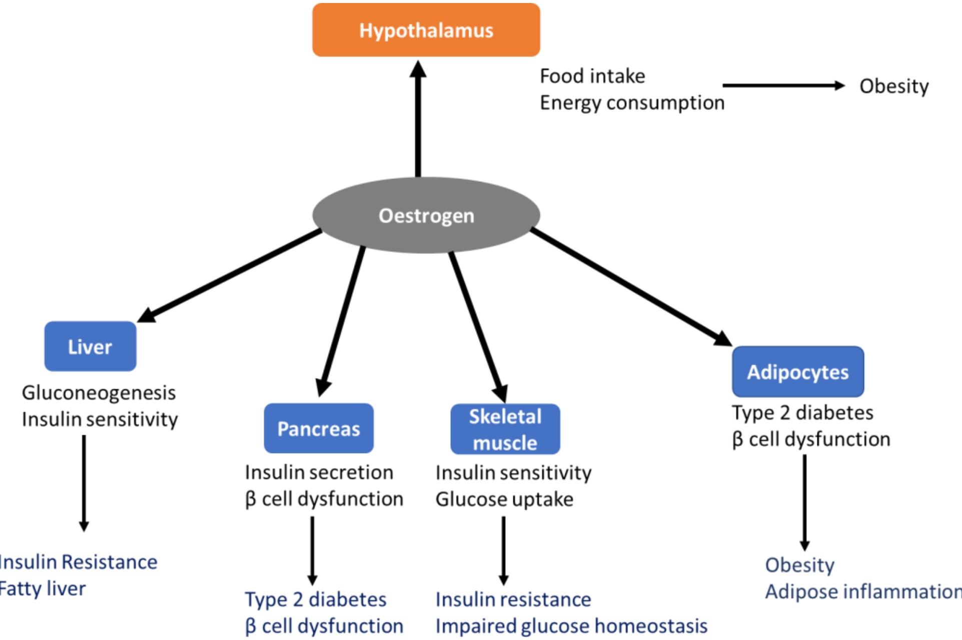


Figure 1.8 Oestradiol deficiency impacts multiple organs in the females and acts on several systems and organs including the Central Nervous System (CNS), liver, β cells of the pancreas, adipocytes and skeletal muscles. As a result, metabolic syndrome, obesity and type 2 diabetes develop [369]

1.6.3.2 Testosterone in disease

The association between testosterone and multiple health disorders is well-documented [370]–[372]. Hypogonadism is a clinical syndrome in which the testes fail to produce sufficient levels of testosterone and sperm cells [373]. The disorder may arise either from a genetic origin (congenital) [374] or can be acquired [375]. Lower circulatory levels of testosterone lead to loss of male secondary characteristics, infertility and muscle weakness. The most common manifestation in congenital disorders is Klinefelter's syndrome [376]. On average, men lose 1%-2% of circulating testosterone every year with age progression [377]. Once serum testosterone levels drop from the adequate healthy male age reference, symptoms may rise and the onset of late-onset hypogonadism (LOH) is observed [378]. Symptoms such as depressions, loss of libido, fatigue, erectile dysfunction and reduced muscle strength may emerge [379]. Testosterone production is linked with multiple disorders such as diabetes mellitus, obesity, alcoholism, liver disease, hemochromatosis, and sickle cell anaemia [372]. Furthermore, diminishing serum testosterone levels are linked to the most health threatening non-communicable

disorders such as cardiovascular disease, the leading cause of mortality around the world [380].

1.6.3.3 Progesterone in disease

The role of progesterone in sexual dimorphism between men and women is less clear and not investigated as well as either oestradiol or testosterone [381], [382]. However, progesterone is involved in modulating the immune system heavily. It acts as a modulatory substance exerting its influence on intracellular and membrane-associated progesterone receptors in immune cells, immune organs, and autoimmune disease tissues [383]. Evidence exists of the association of progesterone with autoimmune disease such as rheumatoid arthritis, where low progesterone levels are thought to be a contributing factor to this particular disease [384]. Progesterone was found to be associated with lung disease exacerbations [385]. Detection of nitric oxide in the lung is considered to be a biomarker of airway inflammation of asthma, which is positively associated with higher progesterone production levels [386]. Serum progesterone is positively associated with worsening condition in lung function of asthmatic women during the luteal phase, the phase in which serum progesterone reaches its peak [387].

1.6.4 Accounting for sexual dimorphism in medical treatments

Gender as a variable in contracting infectious diseases has been neglected in animal and clinical studies which aim to understand host-pathogen interactions and development of treatments [388], [389]. Animal studies usually exclude female subject, in part due to the fluctuating hormone levels during the estrus cycle [390]. Clinically, in 1977, FDA guidelines recommended the exclusion of productive age women from initial human clinical trials such as phase I [391]. This may have led to increased adverse events in women, which was observed between the years 1997-2000 when 8 out of 10 withdrawn drugs resulted in greater health risks for women [392]. In 1994, the National Institutes of Health (NIH) issued new guidelines taking into account gender differences in conducting clinical trials [393]. Despite the relatively recent focus and effort on the inclusion of female subjects in clinical research, some drugs and therapeutic guidelines are exclusively based on clinical trials with males only [391], [394].

1.6.5 Therapeutic utilisation of sex hormones

Therapeutic interventions using sex hormones were part of medical controversies since these treatments were recommended in 1992 [395] . Based on multiple observational and meta-analyses, hormone replacement therapy (HRT) was recommended for the treatment of coronary disease, dementia, osteoporosis for post-menopausal women by administrating estrogen [396]–[398]. The findings and recommendations were reversed earlier in the 21st century due to rising incidence of breast cancer, heart disease and osteoporosis [399], [400] . However, subsequent re-analysis and further investigations concluded that HRT may be beneficial to women within the age of 50-59 years, suggesting that early administration is key to achieve disease prevention [401].

Testosterone replacement therapy can be administered to patients suffering from hypogonadism. Treatment is carried-out via multiple routes; oral, intramuscular, transdermal patches, and gels [375]. Concerns were raised regarding the oral prescriptions due to inability to control testosterone serum levels in the blood post-administration, warnings were given of possible adverse events such as prostate cancer, sleep apnoea and erythrocytosis [402]. Other studies have demonstrated an increased risk of cardiovascular disease following the administration of testosterone, prompting the Food and Drug Administration (FDA) to issue consumer warnings [379].

The FDA approved the use of progesterone, it can be administered via multiple routes [403]. However, it has not been effective in treating conditions such as acute traumatic injuries in phase III clinical trials [404] , due to lack of optimal dosing [405] and inaccurate inferences from pre-clinical data in designing clinical trials [406].

Sexual dimorphism in men and women in relation to infectious diseases will be highlighted in chapter 5 with examples of infections, immune modulation and the overall impact of hormones on experimental models and treatments.

1.7 Aims

- To characterise genotypic and phenotypic traits in a panel of isolates sourced from the UK and the state of Kuwait
- To establish the AMR profile of all clinical isolates
- To assess whether AUM is an adequate *in vitro* laboratory model that recapitulates *P. aeruginosa* responses in human urine
- To compare the proteomic profile of *P. aeruginosa* in AUM in comparison to LB and urine
- To study the impact of sex hormones oestradiol, testosterone and progesterone on *P. aeruginosa* phenotypes.
- To investigate the impact of sex hormones on the proteomic profile of clinical UTI *P. aeruginosa*

Chapter 2

2 Materials and Methods

2.1. UTI isolates storage and hormone stocks

2.1.1 Bacterial isolates

P. aeruginosa urinary tract isolates were sourced from the Royal Liverpool Hospital (n=15) and Kuwait (n=8) (Table 2.1). The average patient age on isolation was 70 years old and 47% were isolated from women in the UK panel. 8 isolates were sourced from Kuwait dating back to 2005, unfortunately, no other information was available.

Table 2. 1 Describes the main and known information of the *P. aeruginosa* strains and clinical isolates obtained for this study. N/A= not available, UK= United Kingdom, USA= United States of America

Isolate Name	Source	Date	Country of origin	Gender	Age	Reference
PAO1	Wound	1954	Australia	N/A	N/A	[407]
PA14	Burn Wound	1977	US	N/A	N/A	[408]
LESB58	CF	1988	UK- Liverpool	N/A	N/A	[409]
133042	Urine	11-Oct-13	UK	M	52	This study
133043	Urine	16-Oct-13	UK	F	80	This study
133044	Urine	16-Oct-13	UK	F	69	This study
133065	Urine	18-Oct-13	UK	F	70	This study
133075	Urine	24-Oct-13	UK	M	76	This study
133082	Urine	25-Oct-13	UK	M	89	This study
133083	Urine	25-Oct-13	UK	F	68	This study
133090	Urine	25-Oct-13	UK	M	83	This study
133098	Urine	31-Oct-13	UK	M	58	This study
133099	Urine	31-Oct-13	UK	F	61	This study
133104	Urine	01-Nov-13	UK	M	72	This study
133105	Urine	01-Nov-13	UK	M	50	This study
133106	Urine	02-Nov-13	UK	M	83	This study
133117	Urine	02-Nov-13	UK	F	61	This study
133126	Urine	07-Nov-13	UK	F	75	This study
758	Urine	4-Jan-05	Kuwait	N/A	N/A	This study
783	Urine	10-Jan-05	Kuwait	N/A	N/A	This study
786	Urine	11-Jan-05	Kuwait	N/A	N/A	This study
864	Urine	18-Apr-05	Kuwait	N/A	N/A	This study
888	Urine	15-May-05	Kuwait	N/A	N/A	This study
902	Urine	22-May-05	Kuwait	N/A	N/A	This study
925	Urine	05-June-05	Kuwait	N/A	N/A	This study
1083	Urine	12-Nov-05	Kuwait	N/A	N/A	This study

2.1.1.2 Isolate storage and culture

All isolates were stored at -80 °C in Luria-Bertani (LB) (Sigma Aldrich) (W/V) 10% glycerol (Sigma Aldrich). Isolates were streaked onto LB Agar (Appleton woods) and grown overnight at 37 °C.

A single representative *P. aeruginosa* colony was used to inoculate LB broth (Sigma Aldrich), artificial urine medium (AUM) or pooled human urine in glass or plastic universal tubes overnight. All cultures were grown at 37°C and shaking at 180 rpm unless otherwise stated. The use of glass tubes avoids adsorption of hormones to the vessel surface.

LB agar (Sigma Aldrich) with the following formulation (agar, 10 g/L sodium chloride, 5 g/L tryptone, 10 g/L, yeast extract, 5 g/L) was used. To prepare LB agar, 12g of the LB agar powder was dissolved in 500 ml of distilled H2o. Bacterial cultures were spread inoculated onto the agar and was allowed to grow overnight at 37 °C.

2.1.1.3 Hormone Stock Concentrations

Hormone stocks of oestradiol, testosterone and progesterone were prepared as following; for oestradiol, 2mM stock = 0.0136g (272.38g/mol) were added to 25ml filtered ethanol (Sigma Aldrich). Subsequently, 200µl of 2mM was added to 20 ml ethanol to make up 20µM working stock. The working solutions were used to reduce the concentration further to 20nM in LB. The same procedure was performed to testosterone (0.014g -288.42g/mol) and progesterone (0.0157g -314.46g/mol). In addition, the same procedure was followed to calculate 10µM hormone stocks. All hormones were stored in glass vials.

2.2 Growth & Morphology characteristics

2.2.1 Growth rate analysis

To determine the bacterial growth rate in LB and AUM. Overnight cultures were diluted 1:100 in either LB broth, AUM or urine in polystyrene 96-well plates (Corning® Costar®). Bacterial growth curves were performed in media and with the addition of hormones; oestradiol, testosterone or progesterone. The hormones were added into LB to a final concentration of 20 nM. All assays were performed with four technical replicates and in quadruplicate biological replicates. The growth of each dilution was

monitored for 24 h at 37°C and the absorbance 600nm was recorded every 30 min by the Fluorostar Omega plate reader.

2.2.2 Colony morphology

To assess the morphology of colonies of UTI *P. aeruginosa*, bacteria from overnight liquid cultures were streaked on plates containing LB agar (Sigma-Aldrich) as shown in (Figure 2.1) to obtain single colonies. The colonies were allowed to grow in room temperature for 72h. Image analysis for morphological characteristics such as circular, raised, entire, mucoidy, colour and size (millimetre) was recorded.

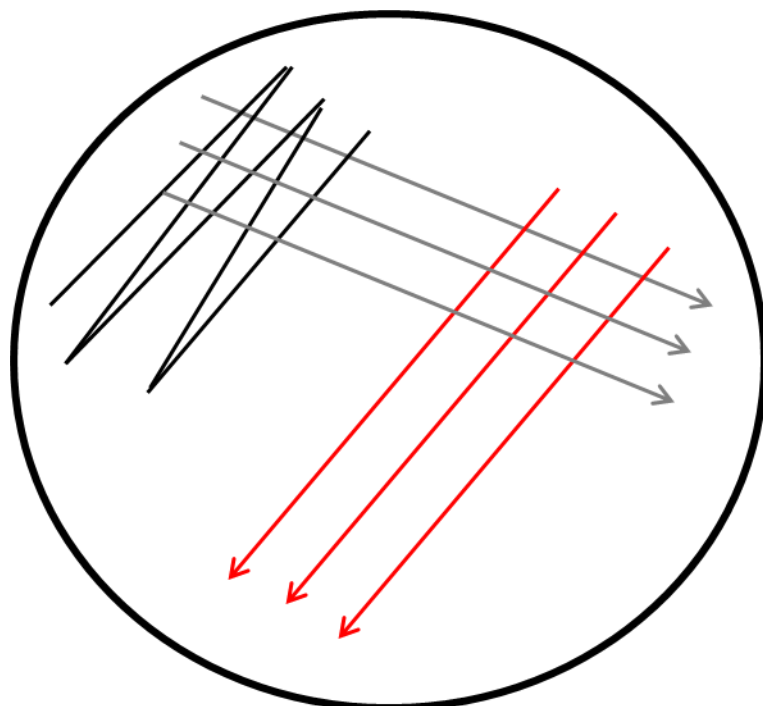


Figure 2.1 Streaking method conducted to study morphological characteristic of *P. aeruginosa* UTI isolates

2.3 Biofilm formation experiments

2.3.1 Biofilm assay (Crystal Violet staining)

The overnight cultures of the 15 clinical isolates were diluted 1:100 in LB or AUM. This was followed by the addition of 200µl of each *P. aeruginosa* containing-LB or AUM solution in quadruplicate to a 96-well plate (Corning® Costar®) and grown at 37°C for 24h or 48h. After the incubation period, the broth was removed, followed by the application of 200µl of phosphate buffered saline (PBS) (Appleton Woods) to the wells twice to wash the biofilms. Removal of PBS was conducted and 200µl of

0.25% crystal violet (CV) w/v in dH_2o (Sigma Aldrich) was added into each well for 10 minutes. Removal of CV was performed, and the wells were rinsed and washed with tap water. Stained biofilms were solubilised by adding 200µl of 95% ethanol v/v (Sigma Aldrich) for 10 minutes and transferred to a new 96-well plate (Corning® Costar®). The absorbance was then measured at OD_{600nm} on the Fluorostar Omega plate reader to determine the biofilm biomass. Hormones were added to some biofilm assays (where stated). In these assays, each isolate was grown as described above with the addition of oestradiol, testosterone and progesterone to a final concentration of 10nM.

2.3.2 Biofilm laser confocal microscopy

For biofilm microscopy, biofilms were prepared following the method described above using glass microscopy-compatible plates (Greiner high and medium binding 96 well plates, Sigma-Aldrich). The biofilms were allowed to grow at 37°C for 24 h. Media and planktonic bacteria were removed gently without disrupting the bottom of the well. Each well was washed gently with LB and then removed. 3.58 ml of PBS was added with 5 µl of two dyes (SYTO 9 dye, 3.34 mM) and (Propidium iodide, 20 mM) from the LIVE/DEAD® BacLight® Bacterial Viability Kit (Thermo Fischer Scientific). Only live cells/biofilms were included in the analysis. The dye solution was used to stain each well with 50 µl. The dye was kept in the wells for 30 minutes then transported to the Centre for Cell Imaging at the University of Liverpool where biofilm microscopy was performed. Specifically, Carl Zeiss microscope with 40x oil lens was used to image the biofilms in Z-stacks. Upon producing 3D images using Imaris, measurement of biofilm structure was performed using Comstat [410]. This software measures multiple biofilm characteristics such as surface area, biomass, roughness co-efficient and maximum thickness.

2.3.3 Pyocyanin assay

To determine pyocyanin production, cultures were grown overnight at 37 °C shaking at 180 rpm. To extract the supernatant containing pyocyanin, 1.5 ml of culture was transferred to Eppendorf tubes and centrifuged at 13,000 for 2 minutes. 100 µl Supernatant was transferred to a 96 well plate and read at 695 nm by Fluorostar

Omega plate reader. For hormone treated cultures, 10 nM of oestradiol, testosterone and progesterone was used.

2.4 Motility Assays

2.4.1 Twitching

To measure twitching motility of the isolates, a colony of *P. aeruginosa* was stabbed in the middle of an agar plate to the bottom. The colonies were then grown over-night at 37°C. The agar was then removed, and the plate was subsequently stained with 0.25 % CV for 15 minutes. Twitching zones were measured using a ruler. The cut-off point in which the isolate is considered motile is 5 mm in diameter.

2.4.2 Swimming motility

The methodology used is based on a swimming motility assay by Ha, *et al,* (2014) [411]. Briefly, media was prepared according to the protocol described. *P. aeruginosa* cultures were grown overnight in LB broth at 37 °C shaking at 180 rpm. Subsequently, a sterile toothpick was used to stab the agar from top to the near bottom of the plate. The agar plates were incubated at 37 °C for 18 h. Three biological replicates were performed for each isolate in the study. Millimetre measurements were taken to assess the diameter of swimming bacteria. To determine whether the isolate is motile, comparison with the reference laboratory strain PA14 were conducted.

2.4.3 Swarming motility

The swarming motility test was performed according the protocol established by Ha, *et al,* (2014) [411]. Once the preparations of plate cultures were completed, 2.5 µl of overnight of *P. aeruginosa* cultures were added to the top of the agar. The agar plates were incubated overnight at 37 °C for 18 h. Three biological replicates were conducted for each isolate. Measurements in mm were taken and the isolates were deemed motile/non-motile depending on the diameter of 6mm.

2.5 Antibiotic resistance and genomic sequencing

2.5.1 Disc diffusion assay

Antibiotic susceptibility testing was carried out following the European Committee on Antimicrobial Susceptibility Testing (EUCAST) protocol and cut-off points (Table 2.2) [412]. Three biological replicates of each clinical isolate were grown overnight on

Mueller-Hinton agar with antibiotics disc (Thermo Fisher Scientific). The measurements in mm were determined and calculated as an average of three readings and compared to the published EUCAST breakpoints to determine if the clinical isolate is sensitive, intermediate or resistant.

Table 2.2 Class of each antibiotic and dose used in this study. S= sensitive R=resistant

Class	**Antibiotics**	**Dose**	**Zone diameter breakpoint (mm)**	
			S ≥	**R <**
Aminoglycosides	Tobramycin	TN 10	16	16
	Gentamicin	GM 10	15	15
	Amikacin	Ak 30	18	15
	Netilmicin	NET 10	12	12
Carbapenems	Imipenem	IMI 10	20	17
	Meropenem	MEM 10	24	18
	Doripenem	Dor 10	25	22
Cephalosporins	Ceftazidime	Caz 30	17	17
	Cefepime	CPM 30	21	21
	Ceftazidime/avibactam	CZA 14	17	17
Fluoroquinolones	Levofloxacin	Lev 5	22	22
	Ciprofloxacin	CIP 5	26	26
Monobactams	Aztreonam	ATM 30	18	18
Penicillins	Piperacillin	PRL 100	18	18
	Piperacillin / Tazobactam	PTZ 110	18	18
	Ticarcillin	TC 75	18	18
	Ticarcillin with clavulanic acid	TIM 85	18	18

2.5.2 Minimum inhibitory concentrations of antimicrobial agents

Minimum inhibitory concentrations (MIC) for one clinical UTI *P. aeruginosa* isolate 758 sourced from Kuwait, was determined using the microdilution method as described in the guidelines of the British Society for Antimicrobial Chemotherapy [413]. Briefly, 100µl of overnight culture of the isolate was diluted to OD600 of 0.05 and was mixed with 100µl of serially diluted colisitin (512 - 0.5 µg/ml) (Sigma Aldrich, UK) in triplicate using cation adjusted MH broth. Microtitre plates were incubated for 1-2 days at 37 °C without shaking and bacterial growth was determined by measuring absorbance at OD600 with a FLUOstar® Omega microplate reader and the MARS Data Analysis Software test.

2.5.3 Genomic DNA analysis

All isolates in this study were sequenced following extraction by Wizard® Genomic DNA Purification Kit (Promega). Briefly, 1 ml of overnight culture was added to a 1.5ml microcentrifuge tube. The sample was centrifuged at 13,000–16,000 × g for 2 minutes in order to pellet the cells and the supernatant was removed. 600µl of Nuclei Lysis Solution was added and resuspended by gentle pipetting. The sample was incubated at 80°C for 5 minutes to lyse the cells, the sample was allowed to cool. Subsequently, 3µl of RNase Solution was added to the cell lysate, followed by tube inversion 2–5 times to ensure adequate mixing. The sample was incubated at 37°C for 15–60 minutes and cooled at room temperature. 200µl of Protein Precipitation Solution was added to the RNase-treated cell lysate, followed by vigorous vortexing at a high speed for 20 S. The sample was then incubated on ice for 5 minutes. Further centrifugation at 13,000–16,000 × g for 2 mins was conducted. The resulting supernatant containing DNA was transferred to a new 1.5ml microcentrifuge tube containing 600µl of temperature isopropanol. This was followed by mixing the tubing until the thread-like strands of DNA were visible from the mass. Subsequently, the supernatant tube was drained on a clear absorbent paper carefully. 600µl of room temperature 70% ethanol (Sigma-Aldrich) was added to the tube, followed by gentle inversion of the tube multiple times to wash the DNA pellet. The sample was centrifuged at 13,000–16,000 × g for 2 mins and ethanol was gently discarded. The pellet was allowed to dry after draining the tube on clean absorbent paper to air-dry

for 10-15 min. 100µl of DNA Rehydration Solution was added to the tube, followed by rehydration of the DNA at 65°C for 1h. The DNA was stored at 2–8°C in preparation for sequencing Centre for Genomic Research (CGR) at the University of Liverpool. Briefly, two isolates were sequenced for each sample in the panel. 100 bp paired reads were then generated from the ends of 500 bp fragments by utilising an Illumina HiSeq 2000 sequencer. FastQ containing sequenced read data were cut-off upon detection of Illumina adapter sequences by utilising Cutdapt version 1.2.1 [414]. This was followed by the selection of option -O 3, which resulted in trimming the 3' of all reads that matched the adapter sequence of 3 bp or more. The Sickle version 1.2 was utilised, the minimum window quality of 20 was chosen as the score to further trim sequences shorted than 10 bp [415].

2.5.4 Identification of antibiotic resistance genes

The Comprehensive Antibiotic Resistance Database (CARD; http://arpcard.mcmaster.ca) [7] was utilised to search for resistance genes on the sequences obtained from CGR. The sequences were inserted in the search tool of the Resistance Gene Identifier (RGI), and the following boxes were ticked prior to analysis were DNA Sequence, perfect and strict hits only, exclude nudge and High quality/ coverage.

2.6 Polymerase chain reaction and RNA extraction for qPCR

2.6.1 DNA extraction

Promega's Wizard® Genomic DNA purification kit was used to extract chromosomal DNA from all isolates included in this study. To summarise, overnight cultures were prepared by inoculating a single colony in 5 ml of LB broth. From this, 1 ml of was transferred to an Eppendorf tube and centrifuged at 13,000 rpm for 2 min to form a bacterial pellet. Resulting pellets were lysed with 600 µl of lysis solution and incubated in a water-bath for 5 min at 80°C. Lysed cells were subsequently treated with Rnase (12 µg) and 200 µl of protein precipitation solution. This was followed by centrifugation at 13000 rpm for 2 min, the accumulated supernatant was transferred to a clear tube consisting of 600 µl of propanol to precipitate the DNA. Further centrifugation at 13000 rpm for 2 min was performed, followed by washing the pellet with 70% of ethanol. Tubes containing DNA suspensions were stored at 4°C.

2.6.2 DNA amplification by PCR and gel electrophoresis

GoTaq ®qPCR master mix was used to perform PCR reactions. Each reaction contained 25 µl of 1x flexi buffer, 200 µM of dNTPs (dntpA, dntpT, dntpC, DntpG), 300 µM of each oligonucleotide primer, 2.5 mM of MgCl2 and 1.25 U Taq polymerase with (Table 2.3). 1 µl of PAO1 DNA was added to the master mix.

Table 2.3 Components used in PCR reactions

Buffer	1X	5X	Temperature	Time
DNTPs	0.05	2.5	95 C	3 min
Buffer	5	25		
Forward primer	0.75	3.75	94 C	20 s
Reverse primer	0.75	3.75	57 C	20 s
Mgcl2	2	10	72 C	40 s
Taq	0.1	0.5	72 C	1 min
DEPC H2O	14.9	74.5		
DNA	1 ul	-		

2.6.3 Gel electrophoresis

To produce agarose gel, agarose powder 1% [W/V] (Bioline) was mixed with TBE 0.5X in a volume of 150 ml, followed by the addition of 3 µl of a 10 mg/ml ethidium bromide solution. 5 µl of DNA produced by previous PCR reactions was loaded onto the gel for 1h to perform electrophoresis in 0.5 TBE solution. To compare sizes pf the bands produced, 1 µl of DNA marker (Invitrogen) was added to the both sides of the gel. (Gelrad-biodoc) translluminator was used to illuminate the bands and photograph the gel.

2.6.4 DNA Purification

DNA was purified using QIAquick PCR purification kit (Qiagen) per manufactures instructions. In summary, 80 µl of Buffer PE (Qiagen) (equivalent to 5 volumes to 1) were added to 20 µl in column attached to a flow-through. The column was centrifuged at 13000 rpm 1 min to bind the DNA. Subsequent washing step was performed by adding 750 µl Buffer PE to the column, the sample was centrifuged at 13000 rpm and eluted by 50 µl pf DEPC water (Invitrogen) into a clear a 1.5 ml tube.

2.6.5 DNA Quantification

Purified DNA samples were quantified using Qubit™ dsDNA BR Assay Kit (Fisher Scientific) per manufactures instructions. Master mix containing 0.5 % of Qubit™ dsDNA reagent and 95.5% of Qubit™ dsDNA BR buffer was prepared for subsequent additions of 2 µl of DNA into separate clear tubes. The concentration of DNA was measured in ng/ul with Qubit 3.0 fluorometer (Invitrogen).

2.6.6 RNA Extraction and cDNA preparation

Multiple steps were performed to extract and prepare cDNA for subsequent qPCR experiments. 1 ml of culture was dispensed into an RNAse free 1.5 eppendorf tube (Thermo Fischer Scientific) and subsequently centrifuged at 13,000 rpm for 2 min. The supernatant was removed, and the pellet was resuspended in 500 µl Triazole (Invitrogen) followed by using Direct-zol® RNA MiniPrep Plus (Zymo Research) kit, RNA extractions were performed in a fume hood. At the end of protocol, the samples were eluted by 50 µl of DEPC water and subsequently stored in a -80 freezer. The samples were quantified using The Nanodrop™ ND-1000 spectrophotometer at 260 nM. Removal of genomic DNA was performed by Dnase using TURBO DNA-free Kit. cDNA synthesis was performed using SuperScript ® III First-Strand Synthesis SuperMix kit as instructed by the manufacturer, synthesis steps were carried out in GeneAMp PCR System 9700. The purity and amount of the yielded cDNA was once again tested by quantification using the Nanodrop™ND-1000 spectrophotometer at 260 nM. Samples were stored in -80° prior to qPCR analysis.

2.6.7 RT qPCR analysis

The mastermix solution for 72 reactions was prepared by mixing 937.5 µl SYBR green (Qiagen), 75 µl of forward primer and 75 µl (Sigma Aldrich) (Table 2.4) and 637.5 of

DEPC-treated water (Invitrogen). To calculate the standard curve, the specific DNA template was diluted (1 in 10) 9 times, the standards used would range from 10^{-4} to 10^{-8}. To conduct the reverse transcription, 23 µl of master mix was added into all the test tubes. Followed by adding 2 µl of standards, DEPC water (negative control) and cDNA samples into the allocated tubes. The samples were inserted into the Rotor Q PCR machine. Then they were preheated for 10 minutes then followed by 40 amplification cycle with the subsequent order (95°C 10 S + 60°C, 15 S + 72°C, 20 S). The analysis was conducted by the Qiagen Rotor gene Q software and the 2-standard curves method.

Table 2.4 Primers used in this study of genes involved in housekeeping, quorum sensing, biofilm formation and exotoxins

Name	Sequence	Annealing Temp (©)	Amplicon size (bp)	Target site	Reference
proC F	CAGGCCGGGCAGTTGCTGTC	74.6	180	*proC*	[416]
proC R	CGTCAGGCGCGAGGCTGTCT	74.5	180	*proC*	[416]
rpoD F	GGGCGAAGAAGGAAATGGTC	66.8	178	*rpoD*	[416]
rpoD R	CAGGTGGCGTAGGTGGAGAA	67.4	178	*rpoD*	[416]
lasR F	AAGGAAGTGTTGCAGTGGTG	62.8	67	*lasR*	[416]
LasR R	GAGCAGTTGCAGATAACCGA	63.0	67	*lasR*	[416]
rhlR F	AACGCGAGATCCTGCAATG	66.3	122	*rhlR*	[416]
rhlR R	GCGCGTCGAACTTCTTCTG	66.2	122	*rhlR*	[416]
pqsR F	TCGTTCTGCGATACGGTGAG	66.3	168	*pqsR*	Unpublished

pqsR R	GCACTGGTTGAAGCGGGAG	68.3	168	*pqsR*	Unpublished
algD F	CGCCGAGATGATCAAGTACA	63.8	157	*algD*	[416]
algD R	TGTAGTAGCGCGACAGGTTCG	67.4	157	*algD*	[416]
pslA F	GCAAGCTGGTGATCTTCTGG	64.9	244	*pslA*	Unpublished
pslA R	TCCCGGAGAACTACAACTCG	64.1	244	*pslA*	Unpublished
pelB F	AGCGCTTGCAACAGATTCTC	64.6	113	*pelB*	[416]
pelB R	AACAGGTTCCAGTGGGTTTC	62.8	113	*pelB*	[416]
cif F	TGAAGAACCAGGCCGACATC	67.5	130	*cif*	Unpublished
cif R	GCACTGGTTGAAGCGGGAG	67.1	130	*cif*	Unpublished
mexX F	AGCTGTTCAAGCAGACCCAGAACA	70.1	171	*mexX*	[417]
mexX R	AGGGTGTCGAAGATGTCGCTGAT	70.1	171	*mexX*	[417]
mexB F	CAAGGGCGTCGGTGACTTCCAG	75.4	272	*mexB*	[418]
mexB R	ACCTGGCAACCGTCGGGATTGA	73.9	272	*mexB*	[418]
flgD F	GTGCAGTCGCTGAACAAGAG	63.9	159	*flgD*	[416]
flgD R	CATACGTTGCTGCTGGAGAC	63.5	159	*flgD*	[416]
phzF F	GGAGATGAACCTGTCGGAGA	64.3	159	*phzF*	[416]
PhzF R	AGCCTGTCCTTGTCGGTCT	63.9	159	*phzF*	[416]

2.7 AUM and Urine media

2.7.1 AUM preparation

A 1L solution of AUM was made using the chemical compounds in (Table 2.5). To start the process, all components were measured into a glass flask with the exception of lactic acid, calcium chloride and Iron II sulphate. Aluminium chloride and citric acid were added separately into the flask. 800 ml of distilled water (ddH2O) was added and stirred with a magnetic rod. As the components started dissolving, stock of 5 mM iron II sulphate was made by adding 0.139 g to 100 ml of ddH2o in addition to a stock with calcium chloride 12.5 mM. Subsequently, 1 ml of 5mM iron II sulphate were added to mixture followed by 199 ml of Calcium chloride $2H_2O$. The lactic acid was added at 0.1 g to dissolve and the pH of the solution was adjusted to 6.5. The solution was sterilised by filtration using a 0.2 µM vacuum filter. A single 500 ml DURAN® bottle containing AUM was stored and used for no longer than 10 days.

2.7.2 Pooled Urine

Urine was collected from healthy controls in accordance with ethical approval obtained from the University of Liverpool. Urine was collected from individuals following consent urine was pooled by combining equal amounts of urine from two males and two females using midstream urine. This was then mixed and filtered using a 0.2 µM vacuum filter. A single 1L DURAN® bottle containing urine was stored and used for no longer than 10 days.

Table 2.5 Contains the components of AUM used in this study with the source of each component

Substance	Quantity (g)	Conc (mmol-1)	Source
Peptone L37	1	-	Sigma Aldrich
Yeast Extract	0.005	-	Sigma Aldrich
Lactic acid D	0.1	1.1	Sigma Aldrich
Citric acid	0.384	2	The British Drug House
Sodium bicarbonate	2.1	25	Sigma Aldrich
Urea	10.21	170	Sigma Aldrich
Uric acid	0.067	0.4	Sigma Aldrich
Creatinine	0.791	7	Sigma Aldrich
Calcium chloride•$2H_2O$	0.367	2.5	Sigma Aldrich
Sodium chloride	5.259	90	Fisher scientific
Iron II sulphate•$7H_2O$	0.0014	0.005	Fisher scientific
Magnesium sulphate•$7H_2O$	0.492	2	Sigma Aldrich
Sodium sulphate•$10H_2O$	3.22	10	Sigma Aldrich
Potassium dihydrogen phosphate	0.953	7	Sigma Aldrich
Di-potassium hydrogen phosphate	1.22	7	Sigma Aldrich
Ammonium chloride	1.34	25	The British drug house
Distilled water	To 1L		

2.8 Proteomic analysis

2.8.1 Preparation of pellets for proteomic analysis

To conduct proteomic analysis, isolate 133098 was chosen due to its consistent phenotypic characteristics and altered antimicrobial resistance in AUM compared to LB (data not shown). The isolate was grown in 7 ml of LB and AUM overnight shaking at 180 rpm and 37 °C. To assess the responses of *P. aeruginosa* to the environment in LB, AUM, and pooled urine media, 5 ml of 5 biological replicates of overnight culture were added to the corresponding media (Figure 2.2). Similarly, to investigate the impact of sex hormones on *P. aeruginosa,* stock solutions of ethanol (vehicle control, Sigma Aldrich) oestradiol, testosterone and progesterone hormones were added to the AUM to a final concentration of 20 nM (Figure 2.3). Growth curves were performed in order to determine the optical density in each media and continuously asses growth. Once the optical density of the cultures reached 0.25 +/- 0.05 OD_{600}, the samples were centrifuged at 4500 rpm for 12 min to form a large pellet. The pellets were washed with PBS and stored at −80 °C before processing for proteomic analysis. Samples were then prepared for proteomic analysis by Dr Stuart Armstrong (University of Liverpool). Briefly, upon the initiation of proteomic analysis, samples were sonicated in 1% (w/v) sodium deoxycholate (SDC) in 50 mM ammonium bicarbonate to lyse the pellets. The samples were heated at 80 °C for 15 min, followed by centrifugation at 12,000×g to pellet debris. The resulting supernatant was retained, and the proteins were reduced with 3 mM dithiothreitol (DTT) (Sigma Aldrich) at 60 °C for 10 min. The sample was allowed to cool, followed by alkylation with 9 mM iodoacetamide (Sigma Aldrich) in the dark for 30 min with all steps conducted with intermittent mixing by vortexing. This was followed by the addition of proteomic grade trypsin (Sigma Aldrich) at a ratio of 50:1 and subsequent incubation overnight at 37 °C. The remnants of SDC were removed by the addition of trifluoroacetic acid (TFA) to a final concentration of 0.5% (v/v). Peptide samples were centrifuged at 12,000×g for 30 min to ensure full removal of remaining precipitated SDC.

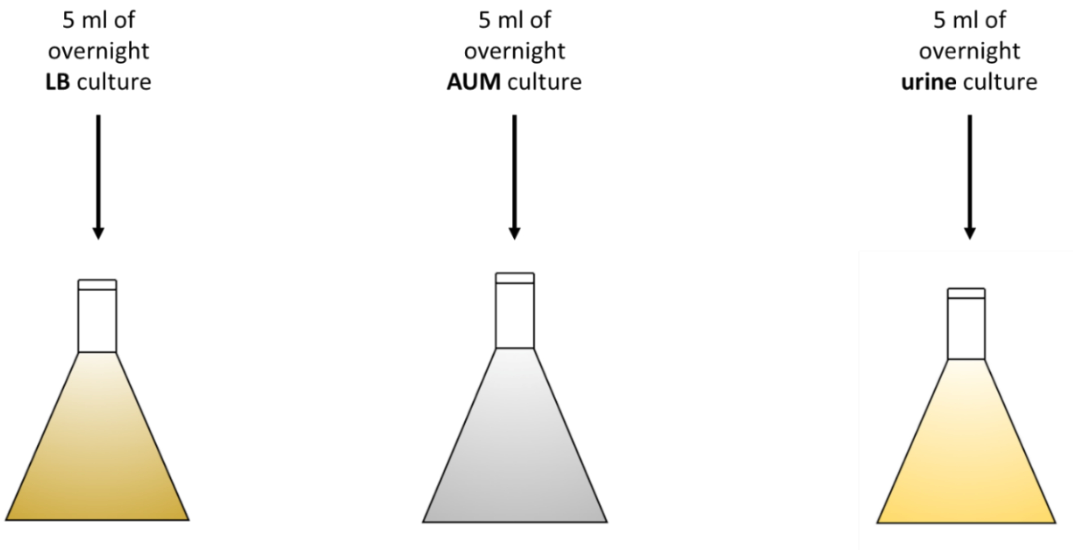


Figure 2.2 The methodology used to inoculate overnight cultures of LB, AUM and pooled urine prior to proteomic processing. 5 ml of *P. aeruginosa* cultures were added to 45 ml of the corresponding media

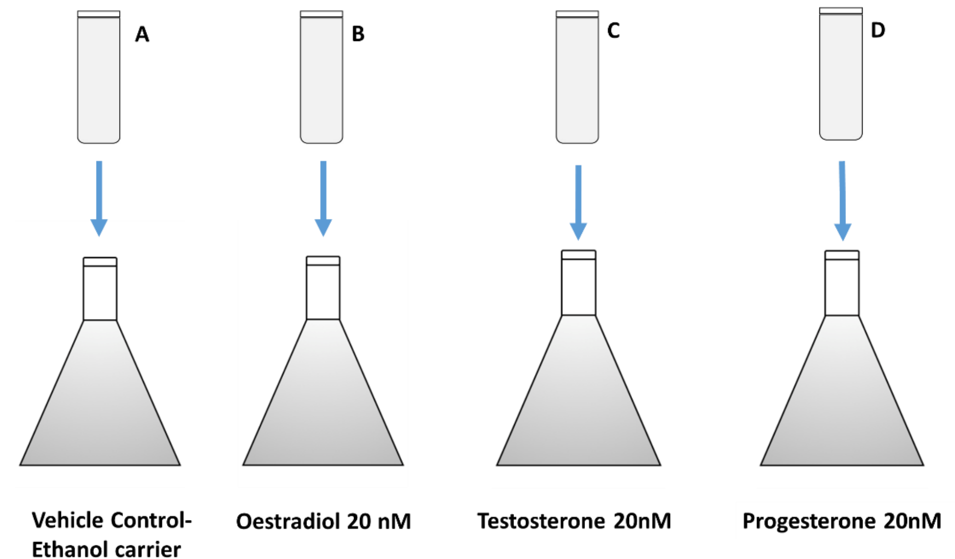


Figure 2.3 The methodology used to inoculate overnight cultures to AUM media prior to proteomic processing. 5 ml of *P. aeruginosa* cultures (A, B, C, D) were added to 45 ml of the corresponding media with 20 nM hormone concentration

2.8.2 NanoLC MS ESI MS/MS analysis of peptides

To initiate processing of the peptides, analysis by on-line nanoflow LC using a machine known as the Ultimate 3000 nano system (Dionex/Thermo Fisher Scientific). Samples of interest were loaded on the trap column with the following specifications

(Acclaim PepMap 100, 2 cm × 75 µm inner diameter, C18, 3 µm, 100 Å) at 5 µl min−1).This was followed by addition of an acquis solution containing 0.1% (v/v) TFA and 2% (v/v) acetonitrile. Upon the 3 min mark, the trap column was set in-line analytical column with the following specifications (Easy-Spray PepMap® RSLC 50 cm × 75 µm inner diameter, C18, 2 µm, 100 Å) and fused with to silica nano-electrospray emitter (Dionex). The column was coupled with a Q-Exactive HF mass spectrometer under an operating constant temperature of 35 °C. The chromatography was conducted with 0.1% formic acid (Buffer A) and (Buffer B) which consists of 80% acetonitrile in 0.1% formic acid. Separation of peptides was performed by a linear gradient of 3.8–50% buffer B over a duration of 90 min with a flow rate of 300 nl per min. The mode of the Q-Exactive HF was set on the data-dependent mode with survey scans obtained at the resolution of 60,000. The Isotope patterns were of the 10 most abundant patterns with charges ranging between +2 to +5 from the survey scan were chosen with an isolation window of 2.0 TH and separated to fragments with high and normalised energy dissuasion. Time for maximum injections of ion for survey scans was set between 100 and 45 ms, respectively. As for the ion target scan, the value was set to 36A for survey scans and 1E5 for the MS/MS scans. The resolution with which MS/MS scans acquired were acquired at a resolution of 30,000. The dynamic exclusion feature was utilised by to minimise sequence repetitions for 20 s.

2.8.3 Protein identification and quantification

To identify and quantify the proteins. Thermo Raw files were transferred into the prognosis QI for proteomics (version 4.1, Nonlinear Dynamics). By utilising default settings and using selected runs as reference, the runs were time aligned. The software identified the peaks by using the default settings involving peaks between +2 and +7 charges only. These data were then converted to mgf files, and tagged for prognosis IQ for proteomics, this was followed by running the data search engine known as the mascot (version 2.3.02, Matrix Science). The translated open reading frames from the reference strain of *P. aeruginosa* were searched against tandem repeat data (Uniprot reference proteome, UP000002438, December 2016) and a contaminant database (cRAP, GPMDB, 2012) (combined 5733 sequences: 1,909,703

residues). Parameters used in this search were as follows, both of precursor mass tolerance and fragment mass tolerance were set at 10 p.p.m and 0.05 Da, respectively. In addition, two tryptic cleavages were allowed. Oxidation parameter was set at a variable modification while the carbamidomethylation was set at a fixed modification. To assess the validity of mascot results, the machine learning algorithm back up the results obtained. Upon utilising the Mascot decoy database function, the false discovery rate was less than 1% and the individual percolator ion scores recorded >13, which indicates to the identify or extensive homology with a ($p < 0.05$). Upon the transferring the mascot search results to Progenesis QI for proteomics, the files were converted to XML files. The intensities of the peptides were normalised against a reference utilised by Progenesis QI for proteomics, the intensities were utilised to emphasize the relative differences in the expression of proteins in the treatment groups. Identification of proteins was restricted only to 2 or more peptides include in this set of data. Again, Prognosis QI for proteomics was utilised to perform statistical analysis which identified statistically significant proteins with $p < 0.05$, $q \leq 0.05$, and relative fold change of ≥ 1.5 of differentially expressed proteins. The Principal component analysis (PCA) plots were generated by using ClustVis proteins which included proteins with 2 or more identified peptides along with $q < 0.05$. Scaling was conducted by applying unit variance to rows; amputated SVD was used to calculate PCA.

2.9 Therapeutics

2.9.1 Bacteriophage-inhibition of *P. aeruginosa* assay

To test the ability of bacteriophages PELP20, PMN, PT6 and 42/1 to infect our panel of UTI isolates, a mixture of 3.5 ml pf liquid top agar (0.6% agar) (Fisher Scientific) and 150 µl of *P. aeruginosa* grown in either LB) (Appleton woods) or AUM was prepared. The mixture was used to inoculate LB agar plates. Phage stocks were adjusted in concentration to 200 plaque forming units per 10 µl to perform a spot assay. 10 µl of each phage was inoculated onto the lawn of *P. aeruginosa* and the plates were allowed to grow overnight at 37 °C. Clear zone formation indicated that the isolate was susceptible to that phage.

2.10 Statistical analysis

All statistical analyses (unless otherwise stated) were performed using Sigma Plot 14 software. Distribution was determined using Shapiro Wilk normality test. Growth curves, biofilm assays were analysed using Kruskal-Wallis analysis of variance and Holm-Sidak post hoc test for nonparametric data and Brown-Forsythe test for Pairwise comparison. MIC data were analysed using Kruskal-Wallis analysis of variance and Brown-Forsythe test.

Chapter 3

3 Characterisation of a Panel of Urinary *P. aeruginosa* Isolates

3.1 Introduction

3.1.1 *P. aeruginosa* characteristics in different human niches

P. aeruginosa is a highly adaptable and versatile opportunistic pathogen with a large genome (5.5 Mb to 7.0 MB). This is larger than other Gram-negative uropathogens such as UPEC (5.2 Mb) [419], *K. pneumoniae* 5.2Mb [420] and *P. mirabilis* 4.063 Mb [421]. *P. aeruginosa* can undergo adaption that enhances survival and virulence in the host. It can act as a pathogen in many different infection types including respiratory, burns and wounds, keratitis as well as urinary tract infections.

3.1.1.1 Respiratory infections

P. aeruginosa can cause chronic respiratory infections in patients with CF, non-CF associated bronchiectasis and patients on ventilators. Infection in CF patients is a prime example of the pathogen's capability to cause severe damage to the respiratory system, leading to increased morbidity, compromised life quality, and eventual mortality [422]. Upon establishment in the CF lung, *P. aeruginosa* can become impossible to eradicate, due to high levels of antibiotic resistance and rapid adaptation to the host environment [423]. Numerous alterations of phenotypic and genetic characteristics take place over the course of the infection to allow the bacteria to further adapt [424], [425]. *P. aeruginosa* secretes important virulence factors such as QS-regulated elastase, pyocyanin and proteases [426], and other factors such as the siderophore pyoverdine [427]. *P. aeruginosa* can lose motility [138] and convert to a mucoid phenotype [426], [428]. Hypermutator strains have been identified and are thought to contribute to adaptation in the CF lung. These isolates have also been linked to the development of antibiotic resistance [424], [429], [430]. For an extensive review on CF pathogen-host interactions see Malhotra *et al,* 2019 [431].

Non- CF bronchiectasis is another situation whereby chronic respiratory infection by *P. aeruginosa* can occur. Bronchiectasis is characterised by the existence of a cough

and sputum that is difficult to clear [432]. It is often exacerbated by the continuous cycle of infections which lead to lung damage, dilation and failure [433]. Risk factors of bronchiectasis consist of old age, poor lung function and prolonged *P. aeruginosa* colonisation [434]–[436]. A meta-analysis conducted on 3683 adult patients revealed that *P. aeruginosa* is associated with increased hospital visits, morbidity, and mortality [437]. Varga *et al,* (2015) analysed a hyper-mutator strain PAHM4 isolated from a chronic bronchiectasis patient and distinguished the similarities and differences between this strain and CF isolates [438]. The role of *P. aeruginosa* in bronchiectasis has been reviewed recently by Chai *et al,* (2019) [439].

3.1.1.2 Burns and wound infections

P. aeruginosa can cause nosocomial burns wound infections [440]. Severe burns lead to devascularisation of the wound environment, which leads to difficulty in clearing bacterial infection due to the inhibition of neutrophils migrating to the wound [441]. Burns patients suffer from an increased susceptibility to infection arising from the breached skin barrier. Such infections can lead to a pro-inflammatory response which is initiated by IL-6 and TNF-α followed by an anti-inflammatory response (IL-10,IL-4) .The avascular nature of the wound decreases oxygen levels and diminishes the nutrient supply to host tissues [442]. Despite these limitations, *P. aeruginosa* can thrive and invade damaged tissues and from here, potentially access the bloodstream [443]. Heparinase produced by *P. aeruginosa* appears to contribute to bloodstream survival and is associated with 100% mortality in a mouse model of burn wound infection. Conversely PA14 lacking *hepP (*which encodes heperinase) were associated with only with 7% mortality of infected mice [444]. Elastase and protease contribute to the breach of host physical barriers through enzymatic action on collagen, thereby further disrupting the integrity of the host membrane defences [445]. Other *P. aeruginosa* virulence factors involved in burn wound pathogenesis are LPS, phospholipase C, ferripyochelin binding protein and effectors of the type III secretion system [445]. As in other types of *P. aeruginosa* infections, antibiotic resistance is a concern and requires immediate development of more therapeutic options. For further recent published literature on the rise of antimicrobial resistance in burn wound patients see [446]. Alternative therapeutics potentially embedded in wound

dressings should be developed to treat/limit *P. aeruginosa* infections in these patients.

3.1.1.3 Keratitis

P. aeruginosa can cause keratitis which is defined as inflammation of the eye due to infection. This can result in the rapid destruction of the cornea [447]. *P. aeruginosa* is the leading cause of bacterial keratitis around the world and a major cause of corneal ulcers in developing countries [448]. *P. aeruginosa* has been associated with contact lens use and the incidence of this infection has increased since the introduction of contact lenses in 1971 [449], [450]. Ocular surgery, trauma and viral infections are predisposing factors [451]. Furthermore, *P. aeruginosa* can be resistant to disinfectants and can adhere to plastic surfaces and form biofilms on contact lenses, intraocular lenses and lens storage cases [452], [453]. Upon infection of the eye, *P. aeruginosa* invades the corneal epithelium and subsequently moves into the corneal stroma. The bacteria binds to corneal epithelial glycoproteins through attaching via pili [453]. LPS also mediates adherence to the cornea or contact lenses [454]. Flagellar motility plays a major role in the dissemination of infections, mutants in flagellar motility genes are attenuated in their ability to colonise the corneal epithelium [455]. Biofilm formation and maturation on the surface of the cornea has been reported [456], [457]. However, the host-pathogen interactions and the role of biofilms has not been extensively researched in ocular infections.

3.1.1.4 Urinary tract infections

In contrast to CF infections caused by *P. aeruginosa*, there is a paucity of information in the literature that address *P. aeruginosa* UTIs. Infections impacting the urinary tract account for the majority of infections in Europe at 40% of all nosocomial infections [458]. Most infections cause inflammation of the bladder, but in some cases, infections can progress to the kidney (pyelonephritis), and particularly in patients suffering from vesico-uretric reflexes (VUR). Patients affected by this disorder could further experience complications such as kidney scarring and failure. UTIs can also be recurrent and persistent, causing more severe infections. Furthermore, dissemination of bacteria into the bloodstream (bacteraemia) and sepsis may lead to fatal infections. One third of bacteremia cases are caused by *E. coli*

in England with over 50% of these are associated with UTI .Uropathogenic *P. aeruginosa* is not as widespread, however, it is frequently detected in complicated UTI such as CAUTI, it has been reported that it is the third most common cause of CAUTI [47]. Multiple virulence factors participate in UTIs, elastase and protease have proteolytic and elastolytic activity in UTI infections and are produced in higher levels than isolates obtained from burn wound infections, skin wounds infections and acute pneumonia [175]. Hamood *et al,* (1996) examined the production of enzymes such as elastase, phospholipase C, toxin A, and exoenzyme S, quantified and compared from isolates derived from trachea, wounds and urinary tract. Most isolates produced all toxins but in different quantities based on the site of infection. Wound and UTI isolates produced significantly higher levels of exoenzyme S than tracheal isolates. As for elastase and phospholipase C, they were produced by most isolates from trachea, wound and urinary tract [180]. Ciragil *et al,* (2004) investigated the production of virulence factors alginate, elastase and alkaline protease from isolates derived from the airways of CF patients and blood, lungs, and urine from non-CF patients [148]. It was observed that UTI isolates produced the least amount of alginate and the highest levels of alkaline protease compared to the other isolates. This may suggest a role for alkaline protease in degrading C2 component of complement system and inhibiting associated phagocytosis [459]. Iron acquisition mechanisms are indispensable for *P. aeruginosa* and the role of siderophores have been reported in CF and burn infections [460], yet their role remains unclear in *P. aeruginosa* uropathogenesis. Pyoverdine and pyochelin are two siderophores produced by *P. aeruginosa* in order to facilitate uptake of iron from the environment. Upon characterisation of 30 UTI and CAUTI isolates, Tielen *et al,* (2011) reported the detection of pyoverdine [90]. Although the majority of *P. aeruginosa* isolates produce these two types of siderophore, a study by Visca *et al,* (1992) revealed that some isolates appeared to rely on only one siderophore as some strains did not produce both siderophores (2.4% and 7.4% were defective in pyochelin and pyoverdine, respectively) [461]. *P. aeruginosa* UTI isolates that produce high levels of haemolysin have been associated with higher bacterial counts and renal colonisation in a mouse model of infection [462]. These findings suggest that *P. aeruginosa* UTI pathogenesis could involve several mechanisms to acquire iron from the host. Other uropathogens

such as UPEC utilise siderophores enterobactin, yersiniabactin, and salmochelin to survive in the urinary tract [463], [464]. For detailed analysis on siderophores and iron acquisition mechanisms see chapters 4 & 5.

P. aeruginosa UTI pathogenesis has recently been reviewed by Newman *et al,* (2017) [269]. Despite the reported genotypic and phenotypic characterisation of *P. aeruginosa* UTI isolates in the literature, this field of interest remains relatively underreported.

3.2 Biofilms

Formation of biofilms is a crucial factor in establishing colonisation and survival in multiple host niches. Biofilm formation on urinary catheters make up 40% of nosocomial infections and are the most common source of Gram-negative bacteraemia in patients [465]. One of the main drawbacks of utilising urinary catheters is the resulting compromise of the natural barriers such as the urethral sphincter [466]. Thus, catheters serve as a basis for biofilm formation and provides a focal point for replication and dissemination of bacteria into the bladder [467]. Treatment is becoming increasingly difficult due to reduced antibiotic susceptibility of biofilms, contributing to the persistence of CAUTI [468]. This is attributed to poor antibiotic penetration, slow growth, nutrient limitation, and the existence of persister cells [469].

P. aeruginosa can possess different biofilm formation characteristics depending on the site of infection. For example, CF isolates produce the exopolysaccharides alginate, Pel and Psl and this has been shown to be important in both *in vitro* biofilm formation assays [470] and *in vivo* [428]. Furthermore, mucoid isolates have been identified that overproduce alginate and these have been linked to chronic infections [471], [472]. Once colonisation occurs and the biofilm is fully established within the respiratory tract, treatment with antibiotics cannot eradicate the infection [473]. Constant immune recruitment of PMNs to surround *P. aeruginosa* biofilms, ultimately leads to lung damage and respiratory failure due to the aggressive inflammatory response [474]. *P. aeruginosa* biofilms are also implicated in chronic wound infections, where penetration of deep layers occurs [475]. Infections with

P. aeruginosa present in a biofilm often cause larger wounds than infections caused by other bacteria such as *S. aureus* and thus can be highly problematic in the healing process [475], [476]. Biofilms on urinary catheters vary in size, as many as thousands of bacteria per ml (>10^5 CFU/ml) can cause infection around the catheter and induce chronic inflammation [477]. The inflammation is driven by recruitment of PMNs and complement components [478]. Gram-negative bacterial uropathogens start developing biofilms upon the colonisation of the catheter [479]. *P. aeruginosa* can utilise flagella to initiate attachment to the catheter. Cellular secretions of bacterial cyclic di GMP then promote formation of biofilms and suppresses multiple cellular functions such as motility. C-di-GMP is considered as a switch between a motile and sessile bacterial lifestyles [77]. Indeed, c-di-GMP binds and acts on the transcriptional regulator FleQ to suppress flagellar movement and inhibits FimX to repress type IV pilus involved in twitching motility [75], [480], [481]. As a secondary messenger, c-di-GMP activates both of the *psl* and *pel* exopolysaccharide operons *in vitro* by binding to the FleQ master regulator [76]. Thus, transforming motile bacteria to sessile biofilms is governed by multiple actions of c-di-GMP acting in concert to promote biofilm formation. Cole *et al,* (2014) examined the ability of PA14 and PA14 mutants in alginate production, to from CAUTI in a mouse model. The findings indicated that c-di-GMP promoted biofilm formation independent of alginate and pel exopolysaccharides, followed by colonisation of the bladder and subsequently, the kidneys [56]. Therefore, the importance of exopolysaccharides in the context of catheter biofilms in unclear.

As well as bacterial-derived factors that promote initial colonisation, *P. aeruginosa* can also utilise host factors to aid this process. *P. aeruginosa* attach to secreted host proteins present on the catheter surface [466]. Tamm-Horsfall protein (THP) is a glycoprotein secreted by kidney tubular cells and the most abundant protein present in urine [482]. THP exists in the mucosal slime and provides protection against invasive *E. coli* in the urinary tract. Mice mutants lacking THP do not clear UPEC as efficiently as the wild-type [483]. However, some studies conducted *in vitro* and *in vivo* found the opposite effect. Harjaj *et al,* (2005) utilised THP-coated *P. aeruginosa* and injected it in murine bladder via urinary catheters, and observed increased

bacterial load and tissue pathology of ascending acute pyelonephritis [462]. THP has also been found to bind to catheters and promotes *P. aeruginosa* biofilm formation [484]. Therefore, the exact role of this host protein remains unclear although it may promote bacterial persistence in the presence of an indwelling catheter.

Urea is another major component of urine which has been shown experimentally to be a contributing host factor which promotes biofilm formation [56]. As the primary solute in urine, urea has been shown to induce the release of eDNA from a subgroup of *P. aeruginosa* cells within the biofilm [56]. Thus, biofilms can be formed in the lumen of catheter without the expression of exopolysaccharides such as Pel and Psl. Alterations of QS systems *las* and *rhl* were mediated by urea and urine, suggesting that urine acts as an inhibitory substance [485]. This indicate that quorum sensing may not be essential during CAUTIs. Further discussion on the role of eDNA and pyocyanin will follow in chapter 4.

3.3 *P. aeruginosa* motility

As discussed previously (section 1.3.2), motility is important in the initial stages of infection in various human niches. *P. aeruginosa* possess several different modes of motility. The exact role in UTIs is unclear.

3.3.1 Swimming motility

Motility is utilised by multiple bacterial species to reach new environmental niches and this includes movement to a potential infection site. *P. aeruginosa* can execute at least 3 types of motility; swimming, twitching and swarming [69]. Swimming motility in liquid is promoted by a single polar flagellar and allows rapid movement [486]. This movement is powered by a motor complex where energy for rotational torque is provided by flagellar subunits. The energy propels the bacterium in a direction controlled by the rotor [487]. Swimming motility is linked with immune activation by TLRs, neutrophil phagocytosis and NETs in the host [488]. As a potential survival strategy in the respiratory tract of CF patients, loss of flagella is common and has been found to confer resistance to phagocytosis and aid in the establishment of chronic infection [489].

3.3.2 Twitching motility

Twitching motility allows movement across solid and semi-solid surfaces and is dependent on type IV pili, fibrous appendages on the cell surface. This process involves repeated extension, tethering and retraction of the pili that results in a jerky movement [490]. Each bacterial cell can have many of these pili appendages that can extend and retract independently, or in a coordinated manner [491]. Despite having pili at each pole*, P. aeruginos*a cells normally utilise one pole to direct movements [491]. Type IV pili are responsible for allowing *P. aeruginosa* to adhere to a diverse range of surfaces such as glass, stainless steel and mammalian buccal epithelial cells [492], [493]. Twitching motility, is hence, considered as a virulence factor promoting micro-colony formation (through initial attachment) and host cell adhesion [492]. This form of motility is utilised to counteract iron depletion/scarcity in mammalian hosts [494]. In the respiratory tract of CF patient, loss of type IV pili has been identified as a possible evolutionary adaptation during chronic infection, perhaps in part mediated as a defence from temperate phage infection which often utilise pili as a receptor. Currently, the role of type IV pili is unclear in *P. aeruginosa* UTI motility, however, adhesive organelles such as the fimbriae in UPEC has been shown to play a role in urothelium adhesion [495].

3.3.3 Swarming motility

Swarming motility is the movement of bacteria across semi-fluid surfaces. It is regarded as the fastest mode of motility to expand and colonise environments and host tissues surfaces [496]. This type of movement is not exclusively dependent on a single organelle or biochemical pathway, instead it involves multiple coordinated cellular activities. *P. aeruginosa* utilize type IV pili, flagella and biosurfactants in this process [492], [497],. Rhamnolipids are the main biosurfactants produced by *P. aeruginosa* and RhlA is the major protein [498]. Current evidence suggests that swarming motility may affect biofilm architecture. Hyper-motile strains form flat biofilms while strains with lower levels of swarming tend to aggregate and form micro-colonies [499], [500]. To address the complexity of the multi-factorial regulation of *P. aeruginosa* swarming motility, Yeung *et al,* (2009) performed insertional mutations in PA14. The study identified 35 transcriptional regulators

involved in in swarming motility and multiple two-component-systems. An inverse relationship between biofilm formation mutants and swarming motility deficient mutants was observed [501].

In contrast to CF infections caused by *P. aeruginosa* [489], [502], motility profiles of UTI isolates are not widely reported. Tielen *et al,* (2011) investigated 30 UTI isolates of *P. aeruginosa* and found that catheter-associated isolates displayed reduced motility in comparison to *P. aeruginosa* isolated from mid-stream urine [90]. However, no correlation was found between each type of motility and biofilm formation. In this study, 90% of isolates displayed swimming, 70% could twitch and 60% showed an ability to swarm [90]. Keratitis isolates showed a similar degree of motility. A study by Priya *et al,* (2005) which analysed the phenotypic differences between 17 ocular *P. aeruginosa* isolates found that 100% possessed the ability to swim, 82% twitched and 64% swarmed *in vitro* [503]. Cigana *et al,* (2016) examined a panel of 338 *P. aeruginosa* CF isolates, most of which were assigned to the most prevalent (P) subtypes identified in an Italian population of CF patients [504]. The majority of P1 isolates were able to twitch (62%) and swim (75%). Other subtypes (P6 and P14) isolates lacked both twitching and swimming. Another study in Italy of 135 CF isolates found that again, motility was generally lower in CF isolates (swimming isolates [40%], twitching [50%] and swarming [45%]) [505].

3.4 Antimicrobial resistance

3.4.1 Intrinsic resistance

3.4.1.1 Efflux pumps

Intrinsic resistance is defined as the innate ability of a given microorganism to withstand the efficacy of antibiotics through existing structural or functional traits [506]. The outer membrane *of P. aeruginosa* contributes to intrinsic resistance owing to its low permeability to multiple types of molecules, including antibiotics [507].The outer membrane of *P. aeruginosa* is highly restrictive, compared to that of *E. coli*, (12- to 1000- fold less permeable) [507], [508]. Efflux pumps play a crucial role in transporting a few antibiotic classes out of the bacterial cell. The protein pumps belong to the resistance nodulation division (RND) super-family of efflux pumps [509]. These are comprised of cytoplasmic membrane transporters, linker

cytoplasmic proteins and outer membrane porin channels [510]. Several efflux pumps contribute to resistance to different antibiotics, for example, MexAB-OprM contributes to the expulsion of β-lactams and quinolones [511], [512]. MexCD-OprJ ejects β-lactams [513]. MexEF-OprN pumps out quinolones [514]. While MexXY-OprM is involved in extruding aminoglycosides [515]. In addition to their baseline activity, mutations can occur that lead to overexpression of efflux pumps. This has contributed substantially to the increasing burden of AMR [516]–[518].

3.4.1.2 Antibiotic inactivating enzymes

Inactivation of enzymes by modifying and breaking down antibiotic molecules is another method by which intrinsic antibiotic resistance is achieved [519]. Numerous antibiotics contain amides and ester chemical bonds which are susceptible to hydrolysis by bacterial enzymes such as β-lactamases enzymes modifying of aminoglycosides [311], [520], [521]. The intrinsic resistance of *P. aeruginosa* is enhanced by the existence of naturally occurring AmpC, a β lactamase that is encoded chromosomally [520]. AmpC utilizes hydrolysis to prevent antibiotic molecules of penicillin and cephalosporins from reaching their targets [522]. AmpC benefits from its location in the periplasm and can be induced using subinhibitory concentrations of β-lactams [522], [523]. In addition, other β-lactamases are chromosomally encoded, and these enzymes can be classified into four groups (A, B, C, D) based on amino acid sequences according to the ambler classification (Table 3.1).

Table 3.1 Ambler classification of β-lactamases and examples of intrinsic enzymes involved in *P. aeruginosa* antibiotic resistance

Ambler class	β-lactamases	Active site agent	Examples	References
A	Penicillinases	Serine	Unknown	_
B	Metallo-β-lactamases	Zinc	Pib-1	[524]
C	cephlaosporinases	Serine	AmpC	[520]
D	Oxacillinases	Serine	OXA-50	[525]

3.4.2 Acquired resistance

In addition to intrinsic resistance, bacterial isolates can acquire further resistance to antibiotics either by mutational alterations or gaining resistance genes via horizontal gene transfer [526]. The combination of intrinsic and acquired resistance complicates potential treatment of *P. aeruginosa* infections [527].

3.4.2.1 Acquired resistance by mutations

Mutational changes reduce susceptibility to antibiotics by, for example, influencing modification of antibiotic targets, overexpression of efflux pumps and antibiotic inactivating enzymes [528]. The mutations occur randomly but can be selected for due to antibiotic pressure during treatment. Furthermore, inactivation of *P. aeruginosa* DNA oxidative repair mechanism through mutations in *mutT, mutS* and *mutY* can increase mutational frequencies (hypermutability), which in turn can increase resistance by causing mutations. For example, mutations that enhance β-lactamase production or overexpression of the efflux pump MexCD-OprJ [529].

Mutations affecting bacterial membrane permeability can lead to enhanced resistance to antibiotics [530]. This occurs due to spontaneous mutations to porins, which are water-filled that allow hydrophilic antibiotics to pass [530], [531]. OprD is such a porin and loss of this channel increases resistance to carbapenems [532], [533]. Fang *et al,* (2014) collected and analysed 61 clinical isolates from different

sources, six of which were from mid-stream urine. The study found that 50 isolates displayed resistance to imipenem by the loss of OprD function either by frameshift mutation or a premature stop codon while the rest of the 11 isolates displayed either reduced *oprD* expression or undetectable OprD porin [95]. Ochs *et al,* (2000) revealed that two loops (2 and 3) contained the entry and binding points for imipenem, mutations in either or both loops lead to conformational changes and increased carbapenem resistance [534].

Efflux pumps eject toxic materials out of the cells to avoid the build-up of harmful molecules such as antibiotics [509], [516]. Overexpression of efflux systems such as MexXY-OprM has been shown to result from gene mutations of the transcriptional regulators, *mexR*, *nalR, nalC* and *nalD*, contributing to reduced susceptibility to β-lactams and fluoroquinolones (FQs) [535]–[538]. Mutations occurring in the *mexZ* gene (a repressor) was also reported to alleviate the levels of overexpression of MexXY-OprM to a few classes of antibiotics, namely, aminoglycosides, β-lactams, and FQs in *P. aeruginosa* clinical isolates [417], [539], [540]. Another efflux system, MexCD-OprJ was found to be overexpressed in several *P. aeruginosa* strains due to mutations in the *nfxB* gene, giving raise to resistance to carbapenems and FQs [513], [541].

Mutational changes can play a role in the protection and modification of bacterial target sites [528]. For instance, alterations of DNA gyrase (GyrA-GyrB) and topoisomerase (ParC-ParE) subunits by chromosomal mutations are one of the common resistance mechanisms to FQs [301]. The mechanisms of action for quinolone antibiotics depend on the inhibition of bacterial DNA replication by affecting DNA gyrase and topoisomerase [542]. Thus, reduction of the binding affinity reduces susceptibility of *P. aeruginosa* to FQs [543]. Polymyxin, which is the last resort class of antibiotics to treat *P. aeruginosa* infections, can be resisted by the bacterium via different mechanisms [544]. First, by modifying and adding 4-amino-L-arabinose (L-Ara4N) to the phosphate group within the lipid A moiety of LPS [545]. Alterations in the two component systems PhoPQ and PmrAB via specific mutations lead to *P. aeruginosa* conferring resistance to cationic polymyxins [546], [547].

3.4.2.2 Acquisition of resistance of genes

P. aeruginosa can acquire further resistance via obtaining transferrable resistance genes. Resistant genes on mobile genetic material such as plasmids, integrons, transposons and phages may be transferred to and between the same or different bacterial species via conjugation, transformation and transduction [430]. Integrons are genetic elements which capture small genetic material called cassettes via site-specific recombination and insert them into a specific location [548]. Integrons have been shown to play a crucial role in disseminating resistance genes between *P. aeruginosa* strains around the world [549]–[552]. Acquisition of β-lactam and aminoglycoside- resistance genes have been reported by several studies worldwide. For instance, several genes have been identified as Class B metallo-beta-lactamases, (MBLs) [553]. Genetic elements such as integrons and plasmids are involved in the transmission of MBLs [554], [555]. A single integron can carry multiple resistance genes. This is demonstrated by two aminoglycoside resistance genes *aacA29a* and *aacA29b,* both are located at the 5’ and ‘3 end on the hydrolysing beta-lactamase VIM-2 gene cassette in *P. aeruginosa* clinical isolates [556]. VIM-2 has been reported in many countries around the world such as Argentina, France, Greece, India and Japan and Spain [557]. In Spain, detection of the MDR and XDR strains carrying VIM-2 with the integron structure IntlI-VIM2-aac6'Ib-qacEΔ1 has been reported [558].

3.4.3 Resistance to β-lactams

3.4.3.1 Extended spectrum β-lactamases

As previously mentioned in section 3.4.1.2, β-lactamase enzymes are classified according to amino acid sequence into 4 sub-groups; A, B, C and D. The enzyme classes A, C and D target an active site of serine for hydrolysis. Group B β- lactamases are metalloenzymes that depend on bivalent zinc for its activity. MBLs resist all β-lactam class antibiotics except for monobactams. In the past few decades, UTI-related extended-spectrum β-lactamases (ESBL) in Enterobacteriaceae and non-fermenting bacterial pathogens such as *P. aeruginosa* and *Acinetobacter baumannii* have been on the rise, particularly in the developing world [559].

3.4.3.2 Class A β-lactamases

Class A β-lactamases are serine enzymes which include *K. pneumoniae* carbapenemases (KPC) enzymes and Guiana extended-spectrum (GES) variants [560]. Transmissible elements such as plasmids and transposons carry KPC encoding genes are on Enterobacteriaceae species such as *K. pneumoniae* [561]. Detection of *P. aeruginosa* carrying KPC enzymes has been reported in China, the Caribbean region and South America, and rare reports from the United States of America [557], [561]. GES enzymes detected in Spain, Turkey, China, and Brazil [557].

3.4.3.3 Class B β-lactamases

Class B β-lactamases, also known as MBLs, are common throughout the world amongst *P. aeruginosa* isolates and the most reported carbapenemases. Unlike other classes of β-lactamases with serine activity, MBLs contain Zn^{2+} ion on the active site and possess strong inhibition via metal chelators. MBLs can hydrolyse carbapenems efficiently and the rest of β-lactams except for monobactams. *P. aeruginosa* containing MBLs are not affected by β-lactamase inhibitors such as tazobactam and clavulanic acid [553]. Integron gene cassette are encoded on genes related to mobile elements and exhibit different compositions, MBL genes occupy these compositions [562]. The most common MBLs are Imipenamases (IMP) and Verona integron-encoded metallo- β-lactamases (VIM) which were discovered in the 1990s in Japan and Italy, respectively (Table 3.2) [563], [564]. These discoveries were followed by more reports of additional MBL-types such as GIM-,SPM,KHM-, NDM-, AIM-, DIM-, SIM-, FIM- and TMB- [557], [565]. , This study will only discuss VIM-type resistance, For extensive reviews on all types see Potron *et al,* (2015) and Hong *et al,* (2015) [553], [557].

Table 3.2 Example of the epidemiology of MBLs around the world

Metallo-β-lactamases	Example encoding gene	Reference
Imipenemases (IMP)	bla_{IMP-1}	[553]
Verona integron-encoded metallo-β-lactamase (VIM)	bla_{VIM-1}	[564]
San Paulo metallo-β-lactamase (SPM)	bla_{SPM-1}	[566]
Germany imipenemase (GIM)	bla_{GIM-1}	[567]
New Delhi metallo-β-lactamase (NDM)	bla_{NDM-1}	[568]
Florence imipenemase (FIM)	bla_{FIM-1}	[569]

3.4.3.4 Verona integron-encoded MBLs

VIM-type *P. aeruginosa* is the most widespread MBL and has been involved in multiple outbreaks around the world [570]. The subtype VIM-2 is the most reported in this opportunistic pathogen [570]. VIM-1 has been reported in Italy upon its detection from a surgical wound [564], followed by further detection in France in four isolates, one of which was isolated from urine [571]. In Greece, a UTI isolate amongst 5 other isolates carrying bla_{VIM} were found to be resistant both to imipenem and meropenem [572]. In the same country, VIM-4 was isolated from a ventriculo-peritoneal catheter and the cerebrospinal fluid. VIM-4 differs from VIM-1 by a single nucleotide and may have diverged from the same ancestor [573]. VIM-4 was also reported in a urine sample isolate collected from a Greek patient in Sweden [574] with further reporting from a children's hospital in Poland, (5 VIM-4 positive *P. aeruginosa* urinary isolates) [575]. VIM-2, it is closely related to VIM-1 as they both share 90% amino acid identity [570] and is disseminated throughout the world and is known to be the most widespread MBL *in P. aeruginosa* [553]. For example, VIM-

2 producing *P. aeruginosa* has been isolated from a urine sample belonging to a Saudi patient diagnosed with prostatitis [576] and from Tunisia from blood and urine samples [577]. In the far east, a South Korean tertiary hospital identified 43 VIM-2 producers over a period of six years mostly from urine and sputum samples [578]. VIM-2 producers have also been detected in Venezuela from blood and respiratory tract samples [579]. In Germany, VIM-2 was isolated from a blood sample of a cancer patient [580]. The first report of outbreak of VIM-2 North America (USA) was published in 2005, where five isolates, one of which was obtained from urine, were identified [581]. In 2004, a novel MBL, VIM-7 was detected. This MBL shares 77% and 74% identity to VIM-1 and VIM-2, respectively [570]. A *P. aeruginosa* pulmonary invasive sample from a cancer patient that carries VIM-7 was identified in the USA [582]. Ever since the discovery of VIM-type MBLs in Italy, they have been reported repeatedly around the world (Table 3.3) [553].

Table 3.3 Examples of variants of VIM- producing *P. aeruginosa* isolates reported around the world since its discovery in Italy in 1999

VIM enzyme Type	Country of isolation	Sample type	Reference
VIM-1	Italy	Surgical wound	[564]
VIM-2	Tunisia	Blood, urine, Catheter	[577]
VIM-3	Taiwan	Blood, urine, Catheter	[583]
VIM-4	Greece	Ventriculo-peritoneal catheter	[573]
VIM-5	Turkey	Respiratory tract	[584]
VIM-6	South Korea	Wound, soft tissue	[585]
VIM-7	USA	Invasive pulmonary sample	[582]
VIM-8	Columbia	Sputum, urine	[586]
VIM-9	UK	Not specified	[557]
VIM-10	UK	Not Specified	[587]
VIM-11	Malaysia	Respiratory tract, abdominal swabs, catheters, blood, urine	[588]
VIM-13	Spain	Not Specified	[589]
VIM-14	Spain	Not Specified	[557]
VIM-15	Bulgaria	Urine	[590]
VIM-16	Germany	Urine	[590]
VIM-17	Greece	Not specified	[591]
VIM-18	India	Sputum	[592]
VIM-20	Spain	Urine, surgical wound, catheter, bone, abscess	[593]
VIM-28	Egypt	Not specified	[594]
VIM-30	France	Not specified	[557]
VIM-36	Belgium	lower respiratory tract	[595]
VIM-37	Poland	Skin	[595]
VIM-38	Turkey	wound, blood, sputum	[596]

3.4.3.5 Ambler Class D

Ambler class D β-lactamases contain OXA-type or oxacillinases are represented by more than 350 genetically diverse enzymes, which are extensively disseminated in Gram-negative bacteria [597]. Generally, OXA-type β-lactamases are categorised into narrow-spectrum and extended spectrum β-lactamases. Narrow-spectrum oxacillinases such as OXA-2 and OXA-10, confer resistance to penicillins and early cephalosporins but can also confer resistance to 2nd generation cephalosporins such as ceftazidime by amino acid substitutions [598], [599].

P. aeruginosa expresses oxacillinases which are β-lactam hydrolysing enzymes [600]. For example, OXA-10, which can be identified in *P. aeruginosa*, hydrolyses cephalosporins, cefotaxime, ceftriaxone, and aztreonam but fails to neutralise ceftazidime and carbapenems [601]. Formerly known as PSE-2, OXA-10 nucleotide sequence was identified by Huovinen *et al,* (1987) [601]. Other examples of oxacillinases include OXA-18 and OXA-198 [602] [603]. In 2018, a study by Bonnin *et al,* (2018) published the detection of three more OXA-198 *P. aeruginosa* tracheal isolates carrying the $bla_{\text{OXA-198}}$ on a 49-kb plasmid. All three patients were deceased in the same hospital [604].

3.4.4 Prevalence of antibiotic resistance and geographical distribution of resistance

Epidemiological studies on AMR in Gram-negative uropathogens show variation in resistance worldwide. For example, lower levels of MDR were found in England than in Eastern Europe, the Middle East and South America [559], [605]. However, in 2000, a multicentre (including hospitals in England) study based on the SENTRY antimicrobial resistance surveillance revealed that uropathogenic *P. aeruginosa* is a cause for concern. This was in part due to *P. aeruginosa* showing less susceptibility to carbapenems at 89.3%, compared to UPEC at 99%. The study, however, does not specify the proportion of isolates originating from England [606]. In the latest surveillance report by SENTRY, which was conducted between 2012-2015 on urinary and intrabdominal *P. aeruginosa* isolates, a total of 64 isolates were collected from the UK and were found to be susceptible to ceftolozane/ tazobactam (100%), ceftazidime (90.6%) and meropenem (84.4%) [607]. Ironmonger *et al,* (2015) conducted the first antimicrobial resistance surveillance in the Midlands (UK) to

detect resistance patterns of uropathogens, one of which was *P. aeruginosa.* The study was performed over a period of 4 years on a population of 5.6 million and collected a total of 786 of *Pseudomonas* spp isolates (4.08% of total uropathogens), detecting 5.7% carbapenemases producers [605]. Public Health England (PHE) started a surveillance program in 2013-2018 to monitor *P. aeruginosa* resistance to ceftazidime and carbapenems in bloodstream infections. The report found that AMR resistance in bloodstream infections remained stable over the period of the study. The report did not specify the percentage of *P. aeruginosa* and their role in bloodstream infections [608].

3.4.4.1 Antimicrobial resistance in the Gulf corporation council

The Gulf Corporation Council (GCC) is comprised of six countries that function as a political and economic union; The state of Kuwait, Kingdom of Saudi Arabia, Kingdom of Bahrain, State of Qatar, United Arab Emirates and Sultanate of Oman (Figure 3.1) [609].

Antibiotic resistance is on the rise across the region. This is attributed to multiple factors, one of which is travelling, a known risk factor for acquisition and transmission of antibiotic resistant bacteria [610], [611]. Increased travel is driven primarily by a large boom in the tourism industry, large inward migration of expatriate workers, and hosting religious pilgrimages such as the Haj and Umra [559], [612], [613]. In addition, multiple studies have been conducted to assess the contributing factors of the rise of antimicrobial resistance in the region. These factors include the high use of antibiotics [614], [615], lack of regional and legislative guidelines for bacterial infection treatments [614], [616], and purchase of antibiotics from pharmacies without prescriptions in certain countries [609], [617].

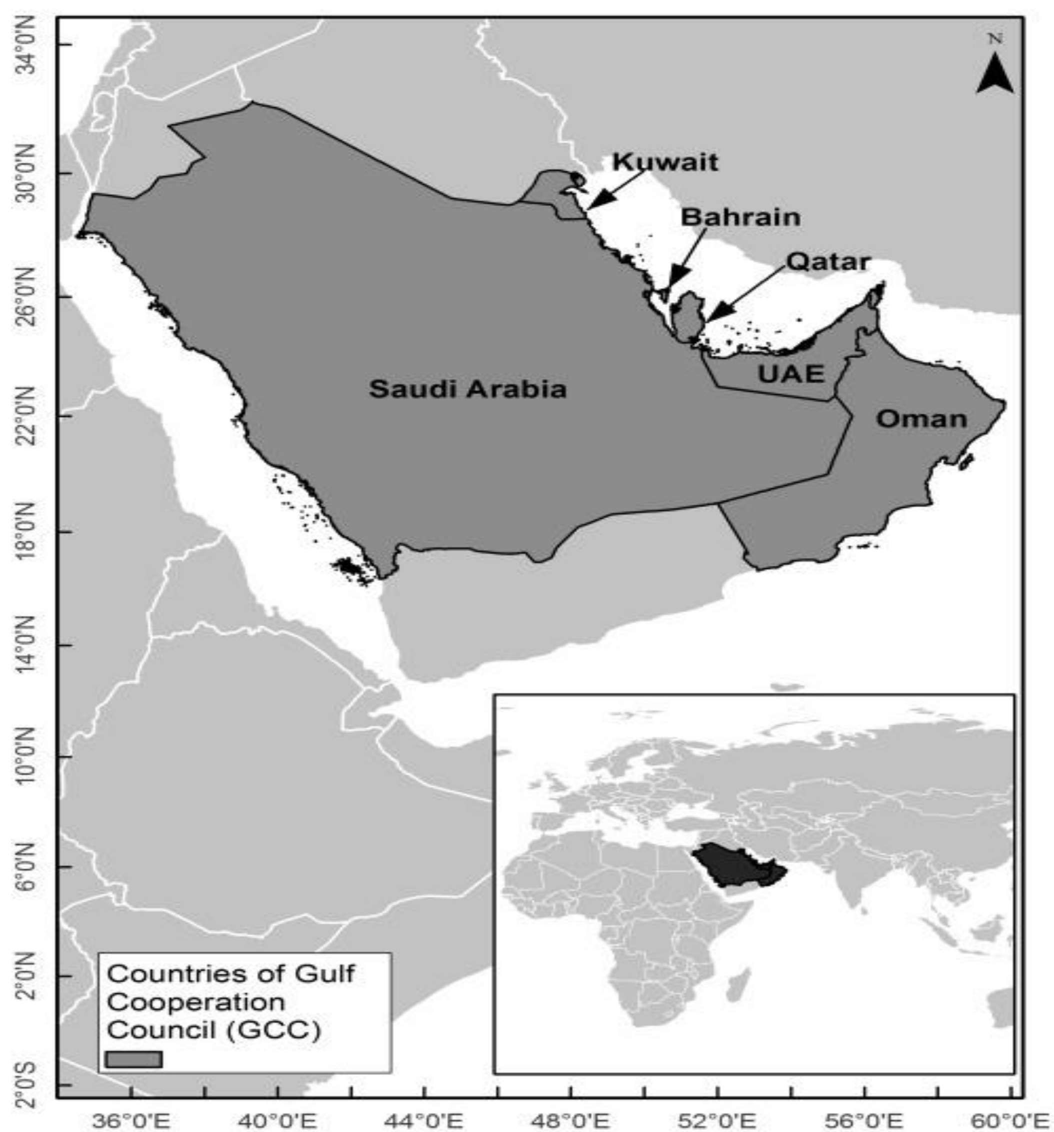


Figure 3.1 The countries of the Gulf Corporation Council comprise of six countries; Kuwait, Saudi Arabia, Qatar, Bahrain, United Arab Emirates [609]

Environmental factors such as naturally occurring high temperatures between 38 °C and 48 °C during the summer months can promote and increase the rate the of horizontal gene transfer between microorganisms carrying AMR genes [618]–[620]. Another concerning factor is the exacerbation of temperatures by climate change and anthropogenic activities, for instance, sea temperature at the Kuwait bay is rising three times the global average [621].

As a result, multiple studies have described the presence of AMR bacteria such as ESBL producing *E. coli* [622], [623], carbapenem resistant *A. baumannii* [624], [625], and pan-drug resistance *K. pneumoniae* [626].

As for *P. aeruginosa*, a retrospective study conducted by al-johani *et al,* (2010) found that *P. aeruginosa* is the second most isolated carbapenem resistant bacteria in an intensive care unit (ICU) in a Saudi hospital. The study also observed a decline in susceptibility from 66% to 24% over a period of 5 years [627]. Carbapenem resistance in the GCC states appear to be associated with VIM-type and IMP-type MBL enzymes [622], [627], [628]. Zowawi *et al,* (2018) carried-out a regional study on *P. aeruginosa* clinical isolates collected from all GCC countries and found that high-risk clones are now detected, particularly, bla_{VIM-} was found in 39% of 95 isolates in the study [629].

3.4.4.2 Prevalence of UTI antibiotic resistance in Kuwait

Kuwait is located in the eastern tip of the Arabian (Persian) Gulf [621]. The country possesses five large district hospitals which provide tertiary care services [630]. Surveillance programmes for AMR are lacking [609].However, sporadic reports of the emergence of AMR bacteria have been on the rise particularly for pathogens such as *A. baumannii* and ESBL-producing Enterobacteriaceae [631], [632].

The first of report of resistant UTI clinical isolates was published in 1986 in *E. coli* and *P. mirabilis.* The authors reported resistance to ampicillin, sulphonamide and trimethoprim/sulphamethoxazole [633]. In 1997, a study reported multiple resistant uropathogens including UPEC, *K. pneumoniae*, *P. mirabilis*, and *P. aeruginosa* in complicated UTIs isolates from 225 patients in a district hospital in Kuwait. In the 10 detected *P. aeruginosa* UTI isolates, aminoglycoside resistance was more common than ceftazidime and piperacillin [634]. Dimitrov *et al,* (2004) conducted a study on community acquired UTI over a period of 7 years between 1995 and 2001 and 1606 isolates were collected. Amongst those, 74 (4.8%) *P. aeruginosa* isolates were reported, with two displaying imipenem resistance [635]. In 2004, 265 (7.5%) of 3592 Enterobacteriaceae and *P. aeruginosa* were found to be ESBL producers. 35 *P. aeruginosa* ESBL were detected, of which 14 were isolated from urine [636]. Al Sweih *et al,* (2005) reported further 193 *P. aeruginosa* uropathogens found to be sensitive to amikacin, ciprofloxacin and piperacillin in two large hospitals in 2002 [637]. In addition, a study conducted over a period of three years (2005-2007) in one of Kuwait's largest hospitals, found that 9.8% of *P. aeruginosa* UTI was acquired in the hospital, in contrast to 4.3% isolated from outpatients. The percentage resistance

of hospital-acquired isolates was 15% and 14% to amikacin, piperacillin and Tazobactam, respectively [638]. These studies provide primary information on UTI *P. aeruginosa* but lack comprehensive analysis on the type of genes responsible for resistance and the emphasis on the role of mutations which increase AMR.

3.5 *P. aeruginosa* genomics

3.5.1 Sequencing of reference strains PAO1, PA14, LESB58

Due to rapid advances in the last two decades, genome sequencing has been extensively used to enhance our understanding of pathogen diversity and adaption [639]. The emergence of high powered and in depth sequencing technology has led to cheaper 'per genome' costs and this has enabled scientists to sequence many pathogens of the same species as opposed to focusing on sequencing genetically distant organisms only [640].

The PAO1 *P. aeruginosa* laboratory strain was isolated from a wound infection in Australia and the genome was subsequently sequenced in the year 2000 using Sanger sequencing [641], [642]. The large and complex genome of *P. aeruginosa* contributes to survival and adaptation in different niches and environments. The size of this genome was 6.3 million base pairs, which is larger than many other bacteria [642], [643]. PAO1 contains 5,557 open reading frames, four putative chemotaxis systems and a great number of genes participating in control systems such as environmental signals and transcriptional regulators [642]. A second commonly used reference strain called PA14 was subsequently sequenced and was found to have 6.5 million base pairs [644], [645]. PA14 contains two pathogenicity islands that may be responsible for the virulence in a wide range of hosts including models such as the nematode *C. elegans*, the insect larvae *Galleria mellonella,* the plant *Arabidoopsis Thaliana*, and mice [646]–[648]. A third important strain related to CF pathogenesis called the Liverpool Epidemic Strain B58 (LESB58) was shown to be transmissible in a landmark paper using molecular methods. Prior to this discovery, the belief that each CF patient harboured individual specific strains was widespread [125]. The reference LESB58 was the earliest LES isolate to be sequenced. The analyses revealed multiple large genomic islands such as prophage clusters, a defective pyocin prophage cluster, and non-phage islands [131].

The genome of *P. aeruginosa* is comprised of a core genome and accessory genome [649]. The core genome is a highly conserved and therefore common to all isolates. The accessory genome includes flexible genes (of which there may be several variants) and mobile genetic elements such as plasmids, transposons, phages and genomic islands. These can play a significant role in determining the pathogenicity in microorganisms, including *P. aeruginosa* [649].

Genomic islands are defined as chromosomal regions which are often strain-specific [650]. Many islands are identified by the atypical G+C content. Pathogenicity islands are genomic islands that encompass distinct genetic elements which encode virulence proteins [650]. The variation observed between *P. aeruginosa* genomes is largely dependent on genomic islands and prophages [651]. Mathee *et al,* (2008) conducted a comparison between the genomes of PAO1 and PA14 to three other *P. aeruginosa* strains isolated from CF patients identified as PA2192, PAC52 and C3719 [652]. The genomes of PA14 and PA2192 were larger and distantly related to the rest of the isolates [652]. The analysis revealed a total of 5021 genes conserved in the genomes possessed by these strains, whereas 52 regions of gene plasticity (RGB) were detected. RGBs allow for the insertion of genomic islands and bacteriophages or removal of DNA segments [652].

3.5.2 Next Generation Sequencing (NGS)

Next Generation Sequencing (NGS) methods are becoming cost-effective and have, in some situations, been used in clinical investigations into transmission and AMR [653]. NGS methods are comprised of two types: short-read sequencing and long-read sequencing. Short-read sequencing (such as Illumina) is considered useful for population-level research with low cost and high-accuracy performance. Long-read methods (such as PacBio) is powerful in *de novo* gene assembly and using this, assemblies of one complete contig are possible.

3.5.3 Population genomics of *P. aeruginosa*

P. aeruginosa populations have been described as displaying an epidemic population structure [654]. Wiehlmann *et al,* (2007) developed a microarray genotyping platform that included analysis of the core and the accessory genome. The paper analysed 240 isolates from diverse habitats and found that the PA14 clone was the

most abundant genotype [645]. The increased availability and utilisation of sequencing has enabled further in-depth studies. The Pseudomonas Genome Database (www.Pseudomonas.com) contains 335 complete genomes and 4512 draft genomes (as of the 2nd of September 2019) [655]. The database provides researchers with genome annotations, cellular components, molecular functions and biological processes [655]. It contains genomes of taxonomical outliers such as PA7, a multi-resistant strain from Argentina, and since its typing and annotation, more isolates were categorised as PA7-like [656], [657]. Population structure of *P. aeruginosa* consisted of group 1 (PAO1-like) and group 2 (PA14-like) and the outlier group PA7-like [658]. LESB58 clusters in group 1. A multi-group study by Freschi *et al,* (2015) characterised 389 strains and found that 309, 68 and 12 strains belonged to groups 1,2 and 3, respectively [658]. Kos *et al,* (2015) utilised NGS to link the genomes of 390 isolates to resistant phenotypes. These clinical isolates were sourced from diverse range of infections including UTIs (n=86) and from different geographical locations around the world [659]. Phylogenetic tree analysis of the isolates revealed a heterogenous population and genome size ranging from 6.0 Mbp to 7.4 Mbp, with PA01-like isolates making up the majority of the samples [659]. The population structure has been recently redefined by utilising NGS sequencing and analysing the pan-genome, leading to the addition of a fourth and fifth group of *P. aeruginosa* isolates [660].

3.5.4 Genomic studies in *P. aeruginosa* UTI infections

The database of sequenced isolates of *P. aeruginosa* isolates appears to be biased towards respiratory infections such as CF. This presumably reflects the amount of research and funding available in this area. Sequenced UTI isolates of *P. aeruginosa* are scarce and this is a potential limitation in current datasets.

Using Single nucleotide polymorphism (SNP) Tielen *et al,* (2011) investigated the genomic variability and the relationship between 30 urethral isolates which led to the detection of 21 genotypically different groups. This suggests that, like *P. aeruginosa* isolates from other sources, the UTI population is highly heterogenous [90]. As mentioned earlier in section 3.5.3, Kos *et al,* (2015) sequenced 86 UTI isolates and found that the average genome size of the isolates was 6.8 Mbp [659]. A UTI isolate

from Germany (called MH38), has been sequenced revealing a 6,889,973 base pair genome. MH38 contains 6,089 protein-coding sequences (CDS)s 254 were found to be unique (Table 3.4). From the unique CDS, 59 phage and 5 transposon associated genes were identified [661]. RN21 was isolated from an acute UTI and was found to have a 7.0 Mbp genome including, again, unique phage-related genes. Some of the phage-related gene displayed strong similarity to the transposable phage D3112. CRISPR phage immunity sequences were also identified [662]. Further genomes from CAUTI isolates have been reported between 6.6-7 Mbp, these strongly suggest that UTI isolates could be a rich source of unique genes, particularly with reference to prophage sequences. Furthermore, although isolates numbers are low, it appears that UTI isolates can contain large genomes of over 7 Mbp.

Genome sequencing has also revealed genes associated with heavy metal and antimicrobial resistance. A Sudan based-study described a draft genome of a multi-drug resistant isolate with an estimated genome size of six mb carrying multiple β-lactamases genes such as *bla*Veb-1, *bla*PAO and *bla*OXA-50 [663]. Overall, UTIs are an understudied source of *P. aeruginosa* isolates and WGS of further UTI isolates may contribute to the population structure and identification of antibiotic resistance genes.

Table 3.4 Draft genome of 4 UTI isolates elucidating key genotypic and phenotypic characteristics such as genome size, GC content and reported key features

Isolate	Genome size	GC content	Key features	Reference
MH38	6.9 Mb	65.83%	Metal resistance Antibiotic resistance Type IV secretion system	[661]
MH19	7.1 Mb	65.95%	Metal resistance Antibiotic resistance Evolutionary homologues of toxins	[664]
RN21	7.0 Mb	65.88%	Phage encoded genes	[662]
UTI	6.0 Mb	64.6%	Antibiotic resistance	[663]

P. aeruginosa contributes to the overall morbidity and mortality of UTIs, with the emergence of highly resistance strains that are specifically involved in CAUTI [665]. Paucity of information regarding *P. aeruginosa* genomics may be detrimental in combating the rise of AMR strains and developing therapeutic options to tackle this threat. Current sequencing projects have resulted in a database that is extensive however, it is biased both geographically (Western origin) and in terms of infection site (respiratory) and therefore additional sequencing could be employed in order to represent these different niches.

In this chapter, the findings will be discussed in relation to reported virulence characteristics in *P. aeruginosa* uropathogens and isolates sourced from other types of infections.

3.6 Aims

1). To phenotypically characterize a panel of urinary tract samples obtained in the United Kingdom.

2). To determine the levels of antimicrobial resistance to 17 anti-Pseudomonal antibiotics of UTI isolates from the UK and the State of Kuwait.

3). To determine the genomic profile of the isolates and the genetic basis of AMR.

3.7 Results

3.7.1 Morphological and growth characteristics

3.7.1.1 Growth of *P. aeruginosa* UTI isolates

Growth curves were performed in order to determine growth patterns over 24 hours for 15 UK clinical isolates and the two reference isolates PA14 and PAO1 (Figure 3.2). The growth of the UTI isolates were highly variable and strain dependent. Comparisons were performed at 4 time points (6,12,18, and 24 h). The only significant difference was observed at the 18h point where the optical density of PAO1 was significantly higher than 133042 (P=0.047). The growth rates and maximum cell density vary between all the UTI clinical isolates

3.7.1.2 Morphology imaging

In other infections, particularly respiratory, colony morphology has been linked to features such as chronic infection (mucoidy), persistence (small colony variants) and alterations in quorum sensing (metallic phenotype). In order to study the colony morphology of the isolates, 23 clinical UTI *P. aeruginosa* isolates (from the UK and Kuwait) were grown over 72 h. These were originally isolated from two different geographical locations; the United Kingdom and the state of Kuwait. Phenotypic characteristics may give an indication of possible mutations in QS regulatory genes such as mutations in *lasR* or energy acquisition mechanism [666]. For instance, the mucoidy phenotypes are associated with alginate overproduction in *P. aeruginosa* and wrinkly shaped colonies indicate an adaptive response linked to oxygen uptake [667]. Colony images were taken after 72 h (Table 3.5). From the UK panel of clinical isolates; isolate 133042 exhibited a pigmented morphology that developed into a wrinkly colony type over time. None of the isolates displayed a mucoid phenotype. The majority of isolates were yellow however blue-green pigmentation was also identified (Isolates 133042, 133065, 133075). The metallic phenotype was observed in isolates 133117, 133042 and 133065. Most isolates maintained a circular shape during growth. Colony size displayed variation from 1-5 mm.

As for isolates sourced from Kuwait (Table 3.6), all isolates exhibited the same circular shape and the yellow pigmentation except isolates 925 and 1083, with both

demonstrating grey and creamy pigments. No mucoidy or wrinkly colonies observed, all isolates appear to have closely similar phenotypes.

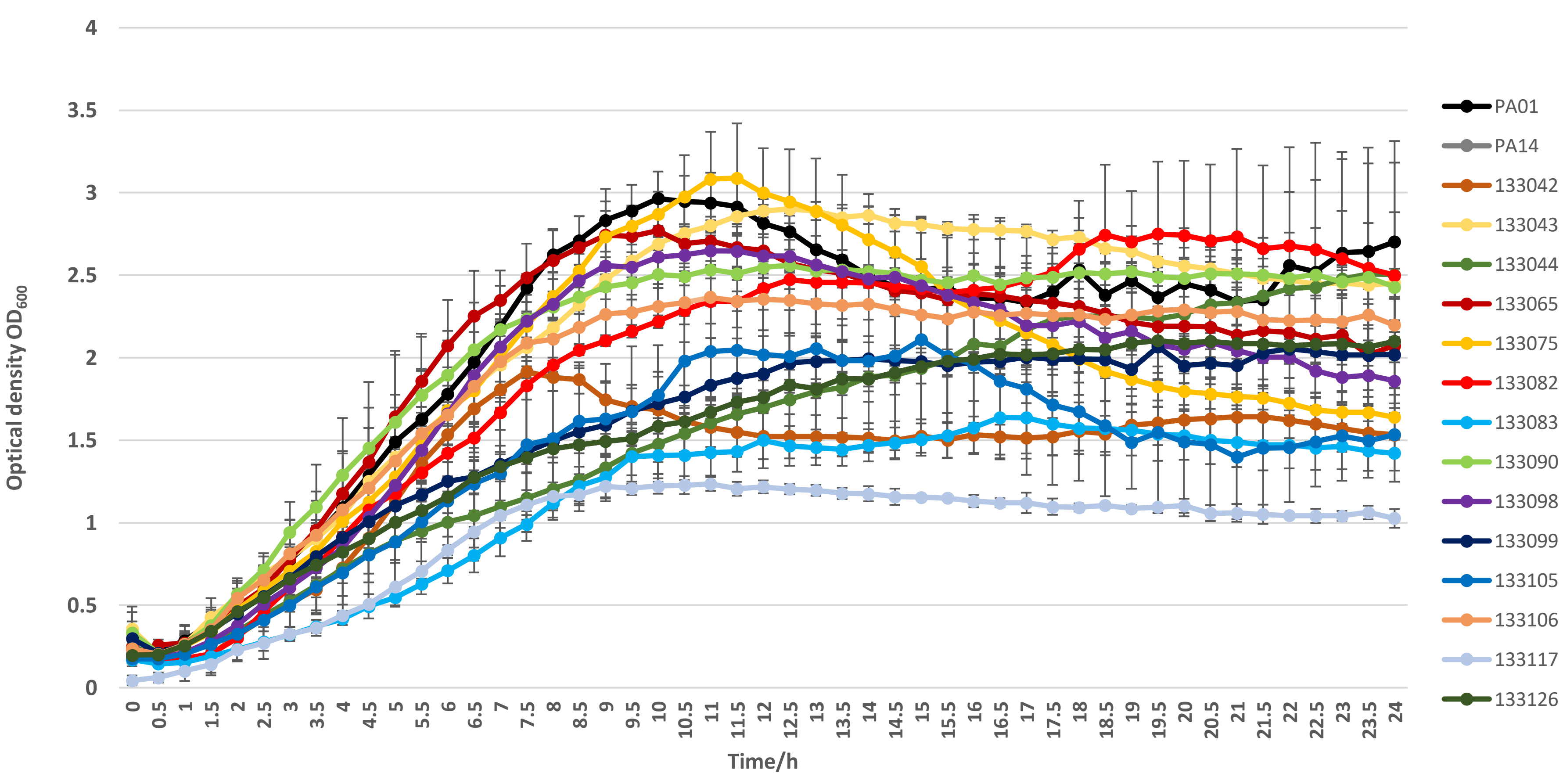


Figure 3. 2 Growth of 14 UTI isolates and 2 references isolates PAO1 and PA14 over 24 h. Optical density was measured at OD600. Pairwise comparison of all isolates involved at 6,12,18 and 24h. Three biological replicates were performed, and points represent the mean (with standard deviation bars)

Table 3. 5 Morphology of the UK panel of isolates after 72 hours. C-circular, Con-convex, E-entire, NM-non-mucoid, R-raised, I-irregular

Isolate	Description	Image	Isolate	Description	Image
PAO1	C, Con, E, NM. Yellow. 3-4 mm.		133090	C, R, E, NM . Yellow 3-5 mm	
PA14	C, R, E, NM. Yellow, 3-4 mm.		133098	I, Con, E, NM. Yellow 3-5 mm	
133042	C, R, E, NM. Green, 2-3 mm.		133099	C, R, E, NM. Yellow 2-3 mm.	
133043	I, R, E, NM. Yellow. 1-2 mm.		133104	C, R, E, NM. Yellow 2-3 mm.	
133044	C, R, E, NM. Yellow. 1-2 mm.		133105	I, R, E, N. Yellow 4-5 mm.	
133065	C, R, E, NM. Blue, 1-2 mm.		133106	C, R, E, NM. Yellow 2-3 mm.	
133075	C, R, E, NM. Grey 3-4 mm.		133117	C, R, E, NM. Yellow 2-3 mm.	
133082	I, R, E, NM. Yellow 2-3 mm.		133126	C, R, E, NM. Yellow 2-3 mm.	
133083	C, R, E, NM . Yellow 2-3 mm.				

Table 3.6 Morphology of Kuwait panel of isolates after 72 hours. C-circular, Con-convex, E-entire, NM-non-mucoid, R-raised, I-irregular

Isolate	Description	Image	Isolate	Description	Image
758	C, Con, E, NM. Yellow. 3-4 mm.		888	C, R, E, NM . Yellow 3-5 mm	
783	C, R, E, NM. Yellow, 3-4 mm.		902	I, Con, E, NM. Yellow 3-5 mm	
786	C, R, E, NM. Yellow, 2-3 mm.		925	C, R, E, NM. Grey 2-3 mm.	
864	I, R, E, NM. Yellow. 1-2 mm.		1083	C, R, E, NM. Yellow 2-3 mm.	

3.8. Biofilm Formation

3.8.1 CV staining

Biofilm formation is one of main, problematic features in CAUTI in nosocomial settings [56], [280]. *P. aeruginosa* is often used as a model to study biofilms [668]. By studying this panel of isolates, the *in vitro* biofilm formation ability of the UTI isolates was determined and compared to laboratory reference strains PAO1 and PA14. Using a 96-well assay to study biofilms adhered to polystyrene wells, it was found that the total biomass of all isolates, apart from 133105, was reduced compared to PAO1. Isolate 133044 displayed a significant reduction in biofilm formation (P=0.036) (Figure 3.3). In comparison to PA14, 5 isolates (133075, 133082,133083, 133105, 133117) formed more biomass, although these differences were not statistically significant. These results show that the majority of isolates are capable of biofilm formation in this assay, however, the extent of the biofilm is variable.

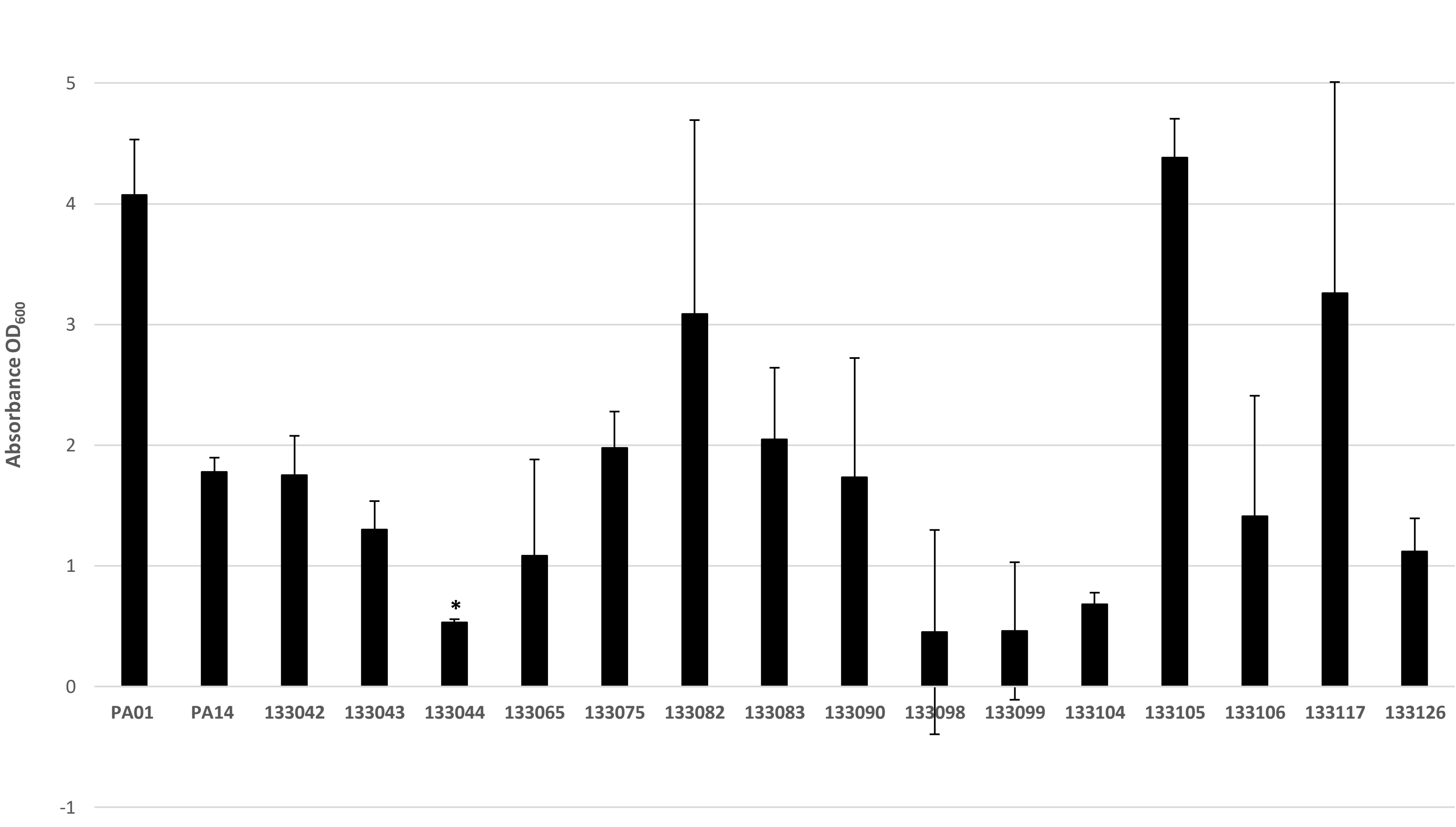


Figure 3.3 Biofilm formation across all clinical isolates. Dunn's statistical test showed a statistically significant difference in the biomass between PAO1 and 133044 only as P= 0.036 which is shown by the asterisk

3.8.1 Biofilms microscopy

Further assessment of biofilm morphology was conducted using confocal microscopy (Figure 3.4). Biofilm microscopy provides further insight and information on the 3D architecture and development of these sessile microbial communities by conducting post-analyses by a software called COMSTAT. Specifically, the software quantifies and compares biofilm structures formed by all the 15 clinical isolates and the reference isolates.

COMSTAT consists of 10 different features which analyses the biofilm quantitatively. 5 variables were chosen in this study; biomass, maximum thickness, roughness co-efficient, surface to biofilm ratio and surface area. The roughness coefficient parameter distinguishes between rough and smooth biofilms. The parameter of surface:biofilm ratio indicates to which fraction of the biofilm is exposed to nutrient flow. As for the biomass; Hydorn *et al,* (2000) defined the biomass as the total volume of the biofilm. Maximum thickness covers a given area regardless of the pores or the holes in the biofilm and correlates inversely with surface to biofilm ratio. The surface area is defined by Heydorn as '' The area summation of all biomass voxel surfaces exposed to the background'' [410].

In terms of biomass, all the UTI isolates produced a greater biomass than the reference isolates except for 133105 (0.92 µm^3/µm^2) (Figure 3.5). This highlights inconsistencies between the microscopy and staining method. Using the staining method, isolate 133105 produced the highest biofilm formation of all of the UTI isolates. Using microscopy, isolate 133075 recorded the highest biomass (3.17 µm^3/µm^2) followed by Isolates 133044 and 133098 (2.73 and 2.84 µm^3/µm^2, respectively). Again, this highlights the inconsistency as both of these isolates were very low biofilm producers in the staining assays. Furthermore, the variation between replicates of the same isolate was very high suggesting that these measurements can be inconsistent and thus, this data must be interpreted with extreme caution.

No significant differences were identified between isolates for any of the microscopy measures. In brief, 60% of all clinical UTI isolates displayed higher surface to biofilm ratio with the highest value recorded by isolate 133090 (26.84 ratio) and the lowest was 133104 (6.14) ratio. No statistical significance was identified (P = 0.934) (Figure

3.6-A). The surface area covered, although identified as higher in 93% of all UTI isolates in comparison to PAO1, there was no statistical difference (P = 0.974) (Figure 3.6-B). There was no significant difference in the biofilm roughness coefficient (P = 0.351) (Figure 3.6-C) or the maximum thickness (P=0.802) (Figure 3.6-D).

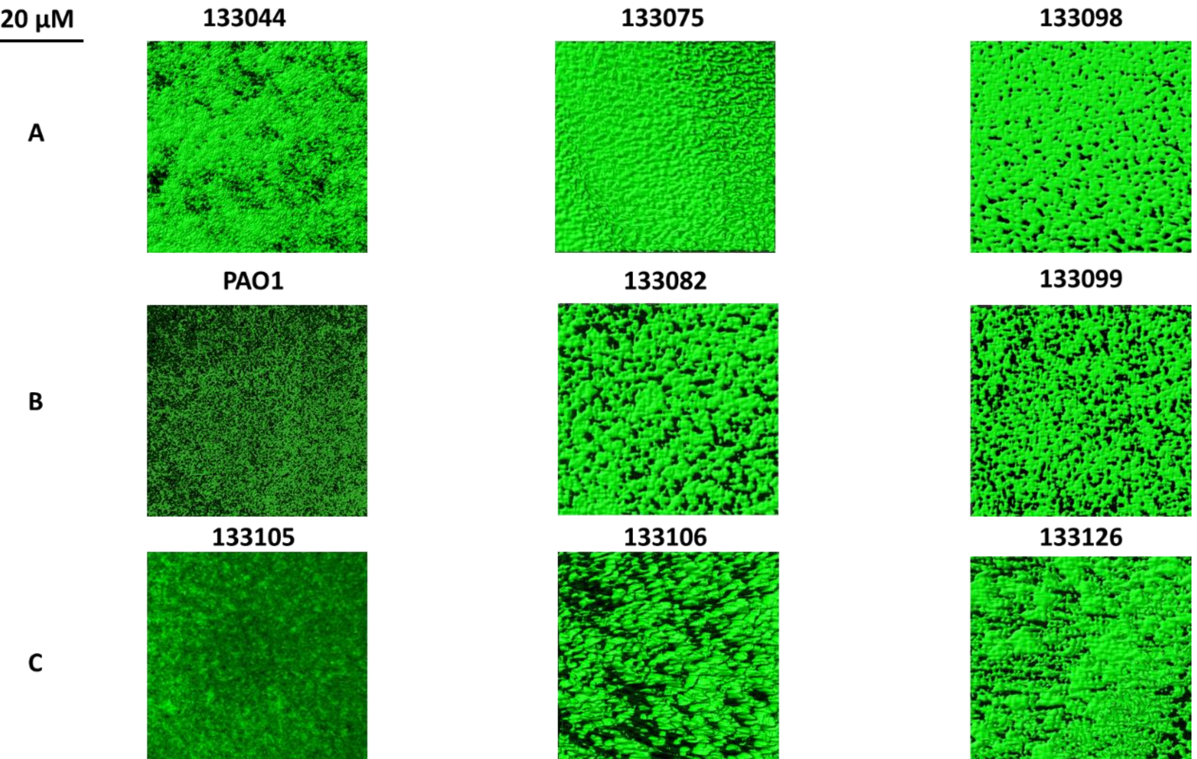


Figure 3.4 Biofilm morphology of UTI isolates of *P. aeruginosa*. Images obtained with Carl-Zeiss confocal microscope at 40X magnification of biofilms formed by the UK panel of *P. aeruginosa* isolates. The images were processed using Imaris and can be used to calculate different parameters of biofilms such as biomass, maximum thickness and surface area by COMSTAT. The scale of the images is 20 µm. **A**: The panel include strong biomass formers. **B:** Intermediate biofilm producers. **C**: Weak Biofilm producers

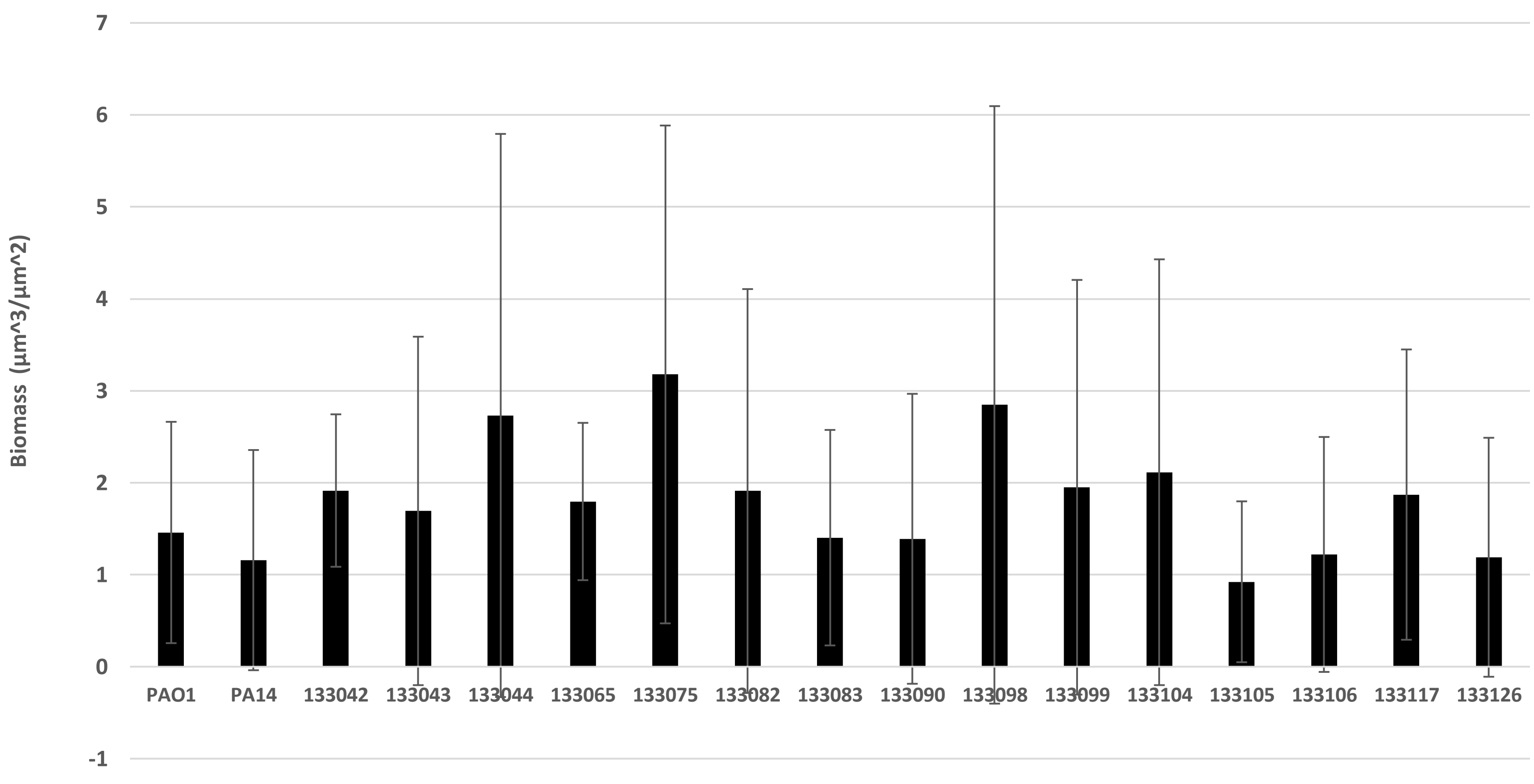


Figure 3.5 The total biofilm biomass of clinical isolates after 48 h growth in LB. Biomass was calculated using Comstat. There are no statistically significant differences between reference and all clinical isolates (n=5) (P = 0.985). Statistical test performed by Kruskal–Wallis test analysis of variance on values

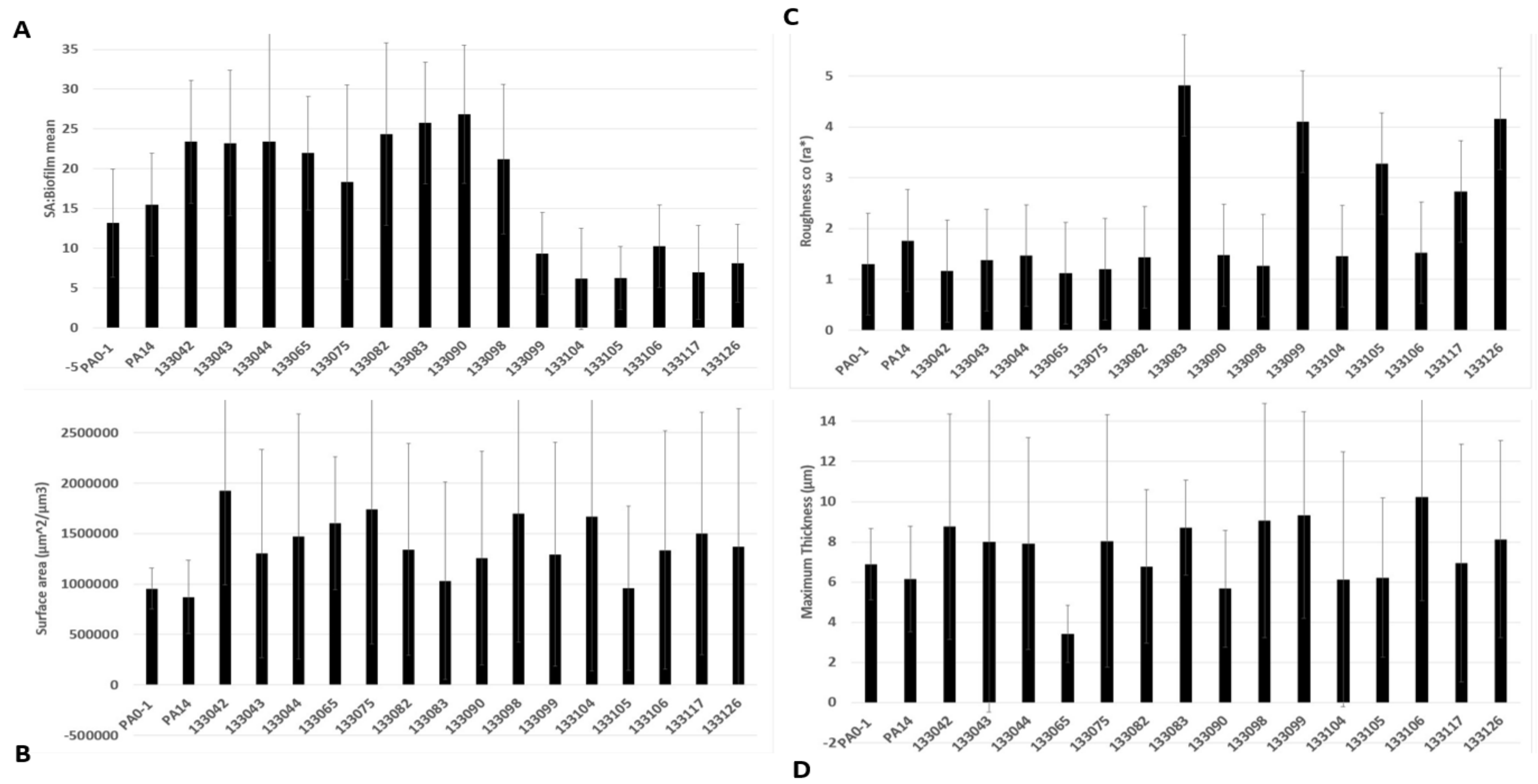


Figure 3.6 The graphs display four parameters calculated by COMSTAT post-microscopy. A): Surface to biofilm ratio with a P = 0.946. B) Surface area of biofilm, P= 0.974. C) Roughness co-efficient with P=0.802. D) Maximum thickness with no significant P=0.351. No statistically significant difference found between reference and clinical isolates. Statistical test conducted by Kruskal–Wallis test analysis of variance on values

3.9 Production of the virulence factor pyocyanin

Pyocyanin is a potent virulence factor utilized by *P. aeruginosa* in urinary tract infections and other types of infection such as CF and keratitis [156]. All the UTI isolates showed decreased pyocyanin production compared to LESB58, which is a known hyperproducer of pyocyanin. For four isolates (133083,10090, 133042 and 133065) the reduction was significant (P=0.003, P=0.015, P= 0.031 and P= 0.041) (Figure 3.7). None of the UTI isolates exhibit the pyocyanin over-production phenotype. There were no significant differences in pyocyanin production between the clinical isolates and PAO1 or PA14. Isolate 133083 displayed the lowest levels of pyocyanin production at 0.002 (absorption at A695). This low level may be indicative of a mutation in the QS system or phenazine production genes [669]. The average production of all clinical isolates is 0.04 (standard deviation of 0.02), which is higher than PAO1, though is less than PA14 (0.063).

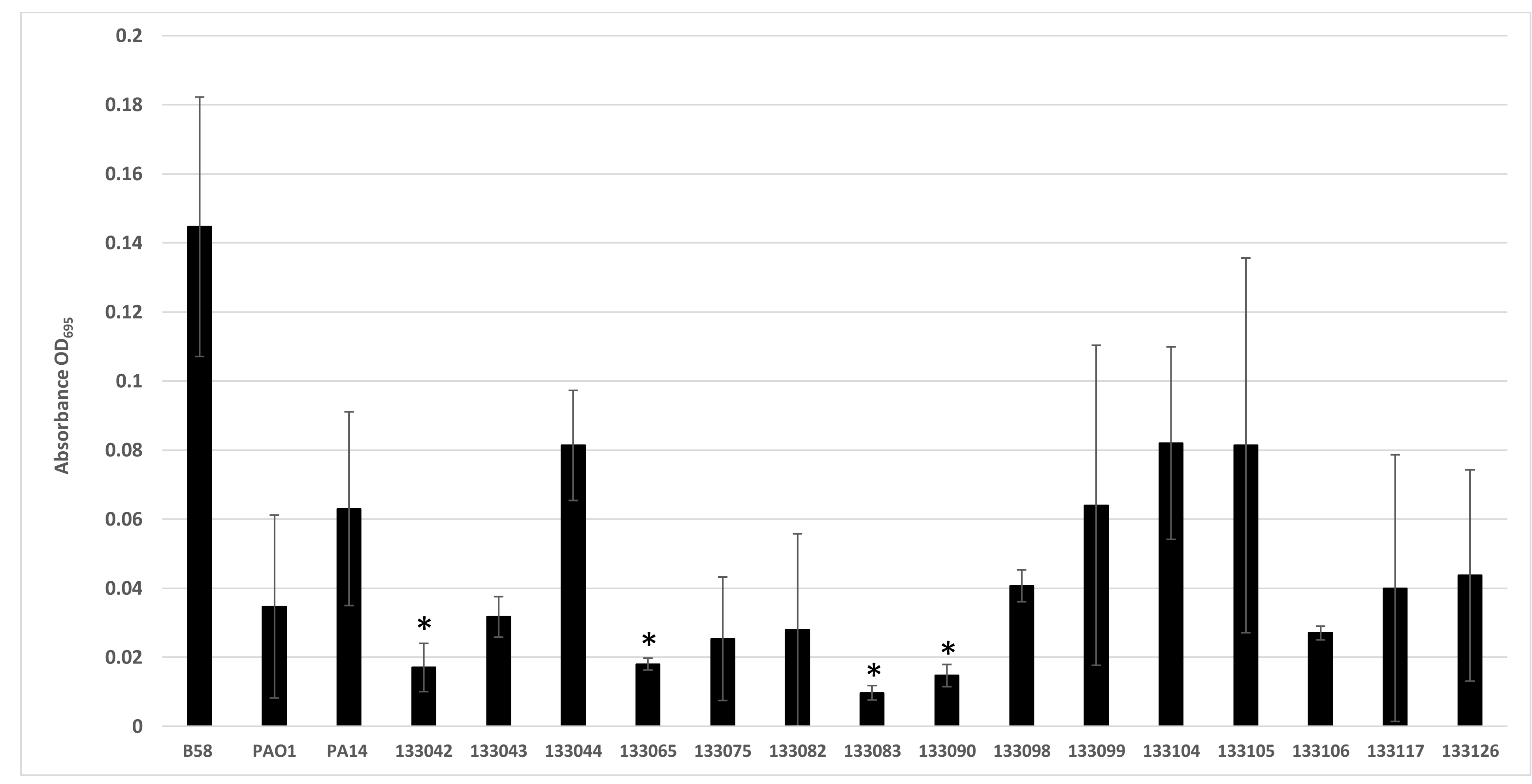
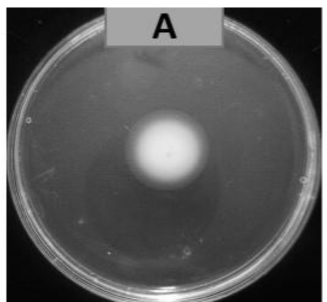


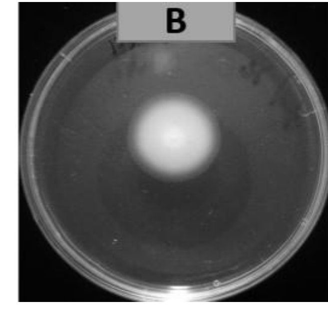
Figure 3.7 Mean pyocyanin production from the clinical UTI isolates of *P. aeruginosa*. Pyocyanin production was determined through measurement of absorbance at 695nm of supernatants. * denotes statistical significance between LESB58 the isolates $P < 0.050$

3.10 Bacterial motility of UTI isolates

3.10.1 Swimming motility

Swimming motility is facilitated by polar flagella present on some *P. aeruginosa* isolates. The flagellum is a virulence factor utilised in swimming but also to initiate biofilm formation [71]. Overnight bacterial cultures were grown in LB medium shaking at 180 rpm and 37°C. By utilising a plate-based method [411], the ability of the 15 UK UTI isolates to swim was analysed in comparison to the reference strain PA14. Based on this, three UTI isolates were significantly reduced in comparison to PA14 (133044,133083 and 13099 with the following respective P values, P= 0.011, 0=0.46 and 0.046). These were classed as non-motile. Thus, 80% of the clinical isolates (n=11) displayed an ability to swim through aqueous media (Figure 3.8 & 3.10).





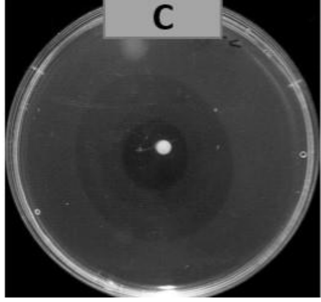


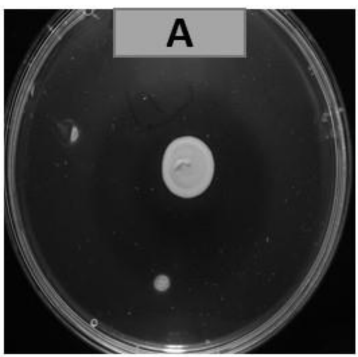
Figure 3.8 Swimming motility of PA14 displayed on image (A). The mean distance travelled by isolate 133106 is the most (20 mm) (image B) and the least travelled is isolate 133099 (4 mm) (image C)

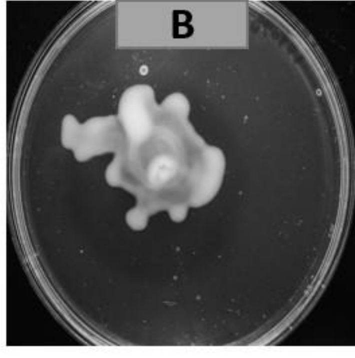
3.10.2 Twitching motility

Twitching motility is facilitated by type IV pili which allows the bacteria to move across surfaces and colonize the host [670]. Twitching motility is involved in forming biofilms. Examination of this type of motility may clarify whether these isolates have the potential to utilize motility to aid CAUTI. The method used was based on Fonseca *et al,* (2004) [671], where CV was utilised to detect twitching movement by staining the bottom of the agar after inoculating with *P. aeruginosa*. The mean distance travelled by all the 15 clinical isolates is 6 mm SD=+/-0.75 mm (Figure 3.11). The cut-off was exceeded by all the UTI isolates except 133044 and 133105 which were non-motile (1 mm). Thus, 86.6% UTI isolates in this study can perform twitching motility.

3.10.3 Swarming motility

Swarming motility is considered the fastest movement across semi-fluid surfaces by *P. aeruginosa* [69]. The average mean distance travelled by the UTI isolates was 12 mm (sd +/- 1.75 mm). The cut-off used to define motile and non-motile isolates was 6 mm. All but two UTI isolates (133083 and 133099) demonstrated the ability to swarm. The greatest swarming was observed for isolates 133082 and 133098, travelling 26 mm and 22 mm, respectively (Figures 3.9 & 3.12).





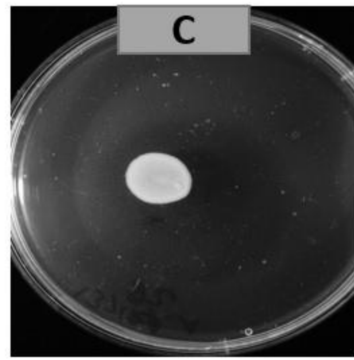


Figure 3.9 A sample of 3 isolates that poses the capacity to perform swarming motility. Strain PAO1 (A) displayed moderate swarming capability, isolate 133098 (B) is the 2nd most motile in the panel, while isolate 133082 (C) is an example of a moderate isolate

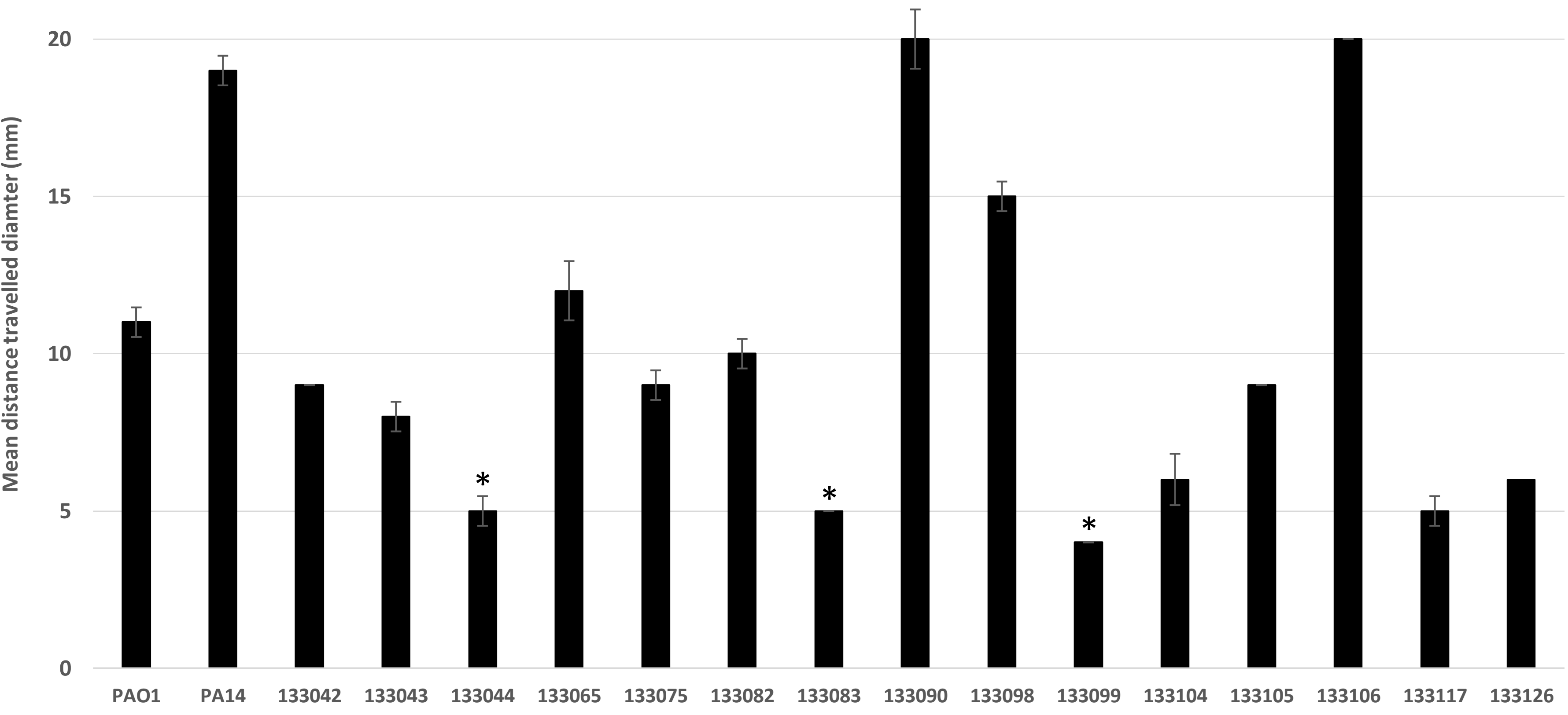


Figure 3.10 The mean value of distances travelled (mm) by swimming in each isolate from the point of inculcation is displayed on the Y axis. The asterisk indicates to a statistically significant difference P< 0.050 between the annotated UTI isolates and PA14

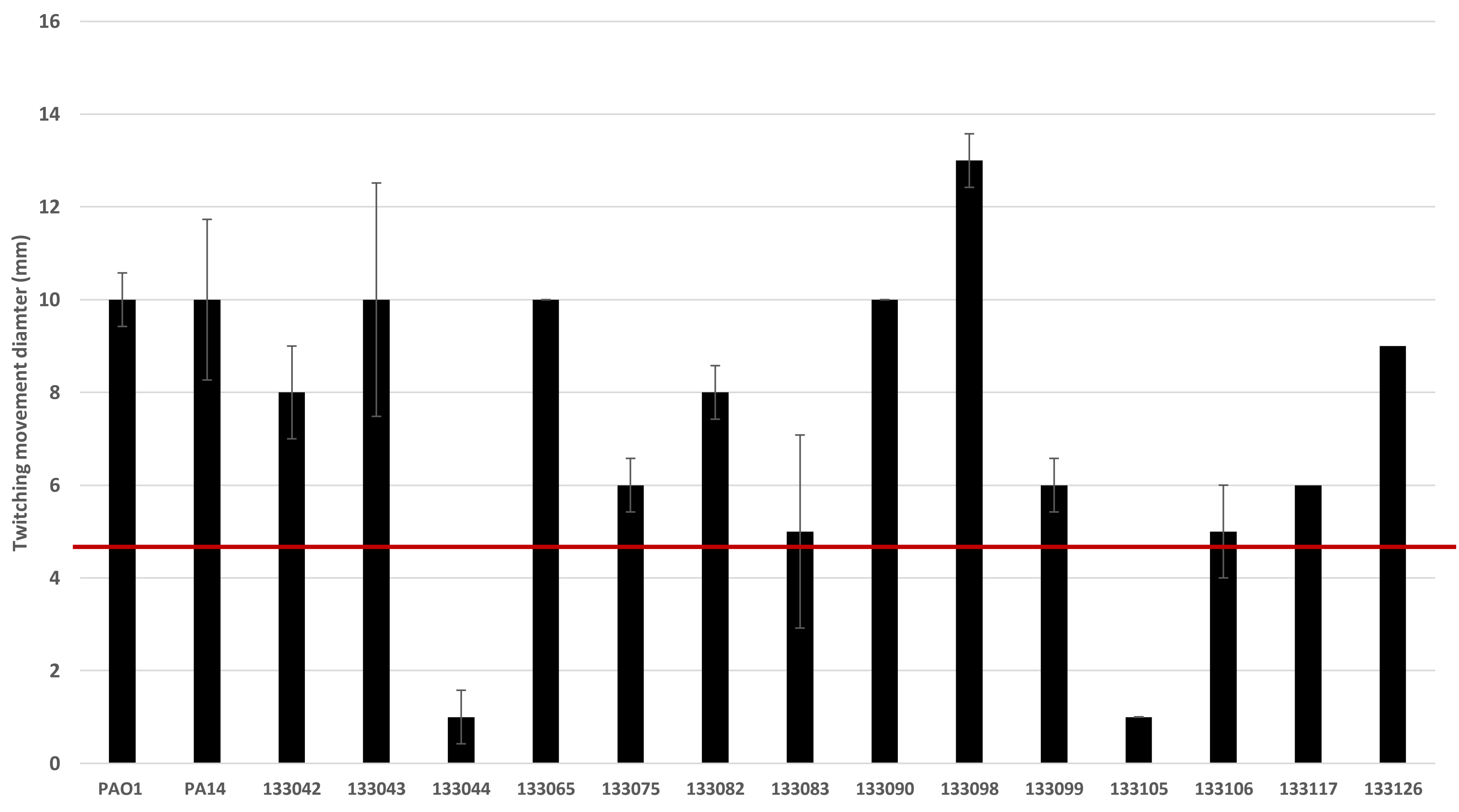


Figure 3.11 Twitching motility. The horizontal line at 5 mm denotes twitching motility and is reached by the two references isolates PAO1 and PA14 and 12 clinical isolates

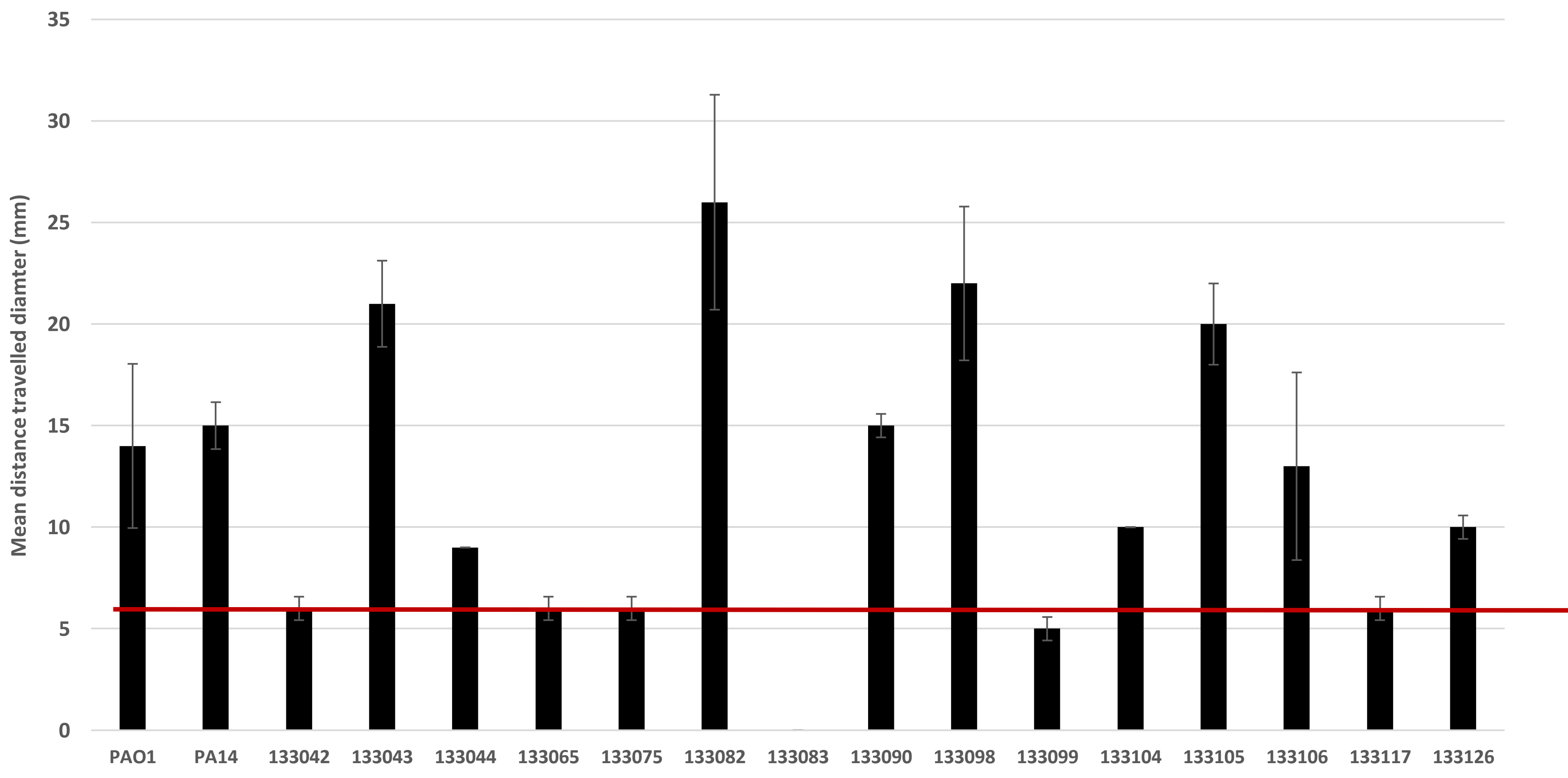


Figure 3.12 The mean average of distance travelled (mm) by swarming motility. The horizontal line is at 6 mm, the cut-off of which an isolate is recognised as motile. 13 UTI isolates can perform swarming motility

3.11 Antibiotic susceptibility testing (AST)

3.11.1 Disc diffusion assay

To examine the ability of the UTI isolates to resists the action of antibiotics, an extensive panel of antibiotics were chosen to test all clinical isolates and the two reference strains PAO1 and PA14 according to clinical breakpoints points published by EUCAST [672]. Table 3.7 shows the results for this susceptibility testing of the UK UTI isolates. Only one isolate, 133044, displayed resistance to aminoglycosides. This isolate showed no zone of inhibition for tobramycin and gentamicin but was susceptible to amikacin. The rest of the isolates displayed zones of susceptibility ranging from 18 to 30 mm to tobramycin, gentamicin and amikacin. Carbapenems are widely used to treat as a last line of treatment of *P. aeruginosa* infections around the world. For imipenem, 2 isolates showed intermediate resistance (133044 and 133105). Furthermore, isolate 133105 was also resistant to meropenem and doripenem, therefore, this one isolate displayed at least some resistance to all three clinically relevant carbapenems. The panel was generally susceptible to cephalosporins.

Ciprofloxacin is a fluoroquinolone antibiotic and is used to treat *P. aeruginosa* infections. 22.6% of the clinical isolates were not susceptible to ciprofloxacin, with isolate 133126 exhibiting intermediate susceptibility and isolates 133065, 133099 and 133126 categorised as resistant. Resistance to levofloxacin (another quinolone) was detected (5/15 isolates with three of these isolates showing no zone of inhibition). Aztreonam targets PBP3 and is classified as a monobactam. 133105 is the only isolate resistant (0 mm) to aztreonam while the rest of isolates displayed inhibition zones ranging from 19 to 30 mm. The isolates were generally susceptible to antibiotics form the penicillins class. However, isolate 133105 was resistant to piperacillin (16 mm), piperacillin Tazobactam (18 mm), ticarcillin (0 mm), ticarcillin with clavulanic acid (0 mm). Other isolates exhibiting resistance to antibiotics in the penicillin class were 133044, 133099 and 133104.

A multi-drug resistant (MDR) isolate is classified as such when resistance occurs to one antibiotic in three or more classes [559]. Although six isolates showed resistance to at least one antibiotic, based on the criterion, only two UK UTI isolates can be

classified as MDR, 133099 and 133105. Overall, these results indicate that although resistance was detected in the isolates, the isolates are susceptible to some of the antibiotics and therefore there are potential treatment options.

In contrast to the UK isolates, the isolates from Kuwait displayed more resistance to antibiotics (Table 3.8). If an isolate is resistant to all antibiotics except two or fewer classes, it is defined as an extensively drug resistant (XDR) bacterial isolate [673]. 75% of the Kuwaiti clinical isolates are classified as XDR (Table 3.8). Isolates 758 and 783 were resistant to all the antibiotics tested. Isolate 786 was only susceptible to imipenem. Isolate 888 was only susceptible to some cephalosporins and tazobactam/piperacillin and isolate 925 was only susceptible to fluoroquinolones (levofloxacin and ciprofloxacin) and gentamicin. Isolate 864 was MDR and displayed resistance to antibiotics from carbapenems, cephalosporins, monobactams and penicillins. The only isolate not classed as resistant was 1083. For this isolate, intermediate levels of resistance were observed to netilmicin. To assess whether isolate 758 is resistant to polymyxins such as colistin, an MIC experiment was conducted. Both isolates were sensitive to colistin at 2 mg/L suggesting that it could be a last resort antibiotic used for treatment against these isolates. However, 2 mg/L represents the cut-off for classification as sensitive (sensitive ≥2, resistant <2) and therefore these isolates must be deemed as a high potential for pan resistance.

Table 3.7 AMR in UK *P. aeruginosa* isolates from UTIs. Size (mm) of zone of inhibition following disk diffusion assays are reported. Resistance is colour coded in red while intermediate values appear in orange

	Aminoglycosides				Carbapenems			Cephalosporins			Fluoroquinolones		Monobactams	Penicillins			
	Tobramycin	Gentamicin	Amikacin	Netilmicin	Imipenem	Meropenem	Doripenem	Ceftazidime	Cefepime	Ceftazidime/ avibactam	Levofloxacin	Ciprofloxacin	Aztreonam	Piperacillin	Piperacillin/ Tazobactam	Ticarcillin	Ticarcillin with clavulanic acid
	TN	GM	AK	NET	IMI	MeM	Dor	Caz	CPM	CZA	Lev	CIP	ATM	PRL	PTZ	TC	TIM
Dose	10	10	30	10	10	10	10	30	30	14	5	5	30	100	110	75	85
133042	24	21	26	18	29	34	35	29	28	28	35	26	30	27	26	26	28
133043	24	23	21	17	25	27	35	23	30	22	32	24	20	22	24	25	20
133044	0	0	18	0	18	28	31	27	22	27	29	24	22	18	25	0	26
133065	26	25	29	22	31	31	40	26	28	27	21	12	22	28	27	33	27
133075	28	28	31	22	25	33	32	25	32	25	37	30	19	24	27	23	21
133082	23	24	27	18	27	26	28	25	25	25	35	28	21	20	27	24	21
133083	22	22	22	19	26	37	35	25	29	25	33	23	26	22	24	28	21
133090	25	23	25	19	27	29	31	26	32	28	34	25	27	22	29	25	22
133098	26	26	27	22	27	38	32	26	30	26	33	30	29	28	26	28	24
133099	21	18	20	12	29	28	28	24	24	25	0	0	20	18	26	23	19
133104	21	18	20	12	29	28	28	24	24	25	0	0	24	18	26	23	19
133105	21	18	20	4	27	28	28	22	22	22	32	0	0	19	23	22	26
133106	25	25	21	20	24	34	34	23	30	23	33	23	25	24	27	21	23
133117	20	20	19	10	28	20	30	24	23	21	0	0	25	20	26	22	21
133126	22	19	21	16	27	36	35	24	31	25	26	26	25	24	27	27	25

Table 3.8 AMR in Kuwait *P. aeruginosa* isolates from UTIs. Size (mm) of zone of inhibition following disk diffusion assays are reported. Resistance is colour coded in red while intermediate values appear in orange

	Aminoglycosides				Carbapenems			Cephalosporins			Fluoroquinolones		Monobactams	Penicillins			
	Tobramycin	Gentamicin	Amikacin	Netilmicin	Imipenem	Meropenem	Doripenem	Ceftazidime	Cefepime	Ceftazidime/avibactam	Levofloxacin	Ciprofloxacin	Aztreonam	Piperacillin	Piperacillin/ Tazobactam	Ticarcillin	Ticarcillin with clavulanic acid
	TN	GM	AK	NET	IMI	MeM	Dor	Caz	CPM	CZA	Lev	CIP	ATM	PRL	PTZ	TC	TIM
Dose	10	10	30	10	10	10	10	30	30	14	5	5	30	100	110	75	85
758	0	11	0	0	9	0	0	0	6	0	0	0	10	0	0	0	0
783	0	10	0	0	10	0	0	0	5	0	0	0	10	0	0	0	0
786	0	0	14	3	24	16	16	10	11	11	0	0	3	9	12	0	0
864	20	20	25	17	11	0	15	15	20	16	25	12	16	14	14	11	8
888	0	0	0	0	0	11	14	23	24	23	0	0	18	7	19	14	7
902	20	20	25	17	25	20	21	10	16	8	22	11	9	0	9	6	0
925	0	17	11	0	10	10	14	0	12	0	24	25	24	0	11	0	0
1083	19	18	20	12	20	29	31	21	22	25	27	20	23	25	27	24	22

3.11.2 Detection of resistance elements

Following WGS, post-analysis was performed using the CARD database to identify genomic determinants of AMR. Overall, as expected, almost all the isolates in both UK and Kuwait panel contained multiple genes encoding efflux pumps (*mex*) and porins (*opr*) (Table 3.9). Resistance to aminoglycosides was found in isolates from Kuwait, particularity the XDR isolates 758 and 783, the most resistant isolates in both panels (Table 3.10). These isolates contained AAC (6')-ii and aaA61, (encoding an aminoglycoside transferase enzyme and an aminoglycoside adenylyltransferase respectively), which both inactivate aminoglycoside antibiotics by enzymatic modification. In addition, APH(3')-Ib, which is a plasmid-encoded phosphotransferase in *E. coli* , was found in both isolates. Other Kuwaiti isolates (786, 888, and 925) also harboured this gene and displayed resistance to aminoglycosides. Isolate 888 contained five aminoglycoside resistance genes; AAC (6')-ii, aadA11, ANT(2'')-Ia, APH(3')-Ib, and APH(3')-Via. In the UK panel of isolates, only 133044 contained genes conferring resistance to aminoglycosides, namely, APH(3''), APH(4), APH(3')-Iib and APH(6)-Id. This correlated with the observed resistance using the disk diffusion tests. Genes conferring quinolone resistance such as *crpP* were found in isolates 133042, 133075, 133117 and 925 (Kuwait isolate). The enzyme product CrpP is a novel ciprofloxacin modifier and has been described recently by Chávez-Jacobo *et a,* (2018) [674]. Ciprofloxacin resistance was found in only one isolate 133117 (*crpP*$^{+}$) in disk diffusion testing. Specific mutations in *gyrA* confer resistance to fluoroquinolones was detected mostly in isolates sourced from Kuwait (62.5%).

Genes carrying β-lactamases are detected across all isolates in both UK and Kuwait. OXA-50 is chromosomally encoded and is found on all isolates. The other OXA-type identified was OXA-10, which is a resistance gene detected in only one isolate, 786. PDC β-lactamases are class C AMR genes that can be found in *P. aeruginosa* [418]. All isolates contained a single PDC enzyme or more, although the type differed. These include PDC-1, PDC-2, PDC-3, PDC-4, PDC-5, PDC-7, PDC-8, PDC-9, PDV-10 and PDC-86. PDC-2 is present is 26 % of the total UTI isolates tested, followed by PDC-3, which is only detected in Kuwaiti isolates 758,783,786 and 888. PDC-5 and PDC-9 were only present in UK isolates, the former is detected in 133044, 133082, and 130083 while

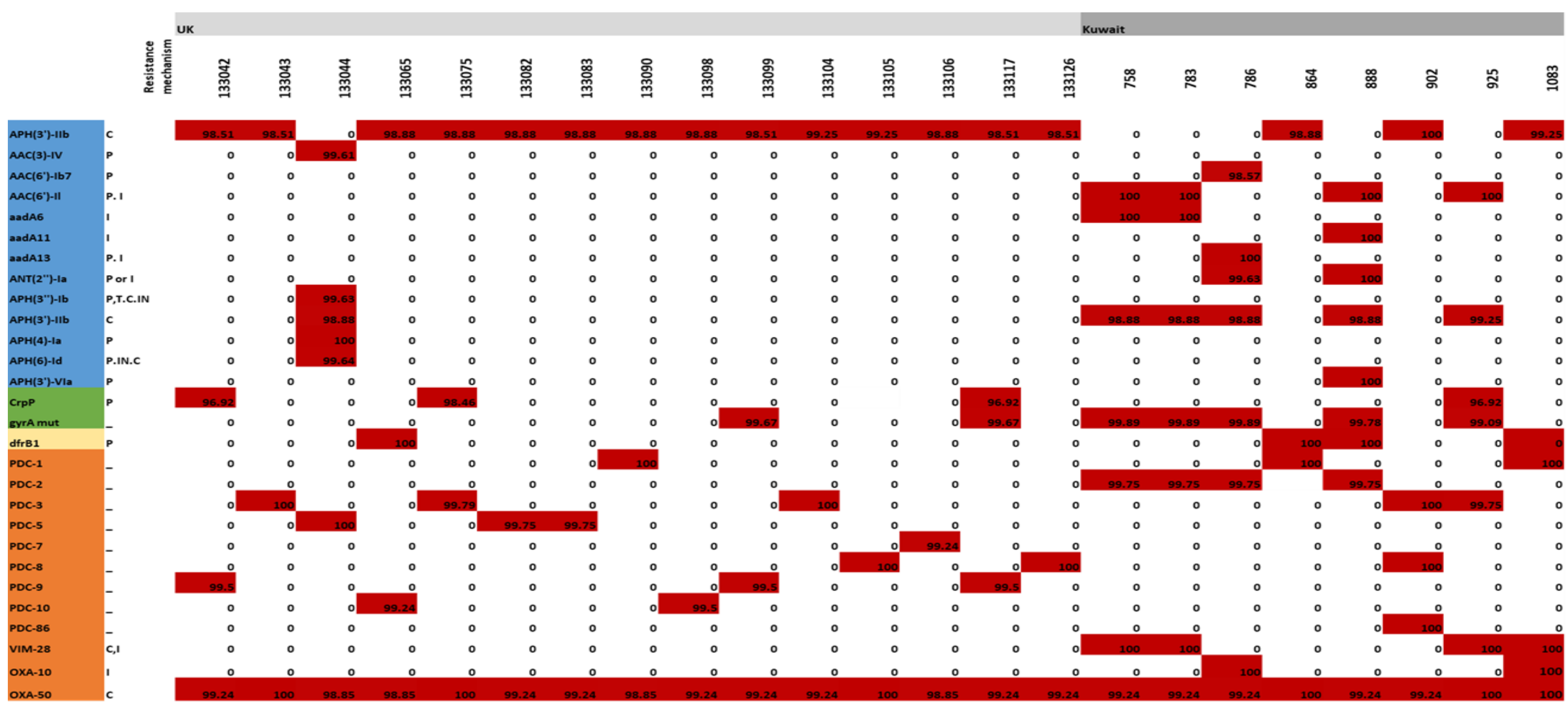
the latter was found in 133042, 133099 and 133117. The rest of the isolates carried PDC-1 (133090, 864), PDC-10 (133065,133098,), PDC-7 (133106) and PDC-86 (902).

A single VIM type gene was identified in three isolates. VIM-28 was detected in these isolates and this gene is highly uncommon in *P. aeruginosa* isolates. Other resistance genes identified were *sul1* which is acquired via horizontal gene transfer and integrated in the bacterial genome [675]. The gene was originally detected in *E. coli* and confers resistance to sulphonamide antibiotics. It is detected in 62% of the isolates sourced from Kuwait, however, was not detected in the UK panel of isolates.

Table 3.9 Genes involved in resistance-nodulation-cell division (RND) antibiotic efflux pump and their respective percentage identity in both of the UK and Kuwait isolates

	UK															Kuwait							
	133042	133043	133044	133065	133075	133082	133083	133090	133098	133099	133104	133105	133106	133117	133126	758	783	786	864	888	902	925	1083
MexA	100	100	100	100	99.9	100	100	100	100	100	99.74	100	100	100	100	100	100	100	100	100	100	99.74	100
MexB	99.9	99.9	99.9	99.81	100	99.9	99.9	99.9	99.9	99.9	99.9	99.9	99.62	99.9	99.9	99.62	99.62	99.62	99.9	99.62	99.9	99.9	99.9
MexC	98.02	100	100	98.87	95.43	100	98.59	98.87	98.87	98.02	100	100	99.15	98.02	99.72	98.87	98.87	98.87	99.15	98.87	100	100	99.72
MexD	94.48	95.43	95.33	94.95	99.76	95.52	95.24	95.52	95.43	94.48	95.33	95.43	95.14	94.48	95.43	95.43	95.43	95.43	95.43	95.43	98.15	96	95.43
MexE	100	100	100	99.28	100	100	100	100	100	100	100	100	99.52	100	100	99.28	99.28	99.28	99.76	99.28	99.76	100	99.76
MexF	100	100	100	100	100	0	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
MexG	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
MexH	99.73	100	100	99.19	100	100	100	99.73	99.73	99.73	100	100	99.46	99.73	99.73	99.73	99.73	99.73	100	99.73	100	100	100
MexI	99.9	100	99.9	99.9	99.73	100	99.81	99.81	99.9	99.9	100	100	99.9	99.9	100	99.9	99.9	99.9	100	99.9	100	100	99.9
MexJ	99.46	100	100	99.46	99.8	99.73	99.73	99.73	99.46	99.46	100	100	99.46	99.46	100	99.73	99.73	99.73	99.73	99.73	98.86	99.73	99.18
MexK	99.8	99.9	99.9	99.8	100	99.8	99.9	100	99.9	99.8	99.9	99.9	99.8	99.8	100	100	100	100	100	100	100	99.9	96.43
MexL	99.53	100	100	99.53	98.7	99.53	99.53	99.06	99.53	99.53	99.53	100	99.53	99.53	100	100	100	100	99.53	100	100	99.53	99.53
MexM	99.22	98.44	98.7	99.22	99.71	98.44	98.7	100	99.22	99.22	99.22	98.44	98.44	99.22	98.7	98.96	98.96	98.96	98.7	98.96	98.7	98.44	98.7
MexN	99.81	99.81	99.52	99.81	100	99.71	99.71	99.9	99.81	99.81	99.71	99.61	99.71	99.81	99.61	99.61	99.61	99.61	99.71	99.61	99.3	99.71	97.3
MexP	99.22	99.74	100	99.48	99.81	99.48	100	100	99.48	99.22	99.48	99.74	99.48	99.22	100	99.48	99.48	99.48	100	99.48	100	100	100
MexQ	99.53	99.81	99.72	99.53	100	99.72	99.72	99.91	99.72	99.53	99.91	99.72	99.53	99.53	99.62	99.53	99.53	99.53	99.72	99.53	100	99.43	99.53
MexR	99.32	100	100	100	100	98.64	100	100	99.32	99.32	100	98.51	99.32	99.32	100	99.32	99.32	99.32	99.32	99.32	100	100	100
MexS	100	100	100	99.71	99.63	99.71	100	99.71	99.71	100	100	100	100	100	100	100	100	100	100	100	100	100	100
MexT	99.63	99.63	99.63	99.63	99.47	99.26	99.63	99.63	99.63	99.26	99.63	99.63	99.63	99.26	99.63	99.63	99.63	99.63	99.63	99.63	100	99.63	99.63
MexV	99.47	99.47	99.47	99.2	99.9	99.47	99.47	99.73	99.47	99.47	99.47	99.73	98.94	99.47	99.2	99.2	99.2	99.2	99.47	99.2	99.47	99.47	99.73
MexW	99.9	98.04	99.9	99.8	93.79	100	99.9	99.9	99.8	99.9	99.9	99.9	99.71	99.9	99.9	99.8	99.8	99.8	99.9	99.8	100	100	99.9
MexX	0	0	0	0		0	0	0	0	0	0		0	0	0	0	0	0	0	0	98.15	0	0
MxY	0	93.5	93.5	93.5	100	93.69	100	93.5	93.5	0	93.59	97.87	0	0	93.69	93.69	93.69	93.69	93.5	93.69	97.3	93.4	96.08
MexZ	99.05	100	100	100	99.77	99.52	99.52	100	99.52	98.57	100	100	99.52	98.57	100	96.67	96.67	96.67	100	100	100	100	100
MuxA	99.77	99.3	100	99.77	100	99.77	99.77	99.77	99.77	99.77	100	99.77	99.77	99.77	99.53	99.53	99.53	99.53	100	99.53	99.77	99.77	99.77
MuxB	99.9	100	100	100	100	100	100	100	100	99.9	100	100	100	99.9	100	100	100	100	100	100	100	100	100
MuxC	100	99.9	100	100	99.06	99.81	100	99.71	100	100	99.9	100	100	100	100	100	100	100	100	100	100	99.9	95.16
nalC	98.59	99.53	99.06	98.59	100	99.06	99.53	100	99.06	98.59	99.53	99.06	98.59	98.59	99.53	98.59	98.59	98.59	98.12	98.59	100	97.62	99.06
nalD	100	99.53	100	100	100	100	100	100	100	100	99.53	100	100	100	100	99.53	99.53	99.53	100	0	100	100	100
OpmB	100	99.8	100	100	99.79	100	98.8	98.8	98.6	100	99.8	100	100	100	99.6	100	100	100	99.8	100	98.8	98.8	98.8
OpmD	99.38	99.79	99.59	99.59	99.59	100	99.79	99.79	99.38	99.38	99.79	99.79	99.59	99.38	99.79	99.38	99.38	99.38	99.59	99.38	99.38	100	100
opmE	99.39	99.39	99.19	99.19	97.51	99.19	98.98	100	99.39	99.39	100	99.19	99.19	99.39	99.19	99.39	99.39	99.39	99.59	99.39	100	99.19	99.39
OpmH	100	96.89	97.3	99.79	98.96	97.1	97.3	97.3	100	100	100	99.79	99.79	100	100	100	100	100	97.1	100	99.59	97.72	99.79
OprJ	99.16	99.16	98.96	99.37	100	99.16	99.16	99.16	99.58	99.16	99.58	99.16	99.16	99.16	99.16	99.16	99.16	99.16	99.37	99.16	100	100	98.72
OprM	100	100	100	100	100	100	99.79	100	99.79	100	100	100	100	100	100	100	100	100	100	100	100	100	99.79
OprN	99.79	100	100	99.79	100	100	99.79	100	99.79	99.79	100	100	99.58	99.79	100	99.79	99.79	99.79	100	99.79	99.12	100	100

Table 3.10 Antimicrobial resistance genes identified in *P. aeruginosa* UTI isolates through whole genome sequencing and analysis using the CARD database. Percentage sequence identity is reported. These genes are involved in promoting resistance to multiple of classes of antibiotics such as aminoglycosides (Blue), Fluoroquinolones (green), trimethoprim (yellow) and β-lactamases (orange) in UK & Kuwait isolates with resistance induced by C= Chromosome, I=Integrons=Transposons, IN=Integrative element



	Resistance mechanism	UK															Kuwait							
		133042	133043	133044	133065	133075	133082	133083	133090	133098	133099	133104	133105	133106	133117	133126	758	783	786	864	888	902	925	1083
APH(3')-IIb	C	98.51	98.51	0	98.88	98.88	98.88	98.88	98.88	98.88	98.51	99.25	99.25	98.88	98.51	98.51	0	0	0	98.88	0	100	0	99.25
AAC(3)-IV	P	0	0	99.61	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
AAC(6')-Ib7	P	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	98.57	0	0	0	0	0
AAC(6')-II	P. I	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	100	100	0	0	100	0	100	0
aadA6	I	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	100	100	0	0	0	0	0	0
aadA11	I	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	100	0	0	0
aadA13	P. I	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	100	0	0	0	0	0
ANT(2'')-Ia	P or I	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	99.63	0	100	0	0	0
APH(3'')-Ib	P,T.C.IN	0	0	99.63	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
APH(3')-IIb	C	0	0	98.88	0	0	0	0	0	0	0	0	0	0	0	0	98.88	98.88	98.88	0	98.88	0	99.25	0
APH(4)-Ia	P	0	0	100	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
APH(6)-Id	P.IN.C	0	0	99.64	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
APH(3')-VIa	P	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	100	0	0	0
CrpP	P	96.92	0	0	0	98.46	0	0	0	0	0	0		0	96.92	0	0	0	0	0	0	0	96.92	0
gyrA mut	_	0	0	0	0	0	0	0	0	0	99.67	0	0	0	99.67	0	99.89	99.89	99.89	0	99.78	0	99.09	0
dfrB1	P	0	0	0	100	0	0	0	0	0	0	0	0	0	0	0	0	0	0	100	100	0	0	0
PDC-1	_	0	0	0	0	0	0	0	100	0	0	0	0	0	0	0	0	0	0	100	0	0	0	100
PDC-2	_	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	99.75	99.75	99.75		99.75	0	0	0
PDC-3	_	0	100	0	0	99.79	0	0	0	0	0	100	0	0	0	0	0	0	0	0	0	100	99.75	0
PDC-5	_	0	0	100	0	0	99.75	99.75	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
PDC-7	_	0	0	0	0	0	0	0	0	0	0	0	0	99.24	0	0	0	0	0	0	0	0	0	0
PDC-8	_	0	0	0	0	0	0	0	0	0	0	0	100	0	0	100	0	0	0	0	0	100	0	0
PDC-9	_	99.5	0	0	0	0	0	0	0	0	99.5	0	0	0	99.5	0	0	0	0	0	0	0	0	0
PDC-10	_	0	0	0	99.24	0	0	0	0	99.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0
PDC-86	_	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	100	0	0
VIM-28	C,I	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	100	100	0	0	0	0	100	100
OXA-10	I	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	100	0	0	0	0	100
OXA-50	C	99.24	100	98.85	98.85	100	99.24	99.24	98.85	99.24	99.24	99.24	100	98.85	99.24	99.24	99.24	99.24	99.24	100	99.24	99.24	100	100

3.12 Genomic DNA analysis

To investigate the genomic background of UTI isolates in this study, Illumina sequencing was conducted to determine the population of structure for both the UK and Kuwait isolates. In both groups, PA14-like isolates were more prevalent at 66.6% and 62.5%. This is in contrast to isolates in the database in which group 1 (PAO1-like) isolates are more prevalent. However, the panel is small and therefore may be skewed by these limited numbers. To demonstrate the distribution of the these UTI isolates, a phylogenic tree was drawn to display the distribution of the population (Figure 3.14). The isolates were spread across both group 1 and group 2. No PA7-like (group 3) isolates were detected. Three isolates from Kuwait were clustered on the same arm of the phylogenetic tree and are very closely related.

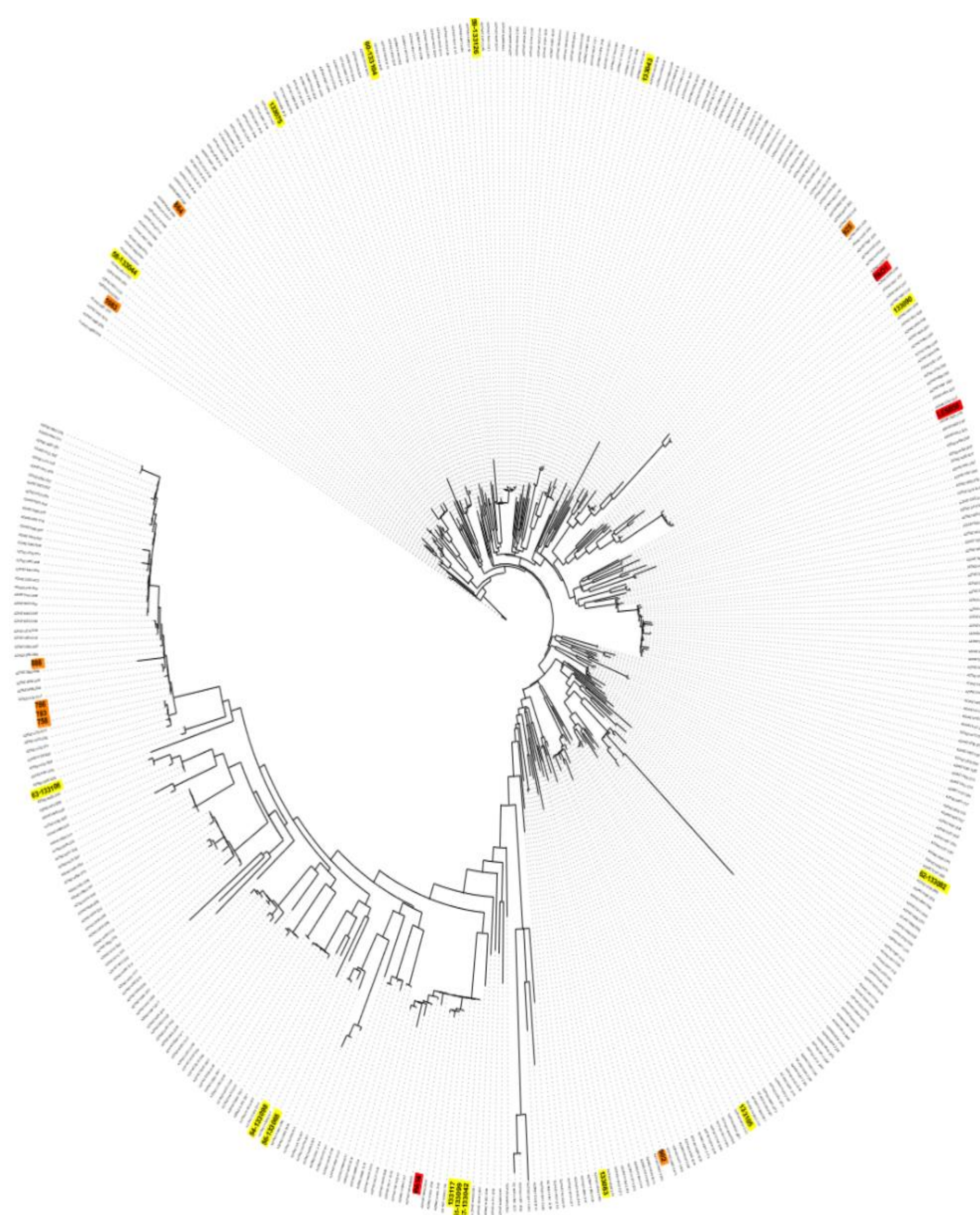


Figure 3.13 Phylogenic tree of the UK and Kuwait panel of isolates according to the genomic composition. This was created by Dr Sam Haldenby at the Centre for Genomic Research, University of Liverpool. The main (PAO1, LESB58, and PA14) strains are colour-coded in red, UK isolates in yellow and isolates sourced from Kuwait in orange

3.13 Discussion

P. aeruginosa has been highly studied yet there are still many gaps in the literature. Many databases and studies are skewed by isolate origin, both geographically and infection site. There is little data on *P. aeruginosa* isolates from UTIs and even less data on isolates sourced from the GCC countries. In this chapter, I have characterised isolates from UTIs using both genotypic and phenotypic methods.

Studying growth in laboratory conditions gives an insight into the behaviour and fitness of the bacterial isolates. Isolates from some sources such as chronic lung infection in CF have been reported as slow growing in the laboratory environment. In this study, the UTI isolates displayed differences in their speed of growth with some isolates growing very fast (133065, similar to the laboratory reference strain PAO1) and others growing much slower and failing to reach a high final density (133117). Many different factors can affect the growth rate of *P. aeruginosa*. Schaber *et al,* (2007) compared growth rates of 5 QS-deficient clinical isolates sourced from respiratory, wound and UTI to PAO1, with no major differences observed at lag, log and exponential phases [676]. It can be concluded that UTI isolates are heterogenous in their ability to grow in the laboratory environment.

Overexpression of alginate exopolysaccharides is a common feature in CF chronic lung infections and has been linked to the inability to clear the bacteria from the respiratory tract [677]. UTI derived mucoid isolates have been previously reported [678], [679]. Like many CF isolates, these isolates were identified as carrying a mutated version of the *mucA* gene. This suggests that the conversion to mucoidy can follow a similar pathway regardless of infection site [677]. However, alginate over-producers are not commonly found in UTI infections, with studies reporting a level of around only 1- 3% [90],[680], [681]. In our study, none of the 25 UTI *P. aeruginosa* isolates displayed the mucoid phenotype, further solidifying previous findings that mucoidy may not play a major role in UTIs due to the possible absence of *mucA* mutations [677].

Colony morphology can also be affected by a variety of other factors including the production of pigments. *P. aeruginosa* can produce a number of coloured compounds

including pyoverdine, pyomelanin, pyorubin and pyocyanin. Dietrich *et al,* (2008) suggested that colony morphology in *P. aeruginosa* can be affected by phenazines production. Smooth colonies have been linked to the production of phenazines whereas isolates lacking this ability can grow as wrinkly colonies [667]. Isolate 133042 exhibited a wrinkly phenotype, which has been attributed to an adaptive response to maximise oxygen uptake by increasing the surface area of the cell [682]. In addition, mutations in the QS regulator gene *lasR* have been associated with a metallic sheen phenotype on nutrient rich media such as LB [666]. This isolate produced very little pyocyanin. Isolate 133117 from UK panel and isolate 925 from Kuwait's panel produced metallic colonies, and this may indicate a possible loss of *lasR* function. However, isolate 133117 could still produce some pyocyanin at a similar level to PAO1. Such mutation confers a growth advantage with carbon and nitrogen sources with the catabolic pathway [666]. QS mutants have been identified in isolates from many different sources. However, they have been linked to adaptation during chronic infection. No information is available on the length of time these patients had been infected for and it would be incredibly interesting to determine whether QS mutants begin to appear in patients with chronic UTIs, similar to that since in CF lung infections [666].

Biofilm formation has been implicated in 65-80% of all microbial and chronic infections [140]. *P. aeruginosa* utilises urinary catheters as a focal point to establish UTI and persist, leading to complicated infections, morbidity, and mortality [56]. Biofilms are commonly involved in CAUTI and they have also been identified in the urothelium and on prostate stones [683]. *P. aeruginosa* is a model organism in which to study biofilm formation and therefore, there is a wealth of literature describing factors associated with this process [118], [684], [685].

Biofilm formation assays grown in 96-well plates and stained using CV, provide a method to measure biofilms that are easy to execute and therefore commonly used [686]. This method was utilised to quantify biofilm for 15 UTI isolates and compared them to reference strains PAO1 and PA14. The biofilm formation of each isolate was determined at 24h [137]. Our results suggest that similar variation exists in this panel of UTI isolates. Isolate 133105 produced the highest level of biofilm, and this

exceeded the amount produced by PAO1 although there was no significant difference. The other isolates produced less biofilm to PAO1 however this was only significant for 133044 (P=0.036). The results in this study indicate that the UTI isolates produce variable levels of biofilms that are isolate-dependent.

Several studies have reported biofilm formation of *P. aeruginosa* isolated from multiple infection sites. *P. aeruginosa* can cause otitis media ear infections in patients with cholesteatoma, (25%-35% of infections) [687], [688]. *P. aeruginosa* isolates from otitis media appear to be good biofilm producers, with 83% of isolates forming significantly higher levels than PAO1. The authors of this study suggested that the ability to form biofilms may play a role in persistent infections in patients with cholesteatoma [689]. A recent study in Calgary, Canada by Doung and colleagues analysed biofilm formation of 108 CF isolates classified as Prairie Epidemic Strain (PES), other epidemic strains (OES) (including 6 LES isolates from the UK) and local non-epidemic isolates. This study demonstrated that local non-epidemic strains produced the highest mean of biofilm biomass followed by PES and OES, respectively [690]. PAHM4, a non-CF bronchiectasis strain forms significantly less biofilm than PAO1 [438]. A study of 101 keratitis isolates found that they produced 17% to 242% of the PAO1 control and the ability to form biofilms was correlated with worse vision outcomes [691]. The Schaber *et al,* (2007) study compared 5 QS-deficient isolates derived from respiratory, wound and UTI to PAO1 to assess biofilm development, the UTI isolate CI-5 was derived from 82-year patient who suffered sepsis secondary to CAUTI. Upon CV staining and comparison of all isolates, the UTI isolate produced the highest biomass amongst all QS-deficient isolates and up to 82% capacity of PAO1 [267]. A study examining uropathogenic *P. aeruginosa* serotypes and their ability to form biofilms *in vitro* via CV staining found variation between the different serotypes. Specifically, O11 serotypes produced the strongest biofilms and were more likely to be resistant to antibiotics while strains belonging to serotype O6 produced the weakest biofilms [692]. The ability of CAUTI *P. aeruginosa* was assessed by Vipin *et al,* (2019) all isolates formed biofilms varying in intensity between 0.20 and 1.11 (OD^{OD580}), indicating the diversity amongst these clinical isolates [693].

Although this method of biofilm quantification is relatively easy and high throughput, the method is limited as it does not provide information on the biofilm architecture or bacterial viability [694]. Biofilm formation *in vivo* does not always involve the formation of the mushroom-shaped structures. Microscopy has been used previously to assess biofilm formation of UTI *P. aeruginosa* [676], [693] . Here, we used confocal microscopy on UTI *P. aeruginosa* to assess biofilm architecture analysed with the post-imaging software COMSTAT [410]. This method of analysis allows the study of different parameters related to biofilm architecture [410]. Five parameters were studied in the reference and UTI isolates; biomass, maximum thickness, surface area, and surface to biofilm ratio. One of the main measurements in COMSTAT, is the measurement of biomass [410]. Despite the different time period of incubation (24h for the plate assay and 48 h for the microscopy), in each assay there was no significant difference between PAO1, and the majority of the isolates tested (14/15 for the plate assays and 15/15 for the microscopy). Furthermore, the results across the two assays were not consistent and isolates that produce the most biofilm in one assay did not in the other. Furthermore, the variability in the biological replicates for both assays, and particularly the microscopy, was large and in hindsight, this variability may have affected these results. It has been shown previously that upon reaching biofilm maturation, some microbial cells disperse due to nutrient depletion and decreased oxygen uptake [695]. Time-dependent analysis of PAO1 biofilm formation in a micro-device control flow system revealed alteration of biomass over a period of a week which resulted in subsequent reduction and dispersal of biofilms after stage V maturation [696]. This phenomenon may have a role in explaining any disparity observed between the plate assay and the microscopy measures of biofilm. To study this further, a time course with points over a 48h period using both methods could be employed.

Biofilm architecture is also known to show variation depending on isolate. PAO1 has been described as producing a mushroom type biofilm [697], whereas other isolates produced much flatter biofilms. In order to determine differences in the architecture, measurements such as roughness, surface area, thickness and biofilm:surface area ratio were analysed. Of particular interest was the roughness coefficient. This is a

dimensionless value which provides a description to variability across a given biofilm [410]. This parameter provides a measure of structural heterogeneity as it differentiates between rough and smooth biofilm. The former has pillars, interstitial channels, and towers of cells separated by spaces lacking any bacterial cells, while the latter is composed mostly of homogenous single layer of cells [698], [699]. No significant differences were observed but again, the variation between replicates was high. This may be due to the time point chosen (48 h). This was chosen to enable the study of a mature biofilm. However, earlier time points may allow more reproducibility and consistency of the results. In our study, based on the roughness coefficient values, no UTI isolates formed rough biofilms in comparison to the rest of isolates, indicating that the biofilm architecture does not differ significantly between isolates. The QS-deficient UTI isolate CI-5 studied by Schaber *et al,* (2007) was also analysed using confocal microscopy and COMSTAT [267], [676]. The analysis after 7 days of biofilm growth revealed a higher maximum thickness parallel to PAO1 by the UTI isolate CI-5 and produced unique towering structures [676]. In our study, maximum thickness was found to be higher in 9 UTI isolates, although the parameter was not significantly higher than PAO1. The consensus in the studies conducted measuring prokaryotic and eukaryotic (fungal) biofilm thickness suggest thicker biofilm tend to give an advantage for the pathogen [676], [700]–[702]. Biofilms may play a role in UTIs however, the methods used in this study are limited to the measurements of biofilm attached to an abiotic surface. *P. aeruginosa* biofilms can also exist in other forms such as aggregates at the air-liquid interface [703] and microcolonies [704], [705]. Most UTI isolates in this study possess intermediate ability to form biofilms, since most isolates registered between 0.1 and 0.2 A_{600}, a similar outcome was observed by Tielen *et al,* (2011) [90].

Motility is a prominent virulence factor utilised by *P. aeruginosa* to cause multiple types of infections [72]. Tielen *et al,* (2011) reported that UTI isolates derived from mid-stream urine were more likely to able to swim than CAUTI isolates, 90% of 30 isolates were able to swim [90]. A study conducted in the Czech Republic revealed that more than 95% of CAUTI isolates have the functional capacity to swim [706]. This was consistent with the findings in this study as 80% exhibited the ability to

swim. Swimming motility initiates attachment and adherence on abiotic surfaces that can lead to biofilm formation including on surfaces such as urinary catheters [67]. Twitching motility is facilitated by type IV pili and is used to move across solid and semi-solid surfaces [492]. Olejnickova *et al,* (2014) examined 139 *P. aeruginosa* UTI isolates for twitching motility which led to the discovery that 75% were capable of twitching [706]. Winstanley *et al,* (2005) tested 63 keratitis isolates for twitching motility, 90% out of the isolates exhibited the ability to twitch *in vitro* [707]. Furthermore, mutants defective in twitching motility were not able to colonise the cornea [708]. In our study 86.6% were able to twitch, indicating that this is another common feature in UTI isolates. Our panel of UTI isolates demonstrated a high degree of swarming motility (86%). Swarming hyper-motile strains tend to form flat biofilms [499]. High percentage (up to 95%) of swarming-motile *P. aeruginosa* CAUTI isolates have been reported [706]. Interestingly, swarming is a key feature for another urinary pathogen, *P. mirabilis* [39]. This may suggest that swarming is a beneficial trait in the urinary environment.

P. aeruginosa produces pyocyanin, an effective phenazine toxin [154]. *P. aeruginosa* UTI isolates consistently produce the most potent pyocyanin compared to isolates derived from other sites of infection. [709]–[711]. Pyocyanin has been shown to be associated with biofilm formation in the urinary tract [168]. Pyocyanin intercalates with eDNA to form biofilms in UTIs thereby promoting cell to cell interactions and affecting cell surface properties. The correlation between pyocyanin and *P. aeruginosa* UTI isolates producing biofilms has been assessed in the Vipin *et al,* (2019) study [693]. In our study, we did not observe a positive relationship between strong biofilm and pyocyanin producers and while none of the UTI isolates we tested were found to over-produce pyocyanin (like the CF strain LESB58), the majority of isolates produced comparable levels to PAO1 and PA14. Tielen *et al,* (2011) showed that 5 -15% of UTI isolates do not produce pyocyanin [90]. In this study, the isolate 133104 produced the highest level of pyocyanin and isolate 133083 produced a very low level of less than 0.01 (A695nm). This low level is comparable to isolates previously identified with mutations in their QS genes (*lasR*) from the CF lung [669].

P. aeruginosa strains are diverse in terms of its population structure. Here we report that the vast majority of isolates are PA14-like. Although the sample size is relatively small, more isolates fall in Group 2. This contrasts with the largest population structure studies which reported that PAO1-like *P. aeruginosa* isolates are the most widespread in the environment [658]–[660]. Further studies could involve analysing a much larger panel of isolates to determine if there is link between Group and UTIs. PA14 is thought to be more virulent than PAO1 in invasion models and has been used extensively in *in vivo* infection models [712]. Three isolates from Kuwait cluster very closely on the phylogenetic tree based on the core genome. Little is known about the epidemiology of these isolates and therefore surveillance of strain types circulating in Kuwait may provide insight into outbreaks or common circulating clone types. This is particularly important in light of the extreme AMR observed. A similar finding was observed by Tielen *et al,* (2011) upon conducting SNP chip typing on 30 *P. aeruginosa* uropathogens, and found 21 different clones [90]. The Kos *et al,* (2015) study sequenced 390 isolates containing 86 UTI isolates, the study did not discuss the relatedness to UTI to either PA01, PA14 and PA7-like groups [659].

The WHO has identified carbapenem resistant *P. aeruginosa* as a critical issue in need of intervention by developing new antimicrobial agents [36]. Upon searching for specific information on the *P. aeruginosa* AMR UTI isolates in both regions covered in this study, it was found that information is either scarce or reported briefly in larger UTI Gram-negative AMR studies [605], [636]–[638]. Based on these results, extreme resistance was identified in isolates from Kuwait and therefore Kuwait may be a hot spot for resistance. However, there may be some bias in isolate selection and therefore a wider study should be implemented. Lack of surveillance programmes, inadequate use of antibiotics, travelling and climate change are contributing factors to the rise of AMR in this region [609]. Genome sequencing of the isolates obtained from Kuwait showed higher resistance than those collected from the UK. Multiple isolates in the panel exhibited resistance to tobramycin, gentamicin and amikacin. This was consistent with the genomics analysis of these isolates as they contained modifying enzymes conferring resistance to aminoglycosides such as AAC (6’)-ii, aadA11, ANT(2'')-Ia and APH(3')-Ib. Some of the acquired genes are plasmid-borne

and may have been transferred by horizontal gene transfer. In addition, *gyrA* mutations and plasmid associated *crpP* enzyme were involved in promoting resistance against ciprofloxacin and levofloxacin in several isolates [674]. dfrB1is a plasmid-associated trimethoprim-resistant dihydrofolate reductase which was initially discovered on in *Bordetella bronchispetica* bacteria, suggesting it might have been transferred at some point to *P. aeruginosa* [713]. Resistance to β-lactamases in the Kuwaiti panel can be attributed to the presence of ESBLs such as PDC-2 and PDC-3. VIM-type resistance has previously been reported in *P. aeruginosa* in the GCC region [629]. VIM-28, an MBL which was first detected in Egypt recently, was found in isolates 758, 783 and 925. VIM-28 gene contains an unusual integron arrangement in the structure, with the gene cassette located downstream of the "*intI1"* gene [594]. Egyptian nationals are one of the largest ethnic minorities in Kuwait [634], [714]. Movement between the two countries may help explain the identification of $^{bla-}$VIM-28 in the two areas. In addition to the intrinsic OXA-50 Class D β-lactamase, which is found in all isolates, OXA-10 was detected in only one isolate 786. To the best of my knowledge, this is the first report of OXA-10 in bacterial isolates from Kuwait. Isolates 758 and 783 are sensitive to colistin using MIC broth microbdilution assay, therefore, there is one antibiotic that could be used to treat infections caused by these bacteria. Due to the extended resistance and the genes identified, it may be plausible that these isolates carry MDR plasmids. Recent studies on isolates from Thailand revealed *P. aeruginosa* carry MDR megaplasmids [715]. Further analysis of the genome sequence data would provide insight into this. The implications of these findings are alarming and require extensive surveillance and AMR stewardship programmes.

Isolates sourced from the UK in this study were much more susceptible to antibiotics with only two MDR isolates detected. The prevalence of AMR *P. aeruginosa* uropathogens is generally considered to be low [55]. Surveillance is not comprehensive. This could be attributed to stricter policies of antibiotic stewardships and wider knowledge amongst the public with antibiotic misuse [716]. According to Ironmonger *et al*, a 4-year long surveillance study detected only 45 non-susceptible *P. aeruginosa* uropathogens to carbapenems in a total of 6985 isolates sourced from the Midlands region of England [605].

The results from this chapter have provided insight into the genomic and phenotypic variation of *P. aeruginosa* isolates from UTIs. The data has highlighted that UTIs can be caused by a diverse selection with regards to both phenotype and genotype. This is not surprising as the *P. aeruginosa* population is diverse, and patients would presumably acquire individual strains from an environmental source. However, this may differ in the hospital setting for patients with long term catheters. The sequencing data has provided information on the population structure of the UTI isolates and further analysis could be performed on virulence genes and mobile genetic elements (such as prophages). This is unfortunately beyond the scope of this thesis. A large variety of antimicrobial resistance genes were identified through sequencing and furthermore, resistance to every class of antibiotics was identified in the UTI isolates using disk diffusion assays. This is of course alarming, particularly the extremely high level of resistance identified in isolates from Kuwait. The exact basis for picking these isolates has been difficult to determine and the isolates were now obtained some years ago. A new study aimed at determining the current prevalence of antimicrobial resistance, both in the UK and in Kuwait, would provide important missing epidemiological data and ensure less biases (or at least known biases). The identification of highly resistant strains of *P. aeruginosa* from UTIs requires further research. In addition, further understanding of how *P. aeruginosa* responds to the urinary environment would allow a deeper analysis of the basis of bacterial pathogenesis and potentially help identify new therapeutic interventions.

Chapter 4

4.Artificial Urine Medium as a Model for UTIs

4.1 Introduction

4.1.1 Components of Urine

Urine is defined as a ‘ transparent, sterile, amber-coloured fluid generated by the kidneys’ [717]. Urine formation occurs via the secretion of waste from the bloodstream into the tubular filtrate along with glucose, excess water, and other waste compounds such as hydrogen ions and drug metabolites [718]. The primary route of excretion of soluble waste in humans is through the urinary tract [717]. According to the urine metabolome database, 4000+ elements and compounds have been detected in urine derived from healthy and ill individuals [719]. Metabolomics is the study of chemical products or metabolites made by cells [720]. This approach has been used in drug discovery, disease marker identification [721]. Previous studies have also utilised 2D-electphoresis in order to construct a urine proteome map [722]. Thus, classification of urine components was established based on functional criteria. These functional groups constitute of 9 groups; kidney secretory and structural proteins, serum and transport proteins, coagulation factors, complement fractions, immunoglobulins with heavy and light chains, proteases and inhibitors, enzymes, metal binding proteins and lipoproteins (Table 4.1) [722]. Urine contains high levels of urea as a by-product of amino acid metabolism [723]. High concentrations of inorganic salts such as sodium, chloride and potassium are found in urine [724]. Furthermore, ammonia, creatinine, organic acids, soluble toxins, haemoglobin derivatives such as urobilin are detected[717], [725]. Hormone and drug metabolites, including these with low molecular weight, have been identified through metabolomics [726].

4.1.1.1 Factors affecting Urine Composition

Urine composition is highly variable amongst healthy and ill individuals and in the same individual over periods of time [727]. Urine is more concentrated in the morning and is influenced by fluid intake, surrounding temperatures and exercise [728], [729]. The normal urinary pH is typically 6.0 to 7.5 but can vary between pH

4.5 to 8. UTIs can also affect the pH of urine which can raise to 9.5. This can be indicative of the presence of bacterial species such as *Proteus spp* and *Klebsiella spp* as a result of urease activity and the generation of ammonia [730], [731].

Table 4.1 Functional classification of components detected in urine using 2-D gel electrophoresis [722]

Functional classification	Examples
Kidney secretory and structural proteins	Uromodulin (Tamm-Horsfall protein), Epithelial-cadherin, Polymeric immunoglobulin receptor, Stomatin-like protein.
Serum constitutional and transport proteins	Albumin, Vitamin D binding protein, Leucine-rich α2 glycoprotein, Transthyretin monomer multimer.
Coagulation factors	Fibrinogen α chain, Kininogen, Fibrinogen β chain, Fibrinogen γ chain.
Complement fractions	Complement factor B (Complement factor Bb, Mannan-binding lectin serine protease 2
Immunoglobulins with heavy and light chains	Ig α-1 chain C region, Ig γ-3 chain C region, Ig γ-2 chain
Proteases and inhibitors	α1 Antitrypsin and α1 Antitrypsin fragment, Pancreatic α amylase; α1 Antichimotrypsin/
Enzymes	Trehalase Ribonuclease, Prostaglandin-H2-isomerase, Dimethylargininase-2
Metal binding proteins	Serum transferrin, Ceruloplasmin, α HS glycoprotein, Copper transporting ATPase 1
Lipoprotein metabolism	Clusterin, Apolipoprotein D, Ganglioside GM2 activator

Dietary habits are known to impact the composition of urine. Siene *et al,* (2002) conducted a study of 10 healthy male subjects consuming a western meat-based diet, compared with those consuming a normal mixed diet and those on an ovo-lacto-vegetarian diet [732]. The study showed that the secretory profiles of urine components were altered, in particular, calcium secretions were highest in participants taking western diets (21%) compared to 13% in people consuming a

vegetarian diet. This suggested that the risk of urolithiasis (formation of stones within the urinary tract) in those consuming a Western diet was increased [732]. In addition to diet, several other metabolic and dietary factors affecting urine composition and renal calculi formation have been implicated (Table 4.2) [722].

Table 4.2 Dietary factors involved in changing urine composition

Dietary factors affecting urine	References
Decreased fluid intake	[733]
High or low calcium intake	[734]
High intake of oxalate	[735]
Animal protein	[736]
Sodium	[737]
Refined sugars	[738]
Potassium	[739]

4.2 Current understanding of bacterial metabolism in urine

Little is known about the behaviour of uropathogenic *P. aeruginosa* and its traits which enable colonisation and infection of humans. However, knowledge gained from studying host-pathogen interactions in *E. coli* has provided insight into mechanisms which may be important determinants of pathogenicity, as they are shared by several Gram-negative bacteria [269]. Bacteriuria is the presence of bacteria in urine and a marker of symptomatic UTI, higher levels of bacteriuria contribute to the of severity of infection [740]. Progression of the infection depends on the survival and fitness of the uropathogen as well as factors impacting the host. Most patients who suffer from persistent UTI are infected with different bacterial strains over time [741]. Interestingly, long-term bladder colonisation appears to select for UPEC with attenuated virulence phenotypes [742]. Different microorganisms have variable fitness which determines their potential to survive in the harsh urinary tract environment [743]. Patients with previous *E. coli* infections are more susceptible to infection and can be colonised by the same or a different uropathogen [744]. Antimicrobial peptides are another defence mechanism which colonising uropathogens must overcome (See section 4.3.1) [745]. In addition, factors

such as defects in TLR receptor signalling, genetic immunodeficiency, recurrent infection or serotype/type replacement influence bacterial progression in the urinary tract. [745], [746]. Survival and growth in urine ensures persistence of uropathogens regardless of the ability to adhere to the urothelium or the dynamics of the inflowing urine, including voiding and the rates of urinary flow rates. Indeed, non-voided uropathogens, which exist in the remnants of urine, grow and multiply to propagate the infection [745]. ABU *E. coli* isolate 83927 has been studied to understand bacterial fitness in the urinary tract, this strain can outcompete UPEC strains 536, CFT073, NU14, and 1177 [747], [748]. The identified adapted metabolic behaviours include alterations in transport and degradation pathways for compounds such as glucuronide, galactonate and galacturonate [747]. In addition, antioxidant defence mechanisms are of paramount importance for survival in the host [749]. ABU *E. coli* 83927 was shown to acquire mutations to adapt its metabolic profile. In an unusual therapeutic approach, Zdziarski *et al,* (2010) inoculated the bacteria into six patients and recaptured the isolates from urine at different time points over several months. Subsequent transcriptomic and proteomic analysis revealed shifts in diverse metabolic pathways such as those utilising amino acids, hexuronates or (deoxy-) ribonucleosides [750]. *Genes guaA* and *argC* were shown to be essential for guanine synthesis in UPEC using transposon mutagenesis. Mutants were found to show attenuated virulence in mouse models of UTI [751]. In addition, *argC* and *carAB* mutations in another transposon study resulted in reduced growth ability of *E. coli*, highlighting the need for arginine to support growth in urine [752].

As for *P. aeruginosa*, utilisation of nutrients can be partially dependent on oxygen availability and limitations in the urinary tract [753]. In aerobic environments, different sugars from carbon sources are utilised by the Entner-Doudoroff-Pathway to perform oxygen respiration through numerous terminal oxidases [754], [755]. The versatile metabolic adaptability of *P. aeruginosa* allows this bacterium to process nitrate or nitrite by denitrification in environments lacking oxygen [756], [757]. *P. aeruginosa* can also produce energy by fermenting arginine in media lacking electron acceptors [753]. In anaerobic conditions, *P. aeruginosa* utilises mixed acid fermentation which prolongs anaerobic survival and assists in the formation of

microcolonies [758]–[760]. In the urinary tract, citrate, lactate and amino acids can be utilised as carbon sources [753]. Tielen *et al,* (2013) investigated the metabolomic profile of PAO1 biofilms grown in AUM under anaerobic conditions [753]. The authors observed that *P. aeruginosa* adapted its central metabolism is favour of utilising citrate, lactate and multiple amino acids derived from peptone. This study identified that genes involved in the tricarboxylic acid cycle such as *gltA*/*prpC* which encode citrate synthetase 1 and 2, were induced under these conditions. *P. aeruginosa* also appeared to synthesise higher concentrations of amino acids including alanine, β-alanine, aspartate, glutamate, glutamine, serine and ornithine within intracellular metabolome in AUM biofilms compared to biofilms grown in 10-fold diluted LB. In contrast, fatty acid biosynthesis was reduced as genes *accA*, *accB*, *accC*, *fabAB*, *fabGD* were downregulated in AUM [753]. Berger *et al,* (2014) utilised 13C-metabolic flux analysis to study PAO1 and 17 other UTI clinical isolates, which were universally able to catabolise glucose by the Entner-Doudoroff-Pathway. The study also reported over-production of NADPH for anabolism by additional supplementation of NADPH, which can be useful against oxidative stresses derived from the host. In addition, the authors concluded that biosynthesis using the tricarboxylic acid cycle , pyruvate metabolism, and the glycoxylate shunt was variable amongst the UTI isolates [761]. These findings provide a good insight into the pathogenic behaviour of *P. aeruginosa*, however, it does not address the similarities and differences of planktonic and biofilm growth of *P. aeruginosa* between AUM and urine. The virulence profile between *P. aeruginosa* grown in urine and AUM may be different. Thus, we will try to address this disparity by conducting growth rate, biofilm formation, gene expression and proteomic analysis between LB medium, AUM and human urine.

4.3. Immunity in the urinary tract

4.3.1 Immune system components in urine

Immune components in urine and the urinary tract determine the way by which an individual deters UTI [762]. Urine contains several compounds and antimicrobial peptides participating in fighting infections, such as lysozyme, defensins, lipocalin, cathelicidin, lactoferrin, ribonuclease-7 and uromodulin (THP) and these can

eliminate bacteria via multiple pathways [17], [762], [763]. The presence of innate immune white blood cells such as neutrophils, macrophages, Natural killer (NK) cells with the related pro-inflammatory cytokines, complement systems and scavenging receptors can help control and clear UTIs (Table 4.3) [762], [764], [765].

Table 4.3 Urine and urinary tract components which confer immunity to uropathogens such as antimicrobial peptides: defensins, lipocalins, cathelicidins, ribonuclease 7. Other compounds with immune functions are featured such as lactoferrin and uromodulin

Immune components	Function	Reference
Lysozyme	Cleavage of Gram-positive bacterial cell wall	[766]
Defensins	Antimicrobial activity against bacteria, chemoattractant for dendritic cells	[767]
Lipocalin	Sequesters iron to prevent bacterial utilisation	[768]
Cathelicidin	Antimicrobial activity against Gram-positive and Gram-negative bacteria, chemoattractant for neutrophils and monocytes	[769]
Lactoferrin	Limits proliferation and adhesion of bacteria, sequesters iron to prevent bacterial utilisation	[770]
Ribonuclease 7	Antimicrobial activity against uropathogens, disruption of the microbial membrane	[771], [772]
Uromodulin (Tamm-Horsfall) protein	Interfering with the ability of uropathogens to bind to the epithelium	[767]

4.3.1.1 Toll-like receptors

TLRs on the surface of immune cells or the epithelia lining the urinary tract recognise conserved molecular patterns known as PAMPs on the uropathogen and drive innate and adaptive immune responses by producing pro-inflammatory cytokines [773]. Four TLRs have been associated with host defences against uropathogens; TLR2, TLR4, TLR5 and TLR11 [774]. TLR2 is an immune receptor to bacterial lipoteichoic acid or lipoprotein and is found in the kidneys and expressed constitutively in the proximal

and distal-collecting tubules [775], [776]. An *in vivo* study involving control and TLR2-deficient mice presented evidence of reduced pro-inflammatory response and leukocyte infiltration in the latter [777]. TLR4 is one of the key TLRs contributing to immune responses against uropathogens. This occurs upon the recognition of LPS of bacteria such as UPEC by TLR4 on bladder cells, which in turn activates neutrophils [778]. TLR4 also recognises UPEC Type I pili which leads to the transcription of genes encoding Il-6 and IL-8 [17], [779]. TLR4 has been shown to interact with the FimH adhesin of UPEC in human embryo kidney cell lines along with animal models [780]. TLR5 recognises flagellin, a structural protein in UPEC's flagella, which triggers the innate immune response in the bladder and kidneys. The importance of this cascade in host defence has been demonstrated in TLR5-defiecent mice, which exhibit a higher bacterial load than wild type mice infected with *E. coli* strain CFT073 [781], [782]. TLR11 is not expressed in the urinary tract of humans or any anatomical location in the body [783]. Zhang *et al,* (2004) characterised TLR11 in mice, and generated mutants mice lacking TLR11 and challenged them with UPEC strain 8NU, knockout mice showed higher bacterial load in the bladder and kidney upon examination the respective homogenates [784].

4.3.1.2 Adaptive immune response

Adaptive and acquired immunity can also play a significant role in combating UTIs. Mice lacking B and T lymphocytes were more vulnerable to UTIs than wild type mice [763], [765]. Carson *et al,* (1999) infected mice lacking γδ -T cell by UPEC strain 1677, and found they were more susceptible to UPEC compared to wild-type mice [785]. Conversely, introduction and restoration of T lymphocytes in the spleen and inactive serum transfer from infected mice to naïve mice enhanced resistance to the colonisation of UPEC isolate NU14 [778], [786]. Studies aimed at designing vaccines against UPEC suggest an important role for humoral and cell mediated immunity via the production of cytokines interferon gamma (IFNϒ), IL-4 and IL-17 in UTI eradication [787]–[789]. The expression of multiple immune components can be variable depending on anatomical location within the urinary tract. For instance, secretory IgA may be the most effective immune component against bacterial colonisation in the bladder, lack of secretory IgA in the urinary tract may lead to recurrent UTIs [763],

[765], [778]. In contrast, systemic production of T lymphocytes appears to be more significant in kidney defences [790].

4.4 The urinary tract microbiome

4.4.1 The urinary microbiome in health and disease

For over a century, the scientific community believed in the dogma of the sterility of the urinary tract and urine [740]. This notion has been challenged in the last decade with emergence of advanced molecular and sequencing techniques aimed at understanding the complex microbiome found in the urinary tract [791]. The current gold standard for urinalysis is the examination of urine culture due to the highest accuracy in which aerobic prototypic phenotypes are identified [792]. Routine culture techniques may not be able to detect fastidious anaerobic uropathogens such as *Ureaplasma* and *Corynebacterium* [740]. Modern sequencing technologies, such as 16S rRNA sequencing, allowed the detection of a diverse urobiome in individuals [793]. The findings of such advanced techniques are supported by modified and enhanced quantitative urine culture protocols leading to the detection of up to 80% of previously undetectable bacteria by the standardised routine protocols, including *Actinomyces naeslundii*, *Fusobacterium nucleatum*, *Gardnerella vaginalis*. Furthermore, microbiome alterations have been detected in certain urinary diseases. In interstitial cystitis (IC), which is a chronic condition identified by persistent pain and at least one symptom such as bladder pain or frequent urination, alterations in the microbiome have been observed [794]. The abundance of *Lactobacillus* is found in 90% of IC samples in comparison to 60% abundance in healthy individuals [740].

4.5 *In vitro* infection models

4.5.1 The aim of artificial mediums *in vitro*

It is widely accepted that laboratory methods based on the utilisation of nutrient rich LB media are not representative of *P. aeruginosa* infections or the pathophysiology of other common uropathogens [795], [796]. Media have been developed *in vitro* to better mimic the conditions in the host. Here we discuss some of the experimental tools developed to enhance our understanding of *P. aeruginosa* infections in other

host tissues, and their importance in our understanding of the use of artificial urine medium (AUM) to study UTIs.

4.5.2 Artificial sputum medium (ASM)

The rise in antimicrobial resistance has led researchers to question the methods/conditions used for *in vitro* studies such as diagnostic testing of planktonic *P. aeruginosa* [797]. For example, *P. aeruginosa* grows in mostly microaerophilic biofilms in chronic infections in the CF lung, calling into question whether the methods of diagnostic testing are representative of the actual infection environment [704]. Many groups have developed and used specific media such as artificial sputum in order to recreate physiologically relevant conditions [798], [799]. These studies provide clear evidence for the application of *in vitro* models that recapitulate the host environment, particularly in the development of novel therapeutics.

4.5.3 Artificial Urine Medium (AUM)

The changing urinary composition in humans can pose a challenge for laboratory investigations of UTI [727]. Typically, urine is pooled from several individuals [800] which could affect the reproducibility of results due to the variability in the proteomic and metabolic profile of urine between individual donors. To combat these effects, Brooks *et al,* (1997) devised artificial urine medium (AUM) to allow a more consistent approach to the investigation of UTI pathogenesis. AUM contains basic urine components such as urea, uric acid and sodium chloride along with other components [800].

4.5.4 AUM as an experimental medium

The importance of AUM as a medium used for research purposes is highlighted in the broad array of applications found in published literature. AUM has been used to study diagnostic tools to uropathogenesis, host-pathogen interactions and the development of therapeutics related to the treatment of UTI. AUM has been used to investigate factors that contribute to UTIs [731]. For example, struvite stones are one of the most common urinary stones and are comprised of magnesium ammonium phosphate ($MgNHPO4 \cdot H2O$). These stones constitute about 10 to 15% of all urinary stones identified in patients globally and are produced by urease producing uropathogens [801]–[803]. The formation of struvite crystals in AUM by

uropathogenic *P. aeruginosa* is inhibited in the presence of vitamin C at 0.5 mg/mL or 1 mg/mL over 15 days, suggesting a strong modulatory effect by ascorbic acid [804]. *P. mirabilis* also induces struvite formation by urease production, whereas aminophosphinic urease inhibitors prevent formation of struvite in AUM [731].

Several studies have used AUM with small differences in its composition, to assess the efficacy of novel antimicrobials targeting Gram-negative uropathogens. AUM containing chitosan (1% chitosan solutions ranges from 50 and 150 kDa) demonstrated antimicrobial capacity to inhibit adhesion of *E. coli* and *K. pneumoniae* on Foley urinary catheters and also prevented the re-growth of both uropathogens [805]. Campana *et al,* (2017) developed biofilms of these uropathogens on urinary catheters for 48 and 72 h in AUM with different chitosan molecules based on molecular weight at pH 5.0, and observed 90.4% and 89.7% removal of biofilm followed by inhibition of regrowth on catheters [806]. Biodegradable ciprofloxacin-incorporated waterborne polyurethane polymers are found to be effective in preventing *P. mirabilis* biofilm formation in AUM and in an artificial bladder model, which consisted of a glass flask maintained at 37°C in a water jacket with a catheter inserted to the base through a silicone tubing [807], [808]. Urease inhibition has also been investigated in UTI-causing Gram-positive uropathogens, where treatment of *S. saprophyticus* in AUM by urease inhibitors cetohydroxamic acid or fluorofamide causes a temporary delay in the raise of pH associated with urease activity [803]. AUM has also been used to test products sold for UTI treatment. However, these over-the-counter plant preparations have shown limited effectiveness in reducing growth of *S. saprophyticus* in AUM [801].

AUM has been involved in studying host-pathogen interactions. Urease causes encrustation on urinary catheters and stents by converting urea to carbon dioxide and ammonia [809]. Encrustation of medical devices can lead to several concerning issues; hindrance of urinary flow, biofilm formation and device-removal associated mucosal damage [810], [811]. Removal of urinary catheters can be performed with ease, but in the case of other medical devices such as urinary stents, surgical removal is required [812]. *In vitro*, AUM containing urease has been utilised as the ideal medium to perform initial research on therapeutic options for biofilm inhibition

treatment, despite the use of urine in some experimental setups. As discussed previously, experimentation with urine has inherent limitations caused by variable composition between healthy and diseased hosts as well as physiological changes that occur with time in the same individual [727], [813]. Assessment of *E. coli* adaptation to the urinary tract and its low metabolite availability has revealed, by qRT-PCR, that nucleotide salvage pathway genes are upregulated in the presence of uracil, xantham and hypoxanthine by using an AUM model [814]. AUM has also been to investigate the link between biofilm formation and UTI antimicrobial resistance. A study conducted in Japan investigated the correlation between biofilm formation in AUM and MBL uropathogenic *P. aeruginosa* and found a significant increase in biofilm formation in MBL producing strains than the non-producers [815]. To characterise the transcriptomic, metabolomic and proteomic profile of PAO1 *P. aeruginosa* in conditions mimicking the UTI environment, Tielen *et al,* (2013) compared biofilm formation in AUM medium and a 10-fold diluted LB media, to utilise it as a control and maintain the growth rates similar to AUM [753]. Transcriptomic analysis revealed that 1019 genes were differentially expressed in AUM while proteomic and metabolic analysis found 19 proteins and 34 metabolites expressed differentially. The dominant response observed at the transcriptomic and proteomic level was to iron limitation as the Fur regulon was upregulated which includes 19 sigma factors and an estimated 80 targeted operons and genes [753].

More recently, there has been a shift towards the use of AUM in diagnostic testing of uropathogens. Laboratory testing of urine specimens produce negative cultures in up to 80% of the tested samples, causing additional workload and significant loss of resources [816]. Most importantly, using urine culture as the diagnostic gold standard causes delays up to two-three days to detect causative agents and antimicrobial susceptibly patterns [817]. Thus, rapid and efficient standardised detection methods are needed. One of the most recent advances in this field is the application of isothermal microcalorimetry (IMC), using AUM as a standardised medium [818]. This study examined four uropathogens in AUM; *E. coli*, *P. mirabilis*, *S. aureus* and *E. faecalis* with IMC, all were detected between 4 to 10 hours after inoculation in AUM [818]. By utilising a portable bacteria-grasping surface-enhanced Raman scattering

(SERS) method, detection of *E. coli, P. mirabilis* and *P. aeruginosa* was significantly faster in LB and AUM (1.5 h) than traditional screening methods. With the electro adsorption principle, researchers managed to grasp the positively charged NH_3^+ on a portable chip with the negatively charged microorganisms [819]. AUM has also been used in cell culture. Madin-Darby Canine Kidney (MDCK) cell polarisation using Transwell™ were optimised using this method. This resulted in more complete polarisation of MDCK renal tubular cell by production of greater levels of tight junction proteins occuldin and ZO-1 [820]. These findings together suggest that AUM maybe a more suitable media to study a wide-variety of related aspects of UTIs such as diagnostic tools, host-pathogen interactions and the development of therapeutics.

4.6 Phages as novel antimicrobials

AUM has previously been used to study bacterial virulence but also the action of antimicrobials. With the rise of resistance, novel antimicrobials are being developed and these could potentially be used to treat UTIs. However, in the development pipeline, it is important to be able to test the activity of any new antimicrobial agent in relevant conditions. Thus, in conjunction with studying changes in bacterial characteristics in physiologically relevant media, it can also be used as a platform in which to assess new therapeutics. Here, I discuss the use of bacteriophages (phages) and will study their activity in AUM.

4.6.1 Therapeutic application of phages against *P. aeruginosa*

Bacteriophages (phages) are one of the most widespread and ubiquitous biological entities in the environment [821]. Phages are classified into lytic and temperate (lysogenic) according to their lifecycle [822]. Lytic phages infect their host (bacteria) and can replicate producing progeny [823]. The process of phage invasion of its host is initiated by adsorbtion to the host cell surface, followed by injection and replication of the phages DNA, eventually resulting in induction of cell lysis and subsequent phage replication and invasion of neighbouring cells [824]. Temperate phages integrate their DNA into the bacterial chromosomal DNA establishing a relatively stable relationship. At later stages during the life cycle these phages can transform into the lytic life cycle [825]. Due to their inability to integrate into host bacteria, currently only lytic phages are of therapeutic interest.

The term “phage therapy” was first coined by Félix d'Hérelle in 1917 [826], followed by early successes in the therapeutic application of phage to treat infections [827]. The use of phages, however, was abandoned in the Western world following the discovery and implementation of antibiotics post World War II [823]. The focus on phages has shifted back considerably due to the emergence of MDR bacterial pathogens and limited therapeutic options [828]. Bacteriophages targeting *P. aeruginosa* were first described in 1960 by Holloway *et al,* (1960) [829] and as of January 2015, 137 complete phage sequences were reported [827].

The vast majority of phages (94%) which target *P. aeruginosa* belong to the order of caudovirales, which consists of 3 families *Podoviridae, Myoviridae* and *Siphoviridae* (Table 4.5) [821], [830]. The taxonomical distinction is based on the shape of the tail [830]. Sequencing analysis revealed that the majority (85%) of *Pseudomonas* phages of caudovirales are *P. aeruginosa* specific. Amongst these phages, it is estimated that 60% are lytic phages while 21.8% are temperate and 18.2% are yet to be classified. These phages have been isolated worldwide from multiple sources with the most common isolation places being wastewater treatment plants and hospitals [827].

Table 4.4 Taxonomy and characteristics of largest order of phages involved in invading *P. aeruginosa*

Phage order	Family	Tail description	Genome size (Kb)	Nucleic acid
Caudovirales	*Podoviridae*	Short noncontractile	41.6 - 74.9	dsDNA
	Myoviridae	long and contractile tail	64.1- 309.2 kb	dsDNA
	Siphoviridae	long and noncontractile tail	34.5 - 61.1	dsDNA

The efficacy of lytic phages as a therapeutic option against *P. aeruginosa* infections is of continued interest to the field. Fur *et al,* (2010) pre-treated hydrogel urinary catheters with *Myoviridae* lytic phages two hours in advance before inoculating the catheters with *P. aeruginosa* which led to the reduction of 2.8 log in comparison to untreated control catheters after 24 hours. The results were reversed between 24-

48 hours of growth in the treated biofilms prompting the investigators to isolate the resistant *P. aeruginosa* cells and study their susceptibility patterns [831]. This allowed them to construct a strategy in which a cocktail of 5 phages applied to pre-treated catheters, resulting in a 3-log reduction of the same *P. aeruginosa* biofilm population versus the untreated controls [831]. Pires *et al,* (2011) utilised two phages (phiIBB-PAA2 and phiIBB-PAP21) capable of reducing the biomass of planktonic cells to infect and reduce biofilms. Upon infecting biofilms with one type of phage, the biofilm was reduced after 2 h and further inhibition took place after 6 h. However, biofilm cells of strain PAO1 developed a resistant phenotype to phiIBB-PAP21 after 24h [832].

In addition to using phages on their own, research has been conducted into combining phages with existing antibiotics in order to potentially enhance any synergistic effect. Torres-Barceló *et al,* (2014) allowed PAO1 to grow for 6h to reach the exponential phase then introduced either streptomycin and/or *Podoviridae* phage LUZ7. Cultures were studied for up to 70 h to assess whether the use of phages can contribute to a reduction of antibiotic doses below the standardised MIC. The results suggested that combination therapy with both the phage and streptomycin was more effective than the individual treatments alone [833]. Knezevic *et al,* (2013) further expanded on this approach by performing kill-curves on *P. aeruginosa* treated with a combination of phages belonging to *Podoviridae* and *Myoviridae* classes coupled with subinhibitory concentrations of ciprofloxacin, gentamicin, ceftriaxone, and polymyxin B. The only effective combination found to inhibit *P. aeruginosa* growth was ceftriaxone and *Siphoviridae* phage σ-1, suggesting that cell is disrupted by the antibiotic paving the way for the phage infection [834]. Zhang *et al,* (2013) extracted RNA phages from wastewater in Missouri, USA and challenged *P. aeruginosa* biofilms with chlorine, the synergistic impact of both treatments proved more effective than individual treatments [835]. Phage cocktails appear to be more effective in a wide range of hosts, simultaneous administration of multiple phages may results in higher reduction of bacterial load [836], [837].

Lehman *et al,* (2015) investigated the utilisation of a multiday model of continuous flow in an *in vitro* model of AUM, to assess the effect of pre-treated hydrogen coating

silicon catheters with mixed *P. aeruginosa* and *P. mirabilis* phages. The application of a cocktail of phages isolated from sewage water was effective in reducing biofilm formation in both pathogens. The authors concluded that AUM is a valid alternative to urine to study the impact of phages on *P. aeruginosa* [838]. This remains the only report which used AUM to study the impact of phages in the urinary tract. Therefore, more research is warranted to assess whether AUM is a viable option for testing phage efficacy.

4.7 Aims

- To compare growth and virulence characteristics of *P. aeruginosa* in AUM with both nutrient rich LB broth and human urine

- To study *P. aeruginosa* proteome changes in AUM compared to urine and a standard laboratory media.

- To compare the resistance of *P. aeruginosa* to therapeutics in AUM to existing nutrient rich laboratory media.

Results 4.8

4.8.1 Growth kinetics of *P. aeruginosa* are similar in urine and AUM

In order to study the factors that might regulate the growth dynamics of *P. aeruginosa*, growth rates of *P. aeruginosa* UTI isolates were measured and compared over a 24 hour period in either AUM, pooled human urine or nutrient rich laboratory media, LB. 3 clinical isolates (133043, 133065 and 133098) (Figure 4.1) were selected as representative strains that demonstrate the global growth patterns observed across the entire isolate panel described in Chapter 3.

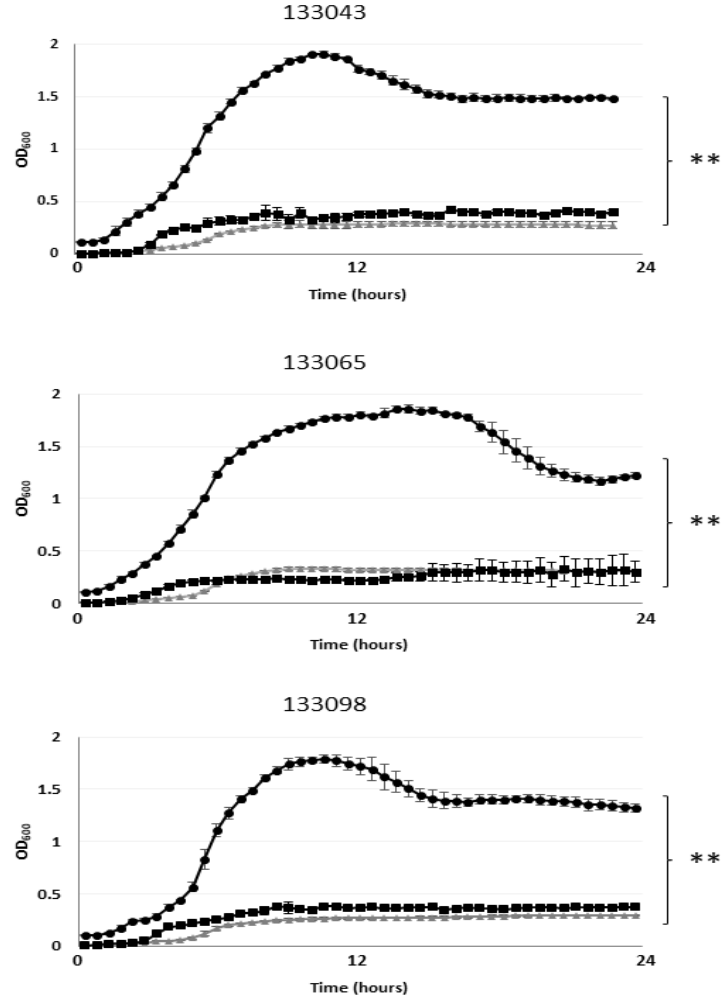


Figure 4.1 Growth curves of 3 representative *P. aeruginosa* UTI isolates chosen from a panel of 15 UTI isolates in LB broth (black circle) artificial urine media (AUM) (grey triangle) and pooled human urine (black square) over 24 hours at 37°C. Significant differences to the growth in LB are shown using **P<0.001

Isolates grown in LB media showed a rapid increase in cell density over the first 12 hours and reached optical densities >1.8 (A600nm). Growth in AUM was significantly lower in comparison to LB. However, this was highly similar to the increases in growth measured in human urine (Figure 4.1). The cell density for all isolates increased to 0.4-0.5 that (A600nm) over the first 6 hours. These data suggest that AUM supports similar levels of growth to pooled human urine.

4.8.2 Biofilm formation in urine is better represented by AUM

As previously discussed in chapter 3, biofilm formation is essential in establishing CAUTI [56], [280]. Thus, to determine whether there are differences in *P. aeruginosa* biofilm formation when grown in AUM, compared to urine or LB, CV staining was performed to estimate the biomass under each condition. For almost all isolates, the highest biofilm formation was observed when grown in LB (Figure 4.4). However, for PA14, the highest amount of biofilm at both 24 h and 48 h could be seen in AUM. At 24 hours, 13/18 isolates showed a significant reduction in biofilm formation in AUM compared to LB. All isolates had reduced biofilm in urine compared to LB at both 24 h and 48 h (Figure 4.2). However, this apparent difference in biofilm formation could be attributed to the differences in growth observed in Figure 4.1. To account for this, the data was reanalysed to correct for total bacterial growth and expressed as per capita biofilm production (Figure 4.3).

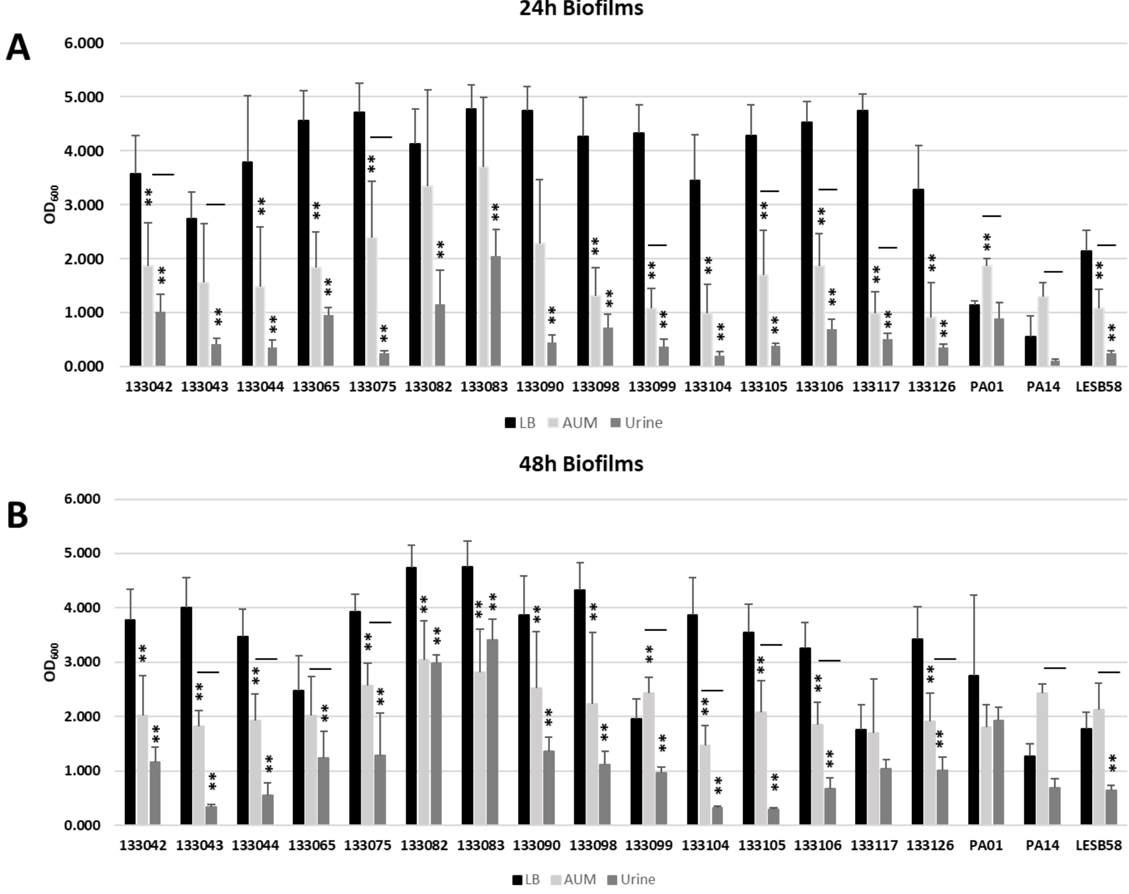


Figure 4.2 Biofilm assay of *P. aeruginosa* UTI isolates in LB (black), artificial urine media (AUM) (light grey) and pooled human urine (dark grey) after 24 h (Panel A) and 48 h (Panel B). Significant differences to the biomass in LB broth are shown using *P<0.05 **P<0.001; significant differences P<0.05 between AUM and pooled urine are denoted with a solid line

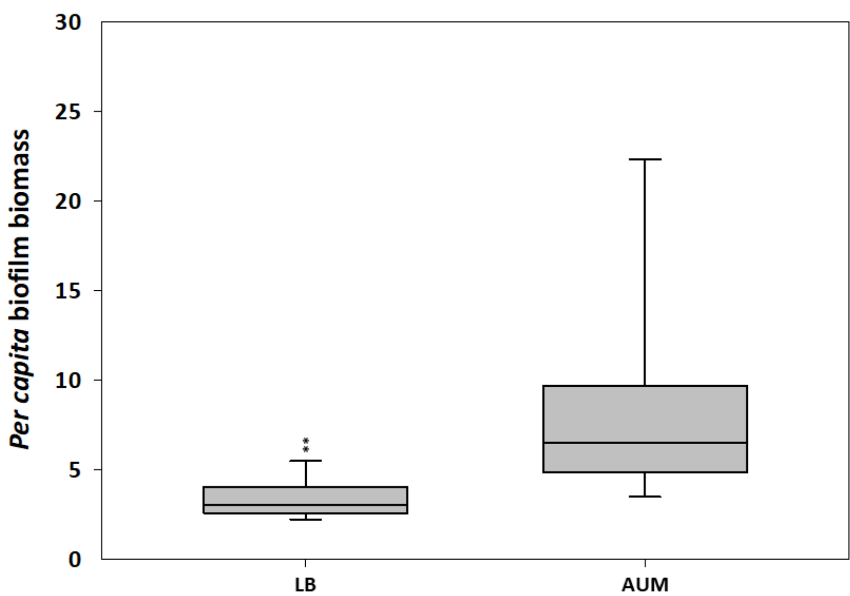


Figure 4.3 Per capita biofilm biomass for 15 clinical P. aeruginosa clinical isolates in LB broth (LB) and artificial urine media (AUM) relative to OD600 after 24 h. Significant differences are shown using **P<0.001

The data shows that under AUM conditions, bacteria had a greater ability to form biofilms compared LB media. This suggested that relatively more bacteria (of the total bacteria) were adhered to the surface of the plastic in AUM than in LB and that AUM conditions may promote biofilm formation. To investigate this observation further, a selected number of clinical isolates were studied using confocal microscopy to study the structure and architecture of the biofilms (Figure 4.4). Isolates grown in AUM appear to form denser and tighter biofilms than those grown in the richer nutrient medium LB. Figure 4.4 displays PAO1 biofilms formed in A) LB and B) AUM. The biofilms formed in AUM are more densely packed with bacteria and are thicker (minimum 25 µm). The trend for more dense biofilms in AUM can also be observed for two clinical isolates (Figure 4.4 C-F). This provides further evidence that AUM promotes enhanced biofilm formation on plastic surfaces.

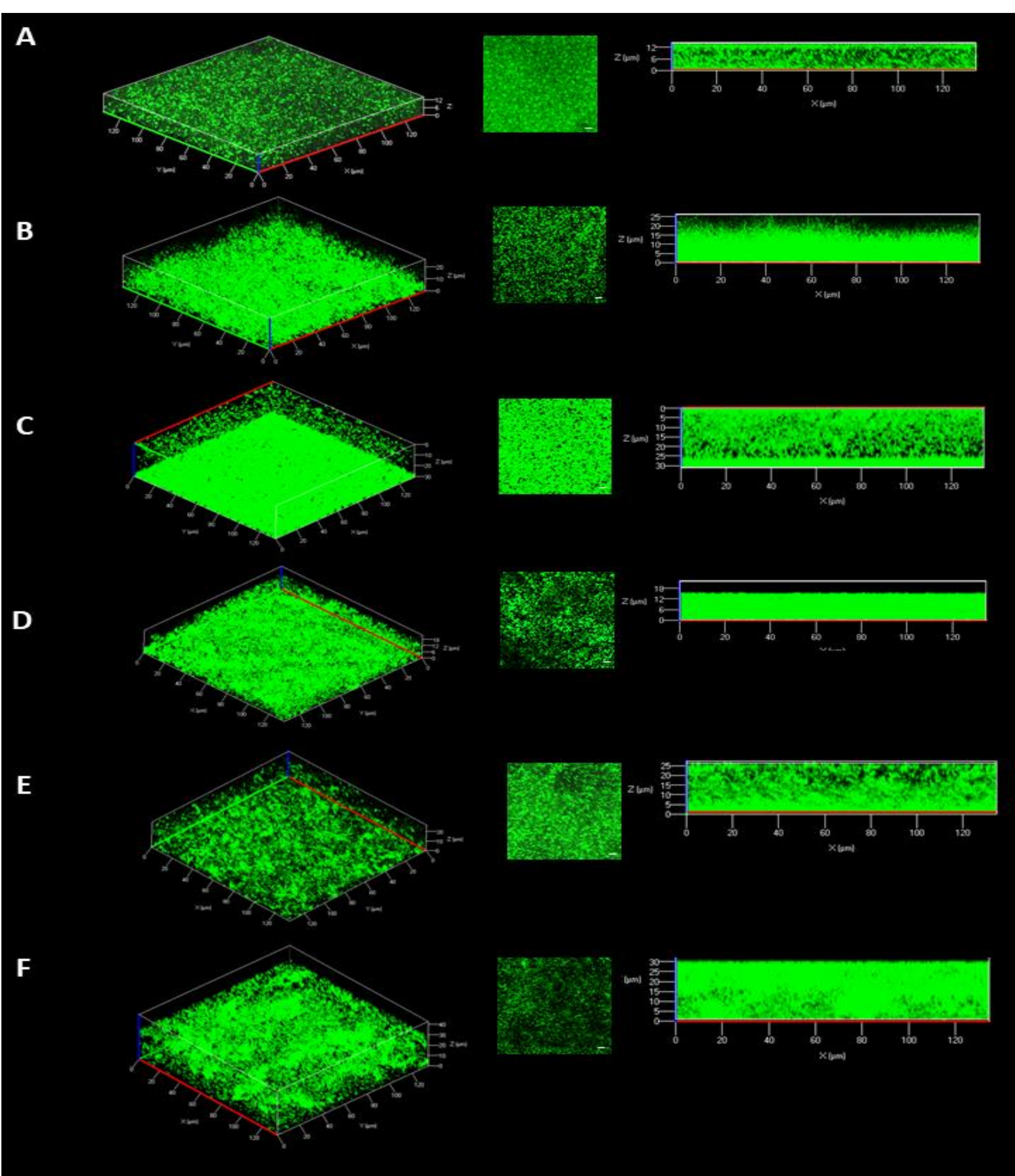


Figure 4.4 Biofilm microscopy of *P. aeruginosa* grown in LB and AUM for 24h. Each panel contains an image of a 3D biofilm, an image *of P. aeruginosa* attached to the bottom of the chamber slide at the base of the biofilm and a cross section to display the profile of the biofilm. A) PAO1 grown in LB, B) PAO1 grown in AUM C) 133117 grown in LB, D) 133117 grown in AUM, E) 133043 in LB and F) 133043 in AUM. The white bar denotes 10 µm

4.8.3 Isolate-dependent pyocyanin production in LB & AUM

This assay was conducted on three reference strains and three UTI isolates (133043,133065 and 133098) to compare pyocyanin production in AUM compared to LB (Figure 4.7). Production of pyocyanin produced in AUM was reduced significantly in LESB58 (P<0.001) and PA14 (P=0.009). In contrast, only isolate

133065 showed significant reduction in LB compared to AUM (P=0.009). The differences in growth were not accounted for in this assay. The response seems to be isolate dependent.

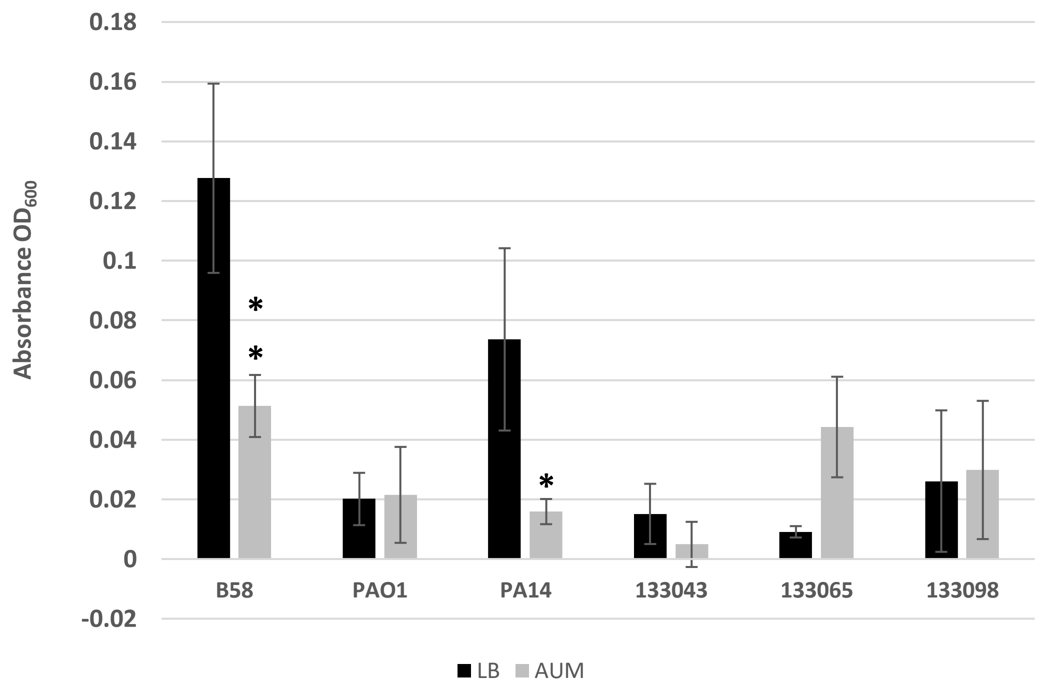


Figure 4.5 Mean pyocyanin production in LB (black) and AUM (Light grey) calculated after measuring absorption at A=695 OD. Significant differences are shown using *P<0.05 **P<0.001

4.8.4 Gene expression is isolate dependent in LB and AUM

In order to determine the relative gene expression between LB and AUM, a small panel of clinical isolates (133043,133065, 133098) and the two reference isolates PAO1 and PA14 were investigated using qPCR. Total RNA was extracted from isolates grown in LB and AUM, gene expression of a panel of genes was determined.

4.8.4.1 Planktonic growth conditions

RNA extraction was performed from LB and AUM cultures. Gene expression was determined for isolates PAO1, PA14, 133043,133065 and 133098. Reference genes *rpoD* and *proC* were used for normalisation purposes since they have previously been used as housekeeping genes in *P. aeruginosa* [416]. Overall, similar trends in gene expression were observed with each housekeeping gene (Figure 4.6 A-B). Genes associated with initial biofilm formation were studied in the five isolates. *pelB* was

expressed >1.5 fold increase in AUM in all isolates excluding 133098. However, the other genes tested (*flgD, algD* and *pslA*) either showed no change or were downregulated. For *flgD*, PA14 showed a downregulation of five-fold. One of the genes involved in alginate production (*algD*) showed a >1.5-fold downregulation for 4/5 isolates with PA14 and 133098 showing over a four-fold downregulation in expression (Figure 4.7). For *pslA*, the response is mixed with some isolates showing a slight increase in expression and other showing a decrease. However, only isolates PA14 and 133065 show a greater the 1.5-fold change (downregulated) (Figure 4.7). A similar isolate-dependent response could also be seen in QS-related genes (Figure 4.7). In AUM, *lasR* expression did not change in PAO1, however, three isolates displayed a strong reduction in *lasR* expression of between -six and -14 -fold. For *phzF* and *rhlR*, all isolates apart from PAO1 showed a reduction in expression of the gene. Expression of *pqsR* did not follow a particular pattern and was isolate dependent (Figure 4.8). For genes *mexX* and *mexB*, the response was again isolate-dependent with some isolates displaying upregulation and others downregulation. The gene encoding the virulence factor, Cif was also studied. For two isolates, there was no change in expression in AUM, however, for the remaining three isolates a downregulation in expression was observed, particularly in PA14 (-six-fold change) (Figure 4.9). Overall, the changes in gene expression were largely isolate dependent. Some trends were observed, such as the upregulation of *pelB* and the down regulation of *algD*.

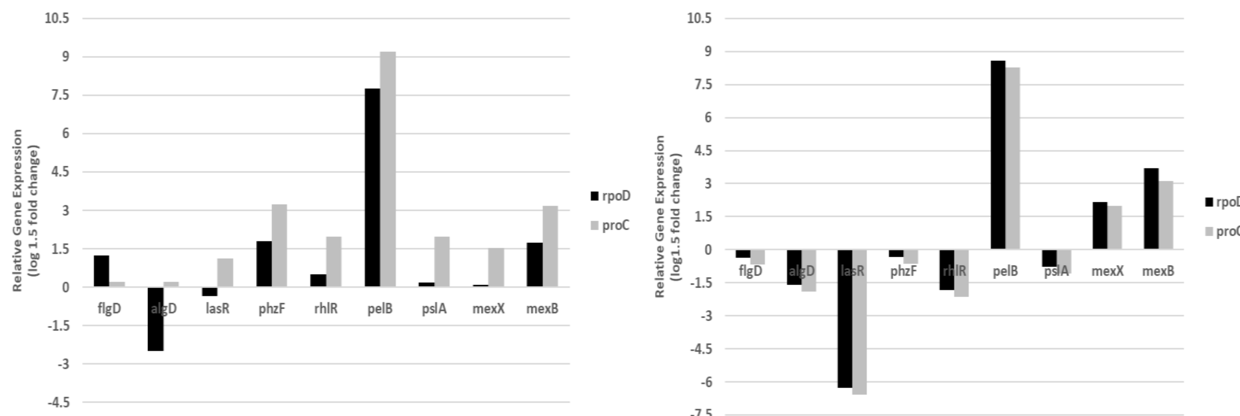


Figure 4 6 Relative gene expression for A). PAO1 growing in planktonic AUM culture and B) UTI isolate 133043, compared to growth in LB, normalised to rpoD and proC genes. Similar trends in gene expression can be seen using both housekeeping genes

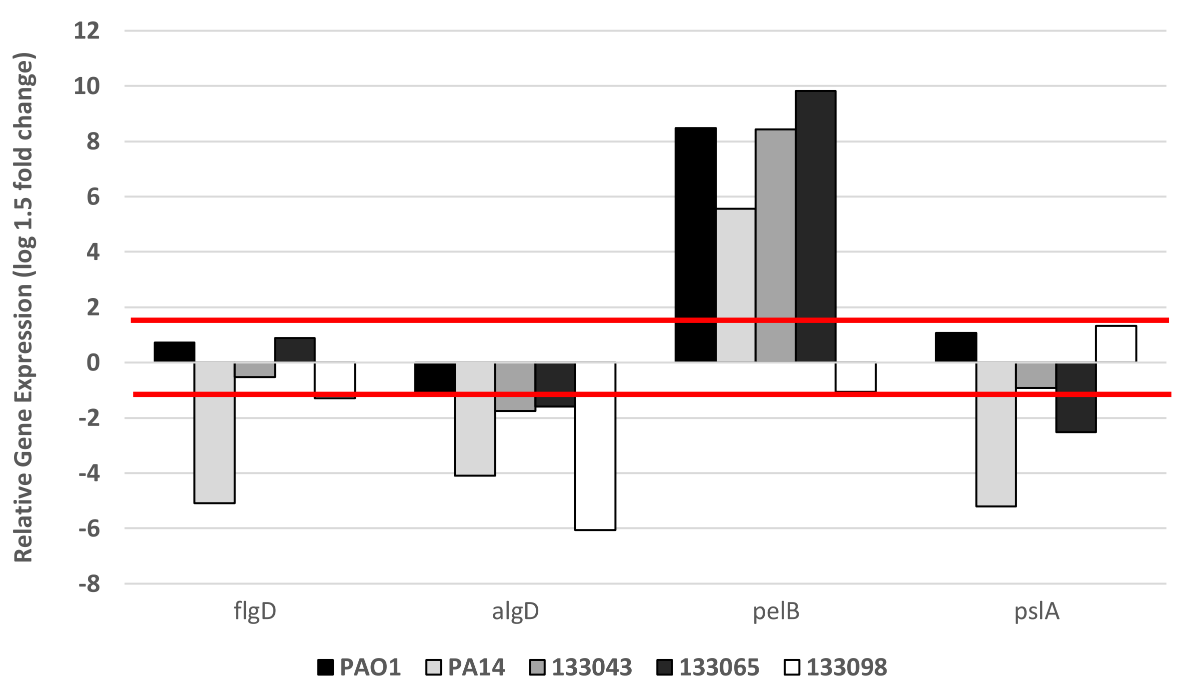


Figure 4.7 Fold change of genes expressed in relation to biofilm formation. Two red lines denote the fold change deemed significant; upregulation 1.5, downregulation, -1.5

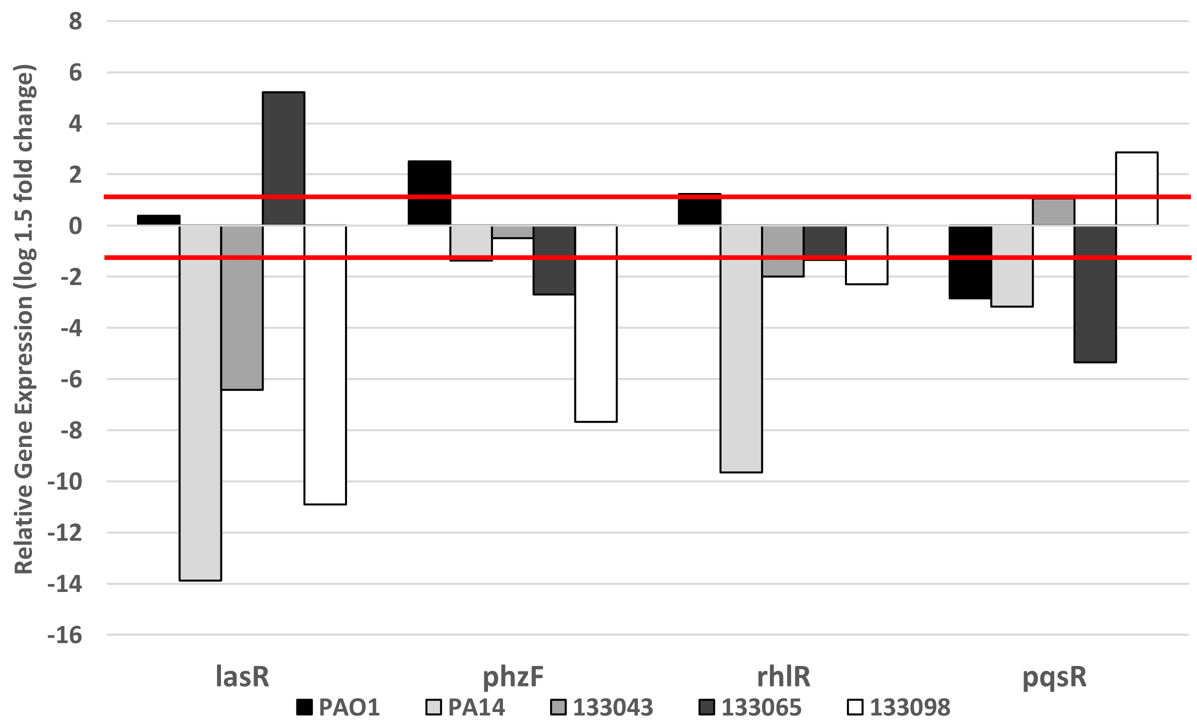


Figure 4.8 Fold change of genes expressed in QS mechanisms. Two red lines denote the fold change deemed significant; upregulation 1.5, downregulation, -1.5

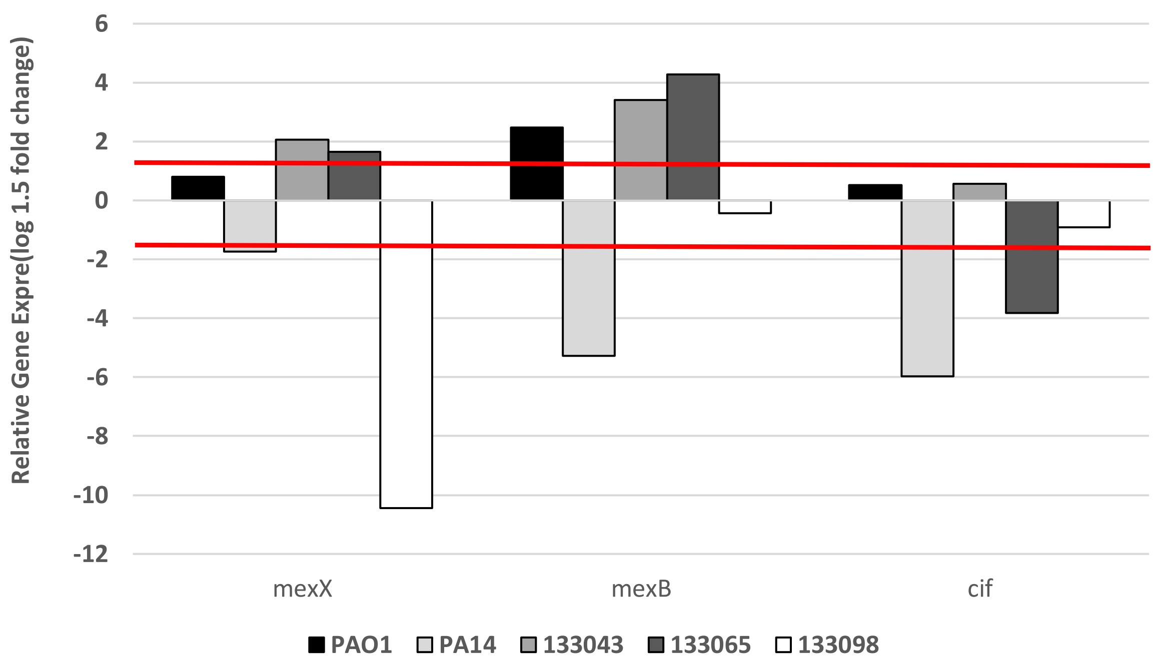


Figure 4.9 Fold change of genes expressed in antimicrobial resistance and virulence. Two red lines denote the fold change deemed significant; upregulation 1.5, downregulation, -1.5

4.8.5 Mapping the proteome of UTI clinical isolate 133098

To address whether the proteomic profile of UTI *P. aeruginosa* is altered in LB, AUM and urine, 5 biological replicates of isolate 133098 were grown in each medium until growth reached the optical density of 0.25 OD (+/- 0.005). This was due to the inability of the bacterium to grow to 0.5 OD in the urine medium in larger culture volumes. Rapid growth in LB was observed in comparison to AUM and urine. However, growth in urine was much slower than AUM. Proteomic studies were performed by Stuart Armstrong and the Centre for Proteomics, University of Liverpool. This was followed by *in silico* proteomic analysis.

PCA was conducted to demonstrate the level of variance and clustering of replicates (Figure 4.10). Furthermore, proteins with significant abundance difference were identified and a heat map was constructed (Figure 4.11) to assess the distribution of the protein abundance profile. The heat maps revealed large areas of differentially produced protein. Protein abundance was compared between AUM and urine in comparison to LB as the control growth media. The criterion by which a protein is deemed significantly abundant is set at > 1.5-fold change and a significant P value

($P<0.05$). Each condition gave rise to a distinct subset of replicates that are tightly clustered.

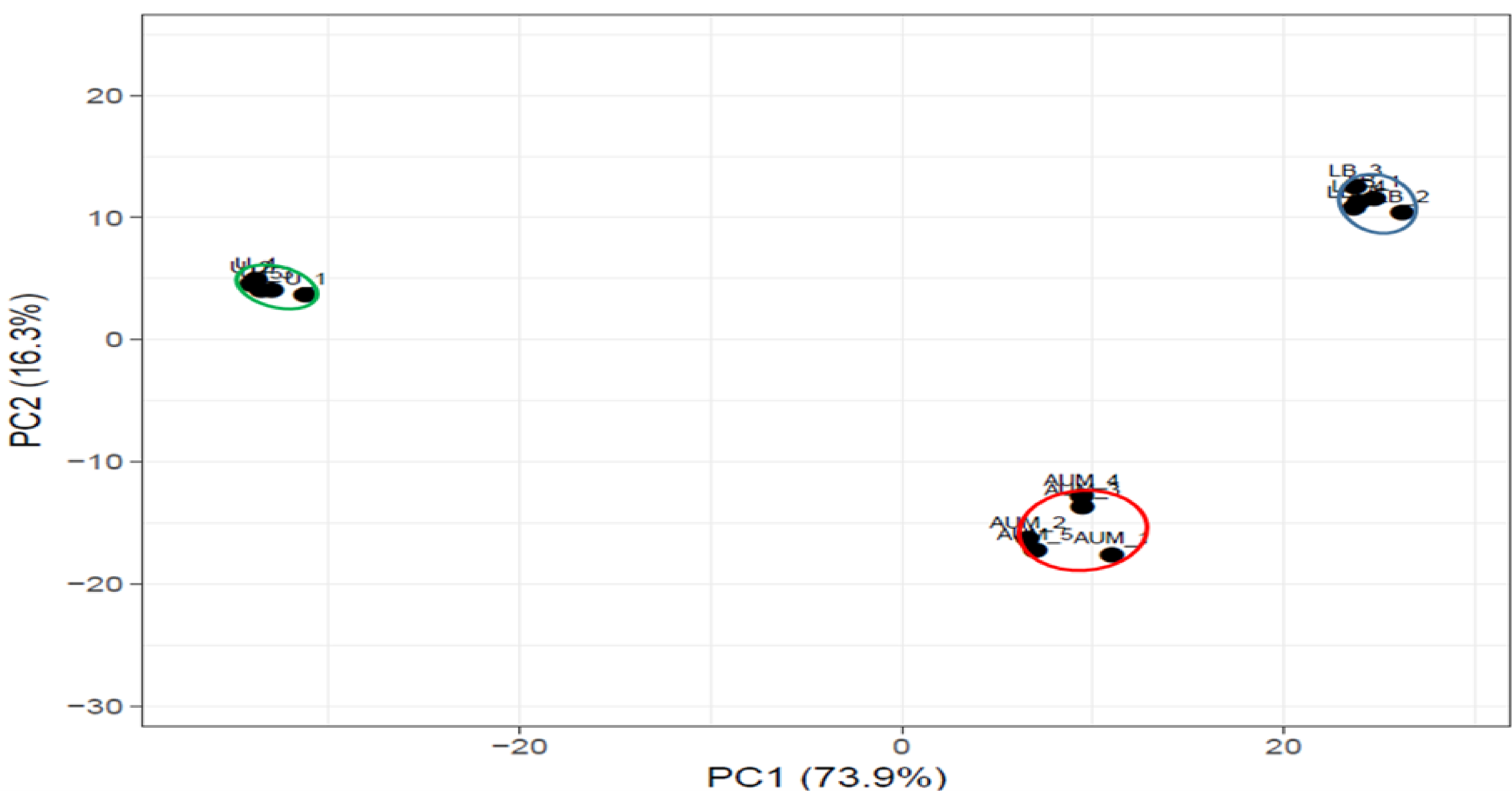


Figure 4.10 PCA of 5 biological replicates in each of LB, AUM and urine present no outliers in the variation of protein abundance in each medium. X axis and Y axis demonstrate principal component 1 and principal component 2 that explain 16.3 and 73.9% of the total variance, respectively

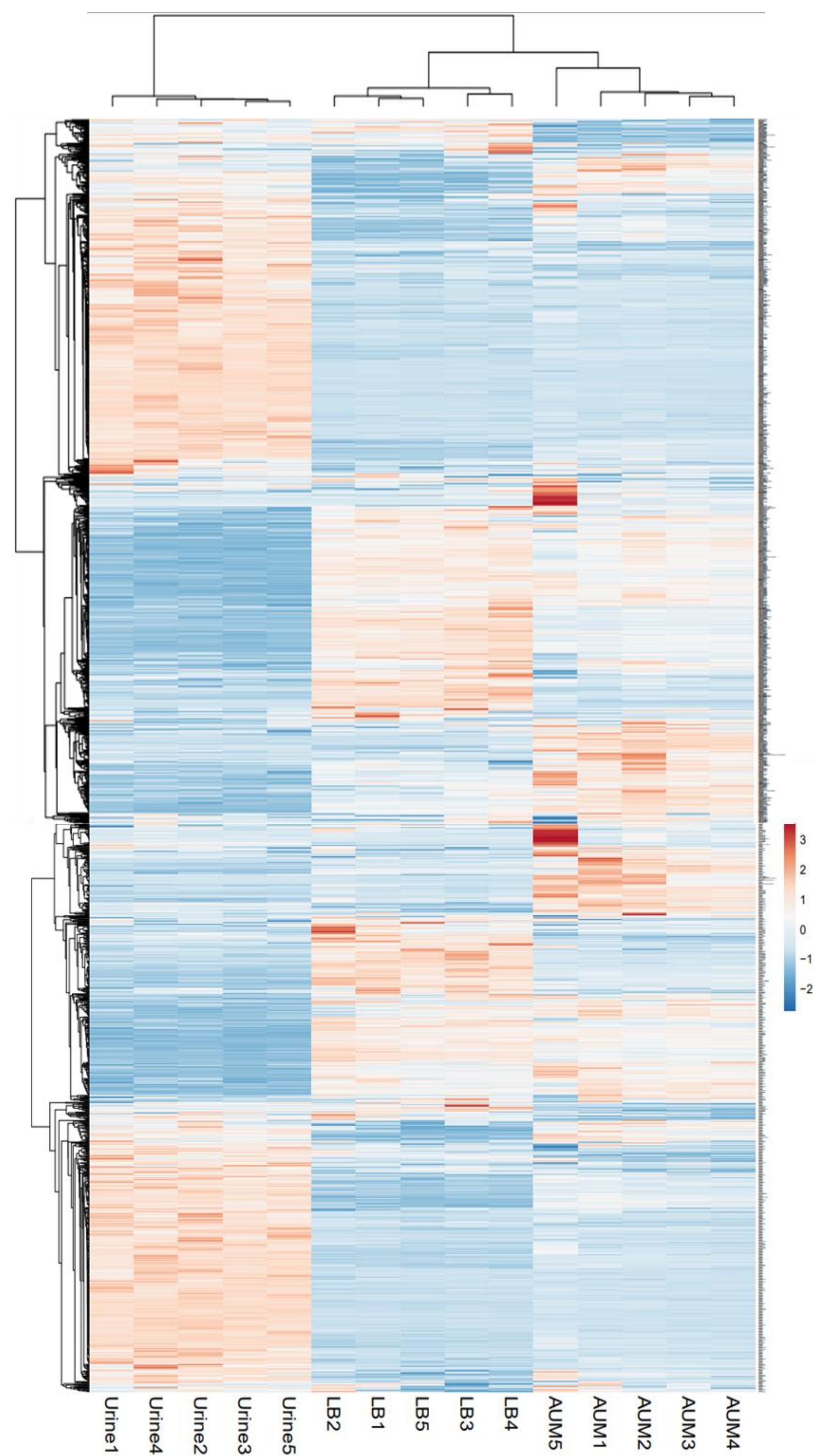


Figure 4.11 Heat map of the distribution of the profile of abundant proteins across all three conditions; LB, AUM and Urine. Red colour demonstrates highest upregulation while dark blue lowest downregulation. Each condition contains 5 biological replicates

4.8.6 *P. aeruginosa* proteome in urine

4.8.6.1 Proteins higher in abundance in urine compared to LB

In silico analysis revealed 1970 proteins which displayed significantly higher abundance in urine compared to LB (Figure 4.14). 30 proteins showed a large upregulation in urine by over 100-fold. Of these, 43% were categorised as either uncharacterised/hypothetical proteins such as PA0543 (infinity fold change, undetectable in LB) and PA2161 (70,022-fold change). Ortholog matches are found in the *Pseudomonas* database to LESB58 (PALES_05411-putative depolymerase) [839] and to *Pseudomonas dentrificans* (ATCC13867 lpqC) [840]. PA2161 is regulated by AlgU, which is the transcription factor that also activates alginate overproduction [841]. Another protein, ExaB is a soluble cytochrome C550 which grows on ethanol with a quinoprotein ethanol dehydrogenase [842], and was found to be higher in urine by 467 fold. Two component systems consists of sensor kinases and response regulators, which sense the surrounding environment and adapt accordingly [162] such as the orphan chemotaxis sensor regulator which increases virulence and antimicrobial tolerance [843]. Proteins PA2572 (regulator) and PA2573 (sensor) were higher in urine by five and 16 folds in comparison to LB, respectively.

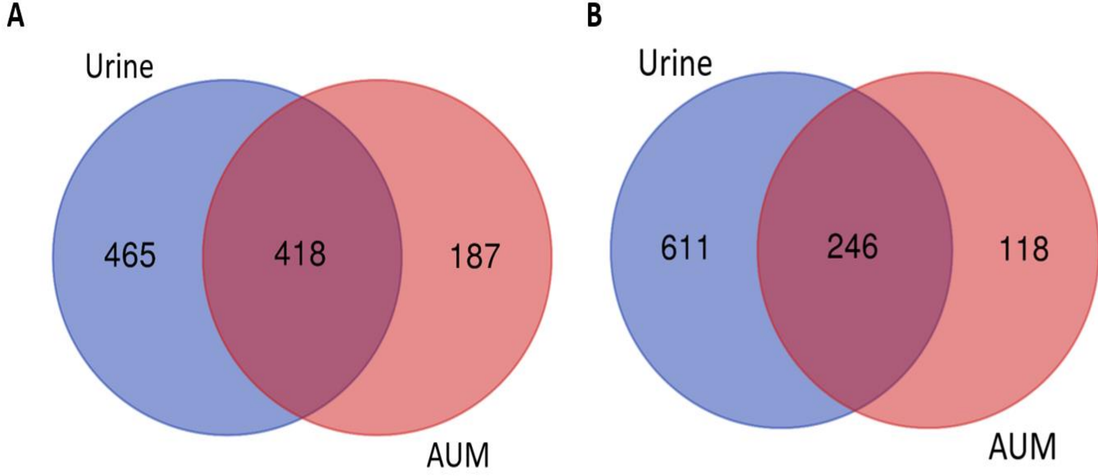


Figure 4.12 A: Upregulated proteins in urine and AUM compared to LB. **B:** Downregulated proteins in Urine and AUM compared to LB

4.8.6.2 Proteins lower in abundance in urine compared to LB

There are 1888 proteins which are downregulated > 1.5-fold and above, including 6 proteins that were down by over 100-fold. 50% of these proteins are hypothetical, one of the notable proteins is ExoS, an effector of the T3SS. When the proteins were compared further to AUM, a trend was observed in which T3SS proteins were more suppressed in urine than AUM as well as LB (Urine<AUM<LB). However, the global downregulation in both conditions may suggest that AUM is a suitable model to study *P. aeruginosa* in UTIs.

Two-component systems such as the PhoP-PhoQ system respond to environmental cues, this system is present in *P. aeruginosa*, *Yersinia pestis*, and *Salmonella enterica* [844]–[846]. Upregulation of the PhoP-PhoQ increases virulence and resistance to anticatatonic peptides [846]. Our results show upregulation in LB. Both of proteins of PhoP and PhoQ were more abundant by 10-fold in LB in comparison to urine.

4.8.6.3 Proteins higher in abundance in AUM compared to LB

As for the results obtained in AUM in comparison to LB, 1293 proteins displayed a significantly higher abundance by >1.5-fold change. The analysis identified that three proteins show over 100-fold increase in AUM in comparison to LB, the proteins were all hypothetical (PA0543, PA2384 and PA2161). These proteins were upregulated by “infinity”, 356 and 209- fold change, respectively. PA2384 protein has been studied and is involved in responses to iron limitation, it acts as a global activator of several important genes involved in iron acquisition and uptake [847]. PA2161 is another uncharacterised protein that has been shown to be AlgU regulated. Elastase showed a 50-fold upregulation in AUM. Elastase is a potent virulence factor and has been shown to promote virulence in the CF lung by degrading mucin and targeting the epithelial tissue [848]. Lactate dehydrogenase (LldA) was also upregulated by 48-fold. Lactate is thought to serve as an important energy source for bacteria, particularly during chronic infection [849]. Increased abundance of this protein may indicate the altered metabolism of *P. aeruginosa* in AUM compared to LB. Other pathways displaying increased protein abundance are phenazine biosynthesis (Table 4.8), pyoverdine production (Table 4.6) and other pathways for which the function is not entirely clear (PA3327-PA3335). This cluster of proteins encoded by the putative

operon PA3327-PA3335 show upregulation of a total of eight proteins believed to control the synthesis of a secreted secondary metabolites which include a gene cluster with a putative efflux transporter [850].

4.8.6.4 Proteins lower in abundance in AUM compared to LB

Many proteins associated with the cell surface and the modification of cell surface properties were down in abundance in AUM compared to LB. These included ArnA, ArnB and Arnc (-20, -21 and -39- fold respectively) and proteins involved is spermidine utilisation (SpeE2 and SpeH at -26 and -16-fold respectively). Downregulation of the Pmr system was observed in AUM in comparison to LB as proteins PmrA and PmrB were found to be upregulated in LB by seven and 14-fold, respectively. A large number of pili-related proteins were also down in abundance in AUM. These included PilW, PilQ and PilU all down by 3-fold and PilM, PilB, PilV and PilN (all down by two-fold).

4.8.6.5 Similarities between AUM and urine compared to LB

418 proteins were upregulated in both of urine and AUM growth media. Notably, proteins involved in the production of the siderophore pyoverdine were all upregulated (Table 4.5). These were similarly upregulated in both AUM and urine. 246 proteins were downregulated in both of AUM and urine. Strikingly, the T3SS system was downregulated in both AUM and urine compared to LB (Table 4.6). Interestingly, the extent to which the proteins were downregulated in urine were higher, as demonstrated by the protein PcvR 35-fold downregulation compared to AUM's 2.7-fold change

Table 4.5 Proteins in pyoverdine pathway upregulated (black) and (red) downregulated

Protein	Urine	Q value	AUM	Q value
PvdO	31.3	2.35E-09	5.5	0.01076928
PvdP	14.5	8.62E-07	3.7	0.08180011
PvdA	1.6	0.00073368	8.8	0.01398713
PvdD	3.1	1.48E-07	7.9	0.01990013
PvdE	2.1	0.00542378	17.8	0.00213072
PvdF	1.8	0.0026495	4.6	0.01477302
PvdJ	1.4	0.00135028	2.9	0.07056501
PvdR	1.3	0.06260262	16.0	0.00158822
PvdQ	2.8	0.00017731	2.8	0.07068926
PvdN	35.8	2.35E-08	35.8	2.35E-08
FpvA	15.4	1.66E-08	3.7	0.00023054
PvdL	1.6	0.00017863	4.6	0.02659835
PvdH	-1.5	9.65E-06	5.5	0.02930295
FpvB	35.9	2.45E-10	1.7	0.00699365

Table 4.6 Proteins involved in T3SS pathway downregulated in Urine and AUM annotated in red

Protein	Urine	Q value	AUM	Q value
ExsA	-8.4	3.91E-05	-1.7	0.09261383
ExsC	-1672.7	4.18E-05	-3.9	0.00461173
ExsD	-6.2	4.36E-07	-2.4	0.00036314
PopB	-7.6	2.94E-07	-6.3	7.29E-06
PopD	-5.9	0.00033522	-3.4	2.22E-05
PopN	-12.9	6.71E-07	-9.0	4.73E-05
PcrV	-35.2	1.29E-06	-2.7	0.0011945
ExoT	-18.0	6.45E-09	-3.9	3.84E-06
ExoY	-7.8	6.69E-07	-2.8	0.00595532
PscF	-1.9	0.00302357	-3.8	0.00010479
PscC	-4.3	1.04E-06	-3.2	0.00070171
PscJ	-11.9	4.89E-08	-3.9	3.64E-05
PcrH	-11.7	4.79E-06	-4.0	6.91E-05
PscQ	-11.0	1.00E-06	-2.6	5.35E-05
PscH	-7.6	4.79E-06	-5.3	0.00137482
PscL	-75.4	4.87E-07	-16.8	6.30E-06
PscE	-23.5	2.12E-08	-2.7	6.83E-05
PscD	-20.1	4.33E-05	-13.7	0.01620284
PscB	-17.7	2.74E-08	-5.5	3.31E-05

4.8.6.6 Differences between AUM and urine compared to LB

Overall, 465 different proteins were upregulated in urine in comparison to 187 in AUM. More proteins were downregulated in urine (611 proteins), while only 118 were downregulated in AUM. One of the main differences observed in protein abundance was of proteins involved in phenazine biosynthesis (Table 4.7). Some proteins involved in phenazine biosynthesis were both down in abundance in urine and AUM. However, there was very little similarity in PhzD, PhzE, PhzF, PhzG and PhzS abundance. In addition, proteins contributing to the PQS system were downregulated in urine versus up in AUM, particularly PqsH with a reduction of 28-fold change (compared to LB) as opposed to a 1.5-fold increase in AUM.

Table 4.7 Fold change of phenazines and pqs pathway protein abundance in urine and AUM media. Grey= upregulation. Red= downregulation

Protein	Urine	Q value	AUM	Q value
PhzM	3.0	0.00118385	15.7	3.97E-05
PhzB	4.2	3.06E-07	5.6	0.00018109
PhzC	1.5	0.08236075	14.8	9.87E-05
PhzD	1.1	0.08526314	21.1	6.57E-05
PhzE	-1.8	0.0004553	8.9	0.00016609
PhzF	1.5	0.00116558	20.8	3.67E-05
PhzG	1	0.06703159	12.7	0.00054675
PhzS	1.8	9.53E-05	11.3	7.29E-05
PqsA	-4.9	2.68E-06	2.5	0.00048118
PqsB	-4.6	6.16E-06	4.2	0.00012162
PqsC	-5.1	2.36E-06	4.0	8.61E-05
PqsE	-1.2	0.05692529	4.1	0.00025938
PhnA	-3.3	0.00017614	4.5	0.00013111
PhnB	-4.0	0.000583	5.4	0.00080591
PqsD	-2.3	0.00027679	4.2	7.62E-05
PqsH	-28.7	8.03E-09	1.5	0.01074726
PqsL	-4.6	6.61E-05	2.1	0.0071457

4.9 The use of AUM as a platform to test therapeutics

To assess the difference between the therapeutic ability of phages in LB and AUM medium, the UK panel of isolates were first treated with 4 phages; PELP20, 42/1, PT6 and PNM to determine sensitivity in LB. PELP20 was the most effective phage and showed activity against 86.6 % of the UTI isolates followed by PNM (53.3%), PT6 (40%) and 42/1 (33.3%) (Table 4.8).

Table 4.8 The panel of UK UTI isolates grown and results of treatment with 4 phages PELP20 ,42/1, PT6, and PNM. +=clear lysis, +/-=lysis with cloudy plaques, -=no lysis

UTI isolates	PELP20	42/1	PT6	PNM
133042	+	-	-	-
133043	+	+	+	+
133044	+/-	+/-	+/-	+/-
133065	+	-	+/-	+/-
133075	+	+/-	+/-	+/-
133082	+	+/-	+/-	+/-
133083	+	+/-	+/-	+/-
133090	+	-	-	+
133098	+	-	-	-
133099	+	-	-	-
133104	-	-	-	+/-
133105	-	-	-	-
133106	+	-	-	-
133117	+	-	-	-
133126	+	-	-	-

For the isolates from Kuwait, PELP20 was only effective against 25% of the total number of isolates. Phage 42/1 was active against the isolate 902, while PT6 was active against 1083 only. PNM failed to produce any plaques (Table 4.9).

Table 4.9 The panel of Kuwait UTI isolates grown and results of treatment with four phages PELP20, 42/1 PT6, and PNM

Isolate	PELP20	42/1	PT6	PNM
758	-	-	-	-
783	-	-	-	-
786	-	-	-	-
864	-	-	-	-
888	-	-	-	-
902	+	+/_	-	-
925	+	-	-	-
1083	-	-	+/-	-

To initially test AUM as a platform to study phage infection to *P. aeruginosa* isolates were pre-grown in AUM. Phage testing was conducted by growing five isolates (133043,133065,13398,133105 and 133117) overnight and then incorporation into a standard plaque assay. In comparison to the phages activity in isolates grown in LB media, only one phage (PELP20) displayed inhibitory activity against some of the isolates grown in AUM, namely 133043, 133065 and 133098 (Table 4.10). Furthermore, isolate 133098 appeared sensitive to phage PELP20 in LB, however, it was resistant in AUM. This small preliminary study suggests that AUM may be useful as a controlled medium to test phage activity.

Table 4.9 A panel of UTI Isolates grown in AUM prior to treatment with phages

UTI Isolate	PELP20	42/1	PT6	PNM
133043	+	-	-	-
133065	+	-	-	-
133098	-	-	-	-
133105	-	-	-	-
133117	+/-	-	-	-

4.10 Discussion

The urinary tract is a harsh environment for bacterial growth and survival [743]. To assess whether AUM mimics urine, *in vitro* comparisons using AUM formulation of Brooks *et al,* (1997) [800], urine and nutrient rich LB were conducted. Urine components vary between healthy and ill individuals and even amongst samples taken from the same individuals in different time points [727]. Thus, we aimed to determine whether this formulation of AUM accurately recapitulates the host-environment encountered by *P. aeruginosa* during UTI pathogenesis. This could then subsequently be used to study other host factors in isolation.

Analysis of growth rates of uropathogenic *P. aeruginosa* suggest that their kinetics are similar in AUM compared to urine, as such, suggesting that AUM may provide a better model to study the *in vitro* growth of uropathogenic *P. aeruginosa* than LB media. UPEC is the most widely studied uropathogen. Though no studies have been conducted to test the growth rate of UPEC using AUM media and the formulation of Brooks *et al,* (1997) [800], studies have been performed in human urine which showed that ABU *E.coli* outcompetes UPEC [748]. Biofilm formation is a virulence factor utilised by uropathogens to aid persistence in the host and is involved in CAUTI colonisation [280], [462]. Despite the higher biomass identified in LB compared to AUM and urine, when per capita biofilm formation was studied, which corrects for cell number, the data suggests that biofilm formation is favoured in AUM. This was reinforced further by the microscopy data which showed densely packed, thick biofilms formed in AUM. According to the results, biomass formed in urine tend to be significantly less than AUM in the majority of the isolates. This could be due to host factors not present in AUM such as THP, which is the most abundant protein in urine and exhibits protective effects against uropathogens such as *E. coli* by interfering with epithelium adhesion [765]. Conversely, studies conducted *in vitro* and *in vivo* of CAUTI *P. aeruginosa* indicated that this glycoprotein promotes biofilm formation on catheters [484]. As mentioned previously, antimicrobial peptides may play a role against invading bacteria. Human cathelicidin LL-37 is present in human urine [851]. This antimicrobial peptide may downregulate the transcription of QS mechanism of *las* and *rhl* by affecting more than 50 genes that are involved

in the development and maintenance of biofilms such as *rhlA*, *rhlB*, the rhamnosyltransferase genes [684]. These events could impact biofilm formation by decreasing the attachment of bacterial cells [684]. Antimicrobial peptides can also affect biofilm formation by promoting twitching motility via the stimulation of genes involved in Type VI pili synthesis [852], [853]. Thus, inclusion of molecules involved in UTI defences in AUM media may lead to better resemblance of biofilm formation in UTI pathophysiology.

Upon conducting pyocyanin assays to a selection of three clinical isolates in LB and AUM, I observed that production of pyocyanin is isolate-dependent, with only one UTI clinical isolate (133065) producing significantly more pyocyanin in AUM. This could be attributed to the specific characteristics of each isolate. Pyocyanin is a type II zwitterion, redox-active, blue pigmented toxin released by *P. aeruginosa* which promote inflammation and impair urothelial cells ability to repair [269]. The abundance of proteins involved in the synthesis of pyocyanin was shown to be more abundant in AUM compared to urine. In AUM, the pyocyanin production pathway was upregulated along with the pqs QS system, which appeared to be inhibited in urine. The underlying mechanisms of such inhibition requires further investigation. Another potential role of pyocyanin in UTIs is in biofilm formation. Pyocyanin is thought to interact with eDNA on urothelium or on the catheters [134].

In order to investigate the profile of proteins (including virulence factors) produced in response to the urinary environment, the abundance of proteins was measured in the three mediums; LB, AUM and urine. Iron acquisition mechanisms are indispensable for uropathogens and bacterial survival in general [26], [753]. Our results support using AUM as a model to test iron uptake strategies comparatively to urine since both mediums appeared to be similarly regulated in terms of abundance of proteins associated with iron uptake pathways. However, urine showed greater abundance of these proteins compared to AUM, particularly FpvB, PvdN, PvdO, which were downregulated by 35.9, 35.8, 31.3-fold, respectively in urine. Thus, upregulation of such molecules may increase bacterial ability to scavenge for iron compounds such as those in lactoferrin in urine [854] and allow the bacteria to survive, invade and colonise the urinary tract. The importance of iron chelating and

acquisition mechanisms in uropathogenesis has been shown in several studies on UPEC. Transcriptomic analysis of an *in vivo* mouse model showed dramatic upregulation of iron acquisition genes such as the outer membrane receptor hemin chuA (13-fold) and the putative heme/haemoglobin transport protein chuS (8-fold) [463]. In comparison to intestinal *E. coli* isolates, UPEC strains from the same patients expressed deferentially higher production of siderophores yersiniabactin and salmochelin [464].

Several proteins were more abundant in AUM in comparison to LB. PA2384 protein function has been elucidated and is involved in responses to iron limitation as a global activator. Zheng *et al,* (2007) performed mutagenesis knockout studies on PA2384 in iron-limited medium and observed the downregulation 71 genes involved in iron acquisition including those of the pyoverdine pathway, pyochelin pathway and Haem uptake mechanism [847]. In addition, upregulation of elastase in AUM is observed in these results. This is consistent with the upregulation of *lasB* in anaerobically grown *P. aeruginosa* PAO1 biofilms [753]. Elastase is a potent virulence factor and has been shown to promote virulence by degrading extracellular matrix components by hydrolysis. The toxin can also disrupt tight junctions of epithelial cells and neutralise several cytokines such as TNF-α and IFN-γ [855], [856]. Thus, a possible role in the urinary tract would be targeting the tight junction within the urothelium. *P. aeruginosa* produces lactate dehydrogenases (LldA), which has been shown to be induced by the L-enantiomer of lactate, a component in the mucin of both the CF lung and in AUM. Lin *et al,* (2018) demonstrated that LldA can contribute to cross-feeding of biofilm formation by catalysing the production of L-lactate in the anaerobic zones within biofilms and transported to aerobic zones in conditions mimicking chronic infections [849].

PmrAB is a two component system which consists of PmrB and is regulated by the other component PmrA [857]. PmrB is a sensor kinase that is activated in response to low Mg^{+2} levels or cationic peptides by modifying the PA3552-PA3559 LPS locus leading to resistance of cationic antimicrobial peptides and cationic polymyxins B [546], [547]. Our results show that the two-component system of PmrAB is down in abundance in AUM. This is in agreement with the earlier phases of CF lung infection

where loss of pmrB function is associated with increased antibiotic susceptibility to trade-off for early colonisation of the host [858]. In addition, proteins which contribute to the biosynthesis of L-Ara4N such as ArnA, ArnB and Arnc were all downregulated by 20, 21 and 39-fold, respectively. The aim of the addition of L-Ara4N to the lipid A moiety of LPS in *P. aeruginosa*, is to evade cationic antimicrobial peptides [859]. Thus, the inability of *P. aeruginosa* to resist this process may result in its clearance from the bladder by cationic peptides in the urinary tract setting. Polyamines such as spermidine (has a 3+ charge) are important molecules for *P. aeruginosa* for LPS stabilisation and protection against antimicrobial peptides [860]. SpeH and SpeE2 were also downregulated, which may also increase susceptibility and cationic peptides , antibiotics and oxidative stress in a natural UTI setting [860].

Comparisons between urine and LB were conducted. Some of the notable upregulated proteins were cytochrome C550, which appears to be important in urine given the substantially higher expression of this protein. Cytochrome C550 is as essential molecule in the oxidation of the ethanol system [842]. Ethanol and its derivatives exist in urine and are utilised as a carbon source by *P. aeruginosa* [861]–[864]. Thus, altered ethanol metabolism maybe a route by which this bacterium adapts in the urinary tract.

Another stark difference in the proteomic profile between AUM and urine is the expression of the two-component system containing the putative response regulator PA2572 and the probable chemotaxis transducer PA2573 [865]. Both proteins are co-expressed in response to environmental cues such as aerobic respiration containing nitrate and to bacterial inoculation into human lung epithelial cells [866]–[868]. This two-component system is involved in regulating a set of 49 genes involved in motility and increased tolerance to antibiotics [865]. These results show that human urine may trigger this two-component system in *P. aeruginosa* to enhance and promote invasion and colonisation in the urinary tract. The PhoQ-PhoP is another two-component system that is involved in resistance to cationic peptides and polymyxins [869]. Transcription of the sensor kinase system occurs in response to low Mg^{+2} or

Ca^{+2} and to cationic peptides [846]. This two-component system was downregulated significantly in urine.

One of the main aims of conducting proteomic analysis was to investigate how different the abundance protein profile of UTI *P. aeruginosa* in AUM and urine. In urine, there was a significant downregulation of the T3SS in urine compared to LB. This effect was also seen in AUM. The T3SS is associated with acute infections rather than chronic infections [870]. Expression of T3SS is triggered by either low concentrations of calcium or contact to the host cell. No host cells were present in any of the models. These results show that both NuoL and ShaC are downregulated in urine, these are involved in the metabolism of nucleotides, amino acids, and are required for T3SS [871]. This is consistent with other findings that metabolic stresses inhibit the T3SS [221], [871], [872]. Urine and AUM conditions may therefore, favour switching to chronic infections where biofilm formation is paramount.

In summary, AUM appears to mimic urine more than LB. 418 proteins were upregulated and particularly in the similar response mounted by the upregulation of iron acquisition mechanisms which was largely similar with a few exceptions such as downregulation of PvdH (1.5- fold change). The other notable similarity is the downregulation T3SS, which is consistent with several studies conducted with other uropathogens such as *E. coli* and *P. mirabilis* [207], [208], [210]. Thus, the T3SS may be universally dispensable amongst uropathogens. However, *in vivo* studies would be needed to study this further.

Based on these proteomic results, AUM is the more suitable laboratory medium to study pathogenesis of *P. aeruginosa* in UTIs (compared to LB). However, some differences in the abundance of proteins reveal the need for optimisation of this medium. One crucial difference is the expression of the PQS system which was up in AUM compared to urine. Functional PQS system is associated with severe inflammation and tissue destruction in an acute pyelonephritis mouse model [129], which further highlights the need for a model that gives more similar responses to urine. Recent attempts have been made to optimise artificial urine composition by adding 0.2% yeast and casamino acids, these results showed optimal growth of Gram-positive and Gram-negative urinary pathogens [745]. None of the formulations,

however, take into account the inclusion of host factors such as cationic peptides and THP which could bridge the gap and yield a closer bacterial response.

In addition to the proteomic analysis presented here, gene expression was investigated in LB and AUM for genes involved in quorum sensing, biofilm formation, virulence, antimicrobial resistance. Gene expression was generally lower in AUM compared to LB in the strains and clinical isolates of *P. aeruginosa* cells in planktonic There was little consistency between the qPCR results and the proteomic analysis, however, the growth conditions differed. The most notable upregulation of a gene in AUM was *pelB* as it was upregulated in PAO1, PA14, 133043 and 133065. PelB is involved in exopolysaccharide production that leads to biofilm formation. *In vitro* expression of either *psl* or *pel* genes is involved in biofilm formation. Little is known about the contribution of Pel exopolysaccharides in UTIs. However, the interactions of pyocyanin and eDNA is thought to be the driving force behind biofilm formation by UTI *P. aeruginosa* in agreement with the published literature [753], alginate production (using *algD* expression as a proxy for this) was downregulated in PA14 and the uroisolate 133098, indicating that alginate is not expressed in AUM. QS genes were not universally upregulated. However, *lasR* was upregulated in 133065 and *pqsR* in 133098. PqsR was higher in abundance in the proteomics experiment too. A functional PQS system is associated with severe inflammation and tissue destruction in an acute pyelonephritis mouse model. There was evidence of *mexB* upregulation in AUM, suggesting a possible increased level of resistance by the utilisation of efflux pumps.

Lytic phages were utilised to determine whether phages have the same impact on UTI *P. aeruginosa* upon pre-treatment in LB and AUM. Four phages displayed variable activity, with PELP20 showing the highest rate of bacterial inhibition in LB. Interestingly, this phage lost its effectiveness against one clinical isolate 133098 when pre-grown in AUM. The other phages, however, were totally ineffective when introduced to AUM containing *P. aeruginosa*. These results indicate that the behaviour of *P. aeruginosa* in AUM may alter the ability of phages to infect their bacterial target. Through proteomic data analysis, it was evident that many pili proteins were significantly down in abundance in AUM compared to LB. These

included PilW, PilQ, and PilU (-3-fold) along with PilM, PilB, PilV and PilN (2-fold). Pili can often act as a receptor for phage infection and therefore depending on the phage, this may alter the ability to infect and kill the bacteria. In addition, the porin OprH, which serves as another receptor, was downregulated by 11-fold. The receptors for phages used in this study are unknown. Thus, studying phage resistance mechanism in AUM warrants more research. This provides further evidence that AUM may provide a more suitable environment for therapeutic testing with phages than other laboratory media. Furthermore, investigation using a cocktail of phages or phage-antibiotic treatment may lead to better outcome [834]–[837].

Chapter 5

5.The Impact of Sex Hormones on UTI P. aeruginosa

5.1 Introduction

5.1.1 The impact of sex hormones on bacterial infections

Several bacterial infections are known to affect men more than women, for instance, gender preference for gastrointestinal tract infections with *Salmonella typhi, Helicobacter pylori, and Clostridium difficile* is biased towards men [873]. In contrast, bacterial infections such as UTIs caused by UPEC, sexually transmitted infections (*Chlamydia trachomatis, Neisseria gonorrhea)* and listeriosis by *Listeria monocytogenes* affect women more than men. The pathogenicity of these organisms is extensively reviewed elsewhere [873], [874].

5.1.1.1 Sex hormones and their role in sepsis

Sepsis is defined as a systemic illness involving microbial invasion in sterile locations in the body such as the bloodstream [875]. Physiologically, overproduction of cytokines such as TNF-α, IL-1β and IL-6 by macrophages is stimulated in sepsis upon the introduction of bacterial LPS. *In vitro,* Moxley *et al,* (2002) examined isolated human peripheral blood where the inflammatory cytokine TNF-α was found to be higher in males than females [876], [877]. This is attributed to the protective effects of oestrogen in relation to immune responses, which have been known since the 1960s [878]. In a series of experiments conducted by Nolan and O'Connell, isolated livers from rats were perfused with female blood, resulting in alteration of the normal vasoconstrictive responses to *E. coli* LPS [878]. In a subsequent study Nolan administrated conjugated oestrogen 1 h prior to LPS exposure and observed a protective effect against the lethality of the endotoxin [879]. In a report by Christeff *et al,* (1994) it was revealed that dose-dependent administration of oestradiol minimised the percentage of deaths due to the presence of LPS among male rats [880]. Post-administration of synthetic oestrogen (ethynyloestradiol) to rats challenged with *E. coli* LPS restored cardiac output and led to the attenuation of hemodynamic changes [881]. These protective effects were observed pre and post challenge with LPS and do not address whether circulating oestrogens in females

provide the same protective effects [881]. Thus, Merkel *et al,* (2001) conducted an experiment using male and female rats with three treatment groups; rats challenged with LPS (extracted from *Vibrio vulnificus*), rats challenged with LPS following gonadectomy, and a third group of rats receiving LPS, gonadectomy and oestrogen supplementation (2 μg/day). In the first treatment group, the fatality rate was 82% in males in comparison to females at 21% during the estrus cycle after the preovulatory oestrogen surge. In the second group, ovariectomised females died in a significantly higher rate to normal females (75% to 21%) and very similar to males (75% to 80%), no oestrogen was detected in ovariectomized females. To determine whether oestrogen was indeed protective, ovariectomized females were administered oestradiol-17β for 5 days (2 μg/day) then injected with LPS (30 to 50 mg/kg of body weight) at the end of supplementation period. As a result, mortality rate dropped to 38% in ovariectomised female rats, a similar rate to the normal rats [882]. This section highlights the first example of sexual dimorphism in bacterial infection in which males appear to be more susceptible to sepsis than females. In the next section, I will highlight the role of hormones in dimorphic responses to *Mycobacterium* infections.

5.1.1.2 Sex hormones in Mycobacterium infections

Mycobacterium infections are one of the most studied respiratory infections in relation to gender-dependent dimorphism [883]. The global prevalence of men affected by *Mycobacterium tuberculosis* is higher than females [884]. Although tuberculosis infections are more common in middle to low-income countries, the general trend of greater prevalence in men compared to women is thought to be approximately a 2:1 ratio, with men also being at higher risk of developing severe outcomes, including mortality [885], [886]. Large epidemiological studies in Asia and Africa, reported that being male is an independent risk factor [887], [888]. The overall higher risk in men, is attributed to the complex interplay between genetic elements, the immune system, sex hormones and environmental factors. However, the link between sex hormones and the immune system appears to play the largest part in the disparity observed between males and females [889]. A study on unvaccinated males by the Bacillus Calmette-Guerin (BCG) vaccine revealed that males mount a stronger immune response through IFN-γ to purified tuberculin derivative, which

could be associated with a dysregulated pro-inflammatory response, and therefore, a worse disease prognosis in males [890]. In addition, further studies indicated that males mount a stronger innate immune response. For example, males showed higher levels of serum C-reactive protein (CRP), than females [891]. A study conducted in Italy, which involved 10,260 patients 0 to 18 years revealed that there was no difference in cases recorded between male and females in the paediatric population [892]. Interestingly, women with oestrogen deficiency due to medical castration (oophorectomy) between the age of 15-30, suffered from an increased risk to tuberculosis mortality [363]. Chan *et al,* (2010) demonstrated that post-menopausal women suffered from a higher risk of developing nontuberculous mycobacteria due to oestrogen deficiency, abnormal expression of adipokines and/or TGF-β [364].

To investigate the pathophysiology of sex hormones on mycobacterium infections, several studies have been conducted in animal models. Male mice were found to be more susceptible than female mice and exhibited less resistance to *Mycobacterium marinum.* Upon the castration of mice, male mice became more resistant to infection, this was reversed by continuous administration of testosterone treatment (5 mg/day) [893]. To assess whether 17β-oestradiol provide protective effects against *Mycobacterium avium* complex (MAC) pulmonary disease, female mice were ovariectomised and were given 17β-oestradiol subcutaneously (10ng/ml). Ovariectomised mice were more susceptible to MAC infection than the controls, however, susceptibility was reduced, suggesting that oestradiol plays a protective role [894]. Progesterone can also influence bacterial infections [873]. A study administered a contraceptive called depot medroxyprogesterone acetate (DMPA), which contains progestin as the active compound, observed that it increases susceptibility of female mice to *M. tuberculosis* by reducing the activity of cytokines [895].

These studies clearly suggest that there is differential susceptibility between males and females. Furthermore, sex hormones appear to influence infection susceptibility in *in vivo* models of infection.

5.1.1.3 The role of hormones in UTIs

Current evidence suggests that women are more susceptible to UTI than men, and when infected, suffer from increased disease severity [11], [896], [897]. The higher prevalence of UTIs in women is primarily attributed to anatomical differences such as the shorter urethra [898]. Indeed, the distance between the urethra meatus and the anus is longer in men than their female counterparts [899]. Furthermore, the peri-meatal environment in men is drier and the release of prostatic secretions provides antimicrobial activity against infections [899]. Differences in the prevalence and severity of UTIs are also attributed to variable levels of sex hormones such as oestrogen and testosterone throughout life [900]. Adult UTI prevalence may not reflect the rate of UTIs in children. The prevalence of infected male infants to female infants is almost twice as much, however, this ratio changes in children up to 2 years old with females accounting for 60% of UTIs [901], [902]. In geriatric populations (>65 years), the rate of UTI prevalence recorded is almost identical 11% to 14% in men to women [903]. Thus, sexual dimorphism between men and women is apparent during the reproductive years, thereby suggesting that sex hormones play a role in host susceptibility to UTIs.

Current studies examining the role of oestrogen-based therapeutics in the prevention of UTI is contradictory and inconclusive in menopausal women [904]. A double-blind randomised controlled trial involving 93 post-menopausal women for a follow-up period of eight months, investigated the impact of administering intravaginal oestriol cream on their susceptibility to UTI. The treatment was deemed successful due to the substantial reduction of the recurrent UTIs (0.5 vs. 5.9 episodes per patient-year) [905]. In a randomised, parallel, controlled trial in Norway using an oestradiol releasing silicon-vaginal ring, the impact of the ring was assessed for 36 weeks and was found to be preventing UTI in the treatment group [906]. Contrary to these findings, a study conducted on postmenopausal women who received an oestriol vaginal pessary and other group which was treated with nitrofurantoin microcrystal antimicrobial agent, found that treatment with the pessary was less effective [907]. Brown *et al,* (2001) used data obtained from randomised trials on the influence of oral hormone therapy (conjugated oestrogens/medroxyprogesterone acetate) on

coronary heart disease, and found that hormone treated patients had a higher rate (although this was not significant) of UTIs in comparison to the placebo group over a mean period of 4.1 years [908]. Another study of a prospective cohort of 1017 postmenopausal women, aged 55 to 75 years, revealed that neither vaginal nor oral oestrogen was protective against cystitis [909]. The Cochrane collaboration, in 2008, reviewed two studies comparing vaginal oestrogen creams to placebo and found that oestradiol had a positive impact on recurrent UTIs depending on the type of the oestrogen and the duration of the study [910].

To study the impact of sex hormones on UTI pathogenesis, several studies have been conducted. Some strains of UPEC possess Dr adhesins which enable the bacteria to adhere to the urothelium by binding to the host receptor CD55 [911]. Such adherence is followed by internalisation into the bladder and kidney cells to form bacterial reservoirs that contribute to recurrent infections. Oestrogen also influences Dr-expressing *E. coli* and its binding to the endometrium and regulates the expression of CD55 in mouse tissues and humans [912]–[914]. As previously discussed in this section, post-menopausal women suffer recurrent infections in the urinary tract and supplementation of oestrogen reduces the risk of recurrent UTIs [915], [916]. Paradoxically, oestradiol is linked to increasing susceptibility to UTIs in reproductive age women [917], [918]. Lüthje *et al,* (2013) sought to investigate the mechanism behind this observation in both mouse models and human samples. Using the serum of healthy menstruating and post-menopausal women, it was observed that tissues from the former had a higher concentration of the antimicrobial peptide cathelicidin LL-37/hCAP-18 (median serum oestradiol levels, 245 to 40 pM) suggesting that oestradiol enhanced the production of this peptide. Lüthje and colleagues administered oestradiol to exfoliating cells extracted from the urine of healthy post-menopausal women and administered oestradiol led to increased detection of mRNAs from antimicrobial peptides (hBD1, hBD2, hBD3, psoriasin ,and RNase 7) in 75% of the women [919]. Oestradiol promotes epithelial barrier function [920], [921], and enhances the function of tight junction proteins such as ZO-1, occludin and E-cadherin [919]. To test whether UPEC CFT073 invades and colonises oestradiol-lacking epithelial tissue, ovariectomised mice were utilised to mimic the post-

menopausal environment within the urinary tract. Increased cellular invasion and higher bacterial load was observed in comparison to sham-treated controls [919]. However, the question remains as to why oestrogen might increase susceptibility to UTIs in young women? The Lüthje *et al* study reported that oestrogen increased the expression of infection-promoting receptors uroplakin Ia (UPIa) and β1 integrin in the earlier phases of the infection [919]. UPIa are located on the umbrella cells on the inner surface of the bladder and are presumed to be important mediators of UPEC entry into the cells, while β1 integrin subunits mediate entry into epithelial cells by interacting with UPEC's FimH [922]. Wang *et al,* (2013) further utilised an ovariectomised murine model to test whether alterations of oestradiol play a role in modulating the passage of UTI in the bladder. Mice lacking ovaries suffered from severe and persistent infection by UPEC strain UTI89 with higher rates of bacteriuria, aggressive inflammatory immune response, and a higher number of intracellular bacterial reservoirs [923]. Administration of 17β-oestradiol (0.01 mg per neck pellet) to a cohort of ovariectomised mice prior to infection resulted in reduced inflammation as levels of serum IL-6, which is associated with a strong inflammatory response, were less abundant than ovariectomised mice than those that were not supplemented by oestradiol [923]. Hormones have receptors such as ERα, ERβ and GRBP30 in various human and mice tissues throughout the body, which adds another layer of complexity to pathogen-host interactions [924]–[926]. ERs induce or repress the transcription of multiple genes involved in immune responses such as cytokines, which suggests that they play major roles in regulating innate immune response infections [927], [928]. Immune modulation of ERs has been studied in multiple viral and bacterial infections including herpes simplex viruses, hantaviruses, *C. difficile* and *P. aeruginosa* [874], [929], [930]. Evidence suggests that ERα is expressed in the kidneys more than ERβ, the variation and expression of these receptors differs around the body and accordingly, the impact of oestradiol differs [369]. In a recent report, Sen *et al,* (2013) sought to identify whether the presence of ERα had any influence on the expression of TNF-α and the modulation of host receptor C55 (targeted by Dr-expressing *E. coli*). This was conducted by administrating of propyl-pyrazole-triol (PPT) which is an agonist that activates the expression of ERα [931]. This was administered to ovariectomised mice and methyl-piperidino-pyrazole

(MPP), an antagonist which blocks the activity of ERα [932], to sham-controlled mice. The severity of UTI was assessed by determining the bacterial load and the expression of CD55 and TNF-α in both the bladder and the kidneys. The drugs were delivered subcutaneously for seven days, followed by transurethral infection by Dr expressing *E. coli* and subsequent sacrifice of the mice two- and six-days post-infection. PPT treatment of ovariectomised mice reduced the bacterial load in the kidneys but increased the load in the bladder with minimal effects on TNF-α and CD55, suggesting that ERα is mediating protection against UPEC. In contrast, MPP treatment impaired the ability of mice to clear the infection in the kidneys but reduced infection in the bladder (lower production TNF-α and CD55), leading the authors to conclude that ERα is indeed involved in the protective action in the kidneys [933].

5.1.1.4 Sexual dimorphism and the role of P. aeruginosa in respiratory infections

Several inflammatory lung diseases are known to exhibit greater severity in females more than males [873]. Females suffer from greater severity of conditions such as asthma, especially post-puberty with more physician visits, hospital visits and an overall 30% higher rate of mortality than affected males [934]–[936]. A similar outcome is seen in chronic obstructive pulmonary disease (COPD), from 1998-2009 the prevalence of this disorder was higher in women than men in all age groups except those from 75–84 and 85+ years in the USA. Overall mortality was also found to be higher in women [937]. Female bronchiectasis patients are hospitalised more often than males with increased risk to bacterial infections such as nontuberculous Mycobacteria due to impaired immunity [938]. In CF, the prevalence of the disease is equal. However, females tend to die younger than their male counterparts by an average of three years [939], [940]. CF mortality is linked to *P. aeruginosa* chronic persistent infection leading to lung failure. Females tend to acquire the pathogen at an earlier age and suffer worse outcomes than males [941]. These differences are sex-based since multiple confounding factors such as morphometric and nutritional factors, were found to be independent from worse CF outcome in females [940].

Major female sex hormones are variable during the menstrual cycle, ovulatory cycle, pregnancy and menopause [942], [943]. The circulating serum levels of oestradiol (17β-oestradiol) range between 20 to 400 pg/ml. However, the expression is

dependent on the main receptors ER-α and ER-β, both of which possess high affinity for oestradiol [944]. In contrast, progesterone ranges in concentration from 300 to 10,000 pg/ml, and acts on two receptors; progesterone receptors A & B (PR-A), PR-B). Interestingly, receptors for both sex hormones are expressed in the lungs [945], [946]. As discussed previously in the context of oestrogen impact on the urothelium and the regulation of innate immune responses, oestrogen can act on ERs in neutrophils and help clear *P. aeruginosa* [947]–[949]. The impact of sex hormones on neutrophils has been reported in several studies. *In vitro* pharmacological doses of oestradiol and progesterone inhibited the activity of neutrophils [950]. A study by Chiang *et al,* (2004) observed that physiological concentrations of oestradiol enhanced degranulation of neutrophils and oxidative stress markers [951]. Female sex hormones have also been found to mediate neutrophil cell apoptosis by delaying cell death and also increase production of ROS [952]. A landmark study investigated the role of sex hormones (oestradiol, oestrone and testosterone) and their perceived contribution to worse outcomes in female CF patients in Ireland [953]. *In vitro* testing of the impact of oestradiol on PAO1 and CF clinical isolates was observed by the induction mucoidy in non-mucoid *P. aeruginosa* compared to testosterone and vehicle control ethanol. Oestradiol also upregulated genes involved in mucoid conversion such as *mucA*, *algD*, and *algT*. In addition, the study assessed the relationship between oestradiol serum levels and exacerbations in women suffering from CF with regular menstrual cycles. The highest spike of oestradiol during the follicular phase was associated with exacerbations [953]. To elucidate the role of 17β-oestradiol in sexual dimorphism in inflammatory lung disease such as CF, Abid *et al,* (2017) conducted a series of experiments [930]. Firstly, oestradiol was administered to wild-type female and male mice then challenged by *P. aeruginosa* PAO1 and a CF-clinical isolate capable of mucoid conversion. Female mice succumbed to death quicker (at least 30 h earlier) with higher bacterial load in lungs than male mice. In a subsequent experiment, oestradiol, progesterone, and a vehicle control were administered to female ovariectomised mice before inoculation with *P. aeruginosa.* Treatment with oestradiol was associated with faster death than the groups receiving the vehicle control or progesterone. In addition, male mice treated with oestrogen died quicker than males administered with vehicle control. To assess

whether the significant differences in death rate in the treated groups with oestradiol were specific to ER, a group of ovariectomised mice received oestradiol with antagonist blocker ICI 182,780 (500 μg/day). This led to a longer survival time, which suggests that blocking nuclear and non-nuclear ERs attenuates poorer survival outcomes than group treated with oestradiol only. To determine whether ER-α and ER-β are involved with oestradiol in worse survival outcome, a group of mice were administered with PPT (ER-α agonist) and Diarylpropionitrile (DPN; ER-β agonist) and compared with the control vehicle group. This resulted in significantly worse survival time after *P. aeruginosa* challenge, leading the authors to conclude that both receptors are indeed involved with oestrogen in mediating worse disease pathophysiology in mice. Furthermore, intact-female mice receiving ICI 182,780 exhibited improved survival upon infection with *P. aeruginosa*, cementing the finding that both ER-α and ER-β are involved with oestradiol in worse response. The researchers also found no direct impact from oestradiol or progesterone on *P. aeruginosa* growth rates or mucoidy, thus, they speculated that it may due to pro-inflammatory responses associated with cytokines, chemokines and neutrophils. Indeed, upon harvesting oestradiol-treated murine lungs 9h after *P. aeruginosa* infection, increases in inflammatory cytokines TNF-α, neutrophil granular proteins myeloperoxidase and neutrophil elastase, were observed in lung tissue in the oestradiol-treated mice. Treatment of neutrophils with oestradiol enhanced oxidative burst but compromised *P. aeruginosa* neutralisation [930]. So, there is clear evidence of a role for sex hormones in modulating the pathogenicity of *P. aeruginosa* infections *in vivo.*

Other studies which investigated the interaction between sex hormones did not account for the possible role of testosterone. Rowland *et al,* (1992) identified oestrogen binding proteins in the cytosol of 14 strains of *P. aeruginosa* [954]. Upon analysis of gene expression of PAO1 *P. aeruginosa,* efflux pumps such as *mexD* and *mexF* were found to be induced by oestrogenic endocrine disruptor 17α-ethynyloestradiol, nonylphenol, and bisphenol-A and leading to the expulsion from such contaminates from the cell. Oestradiol, oestrone and oestrione were found to

be QS inhibitors at high physiological concentrations of 2 mM (0.5 mg/ml), which is higher than normal physiological levels [955].

5.1.2 Iron acquisition mechanisms in *P. aeruginosa*

Bacterial pathogens require iron which is an essential factor, due to its importance to cellular respiration [956]. The host limits iron availability as a strategy to counter colonisation by the bacteria [957], [958]. In mammalian hosts, iron is found in heme proteins (cytochromes and haemoglobin) and attached to extracellular proteins such as transferrin and lactoferrin [959]. Insoluble iron compounds constitute the vast majority of iron in the host, thus, *P. aeruginosa* amongst other pathogens must devise various mechanisms to acquire iron (Table 5.1) [460], [960], [961]. *P. aeruginosa* utilises different strategies to chelate iron in different environments including the type of infection (acute V chronic).The siderophore pyoverdine is utilised in acute infections such as that shown in burn mouse models, while pyochelin has been identified in chronic CF infections [474], [962][275].

Table 5.1 Iron acquisition mechanisms utilised by *P. aeruginosa* and their function during infection

Iron uptake systems	Function	Example	Reference
Siderophores	Synthesis of extracellular Fe^{3+}chelating molecules	Pyoverdine and pyochelin	[960], [963]
Heme proteins	Take-up of heme from host hemeproteins	PhuS	[279]
Xenosiderophores	Hijacking siderophores made by other microorganisms.	Enterobactin	[964]
Feo system	Acquisition of Fe^{2+} via the FeOABC system	Phenazines utilisation	[965]

5.1.2.1 Siderophores; Iron chelating molecules

Siderophores are soluble, organic ligands excreted as low-molecular compounds to chelate Fe^{3+} with high specificity and affinity. Two major siderophores known to be highly involved in the virulence of *P. aeruginosa* are pyoverdine and pyochelin [275], [966]. Pyoverdine is yellow-green, fluorescent siderophore that is produced in low-iron environments and consists of a chromophore which contains a conserved region and highly variable peptide chain [460], [967]. The chromophore is also shared amongst multiple fluorescent *Pseudomonas* species such *as P. aeruginosa, P. fluorescens, P. syringae* and participates in the chelation of the Fe^{3+} [968]. The variable peptide chain consists of 6-12 amino acids and is involved in the production of at least three different pyoverdines (I-III) based on amino acid composition [275], [460], [967]. The production of pyoverdine is a complex process which involves 20 proteins contributing to regulation, maturation, transportation and iron uptake, which spans across the bacterial membrane (Figure 5.1) [969].

Pyoverdine binds to iron, for example host transferrin, with a very high affinity and is transferred via a TonB-dependent receptor (TBDR) mechanism into the bacterial cytoplasm (Figure 5.2) [275]. TBDRs play a major role in acquiring ferric substances and ensuring their transportation into the cytoplasm of the cell [35], [36]. *P. aeruginosa* has more than 30 genes that control TBDRs, the majority of which are involved in acquisition of (siderophore-ferric) complexes known as ferrisiderophores [9], [37]. TBDRs are categorised into simple TBDR and TonB-receptor transducers (TBDT) [36]. TBDRs do not possess the cell signalling N-terminal domain [36]. In contrast, TBTDs contain a signalling domain upstream of the tonB box, which enables it to interact with a sigma regulator in the inner membrane [38]. The sigma regulator in *P. aeruginosa* is FpvR [970].

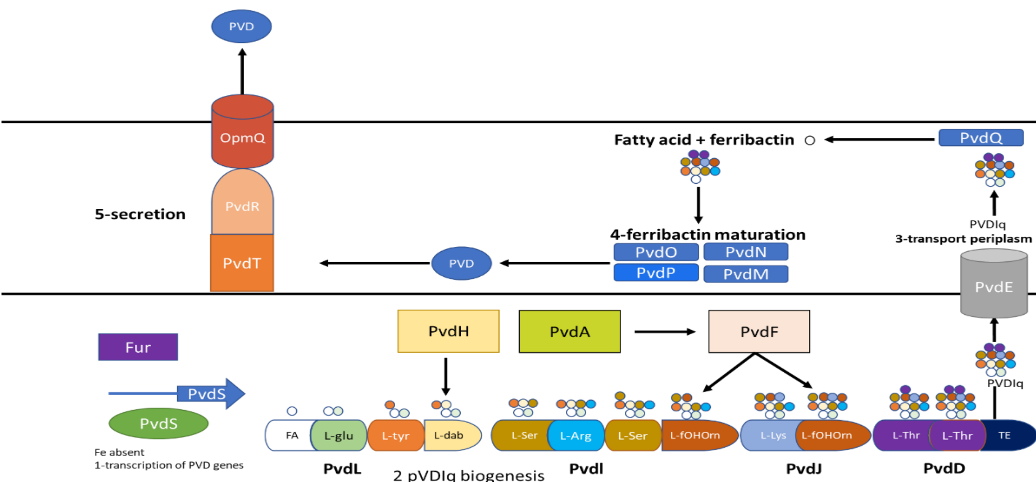


Figure 5.1 Illustration of the steps involved in pyoverdine production and export. Initiation of pyoverdine production occurs once a low concentration of iron is detected. This results in dissociation of the binding of Fur-PvdS promoter complex. The release of the Sigma-factor PvdS helps it bind to the promoter IS boxes of pyoverdine genes, resulting in transcription and then translation of pyoverdine proteins. The peptide synthases are PvdL, PvdI, PvdJ, and PvdF assemble the precursor PVDIq, which begins with a C14 fatty acid's (FA) to L-Glu and is finalised upon the release of PVDIq from the PvdD protein by the thioesterase module (TE). As for the role of PvdH, PvdA, PvdF, these enzymes produce amino L-diaminobutyrate (L-Dab) and L-formyl-OH-ornithine which are merged into PVDIq by the four peptide synthases. PvdE is tasked with transporting PVDIq into the periplasm, followed by the removal of myristoleic acid by the acylase PvdQ. This leads to the maturation of the chromophore and the catalysis of the precursor ferribactin. The role of the periplasmic PvdM, PvdN and PvdO in the maturation of the ferribactin is unknown. However, it is possible that one of the proteins transform L-glutamate into one of the of three residues in pyoverdine. The siderophore is transported outside the cell via PvdRT-OpmQ into the environment. Adapted from [969]

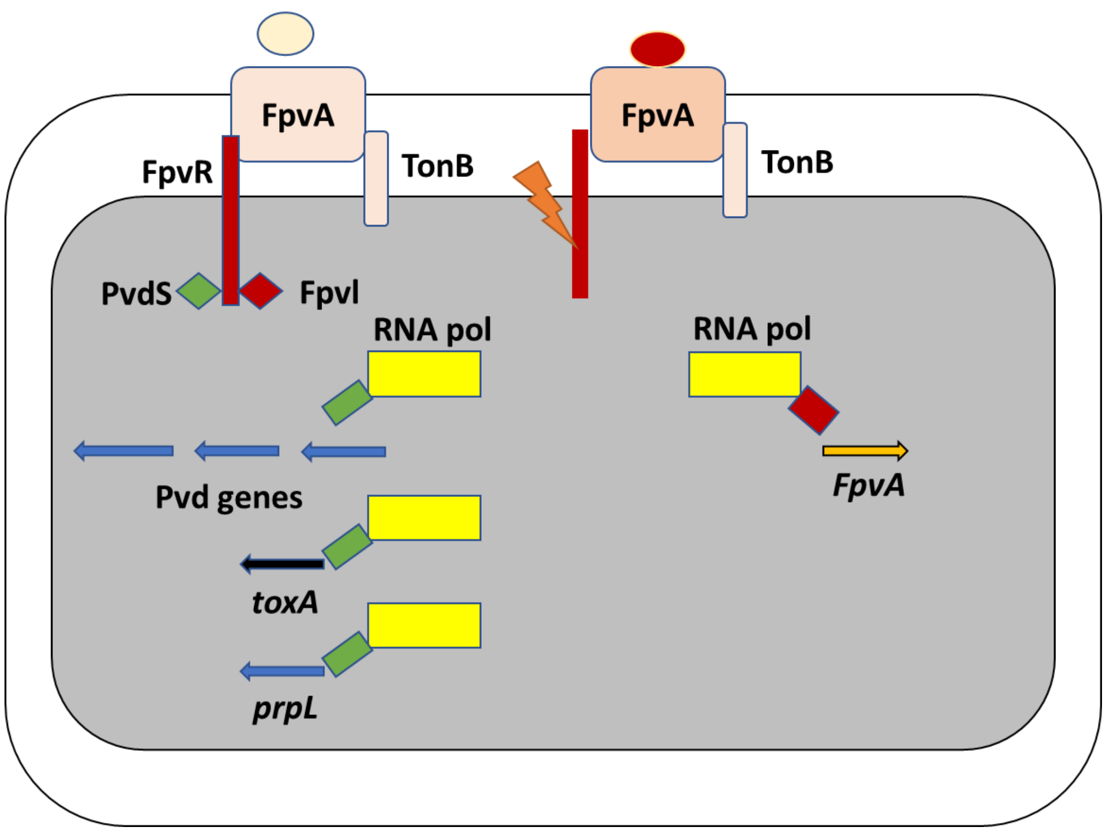


Figure 5.2 The left panel displays an apo pyoverdine (beige dot) prior to engaging FpvA, a TBDR associated with the FpvR anti-σ factor. The role of FpvR is to prevent involvement of both ECF σ PvdS and FpvI by its cytoplasmic domain. Thus, the possibility of sigma factors association with the core RNA polymerase is small, resulting in non-transcription of the *PvdS* and *FpvI.* The right panel displays the event of activation of pyoverdine synthesis; the ferripyoverdine (red dot) binds to the FpvA receptor, which leads to conformational change that induces proteolysis of FpvR. The sigma factors PvdS and FpvI establish the needed association with the core RNA polymerase resulting in the transcription of pyoverdine genes and virulence actors *ToxA* and *prpl* [275]

TBDTs can regulate their own synthesis along with cognate siderophores. FpvA is another example of a TBDT and this protein can sense cognate ferrisiderophores by recognising anti-sigma factor proteins at the membrane resulting in proteolytic cleavage and releasing extracytoplasmic sigma factor (ECF σ) [15], [18], [36]. The expression of ECF sigma factors, which are also known as starvation sigma factors, is controlled by iron via the ferric-uptake regulator (Fur) [39]. Three types of TBDRs are present in *P. aeruginosa*, which correspond to the pyoverdine types; (FpvAI, FpvAII, and FpvAIII). It is estimated that more than 95% of *P. aeruginosa* possess an FpvB

receptor, which allows them to utilise type I ferripyoverdine produced by other isolates [37], [41]. Dingemans *et al,* (2014) analysed *P. aeruginosa* isolates from CF clones from a patient population admitted to the Universitair Ziekenhuis Brussel. It was reported that the *FpvB* gene was deleted during CF adaptation in 17 of the collected 54 isolates, and seven other TBDRs in various isolates were observed upon comparison to DK2 strain [971].

Evidence suggests that pyoverdine is critical for virulence in a murine burn infection model, where TBDR mutants are deemed avirulent [275], [972]. Pyoverdine not only acts as an iron chelator, it also acts as a signal molecule for two other extracellular virulence factors; exotoxin A and protease PrpL [460], [973]. In addition, availability of iron, or lack thereof, is detrimental to the formation of biofilms and its architecture [974]. *P. aeruginosa* on glass surfaces form thin biofilms in presence of lactoferrin with planktonic cells performing twitching motility [975]. In the absence of lactoferrin, thicker biofilms with mushroom-like structures are produced, correlating with pyoverdine production. Mutant strains lacking pyoverdine are not capable or forming mushroom-like structures[974]. A study by Patriquin *et al,* (2008) found that biofilms are in need of iron supplementation more than planktonic cells. Iron deficiency leads to the stimulation of twitching motility via the Rhl QS system [976].

Pyochelin is another siderophore produced by *P. aeruginosa.* Dumas *et al,* (2013) demonstrated that pyochelin is produced first followed by pyoverdine as iron concentrations start to plummet [977]. Production of pyochelin correlates with higher levels of availability of iron in the environment. Pyochelin requires the iron-regulated *pchDCBA* operon to be produced [977], [978]. Pyochelin-iron causes oxidative cell damage and inflammation to the host, this activity is compounded in the presence of pyocyanin [979]–[981]. In CF lungs, sustainability of inflammation could be one of the roles attributed to pyochelin [474]. Increasing levels of pyochelin have also been observed in artificial sputum medium (ASM), which may indicate that it plays a role in CF infections by chelating iron [982].

5.1.2.2 Xenosiderophores: siderophore piracy

P. aeruginosa is capable of hijacking siderophores released by other Gram-negative bacteria such as *E. coli* (Table 5.2). This is exemplified by the capture of the

siderophore enterobactin via two receptors; PfeA and PirA [983], [984]. The importance of these xenosiderophores has not been fully elucidated yet in real-time infections with all related studies conducted *in vitro.* Siderophore piracy may help *P. aeruginosa* fend off competition in polymicrobial infections [985].

Table 5.2 Types of xenosiderophores obtained by *P. aeruginosa* from different bacterial species

Receptor	Xenosiderophores	Reference
FoxB, FiuA	ferrioxamine and ferrichrome	[986]
FemA	mycobactin and carboxymycobactin	[987]
FecA	Fe-citrate	[988]
ChtA	rhizobactin, aerobactin, and schizokinen	[964]
FvbA	Vibriobactin	[989]

5.1.2.3 The FeoABC transport system and the role of phenazines

Fe^{3+} is not the only form of iron scavenged by *P. aeruginosa.* In anaerobic/microaerobic, low pH environments, Fe^{2+} is present in a soluble form [961]. The FeoABC system, which is present in Gram negative bacteria is utilised to transport ferrous iron to the cell cytoplasm following the diffusion of Fe^{2+} through the outer membrane [965], [990]. *P. aeruginosa* employs the Feo system comprising of the permease FeoB with the proteins FeoA and FeoC [965]. Wang *et al,* (2011) demonstrated that deletion of *FeoB* attenuates *P. aeruginosa* virulence and reduces the ability to form biofilms [278]. This transport mechanism of iron could be utilised in microaerobic or anaerobic environment such as the lungs of CF patients [991]. Phenazines such as phenazine-1-carboxylic acid (PCA) and pyocyanin can reduce the Fe^{3+} bound in host proteins to Fe^{2+}, which in turn enables the Feo transport system to acquire these soluble iron molecules (Figure 5.3) [992]. This mechanism is plausible in the CF lungs where *P. aeruginosa* biofilms are formed and high levels of PCA and

pyocyanin present in the latter stages of infection accompanied with progressive health deterioration for CF patient [993], [994]. Pyocyanin has been linked to biofilm formation in UTIs and therefore this mechanism of iron acquisition may also play a role in the urinary tract.

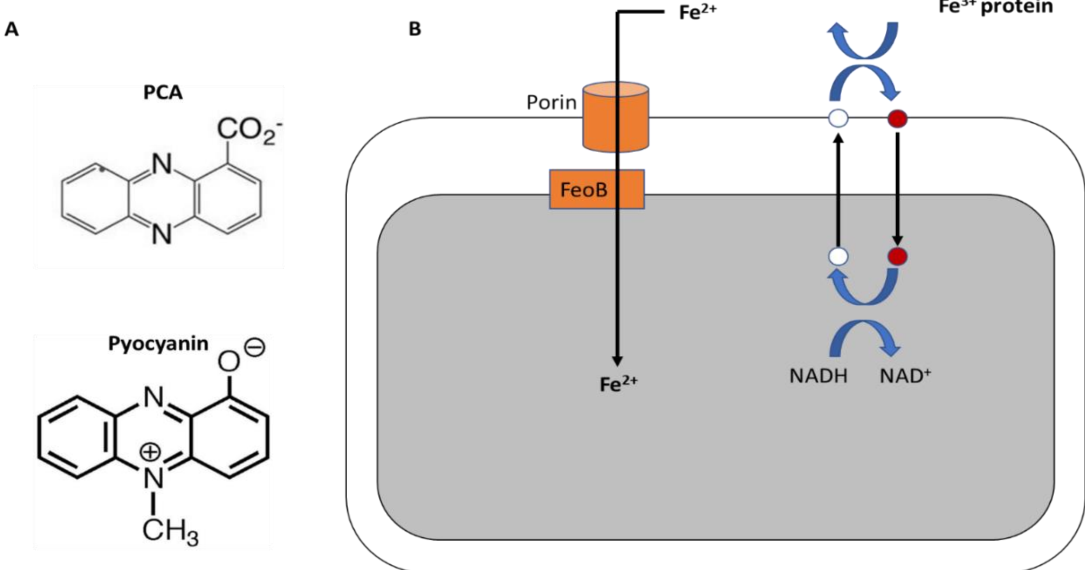


Figure 5.3 (A) Structures of two *P. aeruginosa* phenazines, Phenazines-1-carboxylyic acid and pyocyanin. **(B)** The white circles represent reduced PCA which is excreted out of the cell then becomes oxidised (red circle), leading to the reduction of Fe^{3+} to Fe^{2+}. Phenazines undergoing oxidation via this pathway are further recycled inside the cell and reduced again by simultaneous oxidation of NADH to NAD [275]

5.1.2.4 Extraction of heme from the host by *P. aeruginosa*

Heme proteins in the host are also targeted directly by *P. aeruginosa*. This occurs via two systems known as Has and Phu [995]. Heme is not freely available since it is hydrophilically bound to the host membranes. Therefore, heme extraction occurs from host haemoglobin or hemopexin, both are heme proteins [996], [997]. The Has system acquires heme from the host and extracts Fe^{2+} in a multi-step process (Figure 5.4). This is initiated by the heme protein hemophore and a TBDR known as HasR which deposits the heme into the periplasm [996]. Heme is then transported into the cytoplasm by an ABC transporter and subsequently bound to the heme chaperone PhuS, where heme is oxygenised by HemO and degraded to carbon monoxide (CO), biliverdin and Fe^{2+} [275], [997].

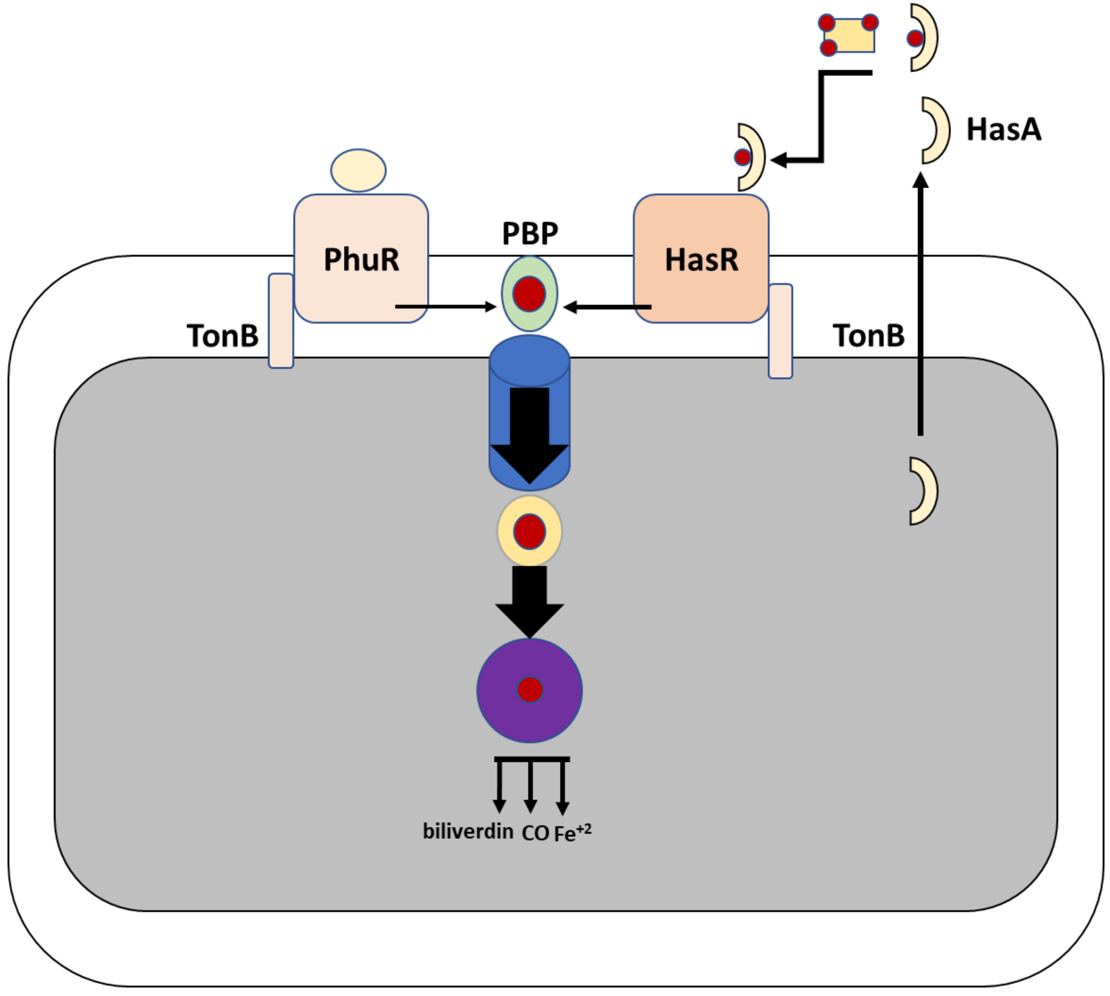


Figure 5.4 *P. aeruginosa* possess two heme uptake system; PhuR and HasR. The TBDR is associated with PhuR and binds directly to hemeproteins to extract heme. The HasR receptor differs, as it binds heme merged to a secreted hemphore protein HasA. Upon introduction into the bacterial periplasm, heme is attached to a periplasmic protein that is driven to an ABC transporter. Once it enters the cytoplasm, heme is chaperoned by PhuS to direct the heme to the heme oxygenase enzyme HemO. Which in turn, cleaves the tetrapyrrole ring, leading to the emergence of biliverdin, CO, and Fe^{2+} [275]

5.1.2.5 Iron acquisition in the urinary tract by UPEC

Iron is in limited supply in the urinary tract [743]. Thus, uropathogens must devise ways to chelate and extract iron from the host for successful invasion and colonisation. Iron acquisition in UTIs is best studied in UPEC [998]. UPEC acquires iron via three mechanisms; import of free iron metals, import of metal-ion complexes in the host, and siderophores [999].

UPEC model strains such as UTI89 and CFT073 contain a system known as Sit, which is similar to the aforementioned FeoABC system In *P. aeruginosa*. In a murine infection model of cystitis, transcriptomic analysis revealed upregulation of the iron transporter SitA upon CFT073 growth in mouse urine [1000]. The *sitA* gene which encodes an ABC type-ferric iron transporter, was found to be upregulated in UTI89 strain to facilitate ferric iron transport [1001], [1002].

UPEC also targets metal ion complexes such as heme by the Chu and Hma systems which are found more in UPEC in comparison to commensal strains of *E. coli* [683], [1003]. A study by Hagan *et al* compared eight UPEC isolates with the CFT073 strain by measuring global gene expression utilising microarrays. seven of the eight isolates expressed the heme-receptor encoded by the gene *chuA* [1004]. In a murine co-infection model, wildtype CFT073 outcompeted an isogenic *hma* mutant by infecting the spleen and the kidney, suggesting that the novel receptor hma is required by UPEC for infection [1003].

As explained in *P. aeruginosa* iron mechanism systems, UPEC also utilises siderophores to capture ferric iron to form ferric iron complexes and transport them via receptors into the cell. Enterobactin, which is the prototypical siderophore produced in all intestinal *E. coli* [464], has been implicated in UTI pathogenesis in humans [1005]. Enterobactin gains access to the bacterial cells via two outer membrane receptors Fiu and CirA, transcription of corresponding mRNAs were found to increase in urine in response to iron limitation [1006]. Chen *et al* conducted comparative genomic analysis of UPEC strain UTI89 to identify genes which undergo positive selection amongst conserved genes, upregulation of entrobactin biosynthesis genes *entD, entF* and uptake *fepE* was observed [1007]. These findings indicate that enterobactin plays a role in UPEC uropathogens.

Salmochelin is a major siderophore in *Salmonella enterica* and UPEC strains [1008]. This siderophore is characterised by a C-glucosylated catechols with an enterobactin analogue as the key component [1009]. Reigstad *et al* compared gene expression between UTI89 intracellular bacterial communities (IBC) isolated from the distal gut and the bladder of mice by laser microdissection. The *iroA* gene which involved in the glycosylation of salmochelin siderophore, was upregulated by 45 ± 3-fold, while *iroN*,

a gene which encodes the salmochelin siderophore receptors was upregulated by 234 ± 28-fold increase in the UTI89 voided from the bladder, in comparison to cecal isolates [1002]. Similar findings were reported in different settings, concurrent *E. coli* isolated from urine and rectum from female outpatients were collected and analysed for salmochelin production and UPECs produced more salmochelin [464]. Johnson *et al,* (2005) collected 65 concurrent urinary and rectal isolates from the same male hosts with febrile UTIs and conducted phylotyping and virulence genotyping. The UPEC isolates contained more virulence genes than rectal isolates, including the salmochelin receptor gene *ironN* [1010].

The third characterised siderophore is aerobactin, which is a citrate-derived siderophore identified first in the Gram-negative bacterial species *Aerobacter aerogenes* [1011]. Aerobactin is a hydroxamate-type siderophore encoded by the operon *iucABCD* and includes the outer membrane encoding gene *iutA* [1012], [1013]. In addition, the conserved *E. coli fhuBCD* genes are involved in aerobactin-associated ferric hydroxmate uptake [1014]. The involvement of aerobactin in UPEC pathogenesis is currently unclear. The Hagan *et al,* (2010) study detected the expression of the outer membrane receptor gene *iutA* in voided UPEC strains isolated from female UTI patients [1004]. Detection of this gene does not signify the involvement of aerobactin in pathogenesis, since some *iutA* carrying isolates do not produce a functional siderophore system [464]. The role of aerobactin in UTI pathogenesis has been disputed, epidemiolocal studies often show contradictory results but a trend can be established in which aerobactin carriers are found more in isolates from the bladder, kidneys and blood [1015].

The fourth siderophore is yersiniabactin, which was identified in *Yersinia Pestis,* the causative bacteria of the plaque known as the black death that swept Europe in the 14th century [1016]. Yersiniabactin is a siderophore and metallophore and genes encoding this iron uptake system are located on a multi-operon pathogenicity island called the high pathogenicity island. It is comprised of four operons expressed by the activation of the transcription factor YbtA [1017]. The yersinia high pathogenicity island exerts a direct impact on the host in UPEC uropathogenesis [999]. Mass spectrometry analysis of yersiniabactin producing UPEC revealed the presence of

copper(II)-yersiniabactin complexes to protect UPEC from copper toxicity [1018]. The Hagan *et al,* (2010) study has also detected the expression of the outer membrane ferric yersiniabactin importer *fyuA* as result of clinical UPEC infection [1004]. Isolation of IBCs containing UPEC by laser microdissection from a mouse infection model of UTI, detected the first expressed enzyme YbtS in the biosynthetic pathway of yersiniabactin and revealed the upregulation of 2000-fold increase in comparison to an intestinal *E. coli* isolate [1002]. The Henderson *et al,* (2009) study which collected concurrent UPEC and rectal isolates from female patients of uncomplicated UTIs, observed production of more yersiniabactin in UTI isolates compared to the rectal isolates upon conducting metabolomic analysis [464]. A similar outcome was observed in males with febrile UTI, as UPEC were found to be carrying the yersiniabactin gene *fyuA* more often than rectal isolates [1010]. Soto *et al,* (2006) conducted a study involving 43 outpatients, in which 27 UPEC isolates caused relapse (same strain) and 53 caused recurrent UPEC infections (different strains). Detection by PCR *In vitro* revealed that strains causing relapse to have the capacity to form biofilms and carry the yersiniabactin gene [1019]. All these findings suggest that yersiniabactin may have a prominent role to play in UPEC pathogenesis.

5.1.2.6 The role of iron acquisition mechanisms in *P. aeruginosa* uropathogenesis

The vast majority of uropathogenic *P. aeruginosa* produce pyochelin and pyoverdine as observed by Visca *et al*, (1992) as only 2.4% were pyochelin-deficient and 7.4% were pyoverdine-deficient amongst 121 UTI isolates [461]. Yadav *et al,* (2000) utilised a *P. aeruginosa* pyochelin and haemolysin producing UTI isolate to infect mice in an acute ascending pyelonephritis model, the infection produced renal lesions (abscesses) in mice. The exact role of both iron uptake systems in infection was not elucidated [57]. This was followed by another study which sought to find association between *P. aeruginosa* virulence factors *in vitro* and their contribution to virulence *In vivo*. 18 UTI isolates which produced pyoverdine, pyochelin and haemolysin were selected to be tested on an ascending mice model of pyelonephritis. An association was found between haemolysin and high bacterial renal counts in mice [58]. Pyochelin and haemolysin were significantly reduced when treated with cranberry juice in a static *in vitro* biofilm model [1020]. Production of pyoverdine *in vitro* was

also reported in 30 *P. aeruginosa* urinary tract isolates, suggesting it has a crucial role in UTI pathogenesis [90]. Different bacterial species also compete one another for iron resources in certain settings [1021]. As a result, enterobacteria such as *E. coli* secrete an inhibitor called escherichelin which acts as a bacterial antagonist to pyochelin synthesis in *P. aeruginosa* [1022], [1023].

Given the lack of understanding about the direct role that hormones play in the pathogenicity of Gram-negative uropathogens, in this chapter I aim to understand the impact of sex hormones on UTI pathogenesis. More specifically, these studies have assessed the effects of testosterone, oestradiol and progesterone on the expression and production of several key virulence factors known to be important in UTI. Proteomic analysis was also conducted using a UTI clinical isolate of *P. aeruginosa* to determine whether sex hormones cause shifts in the proteome that may affect pathogenicity.

5.2 Aims

- To determine global changes in the bacterial proteome in response to sex hormones and identify key pathways.

- To investigate the impact of oestradiol, testosterone and progesterone on *P. aeruginosa* protein abundance.

- To investigate the impact of hormones on virulence factors and biofilm formation.

5.3 The influence of hormones on *P. aeruginosa*

5.3.1 Proteomic analysis of the influence of sex hormone in AUM

In order to assess the abundance of proteins following exposure to oestradiol, testosterone and progesterone, isolate 133098 was chosen to be inoculated in AUM as a model of infection. The isolate was treated with 20 nM concentration with each of oestradiol, testosterone and progesterone. The cultures were grown under static conditions to an OD of 0.25 +/- 0.045.

Initially, a comparison between AUM and AUM-V was performed. AUM-V consisted of AUM plus the vehicle/ carrier that the sex hormones were prepared in (ethanol). 19 proteins displayed a significant increased abundance of >1.5 fold in AUM compared to AUM-V. These included PhzF (56-fold) and PhzM (26-fold), both part of the phenazine biosynthesis pathway. PqsE, which was higher in abundance by 6-fold, is involved in the production of alkyl-quinolones and PA4131, which is a probable iron-sulphur protein. Only 2 proteins were significantly decreased in AUM. These were PA3009 (uncharacterised protein, 2-fold down) and DapE (Succinyl-diaminopimelate desuccinylase, 1.78-fold down). Overall, very few proteins displayed a significant difference between AUM and AUM-V. Despite this, the samples were clearly separated on the PCA plot (Figure 5.5). AUM-V was the more appropriate control for studying the impact of the sex hormones alone and therefore this was used in all subsequent analysis. In addition, observation of protein abundance in all conditions revealed that most proteins were upregulated in the controls, AUM and AUM-V (Figure 5.6).

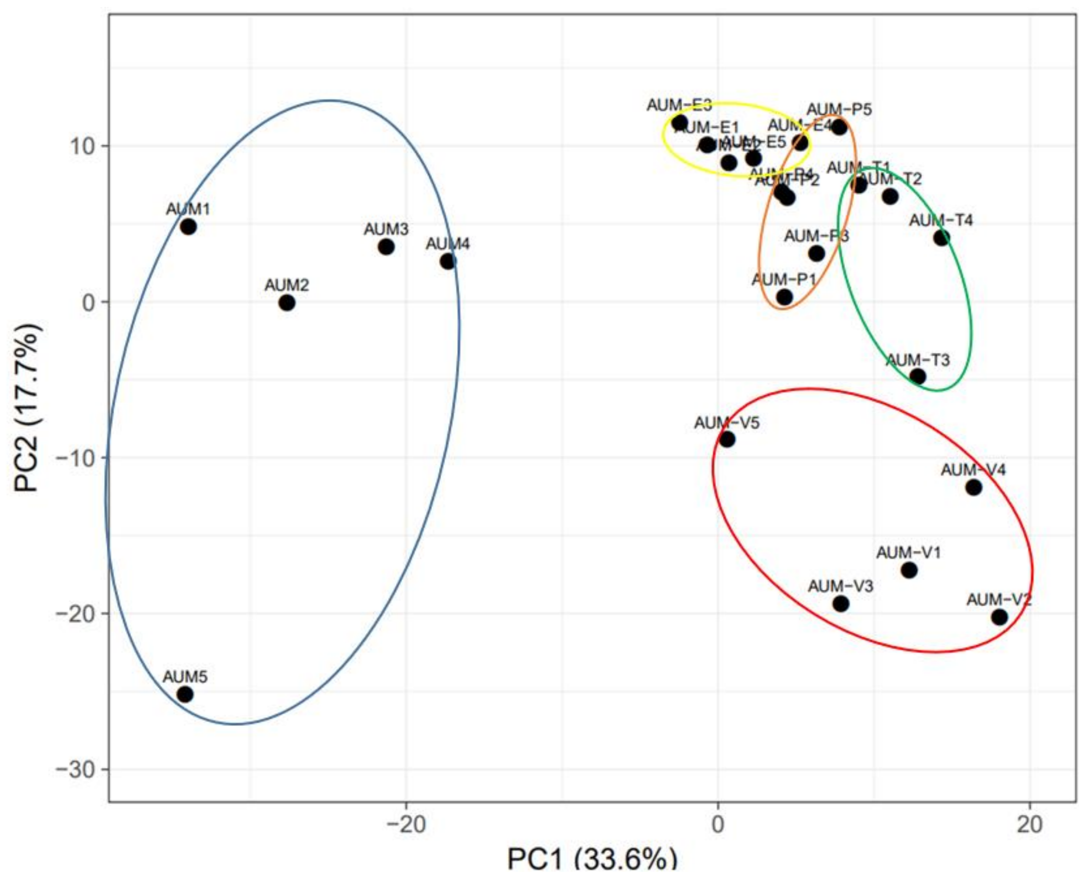


Figure 5.5 PCA of 5 biological replicates in each of AUM, (AUM-V= vehicle control, AUM-E=oestradiol, AUM-T=testosterone and AUM-P=progesterone) displays the clustering of conditions and their related samples

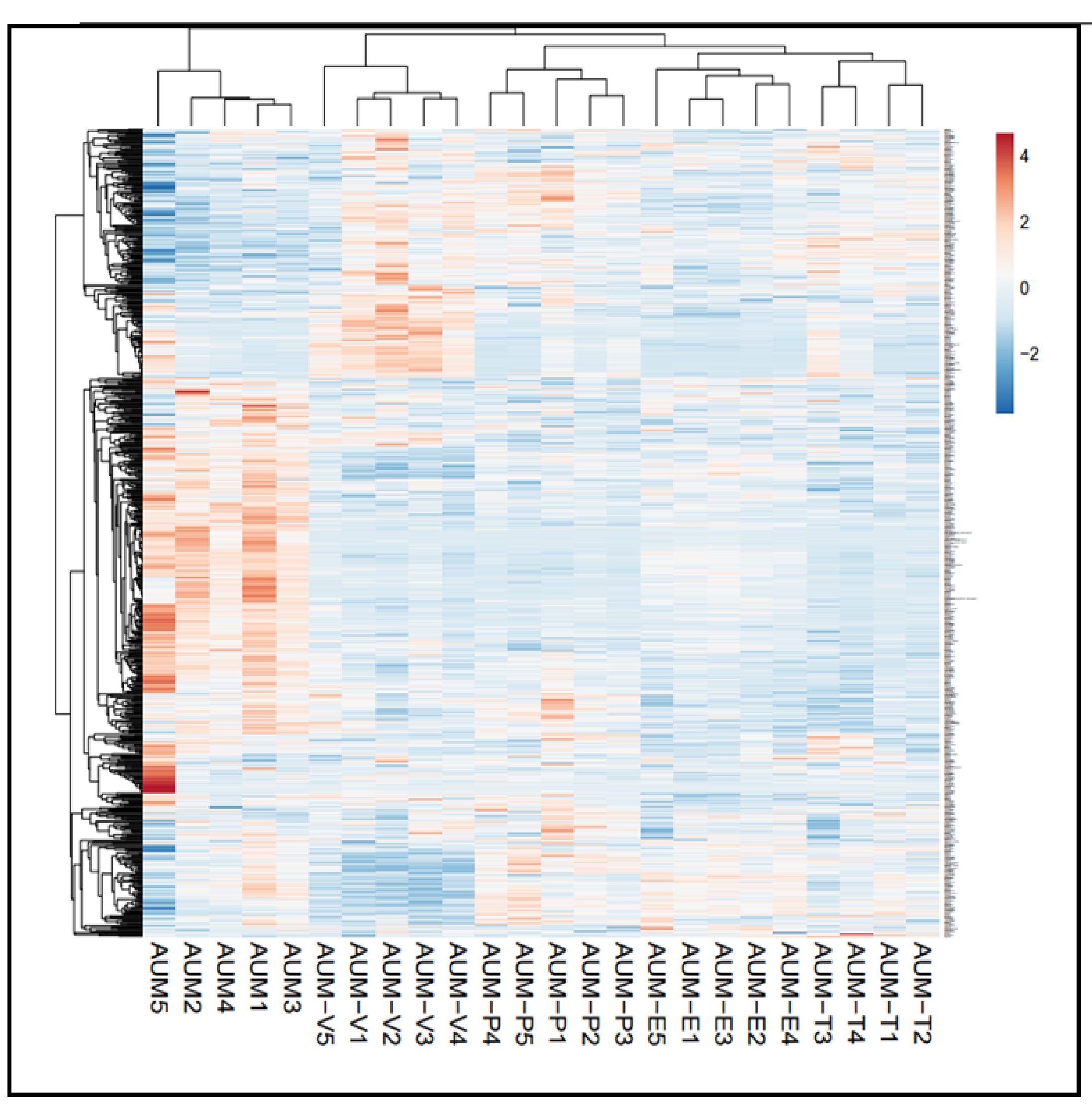


Figure 5.6 Heat map of the distribution of the profile of abundant proteins across all non-treated samples (AUM) and treated samples AUM-V, AUM-E, AUM-T, AUM-P (AUM-V= vehicle control, AUM-E=oestradiol, AUM-T=testosterone and AUM-P=progesterone)

5.3.1.1 Proteins increased under all hormone treatment conditions

In silico analysis revealed 52 proteins which displayed significantly higher abundance in all hormones in comparison to AUM-V. There was also a subset of proteins that were unique to each treatment type with 42, 43 and 82 proteins significantly higher in oestradiol, testosterone and progesterone, respectively (Figure 5.7).

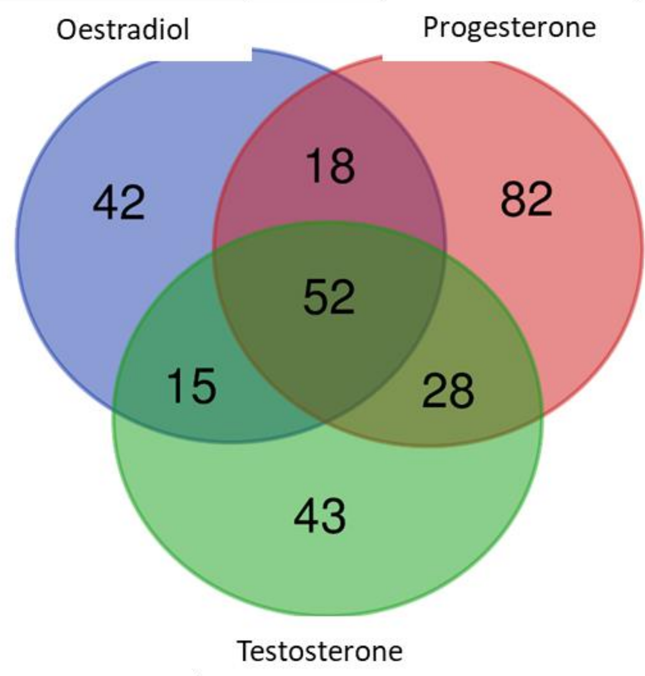


Figure 5.7 Venn diagram of upregulated proteins AUM containing hormone in comparison to AUM- V

Proteins which contribute to denitrification pathways were upregulated in response to the presence of hormones. NirS for instance, is a nitrate involved in haem binding in addition to NirF and NirL, both of which contribute to the biosynthesis of heme d1. These Nir pathway proteins were upregulated across all hormone treatments from 1.8-fold to 8-fold change. KatA is a catalase which protects *P. aeruginosa* against hydrogen peroxide released by the invaded host and self-produced nitric-oxide, and also involved with haem binding [1024]. The protein is upregulated by 2.69, 1.85 and 2.25-fold in oestradiol, testosterone and progesterone, respectively. *leuC* is a gene which encodes a 3-isopropylmalate dehydratase large subunit with a 4 iron, 4 sulphur cluster binding subunit, the protein was upregulated from 1.7 to 2.5 fold-change [655].

5.3.1.2 Proteins decreased under all hormone conditions

67 proteins displayed significantly decreased abundance of more than 1.5-fold in all three hormone conditions. Only seven proteins were uniquely reduced in oestradiol with 45 and 46 proteins decreased in progesterone and testosterone conditions only

(Figure 5.8). Of the 67 shared proteins, a large proportion were identified as being involved in iron acquisition pathways.

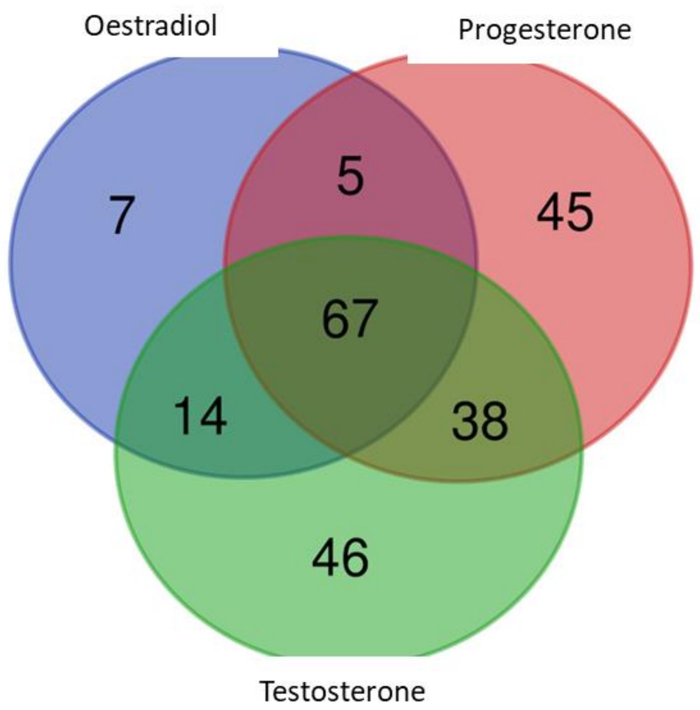


Figure 5.8 Venn diagram of proteins downregulated upon treatment with oestradiol, testosterone and progesterone

Iron acquisition mechanisms are indispensable in *P. aeruginosa* infections or other types of bacterial infections. Thus, we analysed the impact of sex hormones on these mechanisms. Pyochelin is a siderophore produced to counteract iron scarcity in the host. All expressed proteins participating in pyochelin biosynthesis were less abundant across all hormones compared to the AUM-V, as the decrease in abundance ranged from -two to -eight fold less than in AUM-V. (Figure 5.9). A similar result is observed for the pyoverdine synthesis pathway, the synthases enzymes and the receptor proteins were less abundant in the presence of hormones. In addition, heme uptake and proteins associated in iron acquisition in both of the PhuR and HasR systems were also less abundant than AUM-V.

The most marked effect on proteins involved in pyochelin synthesis was found in progesterone since five proteins were down-regulated by eight-fold or more (Figure 5.9). These proteins are PchG, PchF, PchE, PchR and PchE and PchB, all of which are

involved in pyochelin synthesis and regulation. Similar decreased abundance was observed with oestradiol and to a lesser extent testosterone, similarly to the pyochelin pathway, the pyoverdine pathway proteins participate in iron uptake such as PvdJ (pyoverdine biosynthesis), PvdD (pyoverdine synthetase), and PvdR (pyoverdine biosynthesis) were found to be significantly less abundant in oestradiol. Interestingly, oestradiol also had the strongest impact on heme uptake associated mechanisms although inhibition of the system could be seen with all hormones.

P. aeruginosa can also hijack siderophores made by other bacteria. Proteins such as ChtA and FecA contribute to the capture of xenosiderophores and were found to be less abundant in all hormone treated AUM ranging from 11-fold and to two-fold reductions [275]. PfeR is a member of a two-component response regulator pair with the sensor PfeS [1025], the regulator pfeR was downregulated by 78-fold in the oestradiol samples. In addition, PfeR was less abundant by three-fold in testosterone samples and five-fold in the progesterone samples.

Other proteins were also lower such as fumarate hydratase FumC, which is produced in response to iron starvation and is also linked to pyochelin and pyoverdine production [1026]. In addition, SodA is a superoxide dismutase that is reduced in response to iron starvation and intracellular redox increase in *P. aeruginosa* [1027], was reduced by at least two-fold in the presence of oestradiol, testosterone and progesterone. PA2033 is hypothetical protein linked to a novel iron-acquisition mechanism [1028], was found to be downregulated in oestradiol, testosterone and progesterone by > six-fold,>two-fold and > four-fold, respectively. There is a clear impact caused by all three hormones leading to a decreased abundance of proteins associated with iron acquisition.

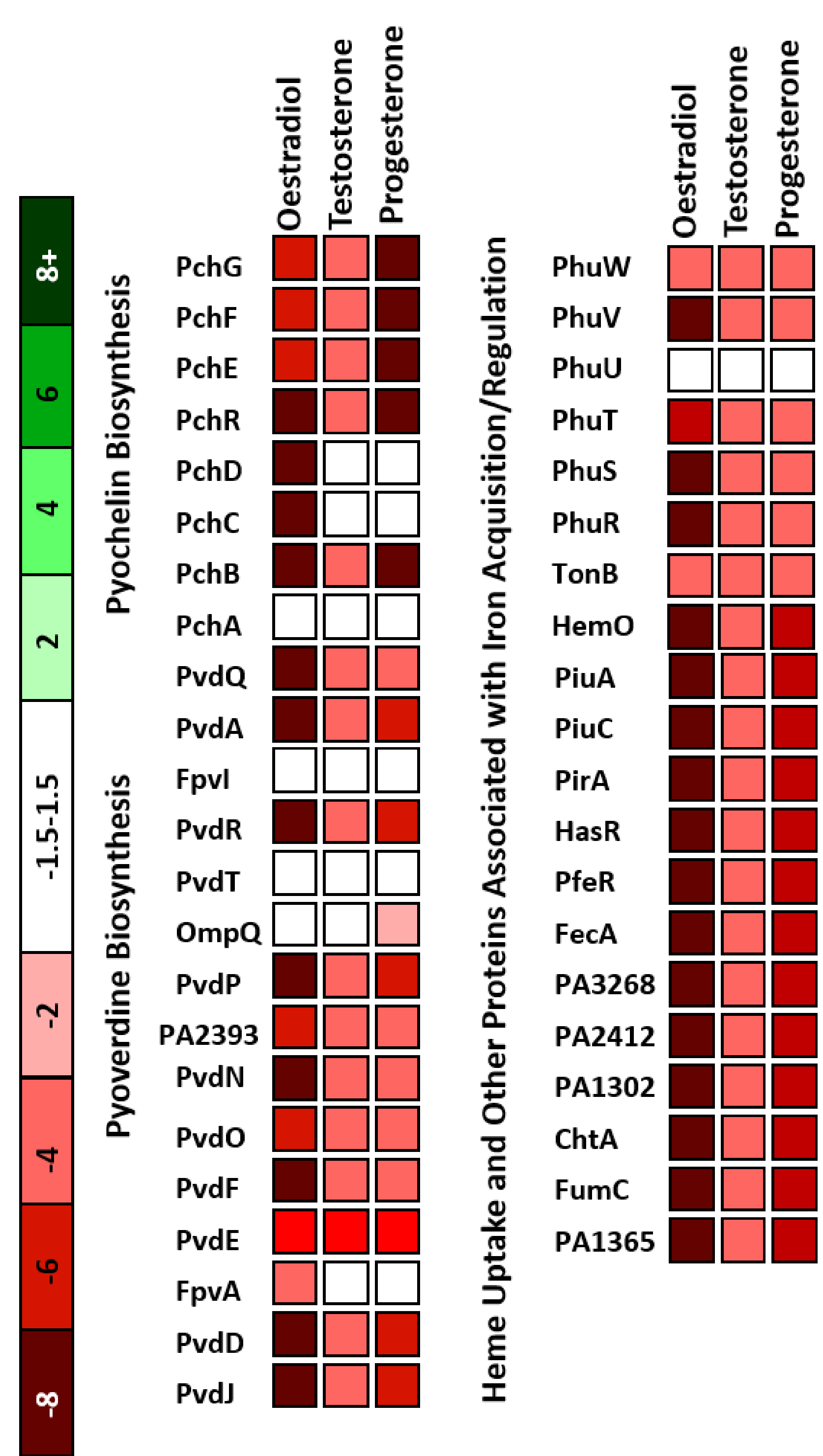


Figure 5.9 Abundance of *P. aeruginosa* proteins involved in iron acquisition across three hormone treatment conditions. The figures display 43 proteins that were either downregulated or failed to increase in the presence of oestradiol, testosterone and progesterone. **(A** displays protein involved in pyochelin and pyoverdine siderophore synthesis and uptake. **B)** The panel of proteins participates in heme chelation and uptake. Fold-change of > 1.5 is deemed significant [275], [655]

5.4 The Impact of oestradiol on *P. aeruginosa*

5.4.1 The Influence of varying oestradiol on bacterial growth

The impact of oestradiol was assessed on growth over 24 h in a small representative panel of isolates; PAO1, PA14 and 133098. No statistical significance was observed by the treatment of oestradiol on each isolate with 20nM of hormone (Figure 5.10 A-C). This suggests that oestradiol has no direct impact on bacterial growth *in vitro*.

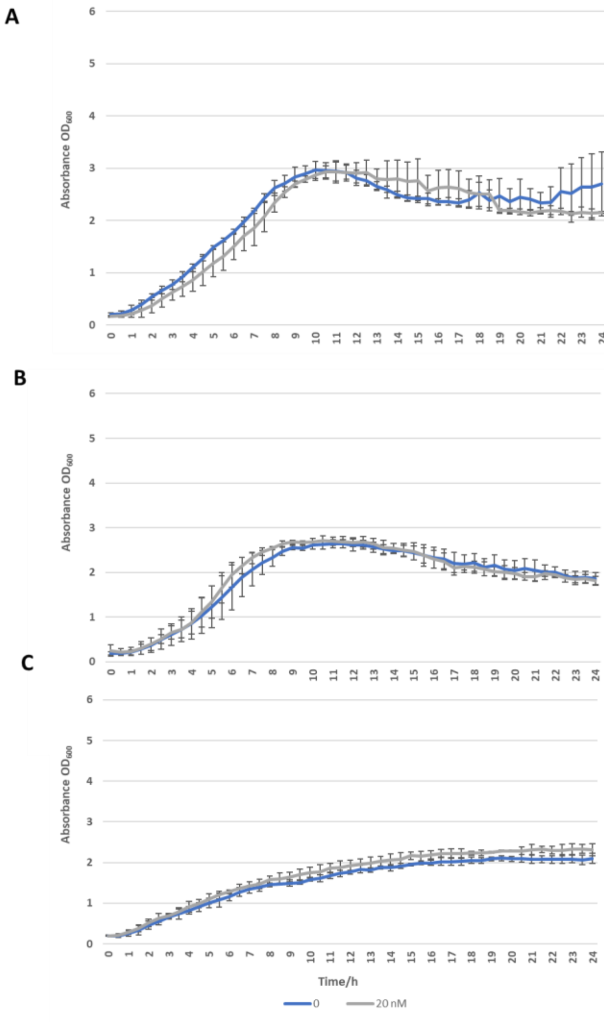


Figure 5.10 Growth of 2 reference strains and a clinical isolate was measured between a control strain and isolate treated with 20 nM oestradiol over 24h. Optical density was measured at A600nm. (A: No statistically significant difference was observed in the growth pattern in PAO1 following testing by equal variance test (Brown-Forsythe) on Ranks at all the examined time points (0, 6, 12, 18h) (P = 0.572) at 24h and for B) and PA14 (P=0.700) at 24h and C) 133098 (P=0.664)

5.4.2 The impact of oestradiol on the *P. aeruginosa* proteome

5.4.2.1 Upregulated proteins in comparison to AUM-V

127 proteins were over 1.5-fold up in AUM-E. Of these, 110 had a significant P value ($P<0.05$). 33 proteins (shaded in grey) were only upregulated in oestradiol and not the other two hormone conditions (Table 5.3).

Table 5.3 Proteins upregulated in response to oestradiol, unique proteins are shaded in grey

Gene names	Max FC	Description	Gene names	Max FC	Description
PA4131	50.21	Probable iron-sulfur protein	PA2771	2.48	Uncharacterized protein O
PA1503	21.46	Uncharacterized protein	PA3419	2.47	UPF0162 protein
PA4129	19.01	Uncharacterized protein	phzS	2.45	Flavin-containing monooxygenase
gcl	8.99	Glyoxylate carboligase	lasD	2.44	Chitin-binding protein CbpD
ccpA	6.73	Cytochrome c551 peroxidase	PA3483	2.38	Uncharacterized protein
lasB	6.06	Elastase	PA2116	2.38	UPF0317 protein PA2116
PA5446	5.62	Uncharacterized protein	PA1260	2.36	Amino acid ABC transporter periplasmic binding protein
PA4130	4.94	Probable sulfite or nitrite reductase	phzM	2.36	Probable phenazine-specific methyltransferase
PA4139	4.76	Uncharacterized protein	PA1518	2.35	5-hydroxyisourate hydrolase
PA4928	4.76	UPF0313 protein PA4928	PA0553	2.34	Uncharacterized protein
ilvA2	4.64	L-threonine dehydratase	pqsA	2.34	Anthranilate--CoA ligase
phnB	4.61	Anthranilate synthase component 2, pyocyanine specific	pqsB	2.34	PqsB
PA4880	4.39	Probable bacterioferritin	PA3187	2.32	Probable ATP-binding component of ABC transporter
bdhA	4.12	3-hydroxybutyrate dehydrogenase	nosZ	2.29	Nitrous-oxide reductase
PA0122	3.95	Uncharacterized protein	gtdA	2.29	Gentisate 1,2-dioxygenase
hcnC	3.72	Hydrogen cyanide synthase subunit HcnC 4129	PA0586	2.28	Uncharacterized protein
PA0510	3.67	Probable uroporphyrin-III c-methyltransferase	PA1516	2.27	Uncharacterized protein
PA5359	3.66	Uncharacterized protein	PA3922	2.23	Uncharacterized protein
PA1500	3.57	Probable oxidoreductase	PA3332	2.23	Uncharacterized PhzA/B-like protein
PA2266	3.45	Probable cytochrome c	pfpI	2.21	Protease PfpI
hcnB	3.39	Hydrogen cyanide synthase subunit HcnB	PA5188	2.20	Probable 3-hydroxyacyl-CoA dehydrogenase
PA4571	3.27	Probable cytochrome c	PA1662	2.20	Probable ClpA/B-type protease
PA1254	3.24	Probable dihydrodipicolinate synthetase	PA2291	2.20	Porin
phnA	3.22	Anthranilate synthase component 1, pyocyanine specific	PA4739	2.19	Uncharacterized protein
pqsE	3.17	Uncharacterized protein	PA2433	2.18	Uncharacterized protein
allA	3.12	Ureidoglycolate lyase	PA0537	2.17	Uncharacterized protein
PA3924	3.03	Probable medium-chain acyl-CoA ligase	pcaF	2.16	Beta-ketoadipyl-CoA thiolase
PA3119	2.99	Uncharacterized protein	phaC1	2.16	Poly (3-hydroxyalkanoic acid) synthase 1
PA1112	2.98	Uncharacterized protein	PA3699	2.15	Probable transcriptional regulator

nirF	2.97	Protein NirF	PA3461	2.15	Uncharacterized protein
gbuA	2.96	Guanidinobutyrase	PA5546	2.12	Uncharacterized protein
PA1517	2.91	Uncharacterized protein	PA5178	2.12	Uncharacterized protein
PA1667	2.83	Uncharacterized protein	phzD	2.12	Phenazine biosynthesis protein PhzD
PA5220	2.82	Uncharacterized protein	PA2567	2.11	Uncharacterized protein
PA2544	2.77	Uncharacterized protein	PA0794	2.10	Probable aconitate hydratase
PA3880	2.74	Uncharacterized protein	PA2169	2.10	Uncharacterized protein
PA4505	2.71	Probable ATP-binding component of ABC transporter	acsA1	2.09	Acetyl-coenzyme A synthetase 1
PA0144	2.70	Uncharacterized protein	ccoP2	2.09	Cbb3-type cytochrome c oxidase subunit
katA	2.69	Catalase	PA5535	2.09	Uncharacterized protein
pqsC	2.67	PqsC	PA4608	2.08	Cyclic diguanosine monophosphate-binding protein
PA1140	2.67	Uncharacterized protein	sdaA	2.08	L-serine dehydratase
opdO	2.65	Pyroglutatmate porin OpdO	PA3602	2.07	Uncharacterized protein
PA1881	2.65	Probable oxidoreductase	PA2633	2.06	Uncharacterized protein
PA2330	2.63	Uncharacterized protein	acnA	2.06	Aconitate hydratase A
PA2481	2.62	Uncharacterized protein	PA2021	2.05	Uncharacterized protein
htpX	2.61	Protease HtpX	chiC	2.04	Chitinase
leuC	2.58	3-isopropylmalate dehydratase large subunit	PA2572	2.04	Probable two-component response regulator
PA4792	2.58	Uncharacterized protein	narH	2.03	Respiratory nitrate reductase beta chain
pqsD	2.57	2-heptyl-4(1H)-quinolone synthase PqsD	fusB	2.03	Elongation factor G 2
mmsA	2.55	Methylmalonate-semialdehyde dehydrogenase [acylating]	PA1880	2.03	Probable oxidoreductase
PA4738	2.53	UPF0337 protein PA4738	PA3712	2.03	Uncharacterized protein
PA5481	2.52	Uncharacterized protein	PA1921	2.02	Uncharacterized protein
nirS	2.52	Nitrite reductase	PA4520	2.01	Probable chemotaxis transducer
amiE	2.48	Aliphatic amidase	phzB2	2.01	Phenazine biosynthesis protein PhzB 2
panE	2.48	Probable 2-dehydropantoate 2-reductase	PA2176	2.01	Uncharacterized protein

Notably, the activation of QS sensing mechanism is linked to an increase of the virulence factors pyocyanin and HCN (Figure 5.11). Production of PQS-associated proteins was upregulated in oestradiol treatments. A two-fold increase in comparison to AUM-V is PqsA-E was observed. The PQS system is implicated in the control of the production of phenazines and HCN. The increase in PQS proteins correlated with an increase in nine proteins linked to phenazines biosynthesis and hydrogen cyanide (HcnB and HcnC). Notably, PA4129 was upregulated in oestradiol, this recently discovered novel protein protects *P. aeruginosa* from the self-produced cyanide [1029]. Overall, oestradiol seems to have a direct impact on *P. aeruginosa* leading to

the increased abundance of proteins associated with the PQS QS system, phenazine biosynthesis and HCN (Figure 5.11). This effect was not observed in the presence of the other two hormones.

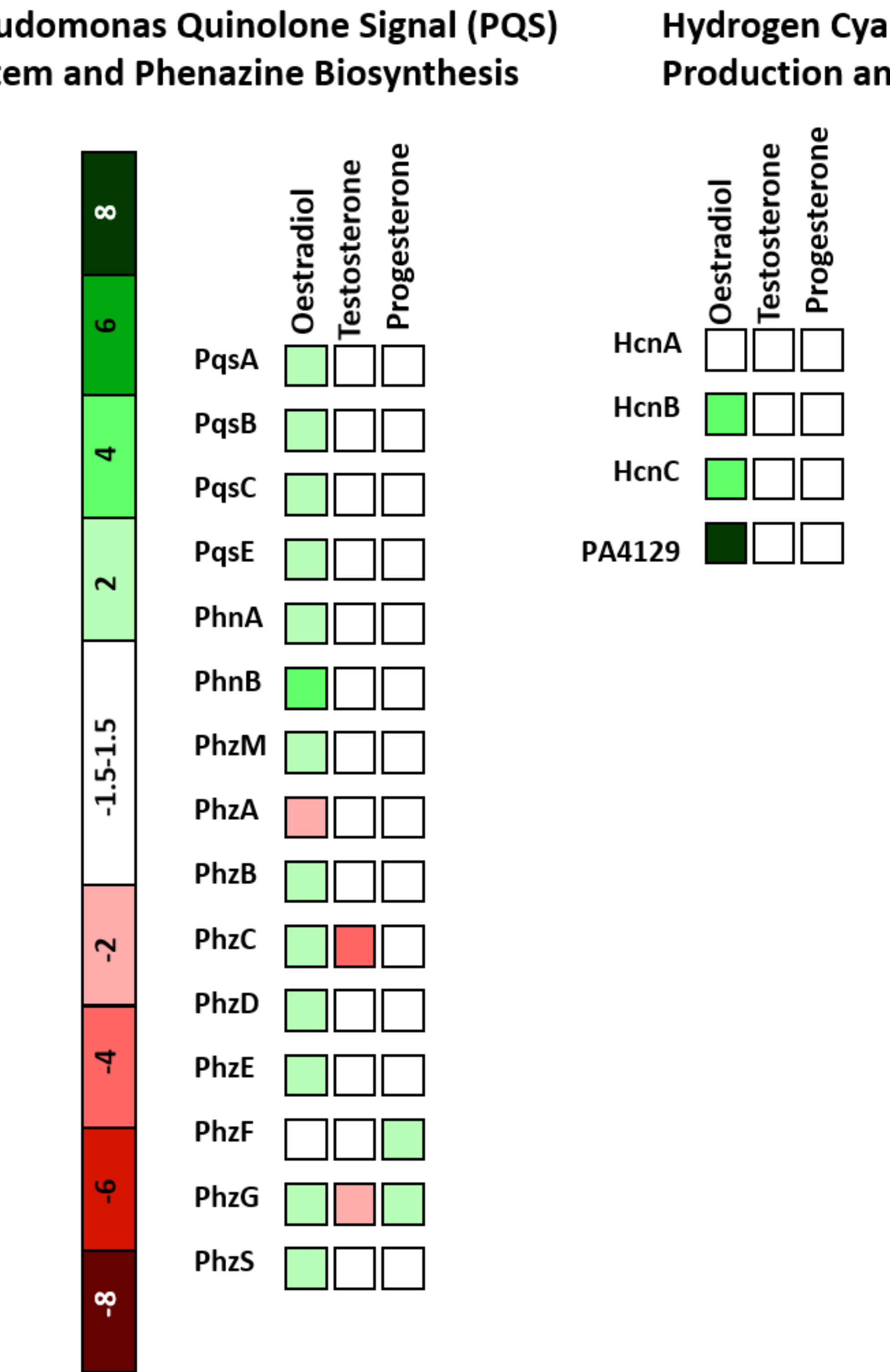


Figure 5.11 Abundance of P. aeruginosa proteins associated with the PQS system, phenazine biosynthesis and the production of hydrogen cyanide. A fold-change of > 1.5 and a significant P value (P<0.05) is significant. Increased abundance is shown in green and decreased abundance is in red

5.4.2.2 Proteins reduced in response to oestradiol

93 proteins were less abundant over 1.5-fold. 82 of these has a significant P value of <0.05. 5 proteins were only down in oestradiol (shaded in grey) (Table 5.4). Some of the proteins displaying a reduced response with oestradiol treatment include PopN, a protein involved in the T3SS (reduced by 2.35-fold change). PriA is a helicase which could be essential in cell division and may have a role in response to antibiotics [655], [1030]. The PriA protein was downregulated by six-fold. FpvA, an outer membrane protein which acts a receptor to ferric-pyoverdine receptor also showed decreased abundance (-2.06-fold).

Table 5.4 Proteins downregulated in response to oestradiol, unique proteins are shaded in grey

Gene names	Max FC	Description	Gene names	Max FC	Description
PA5487	-122.21	Uncharacterised protein	PA2393	-4.24	Probable dipeptidase
PA2034	-87.73	Uncharacterized protein	phuT	-4.22	Heme-transport protein, PhuT
pfeR	-78.25	Transcriptional activator protein PfeR	PA3734	-4.20	Uncharacterized protein
PA2412	-64.54	Uncharacterized protein	PA1490	-4.05	Probable transcriptional regulator
pchR	-46.13	Regulatory protein PchR	pirA	-3.86	Ferric enterobactin receptor PirA
PA2384	-39.19	Uncharacterized protein	PA4705	-3.62	Uncharacterized protein
pvdA	-34.62	L-ornithine N(5)-monooxygenase	PA5149	-3.59	Uncharacterized protein
pvdR	-33.61	PvdR	fumC2	-3.41	Fumarate hydratase class II 1
PA2452	-31.98	Uncharacterized protein	PA4675	-3.32	Probable TonB-dependent receptor
pvdH	-28.78	L-2,4-diaminobutyrate:2-ketoglutarate 4-aminotransferase, PvdH	PA2550	-3.27	Probable acyl-CoA dehydrogenase
hemO	-28.36	Heme oxygenase	pscL-	-3.26	Type III export protein PscL
PA2411	-27.96	Probable thioesterase	PA1365	-3.20	Probable siderophore receptor
pvdE	-21.04	Pyoverdine biosynthesis protein PvdE	tonB-	-3.19	Protein TonB
PA2451	-17.32	Uncharacterized protein	PA0047	-3.12	Uncharacterized protein
pvdF	-16.96	Pyoverdine synthetase F	PA2204	-2.96	Probable binding protein component of ABC transporter
pvdD	-16.69	Pyoverdine synthetase D	PA3866	-2.94	Pyocin protein
PA2402	-14.69	Probable non-ribosomal peptide synthetase	ampDh2-	-2.89	AmpDh2
pvdL	-14.60	PvdL	sodA-	-2.80	Superoxide dismutase [Mn]
pvdJ	-14.08	PvdJ	exoT-	-2.71	Exoenzyme T
fecA	-11.66	Fe(III) dicitrate transport protein FecA	hasR-	-2.68	Heme uptake outer membrane receptor HasR
phuV	-11.33	Hemin import ATP-binding protein HmuV	PA0046	-2.66	Uncharacterized protein
PA4709	-10.72	Probable hemin degrading factor	PA2381	-2.63	Uncharacterized protein
nrdD	-8.70	Class III (Anaerobic) ribonucleoside-triphosphate reductase subunit, NrdD	PA2196	-2.61	Probable transcriptional regulator
pvdQ	-8.04	Acyl-homoserine lactone acylase PvdQ	PA4373	-2.57	Uncharacterized protein
PA2033	-7.91	Uncharacterized protein	PA1699	-2.55	Uncharacterized protein

pchF	-7.23	Pyochelin synthetase	tadA-	-2.42	tRNA-specific adenosine deaminase
pvdN	-7.12	PvdN	rrmA-	-2.39	rRNA methyltransferase
pvdP	-6.85	PvdP	PA0821	-2.38	Uncharacterized protein
pvdO	-6.81	PvdO	PA2528	-2.37	Probable Resistance-Nodulation-Cell Division (RND) efflux membrane fusion protein
phuR	-6.49	Heme/hemoglobin uptake outer membrane receptor PhuR	popN-	-2.35	Type III secretion outer membrane protein PopN
PA3268	-6.44	Probable TonB-dependent receptor	PA1302	-2.34	Probable heme utilization protein
PA4358	-6.21	Ferrous iron transport protein B	glpD-	-2.33	Glycerol-3-phosphate dehydrogenase
pchB	-6.11	Isochorismate pyruvate lyase	icmP-	-2.29	Insulin-cleaving metalloproteinase outer membrane protein
priA	-6.07	Primosomal protein N'	PA3768	-2.27	Probable metallo-oxidoreductase
PA5445	-5.22	Probable coenzyme A transferase	PA3310	-2.20	Uncharacterized protein
PA4514	-5.11	Probable outer membrane receptor for iron transport	rpmE2	-2.18	50S ribosomal protein L31 type B
pchG	-4.71	Pyochelin biosynthetic protein PchG	fpvA	-2.06	Ferripyoverdine receptor
opdP	-4.51	Glycine-glutamate dipeptide porin OpdP	PA3054	-2.04	Uncharacterized protein
PA4357	-4.30	Uncharacterized protein	popD	-2.03	Translocator outer membrane protein PopD
piuC	-4.27	PKHD-type hydroxylase PiuC	PA5022	-2.02	Uncharacterized protein
pchE	-4.25	Dihydroaeruginoic acid synthetase	PA5508	2.00	Probable glutamine synthetase

5.4.3 Biofilm assay to asses oestradiol's impact on biofilms

To determine whether oestradiol had a direct impact on biofilm formation, varying concentrations of oestradiol at 10, 40 and 100m applied to all panel of clinical isolates. A selection of the three isolates PAO1, PA14 and 133098. No statistical significance was observed between oestradiol treatment and the control (Figure 5.12 A-C).

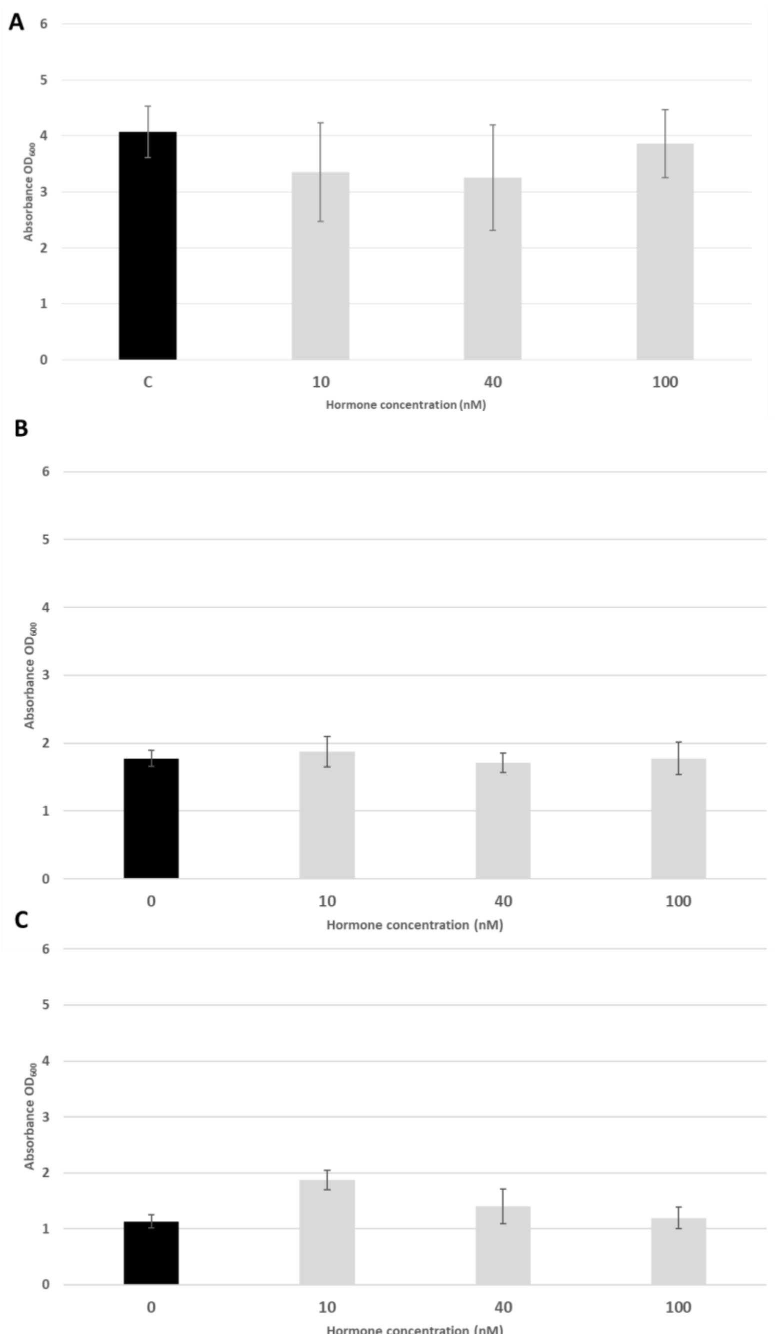


Figure 5.12 The total biomass produced post CV staining in the control (0) and treatment groups of 10 nM,40 nM and 100 nM of oestradiol. A) treatment of PAO1 with oestradiol did not produce any significant differences P = 0.229. B) treatment of PA14 strain resulted in no significant differences P = 0.618. C) No significant differences observed in clinical isolate 133098, P = 0.927. Statistical analysis was conducted with equal variance test (Brown-Forsythe)

5.4.4 Minimal impact of oestradiol on pyocyanin production

A pyocyanin assay was conducted to assess the impact of oestradiol on *P. aeruginosa* in LB media. Only three strains and isolates were reduced in the treatment group of oestradiol, namely, PA14 (P=0.014), 133099 (P=0.028), 133106 (P=0.011). In contrast, isolate 133065 (P=0.039) treatment with oestradiol increased the production of pyocyanin (Figure 5.13). Isolate 133098, the isolate used in the proteomics experiment, did show an increase in pyocyanin in the presence of oestradiol, however, this increase was not significant. It is clear from these results that this response is not universal.

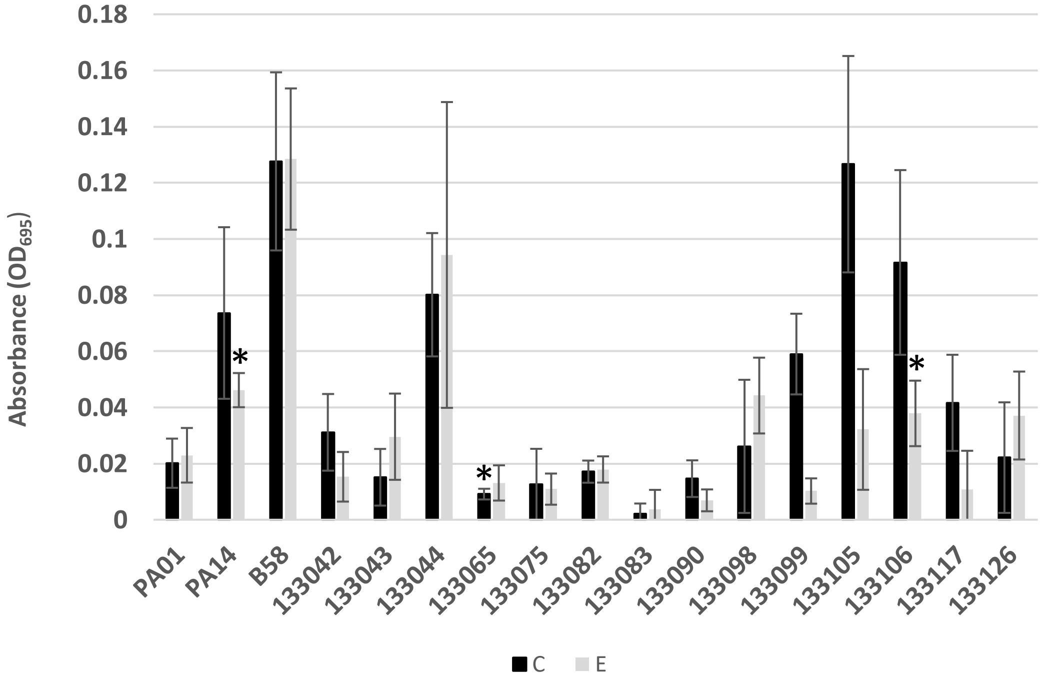


Figure 5.13 Mean pyocyanin calculated of three biological isolates in the C= control group (black) and E=10 nM oestradiol (light grey) of UTI *P. aeruginosa*. Significant differences observed in three isolates. * denotes P<0.050

5.5 The Impact of testosterone on *P. aeruginosa*

5.5.1 Investigation of 20nM testosterone influence on growth rates

To determine whether testosterone had a direct impact on bacterial growth, growth rates in the presence and absence of the hormone were investigated in PAO1, PA14 and 133098. There was some evidence that testosterone increased growth in one isolate. Over the first 12 hours, PAO1 was significantly increased by the presence of

testosterone at 20 nM. However, no statistical significance was observed for the other isolates, PA14 (P = 0.700) (Figure 5.14) and the UTI isolate 133098 P= 0.664 (Figure 5.14 A-C).

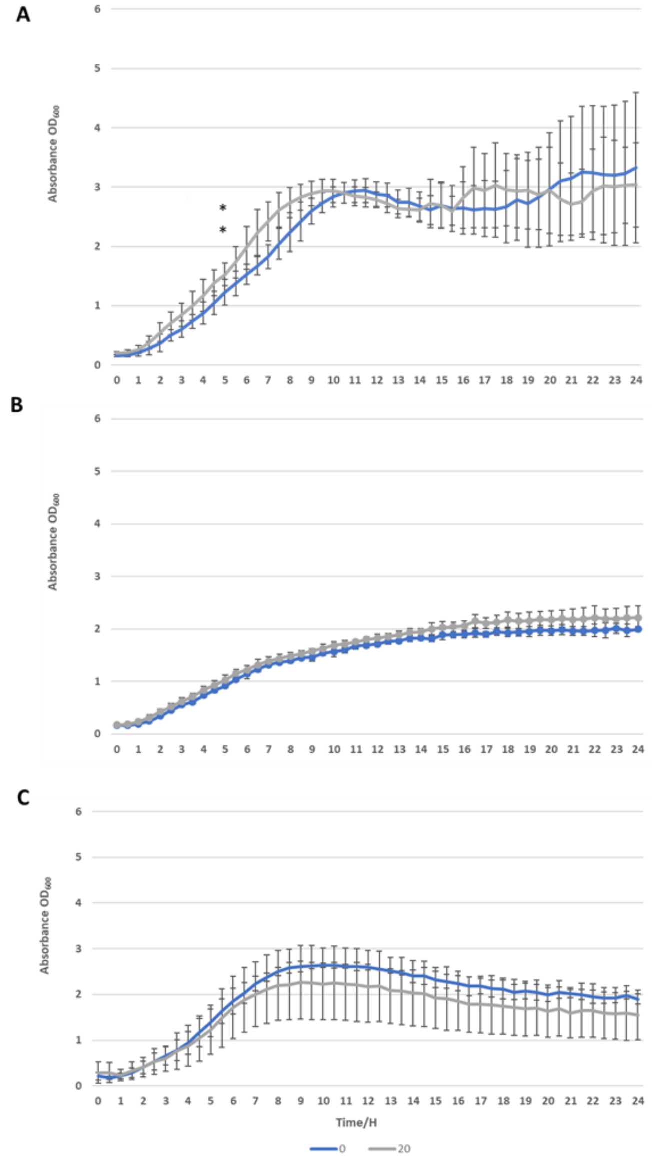


Figure 5.14 Growth pattern of three strains with control lacking hormones and 20 nM testosterone over 24h. **A)** PAO1 growth higher in the oestradiol treated group at 6h with **P = <0.001 using the methods of Holm-Sidak of pairwise comparison, no statistical significance was observed at the end of the growth rates at 24h with (P = 0.400). **B)** No statistically significant relationship observed between the control and the strain PA14 P = 0.572. **C)** Clinical isolate 133098 produced no significant relationship P = 0.898. Equal variance test (Brown-Forsythe) was conducted for all statistical tests

5.5.2 Proteomics of testosterone

5.5.2.1 Upregulated proteins in testosterone treatment

Overall, 64 proteins were significantly upregulated with testosterone treatment, of which 13 were uniquely upregulated in this condition (Table 5.5). One of the notable upregulated proteins is the outer membrane OprM efflux pump [1031], which was upregulated by a 1.78-fold change. *SpeH* gene is an S-adenosylmethionine decarboxylase proenzyme that is part of spermidine biosynthesis and is linked to resisting oxidative stress in the host, the protein was upregulated by 1.84-fold.

Table 5.5 Proteins upregulated in response to testosterone, unique proteins are shaded in grey

Gene names	Max FC	Description	Gene names	Max FC	Description
PA4928	5.32	UPF0313 protein PA4928	PA1517	1.83	Uncharacterized protein
ccpA	4.14	Cytochrome c551 peroxidase	PA1500	1.82	Probable oxidoreductase
PA5103	3.90	Uncharacterized protein	PA3924	1.82	Probable medium-chain acyl-CoA ligase
PA0510	3.15	Probable uroporphyrin-III c-methyltransferase	ccoP2	1.81	Cbb3-type cytochrome c oxidase subunit
PA2330	3.08	Uncharacterized protein	PA2572	1.81	Probable two-component response regulator
gtdA	2.87	Gentisate 1,2-dioxygenase	PA2169	1.81	Uncharacterized protein
PA0122	2.57	Uncharacterized protein	coxB	1.80	Cytochrome c oxidase subunit 2
gbuA	2.57	Guanidinobutyrase	PA1516	1.79	Uncharacterized protein
katE	2.44	Catalase HPII	PA1140	1.78	Uncharacterized protein
PA1733	2.42	Uncharacterized	oprM	1.78	Outer membrane protein OprM
PA1260	2.35	Amino acid ABC transporter periplasmic binding protein	PA0794	1.75	Probable aconitate hydratase
htpX	2.34	Protease HtpX	leuC	1.72	3-isopropylmalate dehydratase large subunit
PA5359	2.34	Uncharacterized protein	chiC	1.71	Chitinase OS=Pseudomonas aeruginosa
PA1921	2.24	Uncharacterized protein	gcl	1.70	Glyoxylate carboligase
ccoN2	2.17	Cytochrome c oxidase, cbb3-type, CcoN subunit	glgP	1.70	Alpha-1,4 glucan phosphorylase
nirF	2.15	Protein NirF	PA3922	1.67	Uncharacterized protein PA3922
PA3416	2.10	Probable pyruvate dehydrogenase E1 component, beta chain	PA4066	1.65	Uncharacterized protein
PA2266	2.10	Probable cytochrome c	PA1817	1.61	Uncharacterized protein
allA	2.05	Ureidoglycolate lyase	rpsT	1.60	30S ribosomal protein S20 5231
PA4792	2.05	Uncharacterized protein	PA2419	1.59	Probable hydrolase
PA4880	2.04	Probable bacterioferritin	PA2567	1.59	Uncharacterized protein
PA5535	2.04	Uncharacterized protein	PA5231	1.59	Probable ATP-binding/permease fusion ABC transporter
PA5534	2.02	Uncharacterized protein	lecA	1.58	PA-I galactophilic lectin
PA0536	2.02	Uncharacterized protein	narG	1.58	Respiratory nitrate reductase alpha chain
mmsA	1.97	Methylmalonate-semialdehyde dehydrogenase [acylating]	PA0915	1.58	Uncharacterized protein
PA3880	1.95	Uncharacterized protein	PA3757	1.57	Probable transcriptional regulator
PA3187	1.95	Probable ATP-binding component of ABC transporter	PA1166	1.56	Uncharacterized protein
nosZ	1.94	Nitrous-oxide reductase	PA1048	1.56	Probable outer membrane
PA4571	1.93	Probable cytochrome c			
PA3417	1.90	Probable pyruvate dehydrogenase E1 component, alpha subunit			
PA2291	1.88	Porin			

PA1880	1.87	Probable oxidoreductase
nirS	1.87	Nitrite reductase
PA2024	1.87	Uncharacterized protein PA2024
katA	1.85	Catalase

5.5.2.2 Downregulated proteins in testosterone treatment

AmpC, a chromosomally encoded protein involved in intrinsic resistance in *P. aeruginosa* [312], was downregulated by 1.94-fold change (Table 5.6). Again, there was evidence of down-regulated T3SS proteins including PscH and PscB which were downregulated by two and 1.5-fold, respectively. Both proteins are type III export apparatus proteins [1032].

Table 5.6 Proteins downregulated in response to testosterone, unique proteins are shaded in grey

Gene names	Max FC	Description	Gene names	Max FC	Description
priA	-105.36	Primosomal protein N'	PA3866	-2.23	Pyocin protein
PA5487	-9.45	Uncharacterized protein	PA5149	-2.22	Uncharacterized protein
PA2550	-7.06	Probable acyl-CoA dehydrogenase	PA2393	-2.19	Probable dipeptidase
fecA	-6.61	Fe (III) dicitrate transport protein FecA	phzC1	-2.19	Phospho-2-dehydro-3-deoxyheptonate aldolase
PA2452	-4.84	Uncharacterized protein	phuT	-2.14	Heme-transport protein, PhuT
PA2451	-4.26	Uncharacterized protein	PA1699	-2.14	Uncharacterized protein
PA1490	-4.19	Probable transcriptional regulator	PA3310	-2.06	Uncharacterized protein
pchR	-3.93	Regulatory protein PchR	tyrS1	-2.06	Tyrosine--tRNA ligase 1
hemO	-3.90	Heme oxygenase	tonB	-2.05	Protein TonB
PA2412	-3.82	Uncharacterized protein	PA2381	-2.04	Uncharacterized protein
pvdR	-3.72	PvdR	pscH	-2.03	Type III export protein PscH
PA3268	-3.56	Probable TonB-dependent receptor	PA0704	-2.03	Probable amidase
pvdL	-3.55	PvdL	PA4675	-2.03	Probable TonB-dependent receptor
pvdH	-3.48	L-2,4-diaminobutyrate:2-ketoglutarate 4-aminotransferase, PvdH	argA	-1.95	Amino-acid acetyltransferase
pvdD	-3.48	Pyoverdine synthetase D	ampC	-1.94	Beta-lactamase
pvdE	-3.47	Pyoverdine biosynthesis protein PvdE	PA0046	-1.94	Uncharacterized protein
PA4643	-3.46	Uncharacterized protein	PA1302	-1.90	Probable heme utilization protein
PA4514	-3.45	Probable outer membrane receptor for iron transport	PA4373	-1.90	Uncharacterized protein
phuV	-3.41	Hemin import ATP-binding protein HmuV	PA5445	-1.87	Probable coenzyme A transferase
pvdA	-3.34	L-ornithine N(5)-monooxygenase	PA3317	-1.86	Uncharacterized protein
PA2034	-3.31	Uncharacterized protein	PA3889	-1.82	Probable binding protein component of ABC transporter
pvdJ	-3.29	PvdJ	PA4615	-1.78	Probable oxidoreductase
PA2402	-3.29	Probable non-ribosomal peptide synthetase	PA1377	-1.76	Uncharacterized protein
PA2033	-3.26	Uncharacterized protein	PA3783	-1.76	Uncharacterized protein
PA1365	-3.20	Probable siderophore receptor	anmK	-1.74	Anhydro-N-acetylmuramic acid kinase
pvdF	-3.19	Pyoverdine synthetase F	PA1068	-1.74	Probable heat shock protein (Hsp90 family)
pchG	-3.12	Pyochelin biosynthetic protein PchG	PA0938	-1.73	Uncharacterized protein
phuR	-3.09	Heme/hemoglobin uptake outer membrane receptor PhuR	PA4317	-1.72	Uncharacterized protein
PA2411	-3.09	Probable thioesterase	PA1315	-1.68	Probable transcriptional regulator
pchF	-3.07	Pyochelin synthetase	prpD	-1.66	Propionate catabolic protein PrpD
hscB	-3.03	Co-chaperone protein HscB homolog	icmP	-1.65	Insulin-cleaving metalloproteinase outer membrane protein
pvdP	-2.82	PvdP	tadA	-1.64	tRNA-specific adenosine deaminase
pchE	-2.77	Dihydroaeruginoic acid synthetase	PA4372	-1.63	Uncharacterized protein
pvdQ	-2.77	Acyl-homoserine lactone acylase PvdQ	PA0086	-1.63	Uncharacterized protein
pchB	-2.68	Isochorismate pyruvate lyase	ppgL	-1.63	Periplasmic gluconolactonase, PpgL
PA2204	-2.66	Probable binding protein component of ABC transporter	PA0663	-1.61	Uncharacterized protein
PA4709	-2.66	Probable hemin degrading factor	PA1494	-1.61	Uncharacterized protein

rcpC	-2.66	RcpC	popD	-1.60	Translocator outer membrane protein PopD
ampDh2	-2.59	AmpDh2	PA1062	-1.59	Uncharacterized protein
pvdO	-2.57	PvdO	PA1832	-1.57	Probable protease
PA0079	-2.53	Uncharacterized protein	PA5022	-1.57	Uncharacterized protein
piuC	-2.53	PKHD-type hydroxylase PiuC	PA4491	-1.57	Uncharacterized protein
pirA	-2.49	Ferric enterobactin receptor PirA	PA4440	-1.56	Uncharacterized protein
pvdN	-2.49	PvdN	fpvB	-1.56	Second ferric pyoverdine receptor FpvB
PA4705	-2.45	Uncharacterized protein	pscL	-1.55	Type III export protein PscL
fumC2	-2.42	Fumarate hydratase class II 1	PA0460	-1.55	Uncharacterized protein
PA4200	-2.40	Uncharacterized protein	PA0840	-1.52	Probable oxidoreductase
sodA	-2.29	Superoxide dismutase [Mn]	exoT	-1.52	Exoenzyme T
hasR	-2.29	Heme uptake outer membrane receptor HasR	pscB	-1.50	Type III export apparatus protein
rrmA	-2.29	rRNA methyltransferase	PA4390	-1.50	Uncharacterized protein

5.5.3 Biofilm assay to investigate the impact of on *P. aeruginosa*

Testosterone appears to have significant impact on the reduction of reference strain PAO1 at 40 nM treatment (P=0.049). This is in contrast to the increased growth that testosterone exerted on PAO1 previously. No other statistically significant relationship observed between testosterone and control groups. However, all concentrations showed a reduction compared to the control (Figure 5.15-A). This reduction was also observed for PA14 (Figure 5.15-B) and 133098 (Figure 5.15-C).

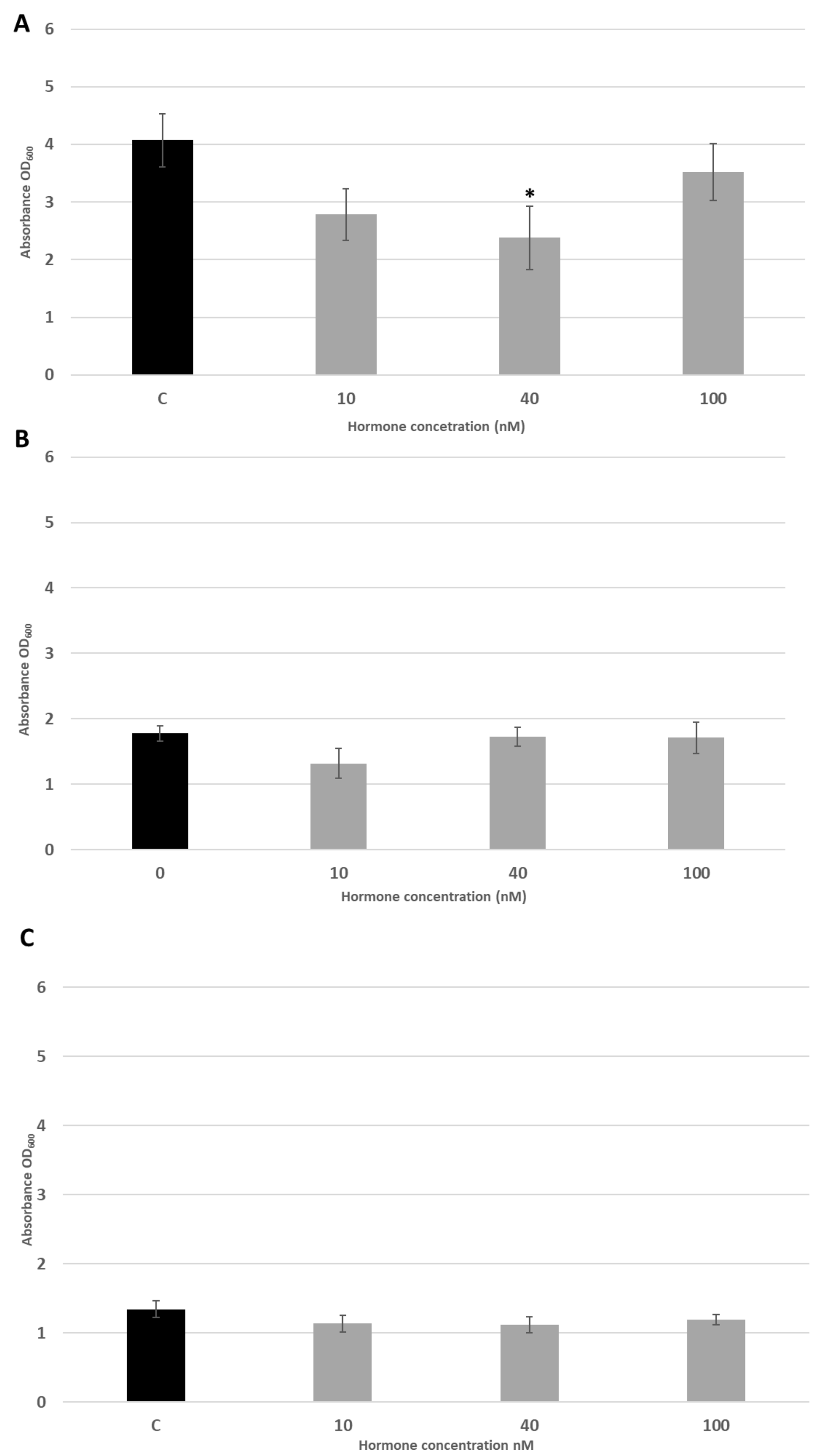


Figure 5.15 The total biomass produced post CV staining in the control (0) and treatment groups of 10 nM,40 nM and 100 nM of testosterone. **A)** treatment of PAO1 with testosterone resulted in a statistically significant reduction between the control (C) and 40nM treatment, * <P=0.050. **B)** treatment of PA14 strain resulted in no significant differences, P = 0.474 **C)** No significant differences observed in clinical isolate 133098, P= 0.888. Statistical analysis was conducted with equal variance test (Brown-Forsythe)

5.5.4 Isolate-dependent reduction of pyocyanin by testosterone

Testosterone showed the highest reduction of pyocyanin amongst all hormones as five isolates were significantly reduced <P=0.05 in comparison to the control (Figure 5.16). Strains and isolates PA14,133099, 133106 all registered P=0.002 while isolates 133117 (P= 0.020) and 13317 (P=0.028).

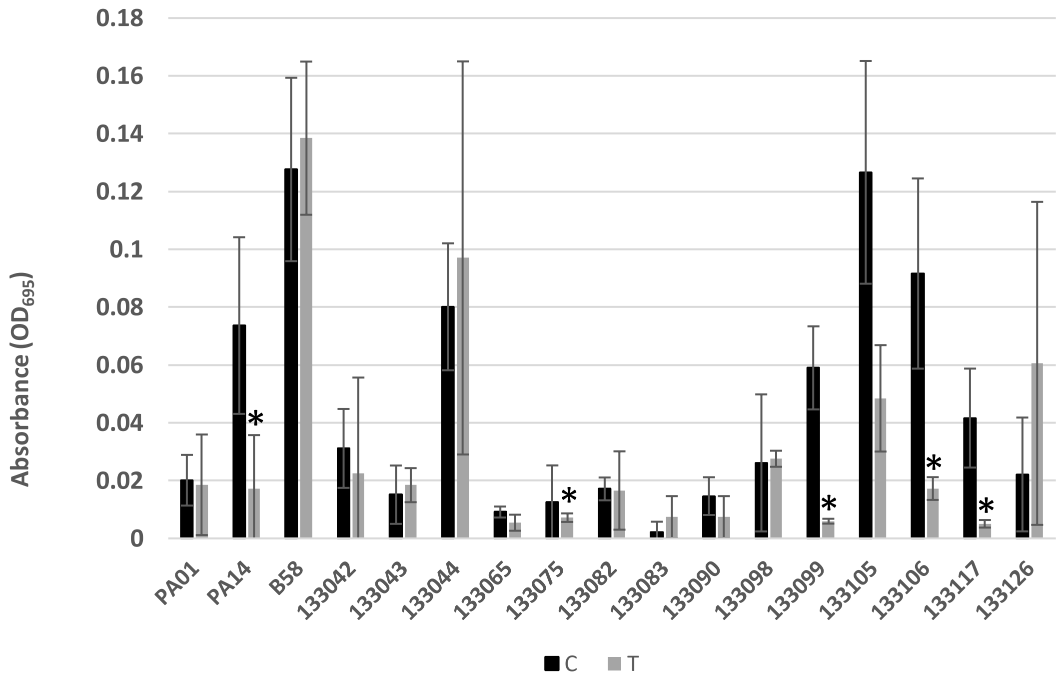


Figure 5.16 Mean pyocyanin calculated of three biological isolates in the control group (black) and 10 nM testosterone (dark grey) of UTI P. aeruginosa. Significant differences observed in 6 isolates. * denotes P<0.050

5.6 The Impact of progesterone on *P. aeruginosa*

5.6.1 The Influence of 20nM progesterone on growth rate

The impact of progesterone was also investigated on growth rates of the three representative isolates PAO1, PA14 and 133098 (Figures 5.17 A-C).

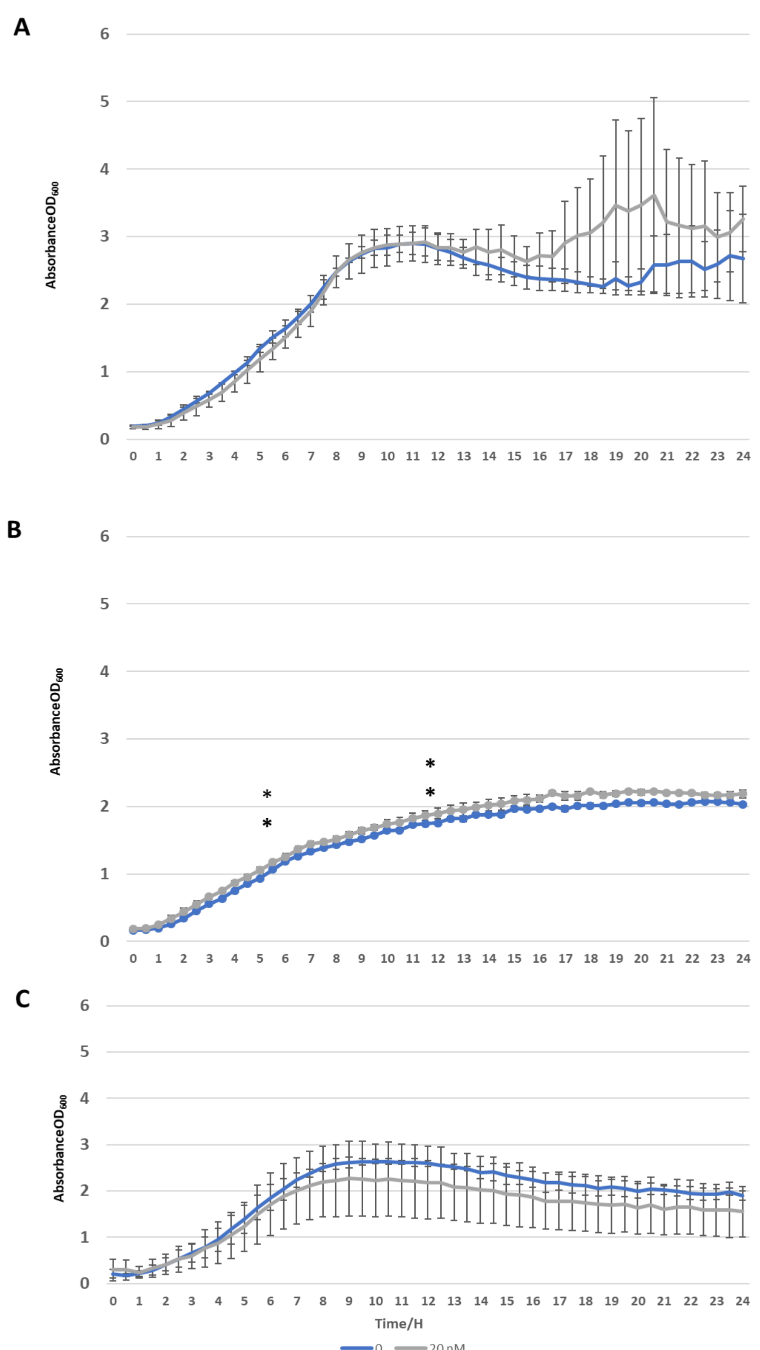


Figure 5.17 Growth pattern of three strains with control lacking hormones and 20nM of progesterone treatment, measured at A600nM optical density. A) The mean of the three biological replicates was calculated and showed no statistical significance P = 0.400 at 24 h. B) Treatment of progesterone resulted in significant differences at the 6th and 12th time points as the growth increased for the strain PA14 at ** P<0.001 but reached P=0.400 at 24h. C) Treatment with progesterone to isolate 133098 yielded no statistical significance at P= 0.230. Statistical tests performed by the Holm-Sidak method and Brown-Forsyth test

5.6.2 Proteomics of progesterone

5.6.2.1 Upregulated proteins in response to progesterone treatment

180 proteins displayed higher abundance in progesterone, 143 of these had a significant P value. Of these 69 were uniquely up in progesterone (Table 5.7). SodB is superoxide dismutase that has been linked to antibiotic tolerance [1033] and is downregulated by 1.6-fold change. NarL which is a two component regulator associated with nitrate sending and has been linked to increased virulence [1034], SigX is an ECF sigma factor involved in regulating responses to the environment [1035].

Table 5.7 Proteins upregulated in response to progesterone, unique proteins are shaded in grey

Gene names	Max FC	Description	Gene names	Max FC	Description
PA0880	Infinity	Probable ring-cleaving dioxygenase	PA1518	1.78	5-hydroxyisourate hydrolase
amiE	14.04	Aliphatic amidase	PA4880	1.78	Probable bacterioferritin
nirL	8.27	Protein NirL	lap	1.78	Aminopeptidase
katE	6.47	Catalase HPII	PA1517	1.77	Uncharacterized protein
ccpA	6.39	Cytochrome c551 peroxidase	PA4200	1.77	Uncharacterized protein
PA4928	5.10	UPF0313 protein PA4928	PA4520	1.77	Probable chemotaxis transducer
PA4131	4.05	Probable iron-sulfur protein	PA3602	1.77	Uncharacterized protein
PA1254	3.93	Probable dihydrodipicolinate synthetase	PA4571	1.77	Probable cytochrome c
PA0510	3.85	Probable uroporphyrin-III c-methyltransferase	PA3347	1.76	Uncharacterized protein
PA0144	3.42	Uncharacterized protein	PA1922	1.74	Probable TonB-dependent receptor
PA3119	3.25	Uncharacterized protein	PA1673	1.73	Bacteriohemerythrin 2920
PA2169	3.04	Uncharacterized protein	azu	1.73	Azurin
PA3880	3.03	Uncharacterized protein	PA4349	1.72	Uncharacterized protein
nosZ	3.02	Nitrous-oxide reductase	PA0743	1.72	NAD-dependent L-serine dehydrogenase
PA5359	3.01	Uncharacterized protein	PA1140	1.71	Uncharacterized protein
PA5532	2.97	Uncharacterized protein	PA1500	1.71	Probable oxidoreductase
PA0122	2.93	Uncharacterized protein	PA1041	1.71	Probable outer membrane protein
PA3416	2.91	Probable pyruvate dehydrogenase E1 component, beta chain	ldh	1.70	Leucine dehydrogenase
PA5535	2.87	Uncharacterized protein	tesA	1.70	Esterase TesA
PA3417	2.86	Probable pyruvate dehydrogenase E1 component, alpha subunit	PA3628	1.69	S-formylglutathione hydrolase
PA2481	2.74	Uncharacterized protein	PA2633	1.69	Uncharacterized protein
chiC	2.62	Chitinase	hmp	1.69	Flavohemoprotein
nirS	2.56	Nitrite reductase	acsA2	1.69	Acetyl-coenzyme A synthetase 2
PA4913	2.52	Probable binding protein component of ABC transporter	PA0269	1.69	Alkyl hydroperoxide reductase AhpD

PA5534	2.47	Uncharacterized protein	lecA	1.68	PA-I galactophilic lectin
gbuA	2.46	Guanidinobutyrase	PA2567	1.68	Uncharacterized protein
PA1665	2.39	Uncharacterized protein pscB	PA3190	1.67	Probable binding protein component of ABC sugar transporter
PA1921	2.39	Uncharacterized protein	PA1880	1.67	Probable oxidoreductase
mmsA	2.39	Methylmalonate-semialdehyde dehydrogenase [acylating]	PA4328	1.67	Uncharacterized protein
ccoP2	2.38	Cbb3-type cytochrome c oxidase subunit	PA4139	1.65	Uncharacterized protein
napA	2.38	Periplasmic nitrate reductase	PA1516	1.64	Uncharacterized protein
PA1881	2.30	Probable oxidoreductase	narL	1.64	Two-component response regulator NarL
PA3922	2.30	Uncharacterized protein PA3922	PA3187	1.64	Probable ATP-binding component of ABC transporter
PA2266	2.26	Probable cytochrome c	acsA1	1.64	Acetyl-coenzyme A synthetase 1
katA	2.25	Catalase	PA3458	1.64	Probable transcriptional regulator
fusB	2.23	Elongation factor G 2	PA1789	1.64	Uncharacterized protein
allA	2.20	Ureidoglycolate lyase	dctP	1.63	C4-dicarboxylate-binding periplasmic protein DctP
nirF	2.18	Protein NirF	PA3858	1.62	Probable amino acid-binding protein
gcvP2	2.15	Glycine dehydrogenase (decarboxylating) 2	rbsB	1.62	Binding protein component of ABC ribose transporter
PA1551	2.15	Probable ferredoxin	PA3723	1.61	Probable FMN oxidoreductase
pcaF	2.14	Beta-ketoadipyl-CoA thiolase	sdaA	1.60	L-serine dehydratase
PA3924	2.11	Probable medium-chain acyl-CoA ligase	sodB	1.60	Superoxide dismutase [Fe]
PA1760	2.06	Probable transcriptional regulator	PA2841	1.59	Probable enoyl-CoA hydratase/isomerase
PA4792	2.02	Uncharacterized protein	PA4333	1.59	Fumarate hydratase class I
phzF	2.02	Trans-2,3-dihydro-3-hydroxyanthranilate isomerase	PA5027	1.58	Uncharacterized protein
ccoN2	2.02	Cytochrome c oxidase, cbb3-type, CcoN subunit	PA4063	1.58	Uncharacterized protein
PA0747	2.01	Probable aldehyde dehydrogenase	arcC	1.58	Carbamate kinase
dksA	1.99	RNA polymerase-binding transcription factor DksA	PA0959	1.57	Uncharacterized protein
PA0586	1.99	Uncharacterized protein	zwf	1.57	Glucose-6-phosphate 1-dehydrogenase
leuC	1.98	3-isopropylmalate dehydratase large subunit	PA1202	1.56	Probable hydrolase
coxB	1.97	Cytochrome c oxidase subunit 2	arcA	1.56	Arginine deiminase
PA2920	1.95	Probable chemotaxis transducer	PA4352	1.55	Uncharacterized protein
PA0794	1.93	Probable aconitate hydratase	PA0459	1.55	Probable ClpA/B protease ATP binding subunit
nirQ	1.92	Denitrification regulatory protein NirQ	PA1287	1.54	Glutathione peroxidase
PA2024	1.92	Uncharacterized protein PA2024	glgB	1.54	1,4-alpha-glucan branching enzyme GlgB
pyrQ	1.92	Dihydroorotase	PA3972	1.54	Probable acyl-CoA dehydrogenase
sbp	1.91	Sulfate-binding protein	PA0915	1.53	Uncharacterized protein
PA0250	1.91	Uncharacterized protein	sigX	1.53	ECF sigma factor SigX
phzG1	1.89	Probable pyridoxamine 5'-phosphate oxidase	PA4787	1.53	Probable transcriptional regulator
PA4611	1.89	Uncharacterized protein	PA5540	1.52	Uncharacterized protein
PA3923	1.88	Uncharacterized protein	PA3712	1.52	Uncharacterized protein

PA5475	1.87	Acetyltransferase PA5475	acnA	1.52	Aconitate hydratase A
PA2433	1.86	Uncharacterized protein	ackA	1.51	Acetate kinase
leuD	1.85	3-isopropylmalate dehydratase small subunit	fdhE	1.51	Protein FdhE homolog
PA1746	1.84	Uncharacterized protein	fdnH	1.51	Nitrate-inducible formate dehydrogenase, beta subunit
PA4502	1.83	Probable binding protein component of ABC transporter	PA5047	1.51	Uncharacterized protein
ccoO2	1.83	Cytochrome c oxidase, cbb3-type, CcoO subunit	rimO	1.51	Ribosomal protein S12 methylthiotransferase RimO
phaC1	1.82	Poly(3-hydroxyalkanoic acid) synthase 1	PA4463	1.51	Uncharacterized protein
PA3415	1.82	Dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase complex	chpB	1.50	Protein-glutamate methylesterase
PA5545	1.81	Uncharacterized protein	bfrB	1.50	Bacterioferritin
PA2504	1.79	Uncharacterized protein	PA3618	1.50	Uncharacterized protein
PA1470	1.79	Probable short-chain dehydrogenase			

5.6.2.2 Downregulated proteins in response to progesterone treatment

155 proteins were down in abundance to progesterone and of these 104 had a significant P value (Table 5.8). 20 of these proteins are significantly reduced in progesterone only. These include AmbE which is lowered by 1.62-fold-change. Amb is L-2-Amino-4-methoxy-*trans*-3-butenoic acid that is a potent *P. aeruginosa* toxin involved in the inhibition of *Bacillus spp* and *E. coli* growth [1036]. OpmQ contributes to pyoverdine transport and was reduced by 1.7-fold change [275]. Ohr is an organic hydroperoxide resistance protein [655], [1037], which is downregulated by 1.7-fold.

Table 5.8 Proteins downregulated in response to progesterone, unique proteins are shaded in grey

Gene names	Max FC	Description	Gene names	Max FC	Description
pchG	-14.01	Pyochelin biosynthetic protein PchG	PA1791	-2.31	Uncharacterized protein
pchF	-12.22	Pyochelin synthetase	PA5445	-2.28	Probable coenzyme A transferase
pchE	-10.47	Dihydroaeruginoic acid synthetase	sodA	-2.27	Superoxide dismutase [Mn]
pchB	-10.42	Isochorismate pyruvate lyase	PA0277	-2.21	Uncharacterized protein
fecA	-9.98	Fe(III) dicitrate transport protein FecA	PA2196	-2.20	Probable transcriptional regulator
pchR	-7.96	Regulatory protein PchR	phuT	-2.17	Heme-transport protein, PhuT
pvdE	-7.21	Pyoverdine biosynthesis protein PvdE	clpV1	-2.16	Protein ClpV1
PA2452	-6.36	Uncharacterized protein	PA3850	-2.12	Uncharacterized protein
fabH	-6.09	3-oxoacyl-[acyl-carrier-protein] synthase 3	PA4675	-2.11	Probable TonB-dependent receptor
pvdH	-5.51	L-2,4-diaminobutyrate:2-ketoglutarate 4-aminotransferase, PvdH	PA3054	-2.09	Uncharacterized protein
pvdL	-5.48	PvdL	piuC	-2.08	PKHD-type hydroxylase PiuC
pfeR	-5.45	Transcriptional activator protein PfeR	fptA	-2.02	Fe (3+)-pyochelin receptor
pvdD	-5.36	Pyoverdine synthetase D	tonB	-2.00	Protein TonB
PA2034	-5.24	Uncharacterized protein	PA1832	-1.99	Probable protease
PA2402	-5.04	Probable non-ribosomal peptide synthetase	PA0938	-1.94	Uncharacterized protein
pvdR	-4.88	PvdR	PA0046	-1.94	Uncharacterized protein
PA4514	-4.67	Probable outer membrane receptor for iron transport	PA3889	-1.90	Probable binding protein component of ABC transporter
pvdA	-4.65	L-ornithine N(5)-monooxygenase	fpvB	-1.87	Second ferric pyoverdine receptor FpvB
PA2033	-4.40	Uncharacterized protein	PA2212	-1.86	4-hydroxythreonine-4-phosphate dehydrogenase 2
PA2411	-4.36	Probable thioesterase	PA5149	-1.85	Uncharacterized protein
PA3330	-4.34	Probable short chain dehydrogenase	nrdD	-1.84	Class III (Anaerobic) ribonucleoside-triphosphate reductase subunit, NrdD
pvdJ	-4.24	PvdJ	PA1377	-1.84	Uncharacterized protein
pvdP	-4.23	PvdP	PA5022	-1.81	Uncharacterized protein
PA2451	-4.19	Uncharacterized protein	tadA	-1.81	tRNA-specific adenosine deaminase
hemO	-4.17	Heme oxygenase	PA1601	-1.77	Probable aldehyde dehydrogenase
PA2384	-4.10	Uncharacterized protein	PA0704	-1.75	Probable amidase
PA1365	-4.09	Probable siderophore receptor	PA0045	-1.74	Uncharacterized protein
PA3268	-3.78	Probable TonB-dependent receptor	ohr	-1.73	Organic hydroperoxide resistance protein
pvdQ	-3.64	Acyl-homoserine lactone acylase PvdQ	PA4373	-1.73	Uncharacterized protein
pvdF	-3.61	Pyoverdine synthetase F	PA1068	-1.73	Probable heat shock protein (Hsp90 family)
phuR	-3.57	Heme/hemoglobin uptake outer membrane receptor PhuR	PA4615	-1.71	Probable oxidoreductase
pirA	-3.47	Ferric enterobactin receptor PirA	opmQ	-1.70	Probable outer membrane protein

fumC2	-3.11	Fumarate hydratase class II 1	PA3768	-1.70	Probable metallo-oxidoreductase
PA2109	-3.10	Uncharacterized protein	PA5113	-1.68	Uncharacterized protein
PA2412	-3.09	Uncharacterized protein	PA0086	-1.68	Uncharacterized protein
PA3866	-3.07	Pyocin protein	exsC	-1.67	Exoenzyme S synthesis protein C
pvdN	-3.00	PvdN	icmP	-1.66	Insulin-cleaving metalloproteinase outer membrane protein
phuV	-2.95	Hemin import ATP-binding protein HmuV	PA1494	-1.63	Uncharacterized protein
PA4705	-2.94	Uncharacterized protein	ambE	-1.62	AmbE
ampDh2	-2.88	AmpDh2	PA0851	-1.61	Uncharacterized protein
pvdO	-2.79	PvdO	PA0660	-1.61	Uncharacterized protein
fiuA	-2.79	Ferrichrome receptor FiuA	PA4491	-1.60	Uncharacterized protein
PA4709	-2.77	Probable hemin degrading factor	PA1132	-1.60	Uncharacterized protein
PA0047	-2.69	Uncharacterized protein	PA3891	-1.59	Probable ATP-binding component of ABC transporter
PA2204	-2.67	Probable binding protein component of ABC transporter	PA4317	-1.59	Uncharacterized protein
PA3318	-2.66	Uncharacterized protein	PA4390	-1.58	Uncharacterized protein
PA0079	-2.64	Uncharacterized protein	ansA	-1.56	L-asparaginase I
PA2550	-2.60	Probable acyl-CoA dehydrogenase	hutH	-1.56	Histidine ammonia-lyase
PA2393	-2.56	Probable dipeptidase	popD	-1.54	Translocator outer membrane protein PopD
hasR	-2.50	Heme uptake outer membrane receptor HasR	gbt	-1.53	Glycine betaine transmethylase
PA1302	-2.50	Probable heme utilization protein	PA3729	-1.52	Uncharacterized protein
PA2528	-2.41	Probable Resistance-Nodulation-Cell Division (RND) efflux membrane fusion protein	ubiG	-1.52	Ubiquinone biosynthesis O-methyltransferase

5.6.3 Biofilm assay assessment of the effects of progesterone on biofilm formation

A reduction in biofilm biomass was observed in the presence of progesterone in all three isolates shown (Figure 5.18 A-C). However, no statistical significance was determined.

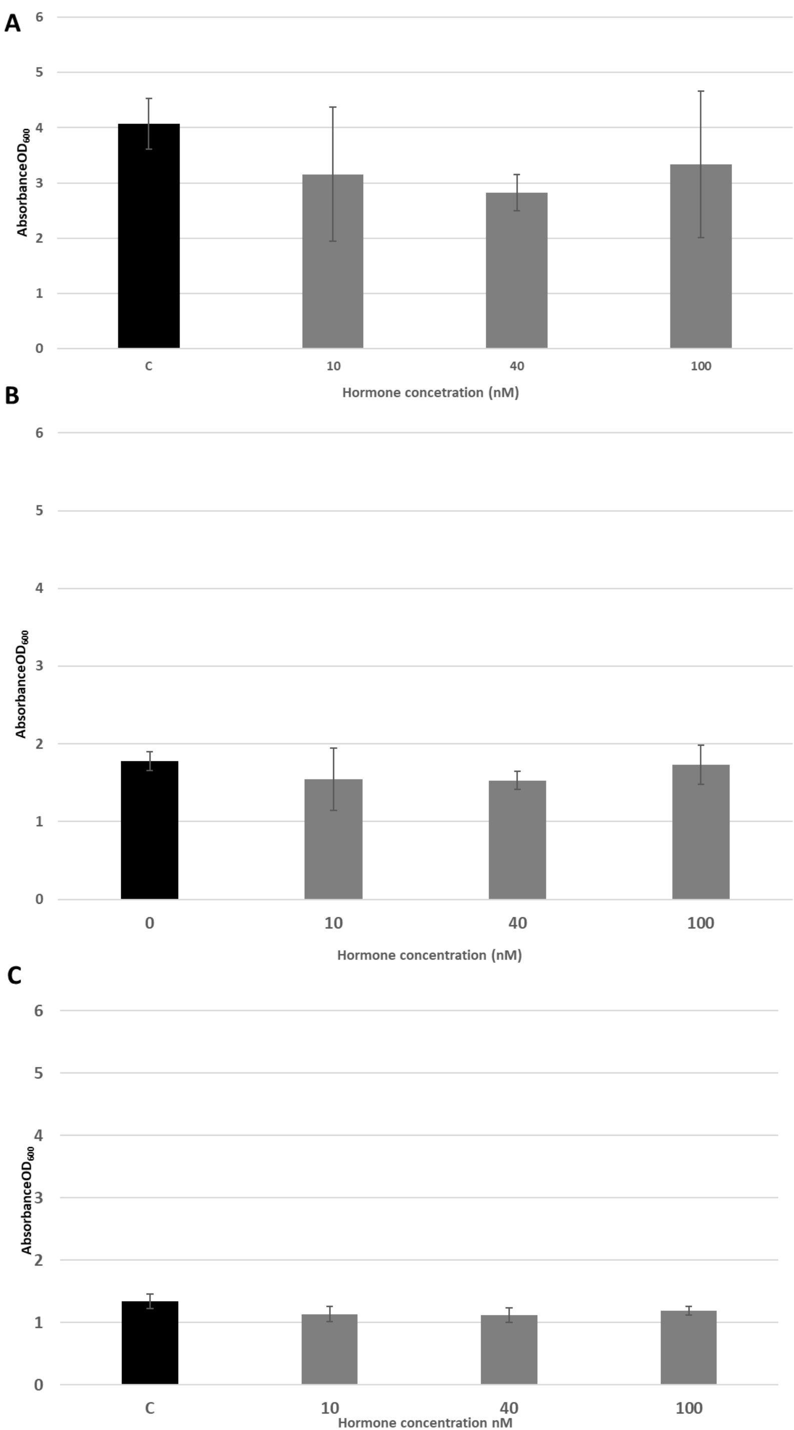


Figure 5.18 The total biomass produced post CV staining in the control (C) and treatment groups of 10 nM,40 nM and 100 nM of progesterone. A) treatment of PAO1 with progesterone produced no significant differences P = 0.582. B) treatment of PA14 strain resulted in no significant differences P = 0.774 C) No significant differences observed in clinical isolate 133098, P = 0.928. Statistical analysis was conducted with equal variance test (Brown-Forsythe)

5.6.4 Minimal impact of progesterone on pyocyanin production

Treatment with progesterone reduced the amount of pyocyanin significantly in the three strains and isolates (Figure 5.19). The reduction of pyocyanin was the most significantly observed in LESB58 strain and clinical isolate 133083 with a P value of < 0.001. However, in isolate 133098, there was no change in pyocyanin production, and this was consistent the proteomics results.

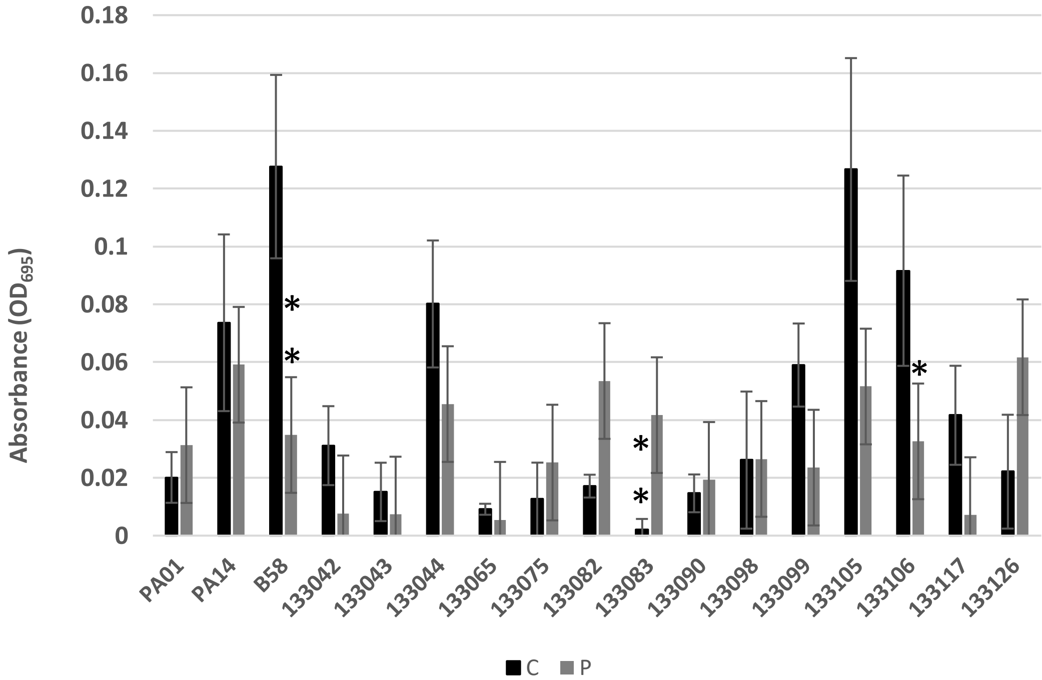


Figure 5.19 Mean pyocyanin calculated of 3 biological isolates of UTI P. aeruginosa compared between the controls (black) and treatment group of 10 nM progesterone (Dark grey). Significant differences observed in comparison in 3 strains and isolates. * denotes P<0.050 and ** denotes P≤ 0.001

5.7 Discussion

Sexual dimorphism exists in relation to susceptibility to viral, parasitic and microbial infections [929], [1038], [1039]. There is growing evidence that sex hormones play a prominent role in affecting such infections in animal infection models and humans [904], [905], [919], [930]. The impact of hormones on bacterial infections has been largely attributed to immune modulation effects. However, evidence of direct impacts on the *P. aeruginosa* PAO1 reference strain has been reported as oestradiol induced mucoidy and upregulated genes involved in its conversion pathway [953]. UTI prevalence is higher in reproductive-age women than men, in part, due to anatomical differences. However, sex hormones have been shown to play a role in modulating infections involving UPEC. As such, diminishing hormone levels in males and particularly, females, may have a negative impact as age progresses past the reproductive stages of life. In this chapter, we sought to assess the direct impact of sex hormones oestradiol, testosterone and progesterone on *P. aeruginosa* in LB and an artificial urine model by conducting several *in vitro* experiments including growth rates, proteomic analysis, biofilm and pyocyanin production.

Our results suggest that hormones do not reproducibly affect growth rates. Although significant differences were determined in the impact of testosterone on the reference strain PAO1 and progesterone on the reference strain PA14 at certain time points, these differences are minimal and do not affect the overall growth rates.

Treatments with sex hormones in AUM inoculated with *P. aeruginosa* revealed common responses of the bacteria in the presence of all three hormones. The proteins involved in iron-uptake were universally down in abundance, with few exceptions. Iron acquisition mechanisms are essential for survival of bacterial pathogens and to invade and colonise the host [1040]. In this chapter, the proteomics data demonstrated that iron chelating systems produced by the opportunistic pathogen are reduced in the presence of the sex hormones oestradiol, testosterone and progesterone. One of those systems involves the production of siderophore pyoverdine, which is produced in response to severe iron starvation in the environment [275]. Urine and the urinary tract are scarce in iron [743], [1041], thus, we used the AUM model to identify whether sex hormones alter the proteomic

profile of UTI *P. aeruginosa* clinical isolate 133098. Approximately 20 proteins are involved in pyoverdine synthesis and these span the inner bacterial membrane, the periplasm and the outer membrane. Examples of these proteins include PvdE, PvdH, PvdL, PvdR, PvdJ, PvdP PvdN, PvdO and opmQ [969]. Upon conducting proteomic analysis, it was observed that the sex hormones used in this study, in particular, oestradiol reduced pyoverdine synthesis. Proteins initiating the process of pyoverdine production such as PvdA, PvdJ and PvdD were all down in abundance. In addition, PvdQ, PvdO and PvdN, which participate in the production and maturation of pyoverdine, were all less abundant in all hormone conditions, particularly, oestradiol treated AUM. The transport complex which includes PvdR was downregulated by 33-fold, 3.7-fold, 4.8-fold in each of oestradiol, testosterone and progesterone, respectively. These findings, together, suggest that oestradiol, and to a lesser degree progesterone and testosterone, play a substantial role in reducing the production of pyoverdine-associated proteins.

Pyochelin synthesis is produced when more iron is available in the surrounding environment relative to the state of which pyoverdine is produced [977]. Reduction of proteins associated with pyochelin production was observed in AUM treated with sex hormones. In order for pyochelin to be produced, it requires PchABCD and the cytoplasmic transcriptional regulator PchR. The levels of these proteins were reduced in the presence of, particularly, oestradiol and progesterone. Haem uptake from the host is conducted via two different pathways, the Phu and HasR systems [995]. In the former, the TBDR PhuR extracts heme directly from haemoproteins, while in the latter, a hemophore with the protein Has extracts haem from haemoproteins to form a complex, which is received by the HasR receptor [996], [997]. Our results show that treatment with all hormones reduced the abundance of all proteins involved in haem uptake. In addition, further reduction of intracellular proteins involved in processing haem such as PhuS and the heme oxygenase are reduced. Furthermore, all sex hormones in this study reduced fumarase C, a protein that is part of the tricarboxylic acid cycle, this protein is produced in response to iron starvation and is involved in alginate biosynthesis and conversion to mucoidy [1026]. This may indicate that,

unlike in CF, the presence of oestradiol in menstruating women may supress *P. aeruginosa* mucoidy [953].

P. aeruginosa can hijack siderophores made by another microorganism to colonise and expand in different ecological niches [275]. *chtA* gene encodes a TDBR to acquire cognate xenosiderophores aerobactin, rhizobactin, and schizokinen, potentially in setting of polymicrobial infection such as CAUTI. [964] All sex hormones in this study reduced the expression of ChtA protein. PfeR is a part of a two component sensor kinase system with PfeS which uptakes enterobactin and imports to the cellular receptor PfeA [1025]. PfeR was reduced significantly in oestradiol treated cultures, thus, this may affect the ability of *P. aeruginosa* to acquire any of these xenosiderophores in UTI-related conditions in polymicrobial infection, potentially providing other UTI pathogens such as UPEC to outcompete *P. aeruginosa* for the hosts limited iron substances.

To the best of our knowledge, our findings are the first to report in-depth proteomic profile of iron acquisition mechanisms of a *P. aeruginosa* clinical isolate in an *in vitro* model mimicking UTI environment in the presence of hormones. The first report of the existence of pyochelin and pyoverdine in UTI *P. aeruginosa* was discussed in a study by Visca *et al*, both were reported to be present in 97.6% and 92.6% respectively in all of the 121 tested UTI isolates in poor iron medium [461]. While the previous study shows that UTI isolates are capable of producing iron-chelating proteins, our study also shows that under urine and urine-like conditions, these mechanisms are upregulated (as shown in chapter 4). However, there is a reduction in iron acquisition mechanisms upon treatment with sex hormones. Previous studies which utilised UTI *P. aeruginosa* producing pyochelin, pyoverdine and haemolysin *in vivo* mouse model demonstrated that haemolysin was involved with higher severity of infection, while the exact contribution of pyochelin and pyoverdine remained elusive [57], [58]. A study by Tielen *et al,* (2011) which examined 30 *P. aeruginosa* UTI isolates provided further evidence of the importance of pyoverdine as all isolates produced it [90]. In a study which utilised proteomics to assess the response strain PAO1 to iron starvation, all classical iron upregulation proteins of haem, pyochelin and pyoverdine were upregulated [1042]. This is in contrast to our results which show

that iron acquisition mechanisms are downregulated, which may suggest that the bioavailability of iron in AUM containing hormones is increased. This is an unusual finding and the exact method is unclear. What is also unclear is how this may affect infection *in vivo*. Our data could suggest that iron bioavailability is good and therefore the bacteria do not need to upregulate the energy intensive production of multiple iron acquisition methods. However, it could be that the hormones may play a protective role in directly reducing iron uptake mechanisms by which *P. aeruginosa* establish colonisation and invasion. This could be further investigated in the future studies by adding different levels of hormone and iron concentrations into AUM or minimal media to determine the impact on bacterial survival and growth. Furthermore, siderophore production assays or qPCR gene expression experiments under relevant conditions could also be performed.

QS mechanisms control the production of virulence factors such as phenazines, elastases and HCN. PQS is the third QS system identified in *P. aeruginosa* and can promote the synthesis of phenazines and HCN [131]. In this study, the abundance profile of PQS proteins is higher in oestradiol-treated samples. Expression of PQS molecules is associated with higher virulence as shown in an acute UTI mouse model [129]. *P. aeruginosa* with a fully functional PQS biosynthetic pathway resulted in higher virulence, tissue destruction and in severe inflammatory responses in mice. Isolates lacking PqsA and PqsH failed to produce PQS in mice and resulted in mild damage and lower bacterial load [129]. In contrast to the reduced proteome abundance of iron acquisition mechanisms, phenazines were upregulated by oestradiol. As previously discussed in chapter 3, phenazines are redox active secondary metabolites that are involved in biofilm formation and act as electron acceptors [1043], [1044]. Oestradiol appeared to increase the expression of all phenazine proteins in the PCA and pyocyanin pathways. PCA biosynthesis in dependent on enzymes encoded by highly similar operons, *phzA1* to *phzG1 (phz1)* and *phzA2* to *G2* (phz2) [155]. Pyocyanin, along with other phenazines, are produced by proteins involved in the modification of PCA by PhzM and PhzS [1042], both of which were upregulated by oestradiol. In the phenotypic pyocyanin assay, however, the response was isolate dependent. The conditions of the experiments were not

identical and further study into the link between oestradiol and pyocyanin could be performed. Additionally, proteins encoding HCN were promoted by oestradiol. HCN is toxic and produced and degraded by a number of bacterial species and it is synthesised via enzymatic decarboxylation of glycine and catalysed by a protein complex called HCN synthase [1045], [1046]. HCN synthase is encoded by the *hcnABC* operon. The expression of HCN synthase is regulated in a tight manner at both the transcriptional and post-transcriptional level [206], [1047], [1048]. The QS systems las and rhl positively regulate HCN in response to high cell density, low oxygen, glycine and iron starvation [202], [1048]. The ANR transcriptional regulator also positively regulates the *hcnABC* promoter in anaerobic conditions [1048]. HCN inhibits ciliary beating function in CF lungs [1049]. In this study, both the las and rhl QS systems were less active in AUM compared to LB and this was not altered in the presence of hormones. Therefore, these systems do not appear to be involved in HCN production in this model. It is possible that both HcnB and HcnC were increased by the female hormone oestradiol via a PQS-induced mechanism. Current understanding of the PQS mechanism is poor, however, it appears that PQS acts both dependently and independently of its cognate PqsR and can possibly target hundreds of unknown protein partners within the cell [122], [1050]. HCN optimal production levels are reached upon transition from the exponential phase into the stationary phase [1051]. HCN has been detected in paediatric CF patients and has been suggested to be identified as biomarker of early *P. aeruginosa* infection [1052], [1053]. Further experimentation *in vitro* and *in vivo* models are required to establish the role of HCN in the pathogenesis of *P. aeruginosa* UTIs. As for the role of oestradiol in promoting mucoidy [953], there is no evidence in this study that oestradiol alters alginate production in the UTI *P. aeruginosa* clinical isolate 133098. This could suggest that the conditions in CF lung and the urinary tract play a different role in the control/production of certain virulence factors. However, it may be that the impact on alginate, like many other features, is isolate dependent and although oestradiol does increase alginate in PAO1, it may not in other isolates. Based on these experiments, oestradiol may increase virulence of *P. aeruginosa* in females (at reproductive age) more than males during UTI infection.

Testosterone is present in men at 10 times higher than in women in the reproductive stages of life [1054]. One of the interesting findings in the proteomic profile is related to antimicrobial resistance in the upregulation and downregulation of OprM and AmpC, respectively. OprM is a porin protein and is a part of the efflux pump MexAB-OprM [532], [1055]. In addition to the involvement of OprM in antimicrobial resistance, the porin is also involved in QS systems (through efflux of products) and response to environmental stresses [1056], [1057]. The MexAB-OprM has been shown to be induced by oxidative stress, such as during triclosan and pentachlorophenol treatment [1058], [1059]. Based on these results, it appears that testosterone can increase OprD and thus, potentially promotes transient antimicrobial resistance. This finding is similar to a study by Li *et al,* (2015) which reported the that synthetic oestradiol promotes the expression of efflux pumps in *P. aeruginosa* [1060]. Many proteins linked to antimicrobial resistance were altered under testosterone treatment. Further studies could determine whether these protein changes result in phenotypic changes in resistance. This could then help to inform treatment choices.

Proteins involved in the T3SS export apparatus proteins PscH and PscB were decreased in abundance in the presence of testosterone, suggesting that testosterone may have a further inhibitory effect on the T3SS.

Progesterone is released in the second half of the menstruation cycle to line the endometrium and prepare reproductive age women for pregnancy [1061]. Very little is known about the interaction of progesterone and its role in bacterial infectious diseases. In addition to the reduction reported to iron acquisition mechanisms, AmbE also showed decreased abundance. This protein is a product of one of the genes in the *ambABCDE* cluster, which encodes AMB, a L-2-Amino-4-methoxy-*trans*-3-butenoic acid that is a potent *P. aeruginosa* toxin capable of inhibiting *Bacillus Spp* and *E. coli* growth [1036]. The decrease of AmbE could potentially be beneficial to *P. aeruginosa* establishing infection in a polymicrobial host settings such as CAUTI and could help to compete with UPEC. Another notable reduction is the decreased abundance of Ohr, which is an organic hydroperoxide resistance protein. The protein is regulated by the *ohrR* gene and its deletion leads to a peroxide sensitive phenotype

[1037]. Based on this, progesterone may perform a protective role against *P. aeruginosa* UTI infection. On the other hand, proteins involved in denitrification and survival in anaerobic conditions such NarL, were increased in abundance. This protein is part of classical two-component regulatory system NarX/NarL which senses nitrate. A Δ*narL* mutant has been shown to lead to a hyper-swarming motile phenotype, promoting biofilm formation [1034]. Thus, the impact of progesterone on biofilm formation could be further investigated in *P. aeruginosa* mutants lacking *narL* to assess its potential role in virulence. SigX is another important protein that was increased in response to progesterone. The protein is an ECF sigma factor which contributes to regulating responses to the environment. SigX mutants exhibit reduced motility, surface attachment and biofilm formation [1035]. Therefore, the role of progesterone on *P. aeruginosa* biofilm formation is unclear and this was consistent with the biofilm microscopy results.

Biofilm formation plays a vital role in virulence of *P. aeruginosa* [129], [462], [683]. Again, variable results were obtained in terms of the isolate type or the sex hormone in use. Upon conducting hormone treatments on biofilms with variable concentrations of hormone (10nM, 40nM and 100 nM), only one significant reduction was observed across the isolates used (PA14 treated with 40nM of testosterone). Thus, this was investigated further with confocal microscopy with the same method utilised in chapter three. No significant reductions in the biomass formed, maximum thickness, surface area, roughness coefficient and surface to biofilm ratio was observed. in the future, a study of castrated male mice and ovariectomised female mice supplemented with hormones of interest could provide more insight into CAUTI. New analytic techniques such as BiofilmX could be used to quantify and characterise biofilms measured using microscopy [1062].

In chapter four\, the proteomic profile of *P. aeruginosa* grown in either LB medium, AUM, or urine was analysed. By adding the sex hormones, the aim was to study the direct impact of hormones in AUM as a model to study *P. aeruginosa* UTI pathogenesis. This enabled the study of a specific effect and determine the role in UTIs, which, otherwise, would not be possible in urine due to endogenous variations in hormones. However, there are some limitations to this study, and these must be

considered. Although the proteomics was conducted in AUM, many of the other assays required LB and had not been optimised in AUM. Thus, for optimal comparisons, these should be conducted in AUM-containing oestradiol, testosterone and progesterone.

While this study shows that there is a direct impact of these hormones on *P. aeruginosa*, the exact mechanism should be investigated further. In particular, it would be interesting to determine whether there are certain receptors on the *P. aeruginosa* outer membrane that can facilitate cell-hormones interactions. Additionally, since the proteomics assay was conducted *in vitro,* this study does not consider potential interactions between hormone receptors in the bladder and the kidney to *P. aeruginosa* UTI infections. For instance, ERα, an oestradiol receptor has been shown to be present in kidney and affects immunes responses to UPEC if supplemented by the agonist (activator) PPT or the antagonist (suppressor) MPP in a mouse UTI model [932]. A similar finding was observed in CF mice where worse outcomes were evident in the presence of oestradiol. Oestradiol induced pro-inflammatory immune responses to *P. aeruginosa* challenge [930]. Thus, more *in vitro* and *in vivo* experiments are warranted.

The levels of these hormones are not consistent throughout life. Testosterone steadily declines in men as they progress into middle-age [1063] and the levels of oestradiol and progesterone fluctuate in reproductive-age women [333], [387]. In addition, the use of oral contraceptives containing estrogen-progestin to prevent pregnancy in premenopausal women affect these levels and therefore, may affect bacterial pathogenesis in infections [953], [1064]. Indeed, the use of oral contraceptive in Ireland has been associated with decreased severity of *P. aeruginosa* infections in CF patients and less treatment with antibiotics, suggesting that oestradiol has a protective effect [1065][953]. During pregnancy, oestrogen and progesterone levels rise constantly to reach their peak in the third trimester and drop shortly after child birth [1066]. Several studies reported the increased frequency of UTIs during pregnancy, in part due to the dilation of the urinary ureters, which could be attributed to be caused by mechanical, hormonal and physical interplay in pregnancy [1067], [1068]. The detection of *P. aeruginosa* MDR isolates have been

reported in developing countries such as Nigeria, where asymptomatic women carrying significant bacteriuria have been reported [1069]. Thus, the higher levels of oestradiol and progesterone may play a protective role. The dual impact of oestradiol and progesterone on *P. aeruginosa* could be further examined in AUM in levels resembling hormone levels in the second and third trimesters. Evidence in the literature suggests that the three hormones studied play an active role in altering the gut microbiome. The relationship between urinary sex hormone levels, UTI *P. aeruginosa* and the urinary microbiome, therefore, may provide more insight on the influence of sex hormones during infections if investigated further.

Overall, this study shows that sex hormones can have a universal influence on some systems such as iron acquisition. However, other systems may be specifically affected by only one of the three hormones. These changes may have implications for bacterial pathogenesis in UTIs. Further studies ought to be conducted *in vivo* with subsequent robust clinical trials. Sex hormones can induce virulence factors in some instances. Production of sex hormones is reduced in both male and female patients as age progresses. Thus, a greater understanding of gender-dependent host-pathogen interactions is needed and may lead to a personalized medical approach.

Chapter 6

6 General discussion and future research

P. aeruginosa is an important opportunistic pathogen that causes a wide spectrum of nosocomial infections [90], [438], [690], [707]. This opportunist bacterium is capable of forming biofilms on urinary catheters and can be highly resistant to antibiotics [58], [485], [659], [753]. One of the infections that *P. aeruginosa* can cause is UTIs, particularly CAUTIs, both of which are generally understudied in the literature and there is a paucity of information and less interest in this field in comparison to *P. aeruginosa* respiratory CF infections [90], [269], [280].

In chapter 3, I sought to develop further understanding of the genotypic and phenotypic characteristics of UTI *P. aeruginosa*, given the limited and sporadic reporting in the literature of such traits [58], [90]. The diverse genomic profile of UTI *P. aeruginosa* has been reported in a few studies either as part of a major sequencing project *of P. aeruginosa* isolates obtained from multiple type of infections or in studies solely focused on UTI isolates [659], [660], [664]. In the former, it was difficult to ascertain to which strain these isolates grouped in the *P. aeruginosa* population structure. While, in the latter, the limited number of studies indicated that *P. aeruginosa* isolates are diverse and from different environmental resources in the same country [90], [662], [664]. Thus, we obtained isolates sourced from the UK and the state of Kuwait. Our findings suggest that these UTI isolates are obtained mostly from different environmental sources. However, a few XDR isolates clustered closely together from Kuwait which could indicate that it may have been a clone isolated from the same patient, spread between patients or from a common environmental source such as the same hospital. These isolates were highly resistant to antibiotics and analysis of the sequence data revealed that many of the genes had previously been identified on plasmids. In this study, it was unclear whether the isolates carried plasmids, however, this could be analysed further. Certainly, the possibility of plasmids contributing to the spread of such resistant isolates requires further investigation. Unfortunately, epidemiologic data for these isolates from Kuwait is

severely lacking. To establish whether common or shared clones UTI patients occur, sequencing of more isolates would be required. This would be important especially given the rise of AMR in the region and the throughout the world [559], [629].

AMR is a major threat worldwide, carbapenem resistant *P. aeruginosa* has been classified as requiring an urgent need for intervention by the WHO in 2017 [36]. These results show a worrying level of extremely resistant UTI *P. aeruginosa* isolates from Kuwait and also MDR isolates from the UK. Fears of antibiotic misuse have been reported in the literature over the past few decades. In Kuwait, the seemingly generous prescription of antibiotics has dismayed healthcare providers due to the government healthcare policies [633]. Kuwait's government has recently implemented strict guidelines to combat the spread of AMR, however, the impact of these guidelines has not been reported yet [609]. Therefore, I propose a long-term surveillance study to monitor the spread of potential AMR *P. aeruginosa* UTI clones within Kuwaiti hospitals.

In terms of phenotypic studies, I have established that the majority of urinary *P. aeruginosa* isolates are motile and can perform swimming, twitching and/or swarming motility [90], [706]. Other studies have reported that isolates derived from midstream urine were more likely to be able to swim than isolates from CAUTI [90]. One of the limitations of our study is the unknown method by which our isolates were obtained, thus, it's not possible to link whether each isolate with certain motility characteristic such as swimming, was derived from midstream urine or urinary catheters. CAUTI *P. aeruginosa* isolates show reduced motility as reported by Tielen *et al* (2011) [90]. To establish whether each type of motility has a role to play infection and progression in acute and chronic UTIs, knock-out mutations of genes involved in flagella and pili genes, followed by infection of murine UTI model are required. Alternatively, a transcriptomic and/or proteomic analysis from isolates derived from each of midstream urine and catheters may provide insights into the utilisation of swimming, twitching and swarming motility. Further studies on isolates from UTIs could also use assays assessing multiple virulence factors such as rhamnolipid assays, cytotoxicity assays, quorum sensing assays, and LPS O-serogroup testing.

Furthermore, it may be more suitable to conduct these assays in AUM (or potentially urine) given the differences in the proteome established in Chapter four between laboratory rich media, urine and AUM.

P. aeruginosa is a model organism for biofilm studies. The ability to form biofilms on catheters contributes to virulence in CAUTIs [280], [706]. The biofilm CV assays *in vitro* in LB rich medium presented in this study showed variation between isolates in terms of biofilm formation ability. Consequently, further investigations sought to further identify differences in the biofilm architecture, however, no differences amongst all the isolates tested were found using confocal laser microscopy. The variation between replicates was high and therefore this brings into question the reliability of this method used for this purpose. This was followed by comparison of biofilms grown in LB rich medium, AUM and urine to establish how closely AUM reflects urine. Biofilms in AUM form flat and densely packed biofilms and therefore it was evident that biofilms in AUM were different to LB and that AUM is a more suitable medium to study urinary isolates of *P. aeruginosa* than LB. However, there were differences in the proteome of *P. aeruginosa* grown in AUM and urine. AUM could be improved and future experiments could involve the addition of components such as THP, a highly abundant glycoprotein found in urine. This has been linked to promoting *P. aeruginosa* biofilm formation [484].

Pyocyanin is a potent exotoxin released by *P. aeruginosa* and is thought to intercalate with eDNA to promote *P. aeruginosa* biofilm formation in the urinary tract [56], [669]. Based on these results, most of the isolates produced pyocyanin to varying levels. In chapter four, a simple pyocyanin assay revealed that clinical isolates produce more pyocyanin in AUM. When proteomic analysis was further conducted, we observed increased abundance of proteins in the phenazine synthesis pathway in AUM compared to urine, which throws into question how important this system is in the host urinary tract. Cole *et al,* (2018) reported that mouse urine inhibited QS and as a result, pyocyanin and rhamnolipid production were decreased [485]. To further elucidate the role of pyocyanin production in UTI pathogenesis, knockout mutations in genes such as *phzS* and *phzM* could be used to assess the ability of relevant UTI

P. aeruginosa isolates in acute CAUTI mouse models or progression on catheters. Currently, the exact mechanisms by which *P. aeruginosa* invade and colonize the urinary tract and the degree of the involvement of phenazines, is unknown and therefore, requires more research.

AUM has not been widely used to assess the phenotypes of UTI *P. aeruginosa* [753]. One of the main objectives of chapter 4 was to observe the similarities and differences of *P. aeruginosa* grown in AUM media in comparison to human urine. In this study only one clinical isolate was used to study the proteomic profile. This was due to both time and cost. However, the possibility of different responses corresponding to different clinical isolates exists based on the genotypic and phenotypic assays conducted in chapter three, thus, future studies could select more isolates to study. These could be based on the relatedness to PAO1 and/or PA14, or phenotypes of interest. Nonetheless, interesting patterns of increased and decreased protein abundance, particularly related to important virulence factors, was observed. AUM mimics urine on its impact on the *P. aeruginosa* proteome with regards to certain systems. For instance, TS33 proteins are heavily decreased in AUM and urine. This finding supports multiple studies on other Gram-negative uropathogens, in which no evidence of T3SS contribution to UTI pathogenesis is found [207], [208], [210]. This suggests that certain components in urine can alter bacterial pathogenesis. Identification of such components could identify targets or avenues for therapeutics. These results also show the increase in proteins associated with iron acquisition mechanisms in AUM and urine are similar, though the extent of the increase differs between the two. This similar trend may indicate that the there is a scarcity of iron in urine and AUM [743]. While I conclude that AUM may be a more adequate medium to study the uropathogensis of *P. aeruginosa*, I also acknowledge the media is limited, largely due to its basic biochemical components [800]. These do not the address the complexity of urine and the existence of diverse immune and metabolic compounds [717], [1070]. The inclusion of the most abundant protein in urine, THP [462], in future AUM *in vitro* experiments may aid to close the gap reflected in the differences between AUM and urine. Furthermore, developing a

new-AUM formulation which contains antimicrobial peptides such as cathelicidin-37, could lead to a more accurate representation of the UTI *P. aeruginosa* proteome.
As part of this wider project, a number of colleagues (Dr. Christina Bronowski and John Newman) conducted multiple experiments utilising AUM to assess the efficacy of antibiotics (data not shown) and found that, when grown in AUM, *P. aeruginosa* tolerance increases to antibiotics including piperacillin/tazobactam and ciprofloxacin. This indicates that diagnostic testing using LB (or other rich media such as Muller-Hinton) may not be accurate and potentially overestimates the action of those antibiotics. This in line with several studies which have questioned diagnostic testing and the relevance to clinical application related to *P. aeruginosa* CF infections [795], [797], [1071], [1072]. Additionally, phages used in this study were less effective towards UTI isolates pre-grown in AUM, which may be linked to decreased abundance of pili and porin proteins that are utilised by phages as receptors [1073].

Finally, I used AUM to further investigate the role of sex hormones on *P. aeruginosa.* Sexual dimorphism is known to play a role in bacterial infection severity and sex hormones have been shown to be contributing factors in animal models and epidemiological studies in humans [873], [930], [953]. As such, evidence of UPEC infection modulation by oestradiol in a mouse model and humans has been demonstrated and shown to lead to a reduction of recurrent UTIs [919]. Furthermore, oestradiol appears to exacerbate *P. aeruginosa* infections in the CF lungs of female patients and modulates a severe immune response in mice, contributing to higher morbidity and lower life expectancy than their male counterparts [930], [953]. In chapter five, I conducted several phenotypic experiments to assess the influence of the sex hormones oestradiol, testosterone and progesterone on growth and virulence of *P. aeruginosa*. Phenotypic assays used to investigate growth and biofilm formation showed no significant difference in traits of all the selected isolates in the study. However, future studies could include the investigation of the impact of hormones on antibiotic susceptibility. MIC assays in the presence of hormones could be performed with antibiotics such as tobramycin. The impact of these hormones on the *P. aeruginosa* proteome resulted in two types of responses; either a universal effect (common to all 3 hormones) in the increase or decrease of proteins from

certain pathways, or hormone-specific responses. Alterations in Iron acquisition proteins was one of the main observations as a significant reduction of associated proteins was observed across all mechanisms involved in sequestering, transport and uptake of iron in the presence with either of the three hormones. This is interesting since iron levels are scarce in this medium, which could mean that sex hormones either increases the bioavailability of iron or directly suppresses these proteins resulting in further restriction of access to the available iron. To test these hypotheses, transposon mutagenesis of major genes in these pathways could be tested in iron scarce medium with the presence of hormones in increasing concentrations. This may provide more insight. Alternatively, gene knockout experiments of iron acquisition genes in *P. aeruginosa* followed by infection with mutant strains in ovariectomised female mice and castrated male supplemented with sex hormones could be performed to elucidate the role further. In animal models, the roles of sex hormone receptors can be further addressed by the utilisation of agonists (PPT) and antagonists (MPP), which act as promoters or blockers of ERα, based on the evidence that the activity of those chemical agonists and antagonists modulate the severity of infection to UPEC pathogenesis [933].

For the hormone-specific impacts on the *P. aeruginosa* proteome, a direct impact on the QS mechanisms was observed for oestradiol since multiple proteins were increased in the PQS pathway, along with increased proteins involved in the production of pyocyanin and HCN. The PQS QS system is linked to increased virulence in an acute mouse model [129]. In samples treated with testosterone, one of the notable findings is the increase of OprM, which acts in concert with MexA and MexB proteins as part of the efflux pump MexAB-OprM. The decrease of AmpC may contribute to changes in *P. aeruginosa* intrinsic resistance to β-lactam antibiotics. For progesterone, SigX an ECF sigma factor which promotes biofilm formation [1035], was increased. Hormone-specific findings also require more research ranging from *in vitro* to *in vivo* experimental models. Recent new methodologies such as Drug Affinity Responsive Target Stability (DARTS) may help in revealing specific interactions between hormones and bacterial target proteins to identify molecules of interest and previously unidentified binding targets [1074].

In conclusion, the mechanisms involved in *P. aeruginosa* causing UTIs are not well understood and require more research. This pathogen is highly adept due to its ability to form biofilms in nosocomial settings on catheters and its ability to acquire resistant genes leading to high rates of economic loss, morbidity and mortality [55], [635], [1075]. In this study, isolates from hospitals in Kuwait displayed worryingly high levels of resistance, thus, urgent epidemiological and surveillance studies are required. *P. aeruginosa* UTI isolates are genetically and phenotypically diverse. Like most other *P. aeruginosa* infections, my findings indicate that most isolates are of environmental origins, and no transmissible strains have been detected. In order to further understand uropathogensis of *P. aeruginosa,* I used an AUM model to compare the characteristics of the bacterium in comparison to the widely used LB media and urine. Our results suggest that AUM is an adequate model to study many *P. aeruginosa* mechanisms associated with pathogenesis. This is epitomised in the similar universal response to crucial virulence determinants such as iron acquisition mechanisms and T3SS in AUM and urine. However, the AUM formulation used in this study appears to have some limitations, specifically with the regards to the PQS QS system. Thus, this AUM formulation may benefit from adding other host factors such as THP protein, the most abundant protein in urine and antimicrobial peptides. Inclusion may lead to a more reflective response of *P. aeruginosa* in the urinary tract. In this study, I addressed some differences based on sex hormones and added oestradiol, testosterone and progesterone to the AUM media and conducted proteomic analysis to elucidate universal and hormone-specific responses. For instance, iron acquisition proteins were decreased globally in comparison to the control, suggesting a possible role for sex hormones in the urinary environment and this may lead to easier acquisition of iron by the bacteria. Overall, *P. aeruginosa* UTI requires more research and understanding. Further studies may lead to a personalised approach and more precision treatment based on individual attributes, rather than a single approach that is practiced to all patients.

7 References

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