Aggregation and Dispersion of Uropathogenic *Escherichia coli* 536

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Abstract

Uropathogenic *Escherichia coli* (UPEC) are the most common pathogens associated with urinary tract infections (UTIs). The formation of, and the dispersal from, intracellular biofilm-like communities (IBCs) is now recognised as an important part of the aetiology of recurrent UTIs. When grown in iron restricted tissue culture medium RPMI 1640, UPEC forms aggregates that are reminiscent of IBCs. We believe RPMI closely mimics a host environment in which biofilm formation is promoted as a survival mechanism. Aggregation and dispersal can be quantified using the Aggregation Index (AI) = (OD Total cells - OD Planktonic cells / OD Total cells). Aggregates are separated from planktonic cells by centrifugation. In culture the aggregates are dispersed by the provision of iron in a process that is inhibited by antibiotics that prevent transcription or translation, suggesting that new gene expression is required to effect dispersal. Aggregates examined by cryo-scanning electron microscopy show a two-dimensional sheet arrangement with bacteria embedded within a matrix suggesting exopolysaccharide production may be the basis of aggregate formation. Aggregates do not form in the presence of cellulase and cellulase rapidly disperses preformed aggregates in the absence of iron. The aggregate matrix stains strongly with Calcofluor White, a fluorescent stain for cellulose. Mutation of the *bcsA* gene encoding the catalytic subunit of cellulose synthase inhibits aggregation leading to the conclusion that the major aggregate exopolymer is cellulose. We hypothesise that successful iron acquisition by UPEC is an important step in recurrent UTIs, providing signals that favour dispersal. The signal to disperse is not transduced by Fur, a recognised iron-dependent regulator of gene expression; *fur* mutants aggregate and disperse normally. A mutation in the phosphodiesterase YhjK, immediately downstream of the cellulose biosynthesis operon severely compromises the ability of aggregates to disperse upon provision of iron. YhjK is hypothesised to affect the balance of intracellular cyclic-di-GMP where low levels favour dispersal. Comparison of UPEC_{ΔbcsA} (no cellulose) and wild type bacteria in antibiotic sensitivity and *Galleria mellonella* infection demonstrated possible advantages of a cellulose matrix in resistance to antibiotics and innate immunity in urinary tract infection. Comparison of UPEC_{ΔyhjK} and wild type bacteria further demonstrated the advantages of co-ordinating the regulation of cellulose mediated aggregation and dispersal in the face of antibiotic challenge and *G. mellonella* infection.
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“......It is quite evident that for the most part, water bacteria are not free floating organisms, but grow upon submerged surfaces.”

Arthur T. Henrics
Journal of Bacteriology
1933, 25: 277-287

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Abbreviations

A              absorbance
bp             base pair
°C             degrees Celsius
cfu            colony forming unit
CSEM           cryo scanning electron microscopy
EDTA           ethylenediamine tetraacetic acid
DMSO           dimethyl sulphoxide
DNA            deoxyribonucleic acid
HEPES          \(N\)-2-hydroxyethylpiperazine-\(N\)-2-ethanesulfonic acid
IBC            intracellular bacterial community
kb             kilo base
kDa            kilo Dalton
g              gravitational force
g, mg, µg       gram, milligram, microgram
l, dl, ml, µl   litre, decilitre, millilitre, microlitre
M, mM, µM      molar, millimolar, micromolar
nm             nanometres
OD\(_{600\text{nm}}\) optical density at 600\(\text{nm}\)
PAGE           polyacrylamide gel electrophoresis
PAI            Pathogenicity Island
PBS            Phosphate Buffered Saline
R              RPMI 1640
RF             RPMI 1640 supplemented with 10 µM FeCl\(_3\)
\(R\)\(_{\text{max}}\) growth of bacteria in RPMI 160 to maximum aggregation
SDS            Sodium Dodecyl-Sulfate
TFA            trifluoroacetic acid
TG             Tris-Glycine
UTI            Urinary Tract Infection
UPEC           Uropathogenic *Escherichia coli*
UV             ultraviolet
w/v            weight per volume ratio
Chapter 1. Literature Review

1.1. Clinical Manifestations and Epidemiology of Urinary Tract Infections

The urinary tract filters and removes the body's liquid wastes. A urinary tract infection (UTI) refers to the presence of bacteria within the urinary system and the site of bacterial presence describes an infection, which may reside within the bladder (cystitis), kidney (pyelonephritis) either of which results in the presence of bacteria in the urine (bacteriuria) (Underwood 2000). The infection may be further classified as "uncomplicated" for those which occur in otherwise healthy individuals with no prior instrumentation of the urogenital tract, or as "complicated" which refers to urogenital tracts which have structural or functional abnormalities, including in-dwelling urinary catheters (Foxman 2002). Uncomplicated UTIs may present with a range of symptoms which may extend from mild irritative voiding to bacteremia, to sepsis and occasionally death. Asymptomatic bacteriuria is characterized by significant numbers of bacteria (>10^8 cfu l^{-1}) in the urine without local or systemic genitourinary tract signs and symptoms; these infections are generally not treated with the exception of infections in pregnant women, where a UTI puts the foetus at risk. Uncomplicated-community associated infections may occur in any individual but those most susceptible are infants, young girls, women and the elderly. UTIs are generally considered relatively minor conditions but in severe cases may require hospitalisation (Foxman 2002).

Symptomatic UTIs are approximately 50 times more common in women than in men. In developed countries it is estimated that greater than 30% of women will have at least one UTI requiring medical treatment before the age of 24 and approximately 50% of women will experience at least one UTI during their lifetime (Kunin 1994, Engel and Schaeffer 1998, Foxman 2002). Recurrent infection rates vary amongst studies but have been estimated to be between 30 ÷ 70% (Hunstad and Justice 2010) with an average of 44% occurring within one month of the initial infection (Ikaheimo et al. 1996, Foxman 2002).

Assessment of the incidence of UTIs is difficult as it is not a reportable disease and the accuracy of a confirmed medical diagnosis is dependent on the presence of symptoms and a positive urine culture. Unfortunately, the ideal protocol is not one that is often practiced and most uncomplicated community acquired UTIs are diagnosed on clinical signs and symptoms and treated without confirmation of the infecting bacteria or with knowledge of bacterial

Uropathogenic *Escherichia coli* (UPEC), is the most common identified cause of community acquired UTIs (70-95%) and causes approximately 50% of nosocomial infections (Donlan 2001, Foxman 2002, Brzuszkiewicz et al. 2006). UTIs may be acute with varying degrees of severity or present as recurrent or chronic infections. Many women experience frequent recurrent infection after the cessation of therapy with either the original infecting organism or re-infection with a new organism. Chronic and persistent infections have a negative impact on the quality of life for many women but the long term sequelae is greatest in young girls where impaired renal function, renal scarring and progressive renal disease may develop into potentially life-threatening illnesses later in life. The cost of treating recurrent urinary tract infections also imposes a considerable cost to healthcare funding.

### 1.1.1 Urinary Tract Infection

The urinary tract is normally a sterile environment that is protected from colonic micro-flora by non-specific host defences, yet urinary tract infections (UTIs) are one of the most common bacterial infections in humans (reviewed by Mulvey 2002). UPEC and other infecting organisms are thought to originate from the host’s intestinal flora and enter the urinary tract via the ascending route. Entry to the urinary tract is gained through the urethra, via the peri-urethra and distal urethra, and attachment within the bladder, normally the primary site of infection. In order to establish an infection the bacteria must first overcome the challenges of the host’s non-specific defence mechanisms such as urine flow, exfoliation of epithelial cells, secretion of Tamm-Horsfall protein and inflammatory response cytokines and chemokines which result in the recruitment of phagocytic cells (Mulvey et al. 2000, Kau et al. 2005, Hannan et al. 2010).

Symptoms of urinary tract infection may be mild to severe depending on the susceptibility of the patient and the virulence of the organism. Generally, symptoms of a bladder infection include a strong urge to urinate that cannot be delayed or a sharp pain or a burning sensation may occur in the urethra when the urine is released. Urine volume may be low and may also be coloured with blood. The urge to urinate recurs quickly. When bacteria enter the ureters and spread to the kidneys, symptoms such as back pain, chills, fever, nausea, and vomiting may occur, as well as the previous symptoms of lower urinary tract infection (Underwood, 2000).
1.1.2 Urinary Tract Infection in Children

Urinary tract infections in children are associated with significantly greater morbidity and long-term sequelae than urinary tract infection in adults. Early childhood infection puts children at increased risk of poor renal growth and function, high blood pressure, impaired renal function, end-stage renal disease, renal scarring, progressive renal disease and increased risk of renal disease in adulthood (Jacobson et al. 1994). Prepubertal girls are three times more likely to have urinary tract infections than boys and many of these young girls will go on to develop recurrent infection within one year of the initial infection. (Jacobson et al. 1994; Foxman 2002). Nuutinen and Uhari report that 86% of children who experienced their first urinary tract infection (89% caused by E. coli) at less than one year of age contracted a recurrent infection during the next three years (Nuutinen and Uhari 2001). This suggests that recurrent and chronic infection begins at a very early age and therefore, amplifies the long-term risks of infection.

1.1.3 Catheter-Associated Urinary Tract Infection (CAUTI)

Catheter-associated urinary tract infections are symptomatic UTIs associated with a urinary catheter. Urinary tracts are the most common site of nosocomial infection where most infections follow instrumentation of the urinary tract, mainly from urinary catheterisation (Gravel et al. 2007). The risk of acquiring a CAUTI depends on the method and duration of catheterisation, the quality of care and host susceptibility. Persistent infections, even after the removal of the catheter and following antibiotic treatment, may lead to complications such as prostatitis, epididymitis, cystitis, pyelonephritis and Gram-negative bacteremia, especially in high risk patients (Wong and Hooton 1981; Matsukawa et al. 2005; Stickler 2008). Approximately 50% of CAUTIs are caused by Escherichia coli, and the remaining by other opportunistic pathogens such as Klebsiella, Proteus, Enterococcus, Pseudomonas, Enterobacter, Serratia and Candida. Many of these organisms are part of the patient’s endogenous bowel flora but may also be acquired through cross-contamination within the hospital setting. Bacterial access to the urinary tract may occur by contamination from bacteria that have colonized the urethra being picked up by the tip of the catheter and pushed into the bladder (Barford and Coates 2009), migration of bacteria along the outside of the catheter or from migration along the internal lumen of the catheter after the collection bag or catheter-drainage tube junction has been contaminated (Matsukawa et al. 2005; Stickler 2008).
The process of insertion of a catheter may cause immediate physical damage to the bladder epithelium resulting in inflammation. Coupled to the presence of bacteria within the urinary tract, immune response activation may also cause damage to the epithelium provoking a synergistic effect and inducing symptoms of infection within the patient (Barford and Coates 2009). The catheter also disrupts normal defences in that drainage of urine begins when the volume of urine is above the catheter balloon, leaving a residual pool of urine within the bladder for bacterial growth. The loss of flushing of urine and failure to remove exfoliated superficial uroepithelial cells from the bladder provides a potential reservoir making colonization of the urinary tract easier for bacteria (Barford and Coates 2009).

1.1.4 Acute and Chronic Infection

Uncomplicated urinary tract infections in women are amongst the most common infections in the community (Hummers-Pradier and Kochen, 2002). In a US survey the self-reported incidence of UTI in women 18 years and older was 10.8% and the cumulative lifetime risk of UTI was 60% (Foxman 2002). Although considered relatively minor, episodes of acute, uncomplicated UTI are associated with considerable morbidity, including 6.1 days of symptoms, 2.4 days of restricted activity and 0.4 days of bed rest (Foxman 2002). Given the large number of women affected by UTI, the societal costs are significant. Although infection has traditionally been regarded as acute and self-limiting, approximately 44% of women with an initial UTI will experience at least one recurrence of symptoms within 6 months, despite antibiotic therapy (Ikaheimo et al. 1996; Hooton and Stamm 1997; Foxman 2002). Infections may occasionally be due to a persistent focus of infection although the majority have previously been attributed to re-infection caused by the initial infecting strain persisting in the faecal flora (Stapleton and Stamm 1997).

Recent studies show that bacteria associated with recurrent UTI often appear to be phenotypically or genetically identical to the bacterial strain that caused the initial infection, suggesting that selected E. coli strains may become adapted for colonizing and infecting their respective hosts (Russo et al. 1995; Stapleton and Stamm 1997). Unfortunately, routine lab testing of urine cultures does not identify the strain of the infecting organism, leaving the clinician with little information regarding the genetic similarity between infecting organisms within the same patient and within the community. Clues to common strain patterns within a community outbreak of urinary tract infection are often detected from antibiotic resistance patterns (Manges et al. 2001; Manges et al. 2008).
Recurrent uncomplicated UTIs are defined as at least three episodes of uncomplicated UTI within 12 months with at least one documented by culture (Mangin et al. 2005; Wagenlehner et al. 2009). In light of increasing bacterial resistance to antibiotics, present day standards indicate the pathogen should first be identified and the antibiotic susceptibility status determined (Mangin et al. 2005; Wagenlehner et al. 2009) before initial therapy, although this rule is often ignored mostly due to economic reasons and the infection treated empirically based on clinical signs and symptoms. The dilemma of this approach is that uropathogen susceptibility profiles are not representative even though such data is required to make valid therapeutic recommendations (Wagenlehner et al. 2009).

Considerations for a UTI treatment regime include the most common causative organism and its known general susceptibility pattern based on clinical studies, ideally within the local geographical location. The pharmacokinetic properties of the antibiotic favour those with high urinary excretion as bacteria are present mainly in the bladder and a lesser extent in the tissue. The outcomes of clinical studies for various antibiotic regimes showing efficacy is an important aspect for choice of treatment of UTIs as short-term regimens are often favoured for better patient compliance, less adverse and collateral effects, lower costs and efficiency comparable to conventional treatment (Wagenlehner et al. 2009). The effects of the antibiotic on the patient are also important factors and most first line antibiotics for UTIs are considered to cause mild to moderate adverse effects such as candidiasis and gastrointestinal disorders, however the use of some first line antibiotics may also carry more severe risks (Lajiness 2008; Wagenlehner et al. 2009). Collateral effects beyond eradication of the infecting organism include selective pressure for resistant mutants from the infecting bacteria and the host's normal flora, which may cause re-infection in the patient or cross-contamination of another population (Wagenlehner et al. 2009). The overall decision to treat a patient empirically must take into account the above concerns as well as specific patient factors such as the severity of the symptoms, allergic history, results of recent microbiological test (if available), risk factors for resistance, and accessibility to medical care (Gupta et al. 2001; Wagenlehner et al. 2009). All antibiotics used for treatment of UTIs cause side effects that may in some way impair a patient beyond the condition for which they serve, further highlighting the negative impact on quality of life associated with UTIs (Rahn 2008). Common antibiotics for treatment of UTIs are discussed in section 1.1.5 and summarized in Table 1.
Despite host defences challenging organisms from the initial point of infection, significant numbers of bacteria can persist within the bladder for days to weeks (Hopkins et al. 1998, Hvidberg et al. 2000). Within a murine model UPEC strains can persist within bladder tissue, even in the presence of antibiotic treatments, although bacterial titres are reduced within the urine (Hvidberg et al. 2000). Persistence appears to be related to the ability of Type 1 piliated E. coli to invade bladder epithelial cells (Connell et al. 1996; Mulvey et al. 1998; Martinez et al. 2000; Mulvey et al. 2001; Anderson et al. 2004; Justice et al. 2004; Brzuszkiewicz et al. 2006; Justice et al. 2006, Mysorekar and Hultgren 2006, Reigstad et al. 2007). This is also seen in clinical settings where treatment of infection with antibiotics is effective in reducing bacterial titres within the urine but fails to clear bacteria from the tissue.

Murine models for UTIs have provided considerable insight into the developmental stages involved in the formation of aggregated bacterial colonies or intracellular bacterial communities, within bladder epithelium. Specific receptor / ligand binding, internalization of the bacteria into host cells and the formation of intracellular bacterial communities (Justice et al. 2004) suggests a powerful evasion strategy to avoid immune attack and consequently antibiotic damage. The release of intracellular bacteria back into the bladder lumen shows bacteria that are often filamentous and cause the death of the host bladder epithelial cell (Justice et al. 2004). A subpopulation of UPEC is able to persist intracellularly for months within a murine model which may serve as a seed for recurrent infections (Mulvey et al. 1998; Mulvey et al. 2001; Schilling et al. 2003). This infection focus, or biofilm is discussed further in section 1.6 and 1.9.2. Similar re-infection is noted in clinical settings where studies have shown that women with recurrent UTI are re-infected with the same organism even months later suggesting the infecting organism is often a single uropathogenic strain that may reside as a vaginal or faecal reservoir or a within the urothelium between recurrent episodes of infection (Russo et al. 1995; Hunstad and Just 2010).

1.1.5 Treatment of Infection

The diagnosis of acute uncomplicated cystitis is usually clinically based (Rahn 2008). A urine culture is the gold standard for diagnosis of bacteriuria and cystitis, yet most infections are treated empirically. Urine dipstick testing and microscopic urinalysis within the clinic often facilitates diagnosis. A "clean catch" sample for urine culture is recommended for patients with complicated or recurrent infections accompanied by a bacterial antibiotic susceptibility test. Recurrence is defined as three positive infections documented in one year
(Barclay and Vega 2008; Wagenlehner et al. 2009). Repeated relapsing infection with the same bacterial strain may also require additional testing to rule out other pathologies or physical abnormalities (Foxman 2002; Mangin et al. 2005).

Uncomplicated infections are generally treated with antibiotics. Other interventions may include rest, hydration (Pollan 1995), and short-term use of urinary analgesics such as phenazopyridine which relieves urinary tract pain (Amit and Halkin 1997). However, phenazopyridine is not an antibiotic and does not kill bacteria. The most common antibiotic medications for uncomplicated urinary tract infections are amoxicillin, a β-lactam antibiotic; trimethoprim/sulfamethoxazole (TMP/SMX), sulphonamides; ciprofloxacin and other fluoroquinolone derivatives; and nitrofurantoin. (Rahn 2008; Lajiness 2008). For recurrent UTI, continuous prophylaxis is recommended with a combination of antibiotics at reduced concentrations (Rahn 2008). A summary of the most common antibiotics used to treat UTIs is listed in Table 1.

Although antibiotic prophylaxis is currently the most effective way to reduce recurrent UTIs it comes with substantial side effects and the efficacy of antibiotics for prophylaxis will be limited by the rate of bacterial susceptibility within the community.

Table 1. Common Antibiotics used to Treat Urinary Tract Infection

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Side Effects</th>
<th>Target Organism</th>
<th>Resistance (NZ 2009)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amoxicillin (β-lactams)</td>
<td>Candidiasis, diarrhoea</td>
<td>Gram negative organisms</td>
<td>51 %</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Enterococcus sp.</td>
<td></td>
</tr>
<tr>
<td>Nitrofurantoin</td>
<td>Headache, vomiting, diarrhoea, vertigo, rash, asthma</td>
<td>Gram negative and Gram positive organisms</td>
<td>1.6 %</td>
</tr>
<tr>
<td>Trimethoprim/</td>
<td>Moderate effects on bowel and vaginal flora, nausea, vomiting</td>
<td>Gram negative organisms</td>
<td>24 %</td>
</tr>
<tr>
<td>Sulfamethoxazole (Sulfonamides)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>Nausea, vomiting, rash, abnormal liver function tests. Increase in Prothrombin Time in patients on Warfarin</td>
<td>Gram negative organisms</td>
<td>8 %</td>
</tr>
</tbody>
</table>

*Data from the ESR Public Health Surveillance (http://www.surv.esr.cri.nz).
1.1.6 Drug Resistance

The management and treatment of urinary tract infection is complicated by the increasing prevalence of antibiotic resistant strains of *Escherichia coli* and the increased resistance to the standard drug treatment for UTIs. The \( \beta \)-lactam antibiotics include penicillins, cephalosporins, and mecillinam (a newer antibiotic more common in Europe) (Sougakoff and Jarlier 2000). \( \beta \)-lactamases, including the Extended-Spectrum \( \beta \)-lactamases (ESBL) are bacterial enzymes that inactivate \( \beta \)-lactam antibiotics. There are approximately 500 different ESBLs described, all of which have mutations within the plasmid-mediated, classical broad-spectrum beta lactamase enzymes. Bacteria carrying these resistance genes are not only resistant to penicillins and cephalosporins but are often also resistant to a wide range of other antibiotic classes including fluoroquinolones, aminoglycosides, and trimethoprim/sulfamethoxazole), thus, limiting effective treatment against infection (Briggs *et al.* 2005).

ESBL production was first noted in the mid 1980s and has since emerged as a major source of antimicrobial resistance in Gram-negative pathogens, most commonly, *Klebsiella* species, *Escherichia coli* and *Proteus mirabilis*, as well as other members of the Enterobacteriaceae family. For UPEC the production of \( \beta \)-lactamase remains the most important contributing factor to \( \beta \)-lactam resistance and increased resistance has been directly linked to the increased clinical use of \( \beta \)-lactam antibiotics (Medeiros 1997). A more recent study indicates that colonization and infection by ESBL-producing bacteria are increasing in the Auckland community and throughout New Zealand (Briggs *et al.* 2005).

The treatment of choice against ESBL organisms is amikacin (an aminoglycoside) (Briggs *et al.* 2005) or a carbapenem, an antibiotic with a chemical structure that makes it more resistant to most \( \beta \)-lactamases, although resistance to these antimicrobials has already been documented (Nathisuwan *et al.* 2001). Drug development has tried to stay ahead of the evolutionary development of \( \beta \)-lactamase but the resultant effect has been a more diverse and potentially more devastating group of \( \beta \)-lactamase enzymes. The ESBLs have evolved to chromosomally-encoded AmpC enzymes (Bush 2001). The AmpC enzymes confer resistance to the more powerful \( \beta \)-lactam antibiotics (oxyimino-cephalosporins and cephamycins) and these enzymes have further progressed to another class of \( \beta \)-lactam antibiotic, the carbapenems, as carbapenem usage has increased due to cephalosporin and fluoroquinolone resistance (Livermore and Woodford 2006).
Infections caused by resistant Gram-negative bacilli, especially ESBL-producers, have numerous economic and clinical implications through increased intensive care unit treatment and the length of hospital stay, as well as increasing morbidity and mortality (Wright and Eiland 2010). These new enzymes confer resistance to the most recent β-lactam antibiotics and present a challenge to clinicians for treatment options for these virulent pathogens, especially when treating community acquired uncomplicated UTIs.

1.2. The Urinary Tract

1.2.1 The Urinary System

The urinary bladder is a cavity lined by transitional cell epithelium (urothelium) which is surrounded by connective tissue and smooth muscle. The urothelium is 7-8 cells thick and has three zones: basal, intermediate and a highly specialized surface layer (Underwood 2000). Urine drains into the bladder by peristaltic movement from the kidneys via the ureters for storage until discharged through the urethra. The specialized umbrella cells of the bladder epithelium contain mannosylated uroplakins (UPIa, UPIb, UPII and UPIII) which are conserved proteins among mammalian species and provide the bladder with its water-impermeable characteristics. The uroplakins organize into plaques that cover most of the luminal surface of the bladder allowing specific interaction with the mannosylated uroplakin and bacteria expressing Type 1 pili (Martinez et al. 2000; Mulvey et al. 2000; Brzuszkiewicz et al. 2006, Hunstad and Justice 2010) which allows bacteria to adhere to the bladder lumen as single cells or as large biofilm-like colonies (Pratt and Kolter 1998).

The kidneys contribute to the body's biochemical homeostasis by eliminating metabolic liquid waste products, regulating fluid and electrolyte balance and influencing acid-base balance. The formation of urine begins in the glomeruli where the filtration of plasma results in filtrate, most of which is reabsorbed in the tubules. Glomerular endothelial cells are fenestrated and allow plasma direct contact with the underlying basement membrane (Underwood 2000). The uroepithelium contain globobiose linked to ceramide lipid on their surface enhancing the interaction of the host cell with some bacterial adhesins and pose an increased risk of developing pyelonephritis.
1.2.2 Host Factors Increasing the Risk of Infection

The susceptibility of a host to an infection is determined by a number of factors. Genetic factors such as the absence of ABO antigens in saliva or gastric secretions (termed a "non-secretor") (Lomberg et al. 1986) and the presence of ABO blood group antigens, especially of the Lewis blood group, on uroepithelium enhance bacterial attachment and have been implicated in increased risk for recurrent urinary tract infections (Sheinfeld et al. 1989). Biological factors such as congenital abnormalities and / or the presence of urinary obstruction or a prior history of UTI (Shortliffe and McCue 2002) also increase the risk of acquiring a urinary tract infection. Behavioral factors such as sexual intercourse, the use of contraceptive products (diaphragms, condoms and spermicides) and the use of antibiotics all contribute to increased risk of infection (Foxman et al. 1995; Fihn et al. 1996). In the aging female, the decrease of estrogen levels may affect the integrity of the epithelium in the urogenital tract and predispose to an increased risk of infection (Foxman 2002).

1.2.3 Host Responses During Infection

Host defences are active during bacterial infection. Non-attached or weakly adherent bacteria are rinsed from the bladder surface by the bulk flow of urine through micturition. The chemical composition of the urine in regard to pH, osmolarity, salts, urea and organic acids can be inhibitory to bacterial growth; concomitantly iron binding molecules such as lactoferrin within the urine scavenge iron and bind an essential nutrient required for bacterial growth (Sorbel 1997). Urinary components such as Tamm-Horsfall protein, secretory IgA, low molecular weight sugars and uromucoid, mask host receptors on the uroepithelium and competitively inhibit receptor – adhesin interactions, lowering the probability of bacterial attachment and colonization (Sorbel 1997; Mulvey et al. 2000).

Bladder epithelium has a slow turnover rate (Hicks 1975) yet patients presenting with UTIs often have exfoliated bladder epithelial cells and associated bacteria in their urine suggesting that exfoliation and clearance of infected and damaged bladder cells can function as a host defence (Fukushi et al. 1979). This hypothesis has been demonstrated in a murine model, that the presence of Type 1 piliated bacteria is able to induce exfoliation, although the degree of exfoliation appears to be dependent on the genetic background of the host (Mulvey et al. 1998). Infection of the bladder with Type 1 piliated bacteria also induces an influx of neutrophils into the bladder lumen where they exert bactericidal activities via the generation of reactive oxygen intermediates and / or the release of preformed anti-microbial peptides.
Bacterial infection of the bladder induces the expression of the adhesion molecule ICAM-1 on the bladder epithelium, which is an important factor for the migration of neutrophils (via ICAM-1 and CD11b / CD18 interactions) to the site of infection (Agace et al. 1995). The presence of bacteria also induces the release of the cytokine IL-6 and chemokine IL-8 from the uroepithelium in response to bacterial lipopolysaccharide (LPS) (Agace et al. 1995) where the cytokines play a number of roles in amplification of the immune response and specifically in regard to neutrophil recruitment (Kopf et al. 1995, Romano et al. 1997). The host immune response produces a number of antimicrobial factors such as nitric oxide and defensins by urothelial cells and immune cells such as mast cells and macrophages to help control a urinary tract infection, yet bacteria are able to persist within the bladder for days to weeks even in the presence of strong host defences and antibiotic treatments (Hvidberg et al. 2000).

1.3. Genotypic Characteristics of Uropathogens

The frequency at which UTIs occur highlights the clinical problem associated with this disease but fails to describe the diversity of the pathogenesis. UTIs present with varying degrees of symptoms, severity and localization, that may also be sporadic, recurrent or chronic; therefore, in order to understand the pathogenesis it is essential to understand the molecular basis of disease diversity (Brzuszkiewicz et al. 2006). The uropathogens are a genotypically and phenotypically similar group of E.coli isolates, which generally fall into a small number of O-sero-groups that represent different subclasses of the pathogen (Johnson 1991) that differ greatly from the majority of faecal E. coli strains.

Escherichia coli are members of the family Enterobacteriaceae. They are facultatively anaerobic Gram-negative rods that colonize the intestines of humans and animals in health and disease, and are also well adapted to life in the natural external environment. As a model laboratory bacterium, the gut-derived K12 laboratory strain (MG1655) has been used to derive a vast amount of genetic and biochemical database information. Distinct E.coli pathotypes such as enteropathogenic (EPEC), enterohemorrhagic (EHEC), enteroinvasive (EIEC), enterotoxigenic (ETEC) and enteroaggregative (EAEC) refer to specific E.coli strains that cause significant morbidity and mortality as human intestinal pathogens. Extraintestinal E.coli (ExPEC) strains refer to other groups of pathogens involved in neonatal
meningitis, sepsis and urinary tract infections. The extraintestinal pathogens differ from diarrheal pathogens in that they are harmless commensal organisms within the gut yet when they invade the cerebral spinal fluid, the blood or the urinary tract they become serious pathogens (Welch et al. 2002). The uropathogens display distinctive features in regard to their adaptations to different habitats and the evolution of their virulence (Chen et al. 2006). UPEC strain 536 a pyelonephritis isolate, UPEC strain CFT073 (Welch et al. 2002) a urosepsis isolate, and UTI89 (Chen et al. 2006) a clinical cystitis isolate have been the primary strains used as model organisms for the study of urinary tract infections. All three strains have been genome sequenced providing a vast amount of information about their genomic make-up. The common genomic properties of these three uropathogens are summarized in Table 2.

### Table 2. Comparison of Commensal E. coli and Three Major Uropathogenic E. coli

<table>
<thead>
<tr>
<th>Strain</th>
<th>E. coli K-12 MG1655 (OR:H48:K-)</th>
<th>UTI89 (O18:K1:H7)</th>
<th>CFT073 (O6:K2:H1)</th>
<th>UPEC 536 (O6:K15:H31)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chromosome size (bp)</td>
<td>4,639,221</td>
<td>5,065,741</td>
<td>5,231,428</td>
<td>4,938,875</td>
</tr>
<tr>
<td>Plasmids</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>GC content (%)</td>
<td>50.8</td>
<td>50.6</td>
<td>50.5</td>
<td>50.5</td>
</tr>
<tr>
<td>Prophage-like elements</td>
<td>10</td>
<td>0</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>PAIs</td>
<td>0</td>
<td>4</td>
<td>13 (60 (&gt;4kb))</td>
<td>5</td>
</tr>
<tr>
<td>tRNA sites</td>
<td>88</td>
<td>87</td>
<td>88</td>
<td>81</td>
</tr>
</tbody>
</table>

*Comparison of non pathogenic MG1655 and uropathogenic E. coli strains UT189, CFT073 and UPEC 536. The table indicates an enlarged chromosome in the uropathogens compared to the commensal E. coli strain MG1655. DNA content contributed by plasmids or prophage-like elements is listed and the number of Pathogenicity Islands in each strain and the tRNA sites indicate where new DNA has been acquired (adapted from Brzuszkiewicz et al. 2006).*

### 1.3.1 Uropathogens and Virulence Factors

Strategic database analysis is one approach used to identify strain-specific adaptations that are under positive selection. This data has been important for the comparison of sequenced uropathogenic strains and the analysis of virulence factors carried on the chromosome of these strains. When compared to the commensal E. coli K-12 MG1655 "backbone" genome, strain-specific adaptations for survival within the urinary tract appear to be common to
uro pathogens. These genes are often acquired from other species / organisms and are found on specific DNA segments within the chromosome or carried on plasmids.

Most genes encoding virulence factors in the uropathogens are located on mobile genetic units within the chromosome, termed pathogenicity islands (PAIs) (Dobrindt et al. 2004, Hacker et al. 2003), which have the ability to spread between bacterial populations by horizontal gene transfer (Ghigo 2001; Hacker et al. 2003; Brzuszkiewicz et al. 2006). Typically, PAIs are distinct regions of DNA, ranging from 10 kb to 200 kb, that are present in the genome of pathogenic bacteria but absent in non-pathogenic strains of the same or related species. The guanine + cytosine (G+C) content of PAIs differs from the core genome indicating that the genetic material has been acquired from elsewhere (Hacker et al. 2003). The PAIs are relatively unstable elements which are often associated with tRNA genes and frequently contain mobile genetic elements such as insertion sequences, transposon and bacteriophage DNA and integrases (Lloyd et al. 2009). Insertion and deletion of PAIs is mediated by the PAI-encoded integrase and functions via site-specific recombination between the flanking direct repeats. Virulence genes present on the bacterial chromosome determine the type of urinary infection a host may acquire (bacteriuria, cystitis or pyelonephritis) and the severity of the disease (Oelschlaeger and Hacker 2002). PAIs carry most of the known UPEC virulence factors and a number of putative genes of unknown function. Comparative genome analysis reveals that horizontal gene transfer, gene loss and insertion sequence element-mediated chromosomal rearrangements play important roles in bacterial evolution and demonstrate that the UPEC chromosome is dynamic and constantly in flux (Brzuszkiewicz et al. 2006). It has been suggested that the instability of the PAIs may also contribute to the transition from acute to chronic infection and that the ability to modulate genome structure is an important feature for adapting to changing environmental conditions (Middendorff et al. 2004).

1.3.2 UPEC 536

E. coli 536 (O6:K15:H31) is a pyelonephritis isolate. Both CFT073 and UPEC 536 belong to the same serogroup yet differ in several virulence-associated traits that may correlate with their pathogenesis. The genome of UPEC strain 536 contains over 4,938 kb, carries one cryptic prophage region and contains no plasmids (Brzuszkiewicz et al. 2006). The genome encodes 4,747 putative coding sequences of which approximately 77% have similar orthologues in MG1655 and approximately 89% of all ORF in E. coli 536 have similar
orthologues in the UPEC CFT073 genome. The remaining ORFs present in the 536 genome are mainly located within a region of the cryptic prophage or within the five major (> 10 kb) and three minor PAIs and constitute the majority of the 536-specific genes (Brzuszkiewicz et al. 2006). The major PAI-associated virulence factors of UPEC 536 are summarized in Figure 1.

Figure 1. The Pathogenicity Islands and Hemin Utilization System of UPEC 536. Virulence factors associated with the PAI. The approximate size and insertion site for the PAI are indicated by the tRNA genes associated with the insertion site.

PAI-associated virulence has been demonstrated in a murine model of ascending UTI where individual loss of PAI I, II, or III resulted in attenuation of the strain confirming that PAIs encode factors that effect in vivo virulence and support the establishment of UTI (Nagy et al. 2006). Single PAI deletion did not significantly affect strain survival in a urosepsis murine model whereas, simultaneous loss of PAI I and PAI II displayed attenuated survival and the PAI encoding α-haemolysin was determined essential for induction of urosepsis (Brzuszkiewicz et al. 2006). Haemolysins are exotoxins produced by the bacteria that induce lysis of red blood cells and liberate iron as haemoglobin to support bacterial growth. UPEC 536 carries two hly determinants (hly I and hly II) which may be responsible for the higher virulence sepsis relative to UTI89 and CFT073 (Knapp et al. 1986). Other UPEC 536 PAI-associated virulence factors include a number of iron acquisition systems, enterobactin (ent),
yesiniabactin (fyuA), salmochelin (iro) two hemin uptake systems (chu and hma), a capsule and adhesion organelles (reviewed in Mulvey et al. 2000; Brzuszkiewicz et al. 2006).

Toxins associated with UPEC 536 and other uropathogens are associated with considerable cellular damage. Vacuolating autotransporting toxin (vat) is a member of the serine protease autotransporter of the Enterobacteriaceae (SPATE) family. Vat, which was first described in avian Escherichia coli, and induces the formation of intracellular vacuoles resulting in cytotoxic effects similar to those caused by the VacA toxin from Helicobacter pylori (Parreira and Gyles 2003). Although UPEC 536 does not carry this toxin the genome of some uropathogens carry genes homologous to vat, yet its role in uropathogenesis is unclear. The secreted autotransporter toxin (Sat) expressed by CFT073 is a vacuolating cytotoxin of bladder and renal epithelium (Guyer et al. 2002). In vitro studies demonstrate elongation of cultured bladder cells and loosening of cellular junctions, whereas, in vivo studies show dissolution of the glomerular membrane and vacuolation of the proximal tubule cells (Guyer et al. 2002) highlighting the damaging effects toxins can have on the uroepithelium. Cytotoxic necrotizing factor 1 (Cnf1) is a toxin produced by approximately 40% of UPEC strains, although not UPEC 536, that mediates its effects via the activation of small GTP-binding proteins (Rho, Rac, and Cdc42 GTPases) in eukaryotic cells (Fabbri et al. 2010). It has also been shown to interfere with immune defenses, specifically by altering the neutrophil cytoskeleton and decreasing the phagocytic ability of neutrophils (Hofman et al. 2000). See Table 3 for a summary of strains possessing these toxins.

PAIs encode several cell surface proteins involved in aggregation. UTI pathogenesis and long-term UTI persistence has been linked to Antigen 43 (Ulett et al. 2007). This protein, encoded by the flu gene, belongs to the family of autotransporter proteins and is present in many Enterobacteriaceae. A phase-variable protein, Antigen 43 is self-associating and produces large bacterial aggregates. It also promotes adherence to some human cells and enhances biofilm formation (Klemm et al. 2004). The presence of Antigen 43 has been shown to help bacteria withstand bactericidal agents such as hydrogen peroxide (Schrembri et al. 2003) and withstand immediate killing by neutrophils through the formation of tight aggregates (Fexby et al. 2007). The Hek adhesion protein found on UPEC 536 PAI1l, in UTI89 and other strains of E. coli is another protein involved in aggregation. This outer membrane protein is involved in auto-aggregation, the agglutination of red blood cells and invasion of epithelial cells and is most often associated with strains of E. coli involved in neonatal meningitis (Fagan and Smith 2007) yet its role in urinary tract infection is unclear.
OmpA an integral outer membrane protein of UPEC strains is important in adhesion and entry into the epithelial bladder cell and also functions as a critical determinant of intracellular virulence (Nicholson et al. 2009). The OmpA protein was shown to have involvement in the tight packing of cells within the intracellular bacterial communities (IBCs) and the completion of the IBC pathway (Nicholson et al. 2009).

The genomes of UPEC 536, CFT073 and UTI89 contain genes homologous to those in the core region of the *Yersinia* high PAI (HPI) which is conserved in gene content and location in these uropathogens and other Enterobacteriaceae such as *Klebsiella* and *Citrobacter* (Chen et al. 2006). The *Yersinia* HPI is present in highly pathogenic strains of *Yersinia* and is found on the chromosome of *Yersinia* spp. The pathogenicity island carries a group of genes involved in the biosynthesis, transport and regulation of the siderophore yersiniabactin required for the acquisition of iron *in vivo* for bacterial growth and dissemination (Hancock et al. 2008). DNA sequences of the boundaries suggest the island is of foreign origin and acquired by chromosomal integration from a phage (Carniel 1999).

Comparative genome analysis of these three uropathogens indicates that horizontal gene transfer, gene loss and insertion, and chromosomal rearrangements play an important role in bacterial evolution. The individual differences between strains alters their potential to cause and determine the severity of disease that may result from common as well as strain-specific gene sets. The major virulence factors associated with these three uropathogens is summarized in Table 3.

### 1.4. Phenotypic Characteristics of Uropathogens

#### 1.4.1 Type 1 pili

The rate limiting step in uropathogenesis is bacterial attachment to the host cell (Mulvey 2002). Bacterial structures involved in attachment have been well characterized (Costerton et al. 1999; Donlan 2001; Jenkinson and Lappin-Scott 2001; Donlan 2002; Jefferson 2004) with the most important of these structures being pili or fimbriae, as these organelles define tissue tropism and enhance strain-specific pathogenic potential. The Type I pilus of *E. coli* has been extensively characterized (Martinez et al. 2000; Bryan et al. 2005) is serologically conserved in all members of the *Enterobacteriaceae* and has been shown to play a variety of roles in the
development of urinary tract infection (Jenkinson and Lappin-Scott 2001; Behrani-Mougeot et al. 2002; Wright and Hultgren 2006). The Type I pili, encoded by the fim operon contains a periplasmic chaperone, an outer membrane usher, a tip adhesin (FimH) and a pilus shaft structural gene. The FimH tip adhesin binds mannosylated proteins and displays broad-based specificity by binding mannose-containing glycoprotein receptors on various host cell types. These cell types include those found on human buccal cells (Baddour et al. 1989), proximal tubular cells of the kidney (Salit and Gotschich 1977), epithelial cells of the bladder, lung, intestine (Baddour et al. 1989) and various inflammatory cells (Tewari et al. 1993). It has also been shown to bind proteins such as leukocyte adhesion molecules CD11 and CD18 (Gbarah et al. 1991) and the Tamm-Horsfall protein in urine (Baddour et al. 1989). The interactions between FimH and the receptors expressed on the bladder epithelium are imperative for the uropathogen to colonize the bladder and cause disease (Langermann et al. 1997, Mulvey et al. 1998). Colonization of the bladder requiring the Type 1 pili has been elegantly demonstrated by Martinez et al. (2000). Cultured bladder epithelial cells were infected with Type 1 pili expressing and FimH E. coli strains and monitored by electron microscopy for adhesion and invasion of the epithelial cell. The pili were shown to be necessary for adherence to human bladder epithelium and for modulation and invasion of the bladder epithelium (Martinez et al. 2000). Interaction between the pilus and the host epithelium activates intracellular bacterial signalling pathways which in turn, modulate actin reorganization, phosphoinositide 3-kinase activation and host protein tyrosine phosphorylation. (Martinez et al. 2000). The ability to invade host cells provides a distinct survival advantage over extracellular bacteria and allows pathogens to evade host defences and facilitate their dissemination both within and across cellular barriers (Falkow et al. 1992).

1.4.2 Pap pili

Uropathogens involved in pyelonephritis are associated with the carriage of the genes encoding the Pap pilus. The pyelonephritis-associated P-pilus, encoded by the pap operon carries the PapG tip adhesion. PapG interactions with the host receptor can be divided into three classes - GI, GII, and GIII - where each class has specific binding to different globlobiose glycolipids. The PapG protein of UTI89 has GIII specificity for Gb05 (Fossman Antigen) found in mouse tissue and causes cystitis in humans without more complicated sequelae (Chen 2006). Whereas, the CFT073 and UPEC 536 PapG protein shows specificity for GII adhesion which binds to Gb04 expressed on human kidney epithelial cells and is
associated with human pyelonephritis highlighting the difference in tissue tropism of UTI89 and CFT073 and UPEC 536 strains (Chen et al. 2006).

1.4.3 Other Pili

Both UTI89 and UPEC 536 express S-type fimbriae (sfa), a sialic acid binding fimbriae. The S-pili have similar assembly and structure to the Type 1 pili and Pap pili and carry a SfaS tip adhesin. The tip adhesin interacts with the sialic acid residues on receptors expressed by renal epithelium and vascular endothelial cells (Morschauser et al. 1990; Hanisch et al. 1993). The presence of S-pili has been implicated as a virulence factor in both urinary tract infections and newborn meningitis (Knight et al. 2002). CFT073 and UPEC 536 also carry F1C pili, a structure similar to the S-pili that differ in receptor specificity (Ott et al. 1988; Hacker 1993). The F1C pilus binds β-GalNac-1, 4β-Gal residues on glycolipids expressed on renal epithelial cells and by renal and bladder endothelial cells (Khan et al. 2000). These pili are found on approximately 14% of UPEC isolates (Mulvey 2002) suggesting a role for F1C pili in UTI pathogenesis. Although UTI89 does not carry an F1C pilus it does have an Fl7-like pilus similar to Type 1 and Pap pilus (Kuehn et al. 1992). The specificity of this pilus is for the carbohydrate N-acetyl-D-glucosamine (Girardeau 1980) a major component of peptidoglycan.

The three strains of uropathogens discussed display a variety of defined pili structures. Database sequence analysis also indicates that there may be a number of putative pili encoded within their genome (Welch et al. 2002) yet to be elucidated, all of which suggest a large repertoire of organelles for specific attachment to host cells and the initiation of infection. Pili and other virulence factors associated with these strains are summarized in Table 3.
Table 3. PAI-associated Virulence Factors

<table>
<thead>
<tr>
<th>Virulence Factor</th>
<th>CFT073</th>
<th>536</th>
<th>UT189</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Iron Uptake Systems</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>enterobactin (ent)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>salmochelin (ira)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>hemin (chu)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Fe/Mn (sit)</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>aerobactin (iutA)</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Yersiniabactin (fyuA)</td>
<td>+/-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>hemin (hma)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>Major pili and fimbriae</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type 1 (fim)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>P (pap)</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>S (sfa)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>F1C (foc)</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><strong>Toxins</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ß-hemolysin (hly)</td>
<td>+</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>cytotoxic necrotizing factor 1 (CNF1)</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>vacuolating autotransporting toxin (vat)</td>
<td>+</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>secreted autotransporter toxin (sat)</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Adhesins</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antigen 43 (flu)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Hek (hek)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Outer membrane protein (ompA)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

The presence or absence of genes is indicated by + or - respectively. Adapted from Wiles et al (2007).

1.5. Iron

In bacteria, iron is an essential nutrient for many cellular functions where it acts as an electron donor and acceptor for numerous cellular processes (reviewed in Carpenter et al. 2009). Excess iron is toxic to a bacterial cell as it promotes the formation of reactive oxygen species (by the Fenton Reaction) that causes damage to biological macromolecules. The regulation of intracellular iron levels in many bacterial species is controlled by the protein
Fur (ferric uptake regulator) (Bagg and Neilands 1987; Escolar et al. 1999; Carpenter et al. 2009). When Fur is associated with Fe^{2+} there is repression of transcription of genes involved in iron acquisition and activation of genes involved in defence against oxidative molecules, allowing the cell to withstand stresses associated with high iron conditions (Wosten et al. 2000). Iron is an essential element for growth of E.coli and other bacteria. During an infection, iron is limited, as a large portion of the iron in humans is intracellular, mostly within red blood cells bound as haemoglobin or sequestered in ferritin, while a small amount of iron is tightly bound to high-affinity iron-binding proteins such as transferrin and lactoferrin (Mietzner and Morse 1994; Otto et al. 1992).

Pathogenic bacteria have acquired a variety of mechanisms to remove iron from host sources. Siderophores compose an iron uptake system that involves the synthesis and transport of low molecular weight iron chelators that have a high affinity for iron (K_d=10^{-20}–10^{-50}M) (Crosa 1989). Siderophores, such as enterobactin, are synthesised via a two-step process that results in the formed molecules being secreted from the cytoplasm to the extracellular space via the outer membrane channel TolC complex (Bleuel et al. 2005). Most E.coli strains produce the catechol siderophore enterobactin and / or the hydroxamate siderophore aerobactin, a siderophore found in many UTI causing E.coli (Johnson 1991). Iron-bound siderophores (ferrisiderophores) are recognized by specific bacterial receptors and transported into the cell where the iron is dissociated and either utilized or stored for later use (Bleuel et al. 2005).

The uptake of iron is facilitated by outer membrane receptors and iron transport is carried out by structurally conserved β-barrel proteins which require energy. Receptors require the activity of a trans-membrane protein complex composed of TonB, ExbB and ExbD to transduce energy from the proton motive force (Braun et al. 2006). The N-terminus of the receptor protein is thought to form a gated closed plug which obstructs the pore of the β-barrel in an unbound state. When iron is bound to the receptor the β-barrel may undergo a conformational change allowing the ferrisiderophore to enter the periplasm (Shultis et al. 2006; Faraldo-Gomez et al. 2003). Within the periplasm, ferrisiderophores are bound by periplasmic substrate-binding proteins and actively transported to the cytoplasm via ABC transport systems (Faraldo-Gomez et al. 2003).

An alternative mechanism of iron acquisition is through direct utilization of host iron compounds, especially haem or haemoglobin (Law et al. 1992). Specific TonB-dependent outer membrane receptors bind haem either directly or following its removal from haem
binding proteins by enzymes. The iron is transported into the periplasm where specific ABC transporters carry the iron to the cytoplasm (Wandersman and Stojiljkovic 2000). Specialized haem binding outer membrane receptors have been identified in *Yersinia enterocolitica* (*hemR*) (Bracken et al. 1999), *Poryphyromonas gingivalis* (*hmuR*) (Liu et al. 2006) and *Shigella dysenteriae* (*shuA*) (Burkhard and Wilks 2007). In some bacteria, such as *Yersinia pestis*, haem acquisition is through the action of hemophores, small proteins that scavenge haem and interact with outer membrane receptors similar to siderophores (Rossi et al. 2001).

![Figure 2. TonB Dependent Iron Transport in *E.coli*. Iron acquisition and uptake are receptor and TonB dependent processes. Enterobactin (blue) and haem (pink) cellular machinery are shown. Iron-loaded enterobactin or haem is bound via siderophores (ent) or haem and recognized by outer membrane receptors (FepA or ChuA) and transported into the periplasm with energy transduced by the TonB/ExbB/ExbD complex (brown). TonB interacts with outer membrane transporters at the TonB box motif. Within the periplasm binding proteins (FepB or ChuT) transfer the iron to inner membrane permeases (FepDG or ChuU) where ATPases (FepC or ChuV) deliver the iron to the cytoplasm. When the ferrisiderophore enters the cytoplasm ferric ion (Fe3+) is reduced to ferrous ion (Fe2+). Excess Fe2+ binds to Fur which in turn binds to target promoters and inhibits transcription of siderophore genes. Some transporters are also regulated by σ/anti-σ factor systems such as the ferric citrate iron transporter, FecA (light yellow). FecA regulates the expression of fecABCDE transport genes which is initiated by the binding of ferric citrate to FecA through the interaction of the N-terminal extension of FecA to the inner membrane σ regulator (yellow) and the exocytoplasmic sigma factor (ECF-σ). Both transport and induction of genes require energy transduced by the TonB complex. The TonB box is indicated in black on the outer membrane receptor. OM, outer membrane, P, periplasm, IM, inner membrane, ECF-σ, exocytoplasmic sigma factor, RNApol RNA polymerase. (Adapted from Noinaj et al. 2010, Hagan and Mobley 2007)]

In pathogenic *E.coli* haem utilization is mediated by genes in the *chu* locus, which shares homology with the *Shigella* heme utilization locus (*shu*) (Mills and Payne 1995). The *chu*
iron uptake system for heme/haemoglobin utilization is a 69-kDa outer membrane protein that is present in many pathogenic strains of *E. coli* including CFT073 and UPEC 536 (Nagy *et al.* 2001). Figure 2 summarizes the molecular machinery required for iron transport and haemin utilization in *E. coli*.

Uropathogens utilize an array of outer membrane iron receptors to facilitate siderophore and haem import from the iron-restricted urinary tract. These iron uptake systems contribute to the fitness of the pathogen yet are considered to be functionally redundant. The contribution of specific receptors to urinary tract pathogenesis was investigated using a competition assay within a murine model of ascending UTI. *E. coli* strain CFT073 catecholate receptor mutants (∆*fepA*, ∆*iha* and ∆*iroN*) were equally fit, suggesting redundant function, yet non-catecholate siderophore receptor mutants, aerobactin and yesiniabactin (∆*iutA* and ∆*fyuA*) were out-competed by other co-inoculated mutants indicating these systems contribute more significantly to UPEC iron acquisition (Garcia *et al.* 2011). The importance of various iron acquisition mechanisms has been demonstrated, where the expression of genes encoding five different iron acquisition systems in bacteria was apparent in the urine of mice inoculated with UPEC strain CFT073 to the bladder (Snyder *et al.* 2004). A more detailed study of bacteria infecting mouse bladders was made by Reigstad *et al* where the induction of ferric iron acquisition system genes (*feoA*, *tonB*, and *exbB*) and the *chuA* hemin receptor genes were shown in UPEC cells forming IBCs by qPCR from laser dissected tissue (Reigstad *et al.* 2007). The use of laser dissection further allowed the demonstration of the response of cells in the proximity of IBCs where genes encoding for transferrin receptor were upregulated (Reigstad *et al.* 2007). The requirement for iron acquisition has been tested in mouse infection models with strains deficient in receptor genes for enterobactin (∆*fepA*), salmochelin (∆*iroN*), aerobactin (∆*iutA*), yesiniabactin (∆*fyuA*), and the haem receptor genes *chuA* and *hma*. These models have demonstrated the redundancy of some of the iron acquisition systems as well as the importance of others (Garcia *et al.* 2011). A *tonB* mutant unable to acquire iron via any of the pathways is avirulent (Torres *et al.* 2001, Garcia *et al.* 2011). Specific mutations in individual acquisition systems do not have a large effect upon virulence, however aerobactin and yesiniabactin receptor gene knockouts (∆*iutA* and ∆*fyuA*) show a reduced competitive index as judged by bacterial numbers in the bladder and kidney (Garcia *et al.* 2011). Table 4 provides a summary of iron uptake systems found in UPEC 536.
Table 4. Summary of the Iron Uptake Systems Found in UPEC 536

<table>
<thead>
<tr>
<th>UPEC 536</th>
<th>Gene</th>
<th>Receptor Substrate</th>
<th>Substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Present</td>
<td>fepA</td>
<td>enterobactin</td>
<td>catecholate</td>
</tr>
<tr>
<td>Not present</td>
<td>iha</td>
<td>enterobactin</td>
<td>catecholate</td>
</tr>
<tr>
<td>Present</td>
<td>iroN</td>
<td>salmochelin</td>
<td>catecholate</td>
</tr>
<tr>
<td>Not present</td>
<td>iutA</td>
<td>aerobactin</td>
<td>hydroxymate</td>
</tr>
<tr>
<td>Present</td>
<td>fyuA</td>
<td>yersiniabactin</td>
<td>mixed</td>
</tr>
<tr>
<td>Present</td>
<td>fhuA</td>
<td>ferrochrome</td>
<td>hydroxymate</td>
</tr>
<tr>
<td>Present</td>
<td>hma</td>
<td>haem</td>
<td>NA</td>
</tr>
<tr>
<td>Present</td>
<td>chu</td>
<td>haem</td>
<td>NA</td>
</tr>
<tr>
<td>Not present</td>
<td>fecA</td>
<td>Ferric citrate</td>
<td>ion</td>
</tr>
</tbody>
</table>

1.6. Biofilms

Over the past three decades a great deal of research into the development and the characteristics of biofilms have taken place. Biofilms are described as “matrix enclosed bacterial populations adherent to each other and/or to surfaces or interfaces” (Costerton et al. 1999) or more simply “a thin coating comprised of living material” (Karatan and Watnick 2009). The preferred lifestyle of micro-organisms is within a biofilm where they accumulate at surfaces and interfaces to form microbial aggregates such as films, mats, flocs, or sludge. A biofilm provides a lifestyle for bacteria that differs from that of planktonic cells. Generally, the matrix immobilizes bacterial cells, holding them in close proximity to each other permitting cell-to-cell interactions such as quorum sensing and horizontal gene transfer. The biofilm also retains extracellular molecules and enzymes that can be used to degrade or dissolve entrapped nutrients from the aqueous medium and be used as energy sources (Branda et al. 2005). The contribution of dry mass may be less than 10% of the biofilm whereas the matrix may account for greater than 90% of the aggregate (Flemming and Wingender 2010). The matrix is a self-produced milieu of exopolymeric substances, polysaccharides and proteins that form a scaffold to provide adhesion to surfaces and stability.
for the three dimensional architecture of the biofilm (Sutherland 2001). Within the structure the matrix provides protection from desiccation, biocides, antibiotics, metallic cations, UV radiation and immune defences (Pamp et al. 2009; Sauer 2003). The matrix biopolymers vary between biofilms depending on the microorganism(s) present and the conditions of the environment such as shear forces, temperature and the availability of nutrients (reviewed in Flemming and Wingender 2010).

1.6.1 Biofilm Development

Biofilm formation is a complex developmental process involving a temporal series of events that are regulated in response to environmental and bacterial derived signals (Sauer 2003). For bacteria to undergo transition from free-living to sessile communities requires changes in gene expression to allow adherence to surfaces and interaction with other cells and the development of the unique physiological properties of a mature biofilm (reviewed by Kuchma and O'Toole 2000; O'Toole 2003).

The developmental stages of biofilm formation are dependent on a series of genetically controlled steps. The process is broadly divided into four distinct stages; attachment, microcolony formation, biofilm maturation and dispersal where each stage is defined by structural differences and cellular phenotypic changes (Sauer 2003).

Early stages of biofilm development rely on bacterial attachment to a surface or an interface through interactions between surface molecules and the structural and secreted components of the bacteria. In aqueous environments, physiochemical variables, may affect the rate of bacterial attachment and the rate of biofilm development (Fera et al. 1989). Electrochemical forces between the negatively charged bacterial cell and a surface are decreased in the presence of cations allowing for greater adherence; whereas the physical forces produced by the hydrodynamics of a medium may influence the time a bacterium is in contact with a surface and the efficiency of attachment (Costerton 1995; Sutherland 2001).

Both Gram-positive and Gram-negative bacteria are able to form biofilms although each organism utilizes specific molecules and structures for cell-to-surface and cell-to-cell interactions. Gram-negative planktonic bacteria adhere to a surface and form small clusters or microcolonies using structures such as pili (fimbriae) and outer membrane proteins. Flagella provide motility to the bacterial cell and allow them to swim within a fluid medium or glide over a surface. Besides their role in motility, flagella have also been involved in adhesion,
biofilm formation and colonization (Harshey et al. 2003). Motile UPEC strains have been shown to have a fitness advantage in persisting within a murine urinary tract for a greater length of time when compared to non-motile UPEC mutants demonstrating a subtle yet advantageous role for flagella in virulence (Wright and Hultgren 2006). Surface organelle expression is a complex, highly regulated process activated in response to environmental and nutritional conditions of the bulk medium (O’Toole 2003; Ren et al. 2004; Reisner et al. 2006).

Cell aggregation and exopolymeric substance (EPS) secretion enhances adhesion to a surface and influences the development of biofilm architecture. As a biofilm matures, cell clusters become progressively layered and thicken. EPS surrounds the bacterial colonies to form pleomorphic structures encasing bacteria within the matrix. As the biofilm develops water channels and cavities form within and between the growing structure allowing the exchange of nutrients and wastes throughout layers of cells. The matrix forms a protective barrier consisting mainly of water and branched fibres of polysaccharide chains, strengthened by environmental macromolecules, uronic acids and pyruvates, all of which influence its physical and chemical properties (Flemming and Wingender 2010).

As biofilm maturity continues the cell clusters reach a maximum thickness where the majority of cells lie as small aggregates within the larger biofilm structure. These micro-niches within the biofilm produce unique microenvironments with differing nutrient and oxygen gradients. The close proximity of cells within the microcolony allows for the creation of nutrient gradients and the cycling of various nutrients through biochemical reactions (reviewed in Donlan 2002) amongst bacteria with different phenotypes as well as for the exchange of genetic material.

The role of the matrix is not necessary for initial attachment of microbial cells to a surface but the production of EPS is essential for the development of the architecture of any biofilm matrix (Allison and Sutherland 1987). Cell density in E.coli biofilms is dependent on the expression of the stationary phase enzyme factor, rpoS, a gene expressed during slow growth (Adams and McLean 1999) and increased levels of nutrients leads to much denser biofilms (Moller et al. 1998). Biofilms alter their appearance as toxic metabolites increase, supporting the concept that biofilm architecture is substrate dependent (Nielson et al. 2000). Surface charges also play a role in the matrix structure where hydrophobic cells on and within the
biofilm form flocked structures, whereas hydrophilic cells will not (Freeman and Lock 1995) indicating that microbial and macromolecular composition of the matrix changes over time.

Mature biofilm development and the architecture of the structure are dependent on a variety of factors influenced by the bacteria and the environment. Motile E.coli K12 strains display vertical structures compared to non-motile E.coli strains (BW25113 (motility-impaired), MG1655ΔqseB, and MG1655ΔfliA) which form small flat microcolonies (Wood et al. 2006) suggesting an important role for flagella and motility in the development of the mature 3-dimensional biofilm. Pili, and specifically curli, are also important in 3-dimensional biofilm development and enhance the formation of biofilms with characteristic water channels and pillars of bacteria at both 25 and 37°C (Kikuchi et al. 2005) whereas curli deficient E.coli K12 strains grow less biofilm overall. Further, the role of exopolysaccharide is important for the development of the biofilm. Pseudomonas aeruginosa and E.coli have been shown to express alginate (Davies et al. 1998) and colanic acid (Prigert-Comberet et al. 2000) respectively, after attachment to a surface. Further study of E.coli K12 strains producing colanic acid indicate that colanic acid does not enhance adhesion to abiotic surfaces but is important in biofilm architecture while the biofilms of strains deficient in colanic production lack structure (Danese et al. 2000).

Uropathogens survive the biochemical insults of the urinary tract by producing a biofilm on bladder epithelium or within close proximity of the urinary tract. The biofilm ensures bacterial survival and may be the focus that facilitates the transition from acute to chronic infection.

1.6.2 The Biofilm Matrix

The biofilm matrix provides a dynamic environment where bacterial cells exist within unique micro-niches and make use of the available nutrients within the matrix. The bacteria form aggregates or microcolonies within a matrix where interstitial voids and channels separate the microcolonies. The matrix is a constantly changing environment with regions that harbour different biochemical environments in response to changing conditions. The matrix immobilizes biofilm cells, keeping them in close proximity to each other. The retention of extracellular enzymes within the matrix sequesters dissolved and particulate nutrients from the water and makes them available as nutrients and energy sources (reviewed in Flemming and Wingender 2010; Danese et al. 2000). Intrinsic factors contributing to the changing matrix are determined by the genetic make-up of the bacterial cells, whereas extrinsic factors
are dictated by the physio-chemical environment in which the biofilm matrix is located (Sutherland 2001). Water, the major component of the biofilm matrix, may be bound within the capsules of bacterial cells or be a solvent whose physical properties, such as viscosity, are determined by the solutes dissolved within it. Water also plays a role in binding and mobility within the matrix and is important for the diffusion of nutrients, wastes and the environmental molecules that occur within the biofilm (reviewed Wingender and Flemming 2010).

Many bacteria produce surface or secreted exopolysaccharides that play a vital role in the interaction of bacteria with the environment. Extracellular polymeric substance (EPS) is composed of repeating units of polysaccharide which can be released from the cell into the environment as a slime or remain attached to the cell to form a capsule. Many types of sugars are found in bacterial polysaccharides with variation in the linkages between sugars and the monosaccharide components. The dominant polymeric substances produced by an organism are strain specific and dependent on the conditions in which it is grown (Reisner et al. 2006, Wingender and Flemming 2010). The EPS matrix is the essence of the biofilm as it provides protection from desiccation, heat and acid shock, osmotic and oxidative stress and provides structure to the biofilm.

The physiochemical forces that connect the polymer strands of the matrix include electrostatic interactions between oppositely charged or like-charged polymer strands, physical entanglement of strands and covalent, hydrophobic and hydrogen bonding. The electrostatic interactions that occur between polymer ions are relatively strong and may be influenced by the ionic strength and the pH of the medium. Other forces such as van der Waal forces, the weakest form of intermolecular force, provide the main cohesive interactions between hydrocarbon chains of the macromolecule. In general biofilms display gel-like properties as they may undergo reversible elastic responses and irreversible deformation depending on the forces acting on the matrix (Wingender and Flemming 2010).

Chemical changes such as the addition of acetyl groups, which are common substitutions on exo-polysaccharides increase the cohesive properties of the EPS and alter biofilm architecture increasing aggregation of bacteria into microcolonies and determine the structurally heterogeneous architecture of mature biofilms (Fong et al. 2010). In contrast, deacetylation of polysaccharide induces the transition from random coils to helices (Cui et al. 1999) or may lead to a change in conformation (Rideout et al. 1997) thus strengthening portions of the matrix.
Investigation of biofilm matrices, specifically individual components, is still a difficult process considering the extent of reactions and transitions the biofilm undergoes in an ever-changing environment. Sampling and analysis of matrix components is a snapshot in time of a complex mixture of biological material.

1.7. Components of the Biofilm Matrix

1.7.1 Introduction

Differences in biofilm structure appear to reflect differences in the composition of the extracellular matrix, where extracellular polysaccharides, proteins and nucleic acids have been shown to be key components of the matrix. (Stewart and Costerton 2001; Sutherland 2001). There are several exopolysaccharides commonly found in the E.coli biofilm matrix such as cellulose (Zogaj et al. 2001), colanic acid (Grant et al. 1969), poly-N-acetyl-glucosamine (Wang et al. 2004) and other less significant lipopolysaccharide and capsular polysaccharide such as LPS O-antigens (Raetz and Whitfield 2002), enterobacterial common antigen (Kuhn et al. 1998) and capsular polysaccharides (K-antigen) (Whitfield 2006) all of which work together in varying degrees to shape and provide structure to the biofilm.

1.7.2 Cellulose

Cellulose, the most abundant component of plant biomass, is a homopolysaccharide composed of D-glucopyranose units linked by β-1,4 glycosidic bonds (Figure 3).

Cellulose is synthesized in nature as individual glucose molecules which undergo self-assembly at the site of biosynthesis (Brown and Saxena 2000). Individual cellulose molecules assemble into larger units known as elementary fibrils (protofibrils), which are packed into larger units called microfibrils and these are then assembled into cellulose fibres (Lynd et al.)
An important and characteristic feature of cellulose is its ability to form a crystalline structure. The cellulose polymer chains are stiffened by intra- and inter-chain hydrogen bonds and adjacent sheets overlie one another to be held together by weak inter-sheet van der Waal forces. The crystalline nature of cellulose implies a structural order in which all of the atoms are fixed in discrete positions with respect to one another ensuring the molecules of each microfibril are packed tightly to prevent penetration of enzymes and water (Lynd et al. 2002). The crystalline structure is not the common structure found in nature as most fibres are composed of crystalline and amorphous regions with kinks and twists of the microfibril or voids and large pits (Blouin et al. 1970; Fan et al. 1980), which help to increase the surface area of the fibre. This structural heterogeneity ensures the fibres are partially hydrated by water and pores and pits allow enough space for the penetration of large molecules and cellulolytic enzymes (Stone et al. 1969).

Most knowledge relating to cellulose synthesis in regard to its regulation and genetic arrangement has come from the model organism *Gluconacetobacter xylinus* (Ross et al. 1987, Brown et al. 2007). The molecular structure of the cellulose biosynthesis locus was first elucidated in *G. xylinus*, (Wong et al. 1990) in which two divergently transcribed operons, *bcsABZC* and *bcsEFG*, are constitutively expressed (Gerstel and Romling 2003; Romling, 2005; Romling 2007). The gene products of *bcsABZC* form a cellulose-synthase complex (Ross et al. 1991; Romling 2007). Recently, new evidence suggests that *yhjQ* but not *yhjR* is also essential for cellulose biosynthesis; *yhjQ* has been renamed *bcsQ* (Le Quere and Ghigo 2009). BcsQ displays polar localization and cell-to-cell adhesion is initiated through the production of cellulose at the poles (Le Quere and Ghigo 2009). A summary of the genetic arrangement can be found in Figure 4.

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**E.coli Cellulose Biosynthesis Operon**

![Figure 4. The Genetic Arrangement of the Cellulose Biosynthesis Operon of *E.coli*. The *E.coli* cellulose biosynthetic operon contains two divergently transcribed operons *bcsABZC-bcsEFG*. It has recently been determined that *yhjQ* (*bcsQ*) is also essential for cellulose biosynthesis. The role of *YhjR* is still unknown.](image-url)
Within the biosynthesis operon the \textit{bcsA} gene encodes the catalytic subunit of the cellulose synthase complex. It is an inner membrane protein whose activity is allosterically controlled by the binding of a small molecule called cyclic-di-GMP (c-di-GMP), a ubiquitous second messenger produced and degraded by diguanylate cyclase and phosphodiesterases, respectively (Ross \textit{et al} 1987; Jenal 2004; and Jenal and Malone 2006; Karatan and Watnick 2009). Diguanylate cyclases have a characteristic GGDEF motif which refers to a conserved sequence of five amino acids that stimulates c-di-GMP production whereas; phosphodiesterases have either an EAL or an HD-GYP amino acid motif and degrade c-di-GMP (Simm \textit{et al}. 2004). The definitive site of binding of c-di-GMP in Enterobacteriaceae is still inconclusive. There is speculation that BcsB binds c-di-GMP in \textit{Gluconacetobacter xylinus}, and induces a conformational change in protein structure that leads to activation of the biosynthetic cellulose operon (Mayer \textit{et al}. 1991) although this may not be the case for \textit{E. coli}. The PilZ domain of \textit{P. aeruginosa} was suggested to be an important aspect of the c-di-GMP binding protein (Amikam and Galperin 2006). This hypothesis was further tested and it was determined that a purified PilZ domain bound c-di-GMP with high affinity, as did the PilZ domain of BcsA from \textit{E. coli} albeit with lower affinity (Ryjenkov \textit{et al}. 2006) suggesting PilZ is sufficient for binding of c-di-GMP but sensory domains and other regulatory processes may over-ride the action of the GGDEF domain under the experimental conditions. The \textit{bcsZ} gene encodes an endo-1,4-D-glucanase (Romling 2007) and is required for cellulose biosynthesis. The \textit{bcsC} gene encodes a putative oxidase. BcsC mutants have impaired cellulose production \textit{in vivo} although all metabolic precursors are present, yet display cellulose synthase activity \textit{in vitro} (Zogaj \textit{et al}. 2001). It is probable that BcsC, an outer membrane protein, may serve as an anchoring region or a channel for cellulose export (Babujee \textit{et al}. 2006). The protein carries an N-terminal membrane domain, various domains of unknown function and several tetratrico peptide repeats (TPRs), a peptide consisting of four amino acids joined by peptide bonds - indicating it may also participate in protein-protein interactions (Romling 2007). The regulation of cellulose synthesis has been extensively studied in \textit{Salmonella} but the molecular basis of cellulose biosynthesis has not been resolved (Kai-Larsen \textit{et al}. 2010; Romling and Gomelsky 2005). Cellulose is also a key feature in \textit{E. coli} biofilm formation, where treatment with cellulase disperses existing biofilms (Zogaj \textit{et al}. 2001; Zogaj \textit{et al}. 2003, Romling 2002, Da Re and Ghigo 2006).

The first gene downstream of the cellulose operon on the UPEC 536 genome is \textit{yhjK} (ECP_3629) a putative diguanylate cyclase which has a central GGDEF motif and a C-
terminal putative phosphodiesterase EAL domain (Rudd 2000). YhjH found in *Salmonella Typhimurium*, is similar to YhjK, and has been shown to be involved in regulating swimming motility, suppression of cellulose synthesis, increased c-di-GMP degradation and carries an EAL domain (Ryjenkov *et al.* 2006).

Cellulose producing bacteria can be visualized using specific fluorescent chemical dyes such as Calcofluor White and a less specific Congo Red dye useful for detection of cellulose and curli expression. The use of these dyes as screening tools has demonstrated cellulose to be an abundant polysaccharide within the Enterobacteriaceae (Beloin *et al.* 2008). Cellulose producing bacteria are associated with the production of robust biofilms that vary in their phenotypic and morphological characteristics depending on the conditions under which the strains are grown.

### 1.7.3 Colanic acid

Colanic acid (CA) or M-antigen is an exopolysaccharide produced by *E.coli* and other members of the Enterobacteriaceae. It is a branched polymer of fucose, galactose, glucuronic acid and glucose (Hanna *et al.* 2003) (Figure 5).

![Figure 5. Structure of the Repeating Unit of Colanic Acid](Garegg *et al.* 1971). The polysaccharide is composed of fucose (β-L-Fuc), galactose (β-D-Gal), Glucuronic acid (β-D-Glc) and glucose (β-Glc) with an O-acetyl group that may reside in position C-2 or C-3 at the unbranched fucose and a pyruvate at the terminus of the side chain of the repeating unit (Verhoef *et al.* 2005).
The surface polysaccharides of \emph{E. coli} may be tightly associated to the outer membrane of the bacterium as in the O and K antigens and enterobacterial common antigen, or loosely associated as colanic acid and poly-\textit{N}-acetyl-glucosamine and referred to as \textit{exo} or slime polysaccharides. These exopolysaccharides are integral components of biofilms acting as the \textit{cement} to bind the various biofilm components (Sutherland 2001). Previous studies indicate that colanic acid synthesis is up-regulated in biofilms (Danese \textit{et al.} 2000, Prigent-Combaret \textit{et al.} 1999) and not synthesized by planktonic cells under \textit{normal} laboratory conditions. Upon production, most of the CA is secreted into the extracellular environment with no attachment to the outer membrane or involvement with initial attachment (Merideth \textit{et al.} 2007, Danese \textit{et al.} 2000) but is involved in the \emph{E. coli} biofilm architecture by influencing the thickness and the spatial arrangement of the structure (Prigent-Cambaret \textit{et al.} 2000).

Colanic Acid is a polyanionic hetero-polysaccharide containing repeated units of D-fucose, D-glucose, D-galactose and D-glucuronic acid sugars that are nonstoichiometrically linked to O-acetyl and pyruvate side chains (Grant \textit{et al.} 1969) (Figure 5). The colanic acid polysaccharide is assembled on the cell membrane by a series of glycosyl transferases at the cytoplasmic face of the inner membrane. Once assembled the single repeat is flipped to the periplasmic side and polymerized by the \textit{wzy} dependent pathway (reviewed in Raetz and Whitfield 2006). The polymer is then thought to be cleaved from the inner membrane anchor, transported across the periplasmic space and excreted into the extracellular environment by a poorly understood process (Merideth \textit{et al.} 2007).

Synthesis of colanic acid is a complex process that involves the 19 gene cluster \textit{wca} (formerly \textit{cps}) (Stevenson \textit{et al.} 1996) and is regulated by a complex signal transduction cascade controlled by the \textit{rcs} (regulator of capsule synthesis) phosho-relay system (Majdalani and Gottesman 2006). Colanic acid is induced in response to osmotic shock (Sledjestki and Gottesman 1996), desiccation (Ophir and Gutnick 1994), increased concentrations of zinc (Hagiwara \textit{et al.} 2003), detergents (Rajagopal \textit{et al.} 2002) and sub-inhibitory levels of \textit{β}-lactams (Sailer \textit{et al.} 2003) suggesting that the presence of colanic acid in biofilms may play a protective role.

\subsection*{1.7.4 Poly-β-1,6-N-acetyl-glucosamine (PNAG)}

The polymer \textit{β}-1,6-\textit{N}-acetylglucosamine (\textit{β}-1,6-GlcNAc) (Figure 6) is a polysaccharide common in \emph{Staphylococcus aureus} and \emph{Staphylococcus epidermidis} biofilms and contributes
to the pathogenesis of the bacterium in wound infections, osteomyelitis and catheter-associated urinary tract infection (Gotz 2002). The β-1,6-GlcNAc polymer in E.coli strain MG1655 has also been found to have a role in cell-cell adhesion and attachment to surfaces (Wang et al, 2004; Agladze et al. 2005).

![Chemical Structure](image)

**Figure 6. Chemical Structure of Poly-β-1,6-N-acetyl-glucosamine**

The production of β-1,6-GlcNAc depends on the *pgaABCD* locus which encodes proteins involved in the synthesis, export and localization of the polymer. PgaA is predicted to be an outer membrane protein, PgaB is involved in polysaccharide modification and PgaC is an inner membrane protein polysaccharide polymerase with two N-terminal and three C-terminal trans-membrane domains. PgaD is a small inner membrane protein with two N-terminal membrane spanning domains of unknown function. The *pga* genes are predicted to encode envelope proteins involved in synthesis, translocation, and possibly surface docking of this polysaccharide (Wang et al. 2004). The *pgaABCD* operon appears to be a horizontally acquired locus and is found in a variety of organisms. Genetic studies and polysaccharide analysis indicate that a membrane bound polysaccharide of *E.coli* is required for biofilm formation (Wang et al. 2004). An *E.coli* K12 strain (JM101) biofilm was able to be dispersed by meta-periodate (an oxidizing agent that creates active aldehyde groups) but not protease, suggesting a role for polysaccharide (Itoh et al. 2005) and that the polymer may be of importance as an adhesin that stabilizes *E.coli* biofilms and other bacteria such as Actinobacillus actinomycetemcomitans and Actinobacillus pleuropneumoniae (Kaplan et al. 2004; Wang et al. 2004). Further, mature twenty four hour *E.coli* biofilms were able to be degraded and dispersed with metaperiodate treatment and with β-hexosaminidase from
Aggregatibacter actinomycetemcomitans (Dispersin B) confirming specific hydrolysis of 1,6-N-acetylglucosamine linkages (Ramusubbu et al. 2005).

1.7.5 Extracellular DNA and DNase Activity

The concept of DNA secretion originated with observations of high content DNA in laboratory cultures of bacteria (Figure 7). Endogenous exDNA was identified as a primary viscous ingredient in halophile slime layers (Smithies and Gibbons 1955) and has since been observed in liquid cultures of a variety of organisms. Early experiments revealed that the exDNA could transform competent strains and suggested that cell death could not always account for the levels of exDNA observed (Smithies and Gibbons 1955). A study investigating the temporal progression of competence in B. subtilis provided a clear distinction between secreted DNA and DNA released by cell lysis which found that exDNA concentrations peaked briefly in late exponential phase declined to almost zero and then began to slowly increase through stationary phase concommitent with cell lysis (Lorenz et al. 1994).

Figure 7. The Chemical Structure of DNA. (www.scientificpsychic.com/.../aminoacids1.htm)

The importance of exDNA to biofilm structural stability has been described in Pseudomonas aeruginosa biofilms (Whitchurch et al. 2002). In this study, early biofilms were more sensitive to destabilization of the biofilm by DNase I than mature biofilms. PCR and
Southern blot analysis indicate that the exDNA was similar to genomic DNA and involved in cell-to-cell interconnections of the matrix in biofilms (Allesen-Holm et al. 2006). The exDNA in *P. aeruginosa* biofilms is suggested to be generated via lysis of a subpopulation of bacteria through the regulation of quorum sensing signalling (Nakamura et al. 2008). The excreted nucleic acid polymer is localized to the stalks of mushroom-shaped structures with increased concentration of exDNA in the outer region of the stalks forming a border between the stalk-forming bacteria and the cap-forming bacteria (Allesen-Holm et al. 2006). Besides providing biofilm stability, exDNA has been described as a multi-faceted component of the *P. aeruginosa* biofilm by contributing to cation gradients, inducing release of genomic DNA and inducible antibiotic resistance to cationic microbial peptides and aminoglycosides (Mulcahy et al. 2008). ExDNA has also been shown to be important in adhesion in some organisms. *Listeria monocytogenes* early biofilm development is dependent upon the presence of exDNA for adhesion (Harmsen et al. 2010). In this study, loss of adhesion induced by DNase I could not be replaced with genomic or salmon sperm DNA but the addition of exogenous peptidoglycan-\(N\)-acetylglucosamine (PNAG) with DNA was able to restore adhesion (Harmsen et al. 2010) highlighting a co-operative role with other components of the biofilm matrix. The degradation of a *P. aeruginosa* biofilm matrix and the release of exDNA from the matrix has been shown to reduce the capacity of the biofilm to induce the release of pro-inflammatory cytokines IL-8 and IL-1\(\beta\), reduce the up-regulation of neutrophil activation cell markers, reduce the number of bacteria phagocytosed per neutrophil contacting a biofilm and reduce the production of neutrophil extracellular traps (NETS) (Fuxman-Bass et al. 2010) indicating that substances within the matrix have immune modulating capabilities. Further study showed that a quorum sensing mutant (*lasI rhfl*) deficient in its ability to produce exDNA had a decreased capacity to stimulate the release of pro-inflammatory cytokines by neutrophils (Fuxman-Bass et al. 2010).

It has also been proposed that bacteria may produce an extracellular nuclease which digests dsDNA for catabolic purposes (Redfield 1993). Extracellular DNA is available in a variety of environments within the mammalian host such as the gastrointestinal tract and the mucosa of normal human lung tissue where exDNA can reach concentrations of 100 \(\mu\)g/ml and as high as 4 mg/ml in cystic fibrosis patients respectively (Potter et al. 1963) which could be utilized by organisms such as *E. coli* as a rich nutrient source (Finkel and Kolter 2001).
1.7.6 Analysis of EPS

There is no universally accepted method for the isolation of EPS. Identification of EPS components is a challenging process and is dependent on the isolation method used for the component of interest, as a component within the EPS may require a specific extraction technique compared to another. The extraction procedure must be adapted to the specific type of biofilm under investigation. Centrifugation, filtration, heating, blending, sonication and treatment with complexing agents and with ion-exchange resins have been described (Neilson and Jahn 1999). It is also difficult to quantitatively isolate EPS from a given biofilm as some of the EPS may remain attached to the bacterial cells and the stratum. Damage to cells during extraction may also cause intracellular material to leak into the matrix (reviewed in Flemming and Wingender 2010). Common isolation techniques inherently select for water soluble EPS and lose insoluble EPS including cellulose, which is an important component of many bacterial matrices. Isolation of cellulose requires harsh treatments with low pH and high temperatures (reviewed in Flemming and Wingender 2010).

Analysis of the EPS has been exploited by numerous methods to investigate the components of a matrix. Fluorescent probes have been useful to identify the presence of certain epitopes, macromolecules or monomers and chemical, biological and physical methods have been used (Majewska-Sawka et al. 2002; Chen et al. 2009). Highly specific polysaccharidases such as Dispersin B, (Ramusubba et al. 2005) have been used to identify the presence of specific polymers. The use of microsensor probes to determine dissolved O$_2$ and pH and the mapping of pH gradients within the biofilm matrix has been a useful strategy to investigate the physiochemical variances within dental biofilms (reviewed in Sutherland 2001). CLSM has become an invaluable tool for determining the structural complexities of the biofilm matrix in regard to architectural aspects of structure such as height, roughness and density (Zhang and Fang 2001; Hall-Stoodley and Stoodley 2009).

The composition and architecture of the EPS matrix provide a complex and dynamic network of macromolecules that impart stability to the matrix. Analysis of its components increases our understanding of the matrix and its interaction with other matrix components, yet new methods that are more sensitive and less destructive still need to be developed.
1.8. Dispersion of Biofilms

1.8.1 Introduction

Biofilms retain high numbers of organisms and considering the dose-dependent pathogenic potential of some organisms are therefore important factors in the transmission of pathogens. The dispersion of bacteria from biofilms may occur through: a) detachment by fluid flow and the hydrodynamic forces that exist within fluids, b) various translocation means such as swarming and/or twitching motility as seen in Pseudomonas aeruginosa, or c) the detachment of clumps of biofilm that may result in surface associated rolling motility as described in S. aureus (Hall-Stoodley et al. 2004). The nutritional status of the environment can have a profound effect on dispersal. In P. aeruginosa biofilms an increase in carbon substrate availability, specifically succinate, glutamate and glucose, induces dispersal with a corresponding decrease in pilus gene expression and an increase in motility (Sauer et al. 2004). Further, regulation of E.coli biofilm dispersion has been associated with the CsrA (carbon storage regulator) protein (Jackson et al. 2002) by post-transcription regulation of gene expression through the binding of mRNA transcripts of regulated genes and increasing or decreasing rates of decay (Lui et al. 1997; Wei et al. 2001).

More recently the role of two opposing enzymes, diguanylate cyclases that catalyze the formation of c-di-GMP and their counterpart, a phosphodiesterase, responsible for the degradation of c-di-GMP, have become a focus of biofilm transition between sessile and motile developmental stages (Jenal 2004; Jenal and Malone 2006; Tamayo et al. 2007).
1.8.2 Intracellular Signalling – cyclic-di-GMP

The nucleotide, cyclic-diguanosine monophosphate (c-di-GMP), is a second messenger in bacteria that was first described as an allosteric activator of cellulose synthase in *G. xylinus* (Ross *et al.* 1987). Over the decades it has been established that this nucleotide, found in all prokaryotic cells, regulates a number of cellular functions such as developmental transitions, aggregation, adhesion, biofilm formation and virulence factors (Jenal 2004; Jenal and Malone 2006; Romling 2005; Romling and Amikam 2006).

Levels of intracellular c-di-GMP are influenced by the opposing action of two enzymes. The first, a diguanylate cyclase (DGC) catalyzes the formation of c-di-GMP synthesis. DGC enzymes are identified by conserved GGDEF motifs within the protein. The second, a phosphodiesterase catalyzes the degradation of c-di-GMP and carries a conserved amino acid EAL or HD-GYP motif in the protein domain (Simm *et al.* 2004; Ryan *et al.* 2006). Hydrolysis of c-di-GMP to phosphoguanyl guanosine (pGpG) is dependent on the presence of Mg$^{2+}$ or Mn$^{2+}$ which then slowly converts back to GMP (Schmidt *et al.* 2005) (Figure 8).

The GGDEF domain was first described in PleD, a regulatory protein controlling swarmer-to-stalk-cell transition in *Caulobacter crescentus* (Hecht and Newton 1995) and the EAL domain first described for BvgR, a repressor of virulence gene expression in *Bordetella pertussis* (Merkel *et al.* 1998). A common theme emerging from c-di-GMP signalling is changes in biofilm characteristics when the expression of GGDEF, EAL or HD-GYP proteins is altered. Increased levels of c-di-GMP enhance attachment, the formation of biofilms and aggregation, whereas low levels promote motility and the expression of virulence factors (Romling 2005; Bobrov *et al.* 2008; Borlee *et al.* 2010; Bobrov *et al.* 2011).
Figure 8. The Synthesis and Degradation of Cyclic-di-GMP. Within the cell, bis-(3'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP) is produced from two molecules of GTP. The reaction is catalyzed by diguanylate cyclases that carry GGDEF motif domains and phosphodiesterases that carry EAL or HD-GYP motif domains. EAL domains cleave the c-di-GMP into pGpG, the first metabolic product of c-di-GMP.

Intracellular concentrations of c-di-GMP are low in planktonic conditions and may also be bound to effector proteins, making accurate quantitative measurement of the nucleotide difficult to determine, although response due to c-di-GMP binding may require less than 50 nM to a few μM (reviewed in Hengge 2009).

The sequencing of bacterial genomes has revealed the presence of numerous proteins with GGDEF, EAL and HD-GYP domains. *Pseudomonas aeruginosa* has 17 GGDEF, 5 EAL, 16 GGDEF / EAL and 3 HD-GYP proteins (Ryan et al. 2006, Galperin and Gomelsky 2006), *Salmonella Typhimurium* harbours 12 proteins with GGDEF domains, 14 proteins with EAL domains (Simm et al. 2004) and *E.coli* has 19 GGDEF and 17 EAL domains (Romling 2005). GGDEF and EAL domains are often found in multi-domain proteins and the same protein
often carries both GGDEF and EAL domains which are located C-terminally from single or multiple sensory and signal transduction domains (Romling 2005). The structure of the GGDEF domain is similar to that of eukaryotic adenylate cyclases (Hecht and Newton 1995; Paul et al. 2008). Second messenger signalling is not new to *E.coli* as a control for intracellular functions. Cyclic AMP (cAMP) in *E.coli* is synthesized by a single adenylate cyclase that is controlled by a complex signal input system and degraded by a specific phosphodiesterase. The cAMP allosterically controls the transcription factor cAMP receptor protein (CRP) and acts on numerous targets that have similar cAMP-CRP binding sites for transcriptional regulation (Botsford and Harman 1992; Holland et al. 2008). In the case of c-di-GMP signalling the number of proteins carrying the required GGDEF, EAL and/or HDGYP motifs and the sensory domains associated with each protein produce a diverse repertoire of combinations to control numerous cellular functions, making c-di-GMP a complex signaling system (Figure 9).

![Diagram of c-di-GMP Binding](image)

**Figure 9. Physiological Functions of c-di-GMP Binding.** Increased c-di-GMP concentrations promote sessility and the expression of adhesive structures such as fimbriae (Kader et al. 2006) and extracellular polymeric substances such as cellulose (Romling 2002). Low c-di-GMP levels are associated with motility (Simm et al. 2004) and other planktonic features.

New insights into the proteins which carry the effector site for c-di-GMP binding are coming to light. Currently four c-di-GMP binding effector proteins families are known. The PilZ family of proteins (Amikam and Galperin 2006) including the PilZ domain found in *bcsA* of *E.coli*, the transcription factor, FleQ of *P. aeruginosa* (Hickman and Harwood 2007) that acts as a repressor but is inactivated upon c-di-GMP binding, PelD of *P. aeruginosa* (Lee et al. 2007), involved in polysaccharide formation, is activated by c-di-GMP binding and the PopA protein of *Caulobacter crescentus* which indirectly controls the degradation of a global regulator involved in cell cycle progression (Duerig et al. 2009). The binding of c-di-GMP
has a number of outcomes that can be manifested in various phenotypes. C-di-GMP has been shown to effect the transcription of genes (Hickman and Harwood 2007), the enzymatic activity within operons (Amikan and Galperin 2006), the cellular structures of the bacterial cell such as flagella by the interference of motor function (Christen et al. 2007) and proteolysis for phase transition in the cell cycle of *C. crescentus* (Paul et al. 2008). The effects of c-di-GMP signalling may induce localized changes in protein expression as seen in the LapA adhesin on the outer membrane of *P. fluorescens* (Newell et al. 2009) although the specific diguanylate cyclase involved in signalling is still unknown. In an attempt to identify diguanylate cyclase of *P. fluorescens*, mutations were made in 30 putative diguanylate cyclases yet, only four resulted in a reduction in biofilm formation under the conditions tested (Newell et al. 2011) suggesting c-di-GMP effects may be localized and specific or total intracellular concentrations may reflect more general effects. The role of c-di-GMP intracellular signalling and the physiological outcomes appear to be a highly complex network of interactions that are slowly being elucidated.

### 1.9. Biofilms as Reservoirs for Infection

Biofilms provide important environmental and host reservoirs for pathogenic bacteria. Characteristics of biofilms, such as resistance to antimicrobials and increased opportunities for genetic exchange, may also increase the pathogenic potential of biofilm bacteria and promote the survival of organisms in the environment (Parsek and Greenberg 2000). The biofilm matrix contains nucleic acids that play a role in the structural composition of the matrix but also have the potential to facilitate horizontal gene transfer via normal transformation processes (Christensen 2001; Hausne and Wuertz 1999) and enhance the probability of transfer of drug resistance and virulence genes. The biofilm produces a heterogeneous bacterial population due to the numerous micro-niches within its structure. As a result different environmental zones develop within the biofilm with respect to aeration, pH, and nutrition (Hall-Stoodley et al. 2004; Lenz et al. 2008, Fux et al. 2005) which may contribute to the development of recalcitrant characteristics of biofilm bacteria.

Nosocomial pathogens, especially biofilm-associated clinical strains are resistant to conventional hospital cleaning practices (Smith and Hunter 2008) and may persist on hospital surfaces for extended periods of time (Kramer et al. 2006) providing a continuous source for transmission of infection. Conduits for drinking water may also represent reservoirs for potentially serious disease especially in immune-compromised individuals (Park et al. 2001).
The host also harbours biofilm reservoirs that may manifest as chronic infections. Bacterial infections rely on a host–pathogen interaction where biofilms may play a significant role in the transition from acute to chronic or persistent infection (Costerton et al. 1999; Anderson et al. 2004; Cegelski 2008; Toretta et al. 2011). Biofilms are now recognized in a number of chronic infections including peritonitis, device-related infections, cystic fibrosis related pneumonia, chronic urinary tract infections, recurrent tonsillitis, chronic rhino-sinusitis, chronic otitis media and chronic wound infections (Hall-Stoodley and Stoodley 2009). The difficulty in diagnosing biofilm infections is that culturing methods, which are adequate for acute infections are inadequate for the determination of chronic biofilm-related infection (Toretta et al. 2011). This has lead to a new diagnostic criterion to describe biofilm infections that takes into consideration biofilm characteristics. The presence of bacterial aggregates, matrix and viable (culture negative) cells, infection focus, recalcitrance to antibiotic therapy and localized inflammation may help diagnose and treat biofilm-related infections more effectively (Parsek and Singh 2003).

Biofilms confer resistance to antibiotics and to immune killing due to protection by the matrix and other previously discussed biofilm characteristics (section 1.6). Consequently, bacterial biofilms are able to exist in close proximity to an infection site until signals induce the bacteria to disperse and re-infect (Reigstad et al. 2007). Biofilm bacteria have the potential to promote infection or a relapse / recurrence of acute symptoms when large numbers of bacteria disperse from the biofilm. The dispersal of bacteria from biofilms may be a passive event influenced by the flow rates and/or hydrodynamic forces of a fluid environment (Hall-Stoodley and Stoodley 2005) or the status of the environment, such as nutrient availability or the presence of antimicrobials (Daly et al. 1998). Bacteria may also actively separate from the biofilm by dissolving the surrounding extracellular matrix through the action of secreted enzymes, such as polysaccharide lyases or by using cell surface-associated enzymes that modulate the surface adhesins (Boyd and Chakrabarty 1994; Lee and Bowden 1996) when induced by an environmental signal(s).

The pathogenic potential due to the lifestyle of biofilm organisms highlights the importance of understanding the mechanisms used by bacteria to transition between biofilm and planktonic states.
1.9.1 Urinary Catheters – A Biofilm Perspective

Urinary catheters are often a required medical treatment for the elderly and the disabled. Indwelling (Foley) catheters also provide bacteria with direct access to the bladder, increasing the risk factor for bacteruria. Bacteria present in the peri-urethral area can migrate into the bladder through the mucoid layer that forms between the epithelial surface of the urethra and the catheter (reviewed in Stickler 2008). Bacterial contamination within drainage bags also allows bacteria access to the bladder via the drainage tube and catheter lumen (Stamm 1991). Initial infections are most often caused by skin flora of the peri-anal region such as *Staphylococcus epidermidis*, *Escherichia coli* and *Enterococcus fecalis* acquired as a contaminant from the patient or caregiver’s normal flora, while other species appear over time (Matsukawa et al. 2005). Treatment with antibiotics for short-term urinary catheter-associated bacteriuria is generally not indicated as infections are often asymptomatic and the risk of promoting antibiotic resistance is high, although screening for and treatment of asymptomatic bacteriuria before transurethral manipulation or instrumentation where short-term catheterization is required is recommended (Nicolle et al. 2005). Screening for, and treatment of, asymptomatic bacteriuria is not recommended for pre-menopausal, non-pregnant women, diabetic women, older persons living in the community, elderly institutionalized people, persons with spinal cord injury or catheterized patients while the catheter remains in situ (Nicolle et al. 2005).

Long-term catheters are generally changed every 10-12 weeks, leaving approximately three months of contaminated mixed species urine flowing through the catheters before a change (reviewed in Stickler 2008) providing an optimum niche for colonization and biofilm development.

Some bacteria produce biofilms that become crystalline in nature, particularly, urease producing *Proteus mirabilis* (Getliffe and Mulhall 1991; Stickler and Zimakoff 1994). Urease hydrolyzes urea to ammonium and carbonate ions that increase the pH of the urine. The alkaline urine induces the precipitation of magnesium and carbonate phosphate crystals providing a stratum for biofilm development. Crystalline biofilms build on the outer surface of the catheter around the balloon and the catheter tip which can cause trauma to the urothelium. Removal of the catheter carries the risk of crystalline biofilm debris coated in large number of bacteria shedding into the bladder where it may initiate stone formation. Biofilm crystal material may also block the flow of urine resulting in leakage of urine from
the edge of the catheter, incontinence and more nursing care (reviewed in Stickler 2008). Blockage of the lumen and retention of urine carries an increased risk of vesicoureteric reflux of infected urine which may lead to pyelonephritis and septicaemia (Liedle 2001; Kunin 1987). Escherichia coli do not produce urease, although like Klebsiella pneumoniae (Rameriz-Castillo and Uribelarrea 2004) and Pseudomonas aeruginosa (Myszka and Czaczek 2009) they do produce large amounts of exopolysaccharide as part of mucoid biofilms that may also occlude the catheter lumen resulting in similar outcomes and mixed species infections exacerbate the condition.

1.9.2 Chronic Urinary Tract Infection- A Biofilm Perspective

Urinary tract infections are often self-limiting and rarely spread further than the urinary tract, yet they have the potential to cause considerable morbidity in children and the elderly. Women are most affected by the disease and the majority of women will experience at least one UTI in her lifetime, and of those many will experience multiple and recurrent infections (Shortliffe and McCue 2002).

The bladder lumen is surrounded by a single layer of differentiated superficial cells overlaying two to three layers of small undifferentiated basal intermediate epithelial cells. The epithelial cells display mannosylated uroplakins, proteins that line the luminal surface of the superficial urothelium. Interaction of the urothelium with Type 1 pili mediates attachment to the host cell and specific interaction with the FimH tip adhesin and the mannosylated uroplakins receptors stimulate a host cell signalling cascade which ultimately leads to internalization of the uropathogen (Martinez et al. 2000; Mulvey et al. 1998). Internalization of the organism within the host cell provides protection from innate and adaptive host defences and may also allow dissemination within and across tissue barriers. Once inside the bladder cell UPEC can replicate intracellularly and re-emerge from the cell, interact with, and invade surrounding and underlying epithelial cells, establish bacterial reservoirs within bladder tissue to facilitate a localized persistence (Mulvey et al. 2001).

Upon internalization loose collections of bacteria form and become encased in a polysaccharide-rich matrix. Within these loosely structured aggregates Type 1 pili and Antigen 43 are present (Anderson et al. 2004). Over a series of growth stages the bacterial aggregates within the umbrella cell are able to escape into the lumen by a process referred to as fluxing where many of the bacteria are in a filamentous form. The bladder urothelium responds to bacterial invasion via TLR-4 which triggers cytokine release and the influx of
polymorphonuclear leukocytes (PMNs) into the lumen (Shilling et al. 2003; Mysorekar and Hultgren 2006) and where the umbrella cells undergo an apoptosis-like death and exfoliation (Mulvey et al. 1998) releasing bacteria into the urine.

In murine models it is observed that when the bladder is sufficiently colonized an intracellular sub-population of bacteria remain in a quiescent state, which may serve as a seed for recurrent infections (Mulvey et al. 2001; Shilling et al. 2003). UPEC Quiescent Intracellular Reservoirs (QIR) becomes established in the intracellular Lamp1+ endosomes of the differentiated superficial cells and/or in the underlying transitional epithelial cells of the bladder urothelium (Mysoreka and Hultgren 2006).

Experimentally, induction of exfoliation of bladder epithelium can be achieved with protamine sulphate, a cationic protein which causes a biochemical inactivation of proteoglycans and glycosaminoglycans (GAG) within the urothelium. Exfoliation by protamine sulphate eradicates bacteria on superficial cells but QIRs may persist within the underlying transitional epithelium. Bacteria within the QIR are viable and capable of re-seeding the uroepithelium (Mysoreka and Hultgren 2006). It is suggested that some people such as spinal cord injury patients and some healthy women with biphasic patterns of GAGs during the menstrual cycle, may have damaged urothelium similar to that seen induced by protamine sulphate that may render bladders more vulnerable to infection of bacteria and account for recurrent infections (Apodaca et al. 2003, Marcolo et al. 2005).

Observation of the multi-stage differentiation cycle while growing in the cytoplasm of umbrella cells as well as the host response of PMN recruitment and phagocytosis within a murine model has been elegantly documented using fluorescent video-microscopy and a biofilm model of persistence within the urinary tract established (Justice et al. 2004). This model is described in Figure 10. Research has provided a better understanding of the evasion strategies used by uropathogens. The infection and re-infection opportunity used by biofilm-associated bacteria explains the difficulty in diagnosing and treating urinary tract infections effectively.
1. Binding – Attachment of cells to bladder epithelium, specific binding FimH to uroplakins, 2. Invasion - Approximately 1-3 hours post infection, 3. Early IBC formation – Loose collection of bacteria within cytoplasm, rod shaped, non-motile, amorphous aggregate, doubling time 30-35 minutes, 4. Middle IBC formation – Organized colonies with biofilm-like traits, simultaneous bacterial differentiation and reduced cell size, tightly packed aggregates, appearance of pods on bladder lumen, 5. Late IBC formation – Twelve hours after infection coccoid-shaped bacteria on outer edge of IBC increase to normal size, become motile, dissociate from IBC, 6. Fluxing/Filamentation – Flagella-type motility into the lumen or onto umbrella cells and may lead to a second round of invasion and differentiation. Similar to detachment/dispersal event in biofilms on abiotic surfaces, 2a - Quiescent Reservoirs – Post exfoliation umbrella cells may contain intracellular, bacteria in small aggregates that resemble those from early IBC development, decreased growth rate, 3a. IBC Pod Exfoliation – Bladder response to bacterial infection is exfoliation of umbrella cells (Adapted from Justice et al. 2004, Anderson et al. 2004).

1.9.3 Diagnosis and Treatment of Biofilms and Chronic Infections

Current methods for diagnosis and treatment of infections examine only isolated planktonic cells, growing as log-phase cultures in nutrient-rich media. Consequently, this approach may limit the development of understanding about the microbial processes under study (Smith 1998; Wolcott and Ehrlich 2008). In chronic infections, planktonic cells appear as transient sub-populations of the biofilm community. Identification of biofilm bacteria requires a new approach to detection and visualization methods for better treatment strategies of biofilm-related disease. Planktonic cells show good antibiotic susceptibility yet biofilm cells will have poor antibiotic susceptibility highlighting the discrepancy that can occur between in vitro test results and in vivo responses to antibiotic therapy. Conventional laboratory testing may only identify a very small percentage of cells residing in a biological eco-system indicating a short-fall in conventional testing of a complex biofilm that may include many...
more species or phenotypes. Assessment of planktonic cells alone may lead to inaccurate or incomplete diagnosis as conventional testing methods do not detect biofilm cells that may be viable but not culturable (Wolcott and Ehrlich 2008). In regard to biofilms and chronic urinary tract infection treatment, antibiotics may achieve sterilization of the urine but fail to remove the causative agent that resides within a biofilm on or within the urinary bladder epithelium, accentuating the need for more rational treatment strategies.

New methods to identify biofilm cells use a range of molecular methods in combination with conventional culturing methods, many of which have proven successful in the identification of bacterial species composition of chronic infections (reviewed in Flemming and Wingender 2010). Routine bacterial analysis may need to expand its approach to identification of bacteria to encompass this very important part of pathogenesis. New strategies for treatment of chronic infection will evolve as mechanisms used by bacteria to cause disease and produce biofilms are discovered. Concomitant therapies that eradicate bacteria and disrupt biofilm formation and/or development may prove more effective than treatment with single or sequential strategies such as antibiotic therapy (Ehrlich et al. 2005). When chronic diseases that affect most tissues and structures within the body are viewed as biofilm diseases the prevalence of the disease becomes significant yet the treatment rationale for biofilm-associated chronic infections may be similar to many infections (Wolcott and Ehrlich 2008).

1.10. Rationale for this Study

In the study of bacterial infections it is important to be aware of the effects of the changes that may occur in the environment of the bacterial population during the progression of an infection. Among the most important of these factors is the availability of iron. In the context of an infection, upon colonization bacteria will most likely encounter an iron-restricted environment (Ganz 2009). Iron will subsequently become available when liberated by the activities of bacteria in successful infections. Uropathogenic *Escherichia coli* (UPEC) exhibits a clear phenotype transition, manifested as the dispersal from aggregates, when iron restricted broth cultures are provided with iron.

Infections of the urinary tract are the most common bacterial infections in humans (Foxman 2002) and UPEC are the leading cause of these infections (Foxman and Brown 2003). Uropathogens form biofilm-like communities within the urinary tract (Rosen et al. 2007; Wiles et al. 2007) that show resistance to antibiotics, that act as a reservoir for infection and
are often responsible for the recurrence of infection (Wright and Hultgren 2006). The battle for iron during a UPEC infection is well documented. Within the bladder cells close to biofilm-like communities, there is increased expression of genes to restrict bacterial access to iron, and biofilm cells up-regulate the expression of genes for iron acquisition (Reigstad et al. 2007). Furthermore, UPEC mutants compromised in iron acquisition are either non-pathogenic or poorly able to compete (Torres et al. 2001; Hagan and Mobley 2009).

In this study, the nature of UPEC biofilm-like aggregates and the role of iron as a regulator of UPEC biofilm dispersion were investigated. The major aims of this study were to:

- use biochemical and genetic approaches to identify the functional components of the biofilm matrix,
- characterise the signals activating dispersion,
- characterise the effects of the aggregate matrix upon antibiotic sensitivity and survival during infection of model organisms
- determine the extent of conservation of the aggregation/dispersal phenotype in UPEC strains beyond the model of UPEC 536.
Chapter 2. Materials and Methods

2.1. Bacterial Strains

2.1.1 Bacterial Strains and Plasmids

A laboratory-adapted UPEC strain 536 for which the genome sequence is available (http://www.ncbi.nlm.nih.gov/sites/entrez) was used as the primary experimental strain. Fresh clinical UTI isolates were acquired from Auckland Hospital. An Escherichia coli wild-type K12 strain MG1655 was used as a non-pathogenic control Guyer et al (1981). Other E. coli strains were used as plasmid cloning hosts, templates for DNA to drive λ red mutagenesis and conjugation. Single-gene knockout strains from the Keio Collection were used to create the respective gene knockout in UPEC 536. A full list of bacterial strains used in this study can be found in Table 5.

A single colony was used to inoculate an overnight culture (RPMI 1640 + 10µM ferric chloride (FeCl₃) and a portion of the culture was stored in 25% v/v glycerol at minus 80°C. All subsequent testing was performed using UPEC strains recovered from minus 80°C storage.
Table 5a. Bacterial Strains used in this Study.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>UPEC 536</td>
<td>Clinical strain isolated from a patient with as pyelonephritis infection. Mrh⁺, Fim⁺, Sre⁺, Hyl⁺ and SmR</td>
<td>Knapp et al. 1986</td>
</tr>
<tr>
<td>MG1655</td>
<td>F⁻, lambda⁻, ilvG⁻ rfb-50 rph-1</td>
<td>Guyer et al. 1981</td>
</tr>
<tr>
<td>S17-1 ąc·pir</td>
<td>Tp⁺, SmR, recA, thi, pro, hsdRM⁺, RP4:2Tc:Mu: Km Tn7 ·pir. Conjugal transfer of plasmids requiring ·pir for replication</td>
<td>deLorenzo et. al. 1990</td>
</tr>
<tr>
<td>CC118 ąc·pir</td>
<td>phoA20 thi-1 rspE rpoB argE (Am) recA1</td>
<td>Herrero et al. 1990</td>
</tr>
<tr>
<td>UPEC 536 ·yaiC</td>
<td>pNQ705yaiC, Cm⁺, yaiC</td>
<td>This study</td>
</tr>
<tr>
<td>UPEC 536 ·bcsA</td>
<td>pNQ705bcsA, Cm⁺, bcsA⁻</td>
<td>This study</td>
</tr>
<tr>
<td>UPEC 536 ·bcsZ</td>
<td>pNQ705bcsZ, Cm⁺, bcsZ</td>
<td>This study</td>
</tr>
<tr>
<td>UPEC 536 ·yhjK</td>
<td>pNQ705 yhjK, Cm⁺, yhjK</td>
<td>This study</td>
</tr>
<tr>
<td>BW25113</td>
<td>·araD-araB)567, ·acZ4787(:rrnB-3), rph-1, ·rhaD-rhaB)568, hsdR514</td>
<td>Datsenko and Wanner, (2000)</td>
</tr>
<tr>
<td>JW0669-2</td>
<td>BW25113, ·æur</td>
<td>Baba et al. (2006)</td>
</tr>
<tr>
<td>JW3499-1 ·bcsZ</td>
<td>BW25113, ·æbcsZ</td>
<td>Baba et al. (2006)</td>
</tr>
<tr>
<td>JW5665-1 ·bcsA</td>
<td>BW25113, ·æbcsA⁻</td>
<td>Baba et al. (2006)</td>
</tr>
<tr>
<td>JW5943-1 ·yhjK</td>
<td>BW25113, ·æyhjK</td>
<td>Baba et al. (2006)</td>
</tr>
<tr>
<td>UPEC 536 ·æur</td>
<td>UPEC 536 ·æur731::kan</td>
<td>This study</td>
</tr>
<tr>
<td>UPEC 536 ·æbcsA</td>
<td>UPEC 536 ·æbcsA787::kan</td>
<td>This study</td>
</tr>
<tr>
<td>UPEC 536 ·æyhjK</td>
<td>UPEC 536 ·æyhjK784::kan</td>
<td>This study</td>
</tr>
<tr>
<td>UPEC 536 ·æbcsZ</td>
<td>UPEC 536 ·æbcsZ786::kan</td>
<td>This study</td>
</tr>
<tr>
<td>Clinical Isolate Strains</td>
<td>Pathology</td>
<td>Source</td>
</tr>
<tr>
<td>--------------------------</td>
<td>-------------------------</td>
<td>-------------------</td>
</tr>
<tr>
<td>OF 5409</td>
<td>Cystitis strain</td>
<td>Auckland Hospital</td>
</tr>
<tr>
<td>OF 6636</td>
<td>Cystitis strain</td>
<td>Auckland Hospital</td>
</tr>
<tr>
<td>OF 5862</td>
<td>Pyelonephritis</td>
<td>Auckland Hospital</td>
</tr>
<tr>
<td>OF 6020</td>
<td>Cystitis strain</td>
<td>Auckland Hospital</td>
</tr>
<tr>
<td>OF 6786</td>
<td>Cystitis strain</td>
<td>Auckland Hospital</td>
</tr>
<tr>
<td>OF 6860</td>
<td>Pyelonephritis</td>
<td>Auckland Hospital</td>
</tr>
<tr>
<td>OF 6762</td>
<td>Cystitis strain</td>
<td>Auckland Hospital</td>
</tr>
<tr>
<td>OF 6703</td>
<td>Cystitis strain</td>
<td>Auckland Hospital</td>
</tr>
<tr>
<td>OF 5179</td>
<td>Cystitis strain</td>
<td>Auckland Hospital</td>
</tr>
<tr>
<td>OF5625</td>
<td>Asymptomatic bacteruria</td>
<td>Auckland Hospital</td>
</tr>
<tr>
<td>OF 6869</td>
<td>Cystitis strain</td>
<td>Auckland Hospital</td>
</tr>
</tbody>
</table>
Table 6. Plasmids used in This Study

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pNQ705</td>
<td>4.5 kb, mobRP4, Cm&lt;sup&gt;R&lt;/sup&gt;, oriV</td>
<td>Milton et. al (1996)</td>
</tr>
<tr>
<td>pTrc99A</td>
<td>4.2 kb, Expression Vector; P&lt;sub&gt;lac&lt;/sub&gt; rnrB T&lt;sub&gt;1&lt;/sub&gt;, T&lt;sub&gt;2&lt;/sub&gt;, lacI&lt;sup&gt;R&lt;/sup&gt;, pBR322 ori, Amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Amann et al. (1988)</td>
</tr>
<tr>
<td>pCR®-BLUNT</td>
<td>3.5 kb, pUC ori, P&lt;sub&gt;lac&lt;/sub&gt;, lacZ&lt;sup&gt;U&lt;/sup&gt; ccd B, Km&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pDONR221+TOMATO</td>
<td>4.86 kb, a PCR product containing the tomato gene flanked with att sites *Gateway&lt;sup&gt;TM&lt;/sup&gt; cloned into pDONR221</td>
<td>S. Wiles (2010)</td>
</tr>
<tr>
<td>pACBSCE</td>
<td>arabinose promoter, ϕred genes, SceI enz, TS ori, Cm&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Lee et.al (2009)</td>
</tr>
<tr>
<td>pKD46</td>
<td>ϕred genes cloned into pKD16, a derivative of INT-&lt;sup&gt;ts&lt;/sup&gt;, carrying araC and araBp from pBAD18, Amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Datsenko and Wanner, (2000), Haldimann and Wanner (2001)</td>
</tr>
<tr>
<td>pNQ705&lt;sub&gt;yaiC&lt;/sub&gt;</td>
<td>Containing an internal fragment of yaiC cloned into pNQ705 as a SacI/XhoI fragment</td>
<td>This Study</td>
</tr>
<tr>
<td>pNQ705&lt;sub&gt;bcsA&lt;/sub&gt;</td>
<td>Containing an internal fragment of bcsA cloned into pNQ705 as a SacI/XhoI fragment</td>
<td>This Study</td>
</tr>
<tr>
<td>pNQ705&lt;sub&gt;bcsZ&lt;/sub&gt;</td>
<td>Containing an internal fragment of bcsZ cloned into pNQ705 as a SacI/XhoI fragment</td>
<td>This Study</td>
</tr>
<tr>
<td>pNQ705&lt;sub&gt;yhjK&lt;/sub&gt;</td>
<td>Containing an internal fragment of yhjK cloned into pNQ705 as a SacI/XhoI fragment</td>
<td>This Study</td>
</tr>
<tr>
<td>pTrc99A&lt;sub&gt;yaiC&lt;/sub&gt;</td>
<td>An IPTG inducible expression vector. yaiC cloned downstream of pTrc NcoI/HindIII</td>
<td>This Study</td>
</tr>
<tr>
<td>pTrc99A&lt;sub&gt;bcsA&lt;/sub&gt;</td>
<td>An IPTG inducible expression vector. bcsA cloned downstream of pTrc EcoRI/HindIII</td>
<td>This Study</td>
</tr>
<tr>
<td>pTrc99A&lt;sub&gt;bcsZ&lt;/sub&gt;</td>
<td>An IPTG inducible expression vector. bcsZ cloned downstream of pTrc NcoI/HindIII</td>
<td>This Study</td>
</tr>
<tr>
<td>pTrc99A&lt;sub&gt;yhjK&lt;/sub&gt;</td>
<td>An IPTG inducible expression vector. yhjK cloned downstream of pTrc NcoI/HindIII</td>
<td>This Study</td>
</tr>
<tr>
<td>pYrc99A&lt;sub&gt;yhjK(alt)&lt;/sub&gt;</td>
<td>An IPTG inducible expression vector. yhjK(alt) cloned downstream of pTrc NcoI/HindIII</td>
<td>This Study</td>
</tr>
</tbody>
</table>
2.1.2 Chemical Reagents

All general chemical reagents were analytical grade and purchased from Scharlau, Sigma, Invitrogen, AppliChem, Acros Organics, or Becton Dickenson unless otherwise stated.

2.1.3 Water

All water used for reagents and molecular experiments was UltraPure (Gibco, Invitrogen) or distilled water.

2.2. Media and Reagents

2.2.1 Media

Culturing of bacteria to single colonies used Columbia Horse Blood Agar, Nutrient Agar (Fort Richard, Auckland) or on freshly prepared LB agar (section 2.2.2) or M9 (section 2.2.3) agar with supplements as required.

2.2.2 Luria Bertani media

Difco™ LB (Lennox) was prepared according to manufacturer’s instruction and consisted of 10 g tryptone, 5 g yeast and 5 g sodium chloride in 1L of distilled water. LB was sterilised by autoclaving at 120°C, 15 psi for 20 min. LB agar was prepared by the addition of Bacteriological Agar (BD) at 1.5% (w/v) to LB broth prior to autoclave sterilization.

2.2.3 M9 Minimal media

M9 minimal media was prepared as described in Sambrook *et al.* 2001. 200 ml of 5x M9 salts (64 g l⁻¹ Na₂HPO₄·7H₂O, 15 g l⁻¹ KH₂PO₄, 2.5 g l⁻¹ NaCl and 5.0 g l⁻¹ NH₄Cl), 20 ml of 20% (w/v) glucose, 500 µl of 20% (w/v) MgSO₄, 200 µl of 5 mg ml⁻¹ thiamine was added together to a final volume of 1L sterile water or sterile molten 1.5% (w/v) agar.

2.2.4 Congo Red Agar

M9 Minimal media agar was prepared (see 2.2.2) and supplemented with 0.001% (w/v) Congo Red dye (benzidinediazo-bis-1-naphthylamine-4-sulfonic acid) prior to pouring plates (Surgalla and Beesley 1969).
2.2.5 Super Optimal Broth (SOB) for Chemically Competent Cells

SOB media was made as described by Hanahan (1983), where 5 g l\(^{-1}\) yeast extract, 20 g l\(^{-1}\) tryptone, 0.584 g l\(^{-1}\) NaCl, 0.186 g l\(^{-1}\) KCl and 2.4 g l\(^{-1}\) MgSO\(_4\) were added together and diluted to 800 mL with water. The pH was adjusted to 7.5 with 1M NaOH (approx. 25 mL) and diluted to 1L with water.

2.2.6 Super Optimal Catabolite (SOC) for Bacterial Transformations

SOC media was prepared as described by Hanahan (1983), where 1L of SOB media was prepared and cooled to below 50ºC and then 20 mL of sterile filtered 20% w/v glucose added.

2.3. Buffers

2.3.1 CCMB80 Buffer for Chemically Competent Cells

CCMB80 Buffer was used as described by Hanahan (1983). For one litre of CCMB80 buffer, 10 mM KOAc pH 7.0 (10 ml of a 1M stock/L), 11.8 g l\(^{-1}\) CaCl\(_2\).2H\(_2\)O, 4 g l\(^{-1}\) MnCl\(_2\).4H\(_2\)O, 2 g l\(^{-1}\) MgCl\(_2\).6H\(_2\)O, 100 ml l\(^{-1}\) glycerol was added together and water added to 900 mL. The pH was adjusted to 6.4 with 0.1M HCl. Water was added to a final volume of 1 L. The solution was filter sterilized and stored at 4°C.

2.3.2 CTAB Solution

For 100 mL, of solution, 4.1 g NaCl was dissolved in 80 ml water prior to the addition of 10 g hexadecyl trimethyl ammonium bromide. The solution was warmed to 65°C to fully dissolve powders and adjusted to a final volume of 1 L.

2.3.3 Alkaline Lysis Plasmid DNA Extraction Solutions

Solution #1

4.52 g (50 mM) Glucose, 6.25 g (25 mM) Tris and 0.5g (10 mM) sodium EDTA were added together and dissolved in 250ml water. The pH was adjusted to 8.0 with 10M NaOH and the solution stored at 4°C.

Solution #2

A 0.4ml (10M) aliquot of NaOH was added to 2ml 10% SDS and dissolved in a total volume of 20 mL. This must be prepared fresh.
Solution #3

Sixty millilitres of 5M Potassium Acetate and 11.5 mL Glacial Acetic Acid were mixed and made up to a final volume of 100 mL with water. Solution #3 was stored at 4°C.

2.3.4 10X PCR Buffer

To 7.8 ml MQ water add 10 ml 1M (500mM) KCl, 2 ml (100 mM) Tris-HCl pH 9.0 and 200 µl (1% v/v) Triton X-100. The solution was aliquoted in 1 ml portions and stored at -20°C.

2.3.5 Phosphate Buffered Saline

One PBS tablet (Sigma) dissolved in 200 ml of water yields 0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride, pH 7.4 at 25°C.

2.3.6 10X TAE Buffer

Buffer was made by dissolving 48.4 g Tris, 3.75 g sodium EDTA, 6.8 g sodium acetate and 16.9 mL GlacialAcetic Acid in water to a final volume of 1L.

2.3.7 Dimethyl Methylene Blue (DMMB) Stain reagents

1. Dimethyl Methylene Blue (DMMB) Stain

Thirty two milligrams DMBB powder was dissolved in 25 mL ethanol and filtered through Whatman #1 filter paper.

2. Formic Acid buffer #1 (FAB #1)

4.7765 g guanidine hydrochloride, 0.5 g sodium formate, 0.5 mL formic acid and 12.5 mL ethanol were added together and diluted to 250 ml with water.

3. Formic Acid buffer #2 (FAB #2)

4.7765 g guanidine hydrochloride, 0.5 g sodium formate and 0.5 mL formic acid were added together and diluted to 50 mL with water.
4. DMMB working solution

The final DMMB working solution was made by the addition of 250 ml FAB #1 and 50 ml FAB #2 to 12.5 ml of filtered DMMB solution and adjusting the final volume to 500 ml with water.

5. Decomplexation Solution (deco)

Guanidine hydrochloride (210.12 g) was dissolved to a final concentration of 4 M with 500 ml of 50 mM sodium acetate buffer (pH 6.8) and 50 ml 1-propanol.

2.4. Methods

2.4.1 Growth Conditions

Cells were grown in RPMI 1640 and supplemented with FeCl₃ to a final concentration of 10 μM where stated, or in Difco™LB (Lennox) medium. Other metal supplements were diluted to concentrations indicated from 10 mM stock solutions of MnSO₄, ZnSO₄, CuCl₂ or NiCl₂ dissolved in 10 mM TrisHCl. Bio-relevant iron supplements were added to a final concentration as follows: 10 μM haemin, 10 μM haemoglobin, 0.5 μM ferritin, 0.6 μM apo-transferrin, 0.6 μM holo-transferrin and 0.6 μM holo-lactoferrin. The following enzymes were added to cultures: amylase at 1600 Uml⁻¹, cellulase at 13.8 Uml⁻¹ and DNAse 1 at 90 Uml⁻¹, Dispersin B at 5 εM and 20 εM (Kane Biotech Inc.). Quorum sensing molecules (Cayman Chemicals), N-butyryl-L-homoserine lactone and N-3-oxo-dodecanoyl-L-homoserine lactone were used at 1 and 10 εM. Antibiotic supplements were ampicillin (Amp) at 100 εg ml⁻¹, chloramphenicol (Cm) at 30 εg ml⁻¹, kanamycin (Km) at 50 εg ml⁻¹, or other supplements: isopropyl-1-thio-(D-galactopyranoside) (IPTG) at 100 εg ml⁻¹, 5-bromo-4-chloro-3 indolyl (-D-galactoside) at 40 εg ml⁻¹. Incubations were performed at 37ºC (unless stated) otherwise with shaking at 200 rpm in 50 ml V-bottom polypropylene (Sarstedt) tubes or 5ml polypropylene flat-bottom vials with 80% air space. Bacteria on agar plates were grown at 37ºC (unless otherwise stated) in moist atmosphere containing 5% CO₂.

2.4.2 Measurement of Bacterial Growth

Bacterial broth cultures were measured by spectrophotometer at OD₆₀₀nm with a path length of 1 cm on the Novaspec II (Amersham) or Ultrospec 2100 Pro (Sweden).
2.4.3 Enumeration of Viable Cells

Enumeration was carried out using the method described by Miles et al. 1938. The inoculum/sample were serially diluted to $10^{-7}$. Three plates were set-up for each dilution series (an average of three readings per sample was recorded). Appropriate plates were dried and each plate divided into eight segments. A 10 µl drop of appropriate dilution was dropped to the surface of the plate and allowed to spread naturally. Plates were left upright on the bench to dry before inversion and incubation at 37°C for 18-24 hours. Colonies forming units (CFU) were counted in the sector where the highest number of full size discrete colonies can be seen.

$$\text{CFU/mL} = \text{average number of colonies for a dilution} \times 100 \times \text{dilution factor}$$

2.5. Aggregation Indices

The Aggregation Index used in this thesis is a modified version of an Aggregation Index described by Malik et al 2003 to separate co-aggregating bacteria from activated sludge. The original method dissociated aggregates by vortexing to attain the total optical density measurement of the cell culture. UPEC 536 aggregates do not breakdown with vortexing. A variety of substances have been employed to reduce viscosity of biofilm suspensions as described by Chen and Stewart 2002, one of which is 0.3M NaCl. A 0.3M salt wash of the UPEC 536 total cell culture and resuspension of cells by vortexing easily formed a homogenous suspension suitable for total optical density measurement within the first seven hours of growth. The modification of this method was rigorously assessed and examined the 610 x g fraction of planktonic cells from aggregates by low speed centrifugation at four different speeds, various dispersion substances, and extensive microscopy to determine the validity of the method and to enable graphical representation of the characteristics displayed by UPEC 536 when grown in RPMI 1640. The study was a major portion of a Masters Thesis (Rowe 2006) which showed that the relative values attained by the Aggregation Index correlate to the growth and aggregation pattern observed in UPEC 536 grown in RPMI 1640 or RPMI 1640 supplemented with iron.
Overnight cultures in RF were diluted 1:100 in the medium indicated and divided into 10 mL aliquots in V-bottomed polypropylene 50 mL tubes (Sarstedt). The tubes were incubated at 37°C with shaking at 200 rpm. At given time intervals, one tube was used to measure aggregation. Bacterial aggregates were pelleted at 610 x g for 2 min and the OD of the planktonic phase was measured at 600nm (OD$_{600nm}$ [planktonic]). The planktonic cells were returned to the original tube and all cells were pelleted at 2450 x g for 5 min. The supernatant was discarded and cells were resuspended by vortexing in 10mL of 0.3M NaCl (Malik et al. 2003). A homogenous suspension of bacterial cells was not produced unless this wash was performed. The total OD (OD$_{600nm}$ [total]) was then measured. The Aggregation Index (AI) was calculated as:

$$AI = \frac{(OD_{600nm[total]} - OD_{600nm[planktonic]})}{OD_{600nm[total]}}$$

The overall dispersion, induced by a given treatment from an aggregated culture over a time period is measured as a relative Aggregation Index reduction calculated as:

$$\frac{(AI_{max} - AI_{final}) \times 100}{AI_{max}}$$

$AI_{max}$ is the point at which maximum aggregates are formed in the culture (approximately 240 minutes) ($R_{max}$) relative to the wild type culture grown in RPMI 1640. $AI_{final}$ is the AI of the culture at the time indicated after the addition of a dispersant to the aggregated culture.

2.6. DNA

2.6.1 Chromosomal DNA Extraction

Chromosomal DNA preparations were carried out with a Zymo Bacterial and Fungal Kit (ZymoResearch) as described by the manufacturer’s instructions or by CTAB extraction as described by Ausubel et al. 1988, Experimental Protocol 22/23. Briefly, 50 ml of overnight culture was grown statically at 30°C in LB and centrifuged at 11,872 x g (Sorvall RC6) for 10 minutes. The bacterial cell pellet was resuspended in 9.25 ml TE buffer (10 mM Tris HCl in 1 mM sodium EDTA, pH8) (USB Corporation) and transferred to a clean 50 ml tube. 0.5
mL of 10% SDS and 5 μl of 25 mg ml-1 Proteinase K (Roche) to the cells were added, mixed and incubated at 37°C until cells appeared lysed (approximately 30 minutes). To the lysed cell solution, 1.8 mL of 5 M NaCl was added and mixed thoroughly by tube inversion, followed by the addition of 1.5 ml of pre-warmed (15°C) hexadecyl trimethyl ammonium bromide (CTAB) was added and thoroughly mixed again by tube inversion. This was incubated at 65°C for 20 minutes. An equal volume of chloroform was added to the solution, mixed well and centrifuged at 13,000 rpm for 10 minutes at RT. The upper aqueous phase was transferred to a clean 50 ml tube and 0.6 volumes of propan-2-ol added to the solution. DNA threads were captured with a bent Pasteur pipette and transferred to a clean Eppendorf tube. The DNA was washed once in 1 ml 70% ethanol and centrifuged for 1 minute at full speed to pellet the DNA. The supernatant was discarded and the pellet air dried for 1-2, hours, resuspended in 500 μl TE buffer, and left to dissolve overnight at 4°C and stored at minus 20°C.

2.6.2 Plasmid Preparation

Plasmid preparations were carried out by Zyppy™ Plasmid Miniprep Kit (Zymo Research) as described in the manufacturer's instructions or by Alkaline Lysis as described by Sambrook and Russell (2001) and outlined briefly below. A 10 ml overnight culture was prepared in appropriate media and antibiotic supplements. Cells (1.5 – 3 mL) were pelleted in a microfuge and resuspended in 100 μl Solution #1 (see alkaline lysis DNA extraction solutions) and incubated on ice for 5 minutes. 200 μl of Solution #2 was added to lyse the cells. 150 μl of Solution #3 was added and the solution held on ice for 5-10 minutes. The chromosomal DNA and cellular debris were separated by centrifugation. The supernatant containing plasmid DNA was transferred to an Eppendorf tube and cleaned with a phenol: chloroform Isoamyl Alcohol (25:24:1) mix (USB Corporation). The solution was centrifuged at high speed for 2 minutes at RT and the top phase transferred to a clean Eppendorf tube. The plasmid DNA was precipitated by the addition of 2 volumes of ethanol and left on ice for 10 minutes. The pellet was washed in 70% ethanol, collected by centrifugation and air dried before being resuspended in 50 μl TE buffer. Large scale preparations were carried out using Qiagen Midi/Maxi T100 and T500 columns as per the manufacturer's instructions. Plasmids used in this study can be found in Table 6.
2.6.3 Polymerase Chain Reaction

Oligonucleotide primers were synthesised by Sigma-Aldrich (Table 7 a-d). Polymerase chain reaction (PCR) amplifications with Taq polymerase were performed as described by Saiki et al. 1988. PCR reactions were carried out in either 20 μl or 50 μl volumes using 1 unit Taq thermostable DNA polymerase (generated as a recombinant protein from an E.coli clone), 1 X PCR Buffer stock (see section 2.2.2.4), 1 μl of each primer (10 μM working stock) (Forward / Reverse) and 1 μl template, 0.7 mM MgCl₂ (25 mM stock) and 1 μl of dNTPs (100 mM stock) (Invitrogen). Annealing temperatures were 5°C lower than the lowest melting temperature of a primer pair and extension times were 1 minute per 1 kb DNA. PCR amplifications with Finnzymes Phusion High Fidelity PCR Master Mix (NEB) were performed according to the manufacturer's instructions.

Reactions were carried out in a Corbett Research Palm Cycler. PCR protocols for heating, denaturation, annealing and extension were variable and depended on the DNA fragment size and the annealing temperature of the primers used. PCR products were separated and electrophoreosed on 0.8% agarose gel stained with 0.1% Red Safe (ChemBio) and visualized under UV light with the GelQuant (BioRad).

Oligonucleotides used for insertional mutations, site directed mutagenesis and cloning were made by Sigma and designed from template sequences found in published references or from the NCBI genbank (www.ncbi.nlm.nih.gov/genbank/). A list of Oligonucleotides used in this study can be found in Table 7 a-d.
<table>
<thead>
<tr>
<th>Insertional</th>
<th>Sequence 5'-3'</th>
<th>Restriction Enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>yaiCIF*</td>
<td>TAAAAACTCGAGGCAGTATTAGCGGGGGAATGTGGG</td>
<td>XhoI</td>
</tr>
<tr>
<td>yaiCIR</td>
<td>CGCCCAGCTCTATCAAATTCTATTGCCTACATAG</td>
<td>SacI</td>
</tr>
<tr>
<td>bcsAIF</td>
<td>GTACCACCTCGAGAACATGCAGAAGCGGGGCAACATC</td>
<td>XhoI</td>
</tr>
<tr>
<td>bcsAIR</td>
<td>AAAAAAGCCTCATCCACGGGCTTACGGCGAATC</td>
<td>SacI</td>
</tr>
<tr>
<td>bcsZIF</td>
<td>AAGAGAGCTCTAAATGGGAGTGGGTGACAGC</td>
<td>SacI</td>
</tr>
<tr>
<td>bcsZIR</td>
<td>TTATAGCTCGAGGCTTATTCATATAGCCGACCCA</td>
<td>XhoI</td>
</tr>
<tr>
<td>furIF</td>
<td>AAAATCTAGACCTGAAAAGTAGCTTCCTCGCTTG</td>
<td>XbaI</td>
</tr>
<tr>
<td>furIR</td>
<td>AAGAGAGCTCGTAAGATGACGTGTGGATAAGG</td>
<td>SacI</td>
</tr>
<tr>
<td>yhjKIF</td>
<td>CGATGAGCTCGTATGGTTGGTGCGCAGTAC</td>
<td>SacI</td>
</tr>
<tr>
<td>yhjKIR</td>
<td>TTATAGCTCGAGGCTAAATGCGCGACGAAAATAGC</td>
<td>XhoI</td>
</tr>
<tr>
<td>bcs operon F</td>
<td>GGGGACAACTTTTGTTAGAAAGGTTGCAATTGCTGATTACGT</td>
<td></td>
</tr>
<tr>
<td>bcs operon R</td>
<td>GGGGACAACTTTTGTTAATAAAAGGTTGGAAATCCAGATTGCAGC</td>
<td></td>
</tr>
<tr>
<td>(short)</td>
<td>GGGGACAACTTTTGTTAATAAAAGGTTGGGCTGCTGCGACAAAAACCGCA</td>
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Underline segments in primer sequence indicate restriction enzyme site

*yaiC denotes *Escherichia coli* c-di-GMP synthase homologous to *Salmonella* Typhimurium *adrA*
<table>
<thead>
<tr>
<th>Deletions</th>
<th>Sequence 5' – 3'</th>
<th>Restriction Enzyme</th>
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<tbody>
<tr>
<td>bcsZ-A</td>
<td>CTGCTCGAGCTATTCCGGTATTACAAAGG</td>
<td>XhoI</td>
</tr>
<tr>
<td>bcsZ-B1</td>
<td>CACGAATTCACCTACGCAACACATTCACTTTC</td>
<td>EcoRI</td>
</tr>
<tr>
<td>bcsZ-B2</td>
<td>ACAAGATTCAAGTTATACCTGACTGGGG</td>
<td>EcoRI</td>
</tr>
<tr>
<td>bcsZ-C</td>
<td>CAGCCCTCGAGGCTTTTCCAGATTCG</td>
<td>XhoI</td>
</tr>
</tbody>
</table>
| bcsA-A    | TGCTCGAGAACGCTACGGTTATTTTATAGG | XhoI |}
| bcsA-B1   | TCAGAATTCATGTAGCTACCTCCCCGACTG | EcoRI |}
| bcsA-B2   | CTGAATTCTGCTCAACAAATGATGATAAC | EcoRI |}
| bcsA-C    | CTGCCTCGAGTTTTTGTCGTCAGACAAA | XhoI |}
| fur-A     | CAACTCGAGGCCGCTGCGTAGTCAGGGCATCG | XhoI |}
| fur-B1    | ATTGAATTCATGCAGCTAATCTCTCTGTTACT | EcoRI |}
| fur-B2    | CACGAATTCAAAATAAGCCAGCCTGAAACAGAAGAAAGC | EcoRI |}
| fur-C     | CTGCCTCGAGTTTAGCTTTTGTTACCGGCGG | XhoI |}
| yaiC-A    | TTACTCGAGTCTACATGAGAAATAAATAGTGA | XhoI |}
| yaiC-B1   | TTTTCTAGACACCACCCCTCTCTCATGGT | XbaI |}
| yaiC-B2   | CCTCTAGAAAGCCGCTGATACCTGATAGT | XbaI |}
| yaiC-C    | TCGCTCGAGTTACCTCCGGCTTTCTTTGCT | XhoI |}
| yjhK-A    | GGAACCTCGAGTGCCAGCGTGGCGGT | XhoI |}
| yjhK-C    | CTACCTCGAGCCCCCTGAAGTAAAGGTCAGCGCG | XhoI |}
### Table 7c. Oligonucleotides used in this Study—Complementation

<table>
<thead>
<tr>
<th>Complementation</th>
<th>Sequence 5'-3'</th>
<th>Restriction Enzyme</th>
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<tbody>
<tr>
<td>yaiCF</td>
<td>TCCT<strong>CATGG</strong>TCCCCAAAATAATGAATGTA</td>
<td>NcoI</td>
</tr>
<tr>
<td>yaiCR</td>
<td>TGCCAA<strong>AGCTT</strong>CAGGCACGCCACTTCGCG</td>
<td>HindIII</td>
</tr>
<tr>
<td>bcsA F</td>
<td>CATGAATTTCTGCACCCGATTGTTGATATTCCGCC</td>
<td>EcoRI</td>
</tr>
<tr>
<td>bcsA R</td>
<td>TCAT<strong>AGCTT</strong>ATATCATCATTGGTGAGCCAAAGCC</td>
<td>HindIII</td>
</tr>
<tr>
<td>bcsZF</td>
<td>TTGA<strong>CATGG</strong>ATGTGTTGCGATGTGACGTG</td>
<td>NcoI</td>
</tr>
<tr>
<td>bcsZR</td>
<td>GTA<strong>AGCTT</strong>AGTTGAAATTTGCGCATTTCC</td>
<td>HindIII</td>
</tr>
<tr>
<td>furF</td>
<td>TCC<strong>CATGG</strong>CTGATAAAATACCGCCTAAAGAAA</td>
<td>NcoI</td>
</tr>
<tr>
<td>furR</td>
<td>AGGC<strong>AGCTT</strong>ATTTGCGTTTGGTCGCTTTC</td>
<td>HindIII</td>
</tr>
<tr>
<td>yhjK ATG</td>
<td>AGACAC<strong>CATGG</strong>GCTGTAAGCTCTGTAACAATCAAGCAGATGG</td>
<td>NcoI</td>
</tr>
<tr>
<td>yhjK TTG</td>
<td>AAGC<strong>CATGG</strong>CAATGGTGAGCCGCGTGTC</td>
<td>NcoI</td>
</tr>
<tr>
<td>yhjK TAA</td>
<td>TGGG<strong>AGCTT</strong>ACCTTCTCAGATCTCCTCTG</td>
<td>HindIII</td>
</tr>
<tr>
<td>bcs operon F(alt)</td>
<td>GGGACAACCTTTGTATAGAAAAGTTGCATTGTTGATATTACGT</td>
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</tr>
<tr>
<td>bcs operon R (att)</td>
<td>GGGACAACCTTTGTATAAAAGTTGGAATCCAGATTGCAGGCCC</td>
<td></td>
</tr>
<tr>
<td>bcs operon yhjK (att)</td>
<td>GGGACAACCTTTGTATAAAAGTTGCGCTGCACAAACCGCA</td>
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</tr>
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</table>

### Table 7d. Oligonucleotides used in this Study—Verification

<table>
<thead>
<tr>
<th>Verification</th>
<th>Sequence 5’–3’</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pNQcat3’F</td>
<td>GATGGCTTCCATGTCGCGCAGAATGC</td>
<td>Milton et al. 1996</td>
</tr>
<tr>
<td>pNQcat5’R</td>
<td>ATTGAGCAACTGACTGAAATGCCTC</td>
<td>Milton et al. 1996</td>
</tr>
<tr>
<td>k1</td>
<td>CAGTCATAGCCGAAATGCCT</td>
<td>Wanna and Datsenko 2000</td>
</tr>
<tr>
<td>k2</td>
<td>CGGCCACAGTGCAGAATCC</td>
<td>Wanna and Datsenko 2000</td>
</tr>
<tr>
<td>pTrc99AF</td>
<td>TGCAGGCTGTAATCACTGCA</td>
<td>Amann et al. 1988</td>
</tr>
<tr>
<td>pTrc99AR</td>
<td>CTGCGACTTCCACTCTCAG</td>
<td>Amann et al. 1988</td>
</tr>
<tr>
<td>M13F</td>
<td>GTAAAAACACGGGCAG</td>
<td></td>
</tr>
<tr>
<td>M13R</td>
<td>CAGGAAACAGCTATGAC</td>
<td></td>
</tr>
</tbody>
</table>
2.6.4 Precipitation of DNA

A solution containing DNA (either for exDNA, cell lysate for plasmids or chromosomal DNA) was obtained by centrifugation at 8000 x g for 3 min. The sample was precipitated with 1.5 volumes of ice-cold ethanol and 5 M NaCl or purified with a Zymo DNA purification kit according to the manufacturer’s instructions. DNA was resuspended in 20 µl TE buffer.

2.6.5 Phenol / Chloroform Purification of Nucleic Acids

An equal volume of phenol / chloroform was added to the nucleic acids to be purified, vortexed and centrifuged at 13,000 x g for 2 minutes. The aqueous phase was transferred to a fresh tube and an equal volume of chloroform added, mixed and centrifuged as above. The aqueous phase was again removed and 0.1 volumes of 3 M sodium acetate (pH 4.3) and 0.7 volumes of propan-2-ol were added. Nucleic acids were pelleted by centrifugation at 13,000 x g for 10 minutes. The pellet was dried and resuspended as described in section 2.5.3.

2.6.6 Restriction Digests

Restriction enzymes were obtained from Invitrogen, New England Biolabs and Roche Diagnostic. Digests were carried out as described in the manufacturer’s instructions. Briefly, restriction digests contained 0.5 – 1.0 µg of plasmid DNA, 1-2 U restriction endonuclease and 1 x buffer (supplied by manufacturer) made to a final volume of 10 – 20 µl with sterile water. Digests were incubated for 3 hours at the appropriate temperature and analysed by agarose gel electrophoresis.

2.6.7 Agarose Gel Electrophoresis

Agarose gels were prepared and run essentially as described by Sambrook et al. 1989. Gels were prepared in 1X TAE Buffer at concentrations of 0.8 – 2% (w/v) agarose. RedSafe™ Nucleic Acid Stain (ChemBio) was added to a final concentration of 10 µg ml⁻¹ before pouring. Samples were prepared in 6X loading dye (3 ml glycerol, 25 mg bromophenol blue and water to 10 ml) and gels run in 1X TAE at 50-120 V. A 1 kb ladder (ZR DNA Molecular Weight Markers, ZymoResearch) was used for a DNA marker.
2.6.8 DNA Purification

DNA was recovered from the agarose gel and purified by Zymoclean™ Gel DNA Recovery Kit (Zymo Research) as described by the manufacturer's instructions. PCR prepared DNA was purified by Nucleic Acid and Protein Nucleospin® Extract II (Macherey-Nagel) as described in the manufacturer's instructions.

2.6.9 Dephosphorylation of DNA

Vector DNA (1-5 µl) for ligation was dephosphorylated in 1/10 volume of 10X Antarctic Phosphatase Reaction Buffer (NEB) and 1 µl (5 U) Antarctic Phosphatase for 15 min at 37°C (total volume 10 µl). The sample was placed in a water bath and the reaction was heat inactivated for 5 minutes at 65°C.

2.6.10 Ligation of DNA

DNA ligation were performed using T4 DNA ligase. Purified DNA inserts were ligated to purified vector DNA at a molar ratio of 3:1 (or as stated). Ligations were carried out in 10 or 20 µl volumes in 1X T4 DNA ligase buffer with 1 U T4 DNA ligase. Reactions were left over night in a RT water bath and dialyzed against water for 15-30 minutes using 0.025 µm nitrocellulose filters (Millipore) prior to electroporation of electrocompetent cells.

2.6.11 Electrocompetent Cells

Electrocompetent cells were made as described by Sambrook and Russell (2001). Inoculum was grown overnight in 2 ml LB broth at 37°C with shaking. Fresh LB broth was inoculated 1:100 and the culture was grown to 0.5-0.8 OD_{600nm}. The culture was chilled on ice for 15-30 minutes before centrifugation at 3220 x g (Heraeus Multifuge 3S-R) at 4°C for 10 min. Cells were washed in a full culture volume and then in a half volume of cold sterile water and resuspended in 100 µl cold 10% glycerol. Cells were pelleted again and resuspended in an appropriate final volume of 10% glycerol (1/1000 of original culture). Aliquots of 50 µl were dispensed to sterile Eppendorf tubes, snap frozen in a dry ice/ ethanol bath and stored at minus 80°C.

2.6.12 Electroporation

Aliquots of electrocompetent cells (section 2.6.13) were thawed on ice. Two µl of dialyzed DNA was added to 40 µl of bacterial cells and left to incubate on ice for 1 minute. Cells and
DNA were transferred to a cold 0.2 mm electroporation cuvette (Invitrogen). The cuvette was placed in the Electroporator chamber and a pulse of 2.5 kV (25 μF and 300 W) was delivered using the GenePulser Xcell (BioRad) according to the manufacturer's instructions. The cuvette was immediately removed and 1 ml of SOC media was added to the cuvette and the mixture transferred to a 5 ml sterile flat-bottom vial. The culture was incubated for 1 h at 37°C (unless otherwise stated) with shaking before plating on to selective media.

2.6.13 Chemically Competent Cells
Chemically competent bacterial cells were made as described by Hanahan et. al 1991. The inoculum was grown overnight in 2 ml LB broth at 37°C with shaking. Fresh SOB broth (100 ml) was inoculated 1:100 and incubated at 28°C with shaking and grown to 0.2 - 0.3 OD600nm in an Erlenmeyer flask. The culture was divided and transferred to two clean 50 ml tubes and centrifuged at 3220 x g (Eppendorf S810R) for 10 min at 4°C. The supernatant was removed and each 50 ml pellet resuspended in 16 ml ice cold CCMB80 Buffer and left to incubate on ice for 20 minutes. Cells were centrifuged again at 3220 x g for 10 min at 4°C, the buffer discarded and each tube resuspended in 2 ml ice cold CCMB80 Buffer and left to incubate on ice for 20 minutes. Aliquots of 50μl were dispensed to clean Eppendorf tubes and stored at minus 80°C.

2.6.14 Transformation by Heat Shock
Chemically competent bacterial cells (2.6.14) were thawed on ice. 1-5 μl purified or ligated DNA was added to 50 μl bacterial cells and left to incubate on ice for 30 minute. Cells and DNA were heat shocked for 1 minute at 42°C in a water bath. Cells were immediately recovered in 250 μl SOC media and incubated for 1 h at 37°C with shaking before plating on to selective media.

2.6.15 Transformation of PCR Product
Transformation of PCR product was undertaken using the Zero Blunt® TOPO® PCR Cloning Kit (Invitrogen) (section 2.5.9) to produce larger quantities of PCR amplified DNA or to hold fragments of PCR product for further testing. The procedure was carried out as described by the manufacturer's instructions.
2.6.16 Plasmid Conjugation

Bacterial conjugation was carried out as described by Winson et al. 1998. Briefly, 5 ml donor culture (S17-1 \(\varphi\)pir strain (de Lorenzo et al 1990) carrying pNQ705 with selected insert) and 5 ml of the recipient strain were grown overnight in LB\(_{Cm}\) and LB broth, respectively. From the overnight cultures, 1 ml of each culture was centrifuged at 13,000 rpm in the microfuge and the supernatant removed. The pellets were gently resuspended in 100 \(\mu\)l of saline and 50 \(\mu\)l of each suspension transferred to a single clean microfuge tube. The mixed culture was spot plated and incubated for 1, 4, 6 and 24 hours. At each time point the conjugated cells were scraped off the plate and placed in a clean microfuge tube. Cells were washed once in 1 ml saline and resuspended in 200 \(\mu\)l saline. Serial dilutions were performed (neat \(10^{-4}\)) and the diluted culture plated to minimal media agar with chloramphenicol selection overnight. Donor and recipient cultures were streaked to LB\(_{Cm}\) and MM agar plates as positive and negative controls.

2.6.17 DNA Sequencing

DNA to be sequenced was purified with Zyppy Plasmid Miniprep Kit (Zymo) or with the Nucleospin® Extract II Kit as described by the manufacturer instructions. Sequencing was carried out at the University of Auckland Sequencing and Genotyping Facility.

2.7. Protein

2.7.1 Whole Cell Protein Samples

Overnight cultures of bacterial strains were diluted 1:100 and incubated at 37°C with shaking at 200 rpm. Broth cultures were centrifuged at 4000 x g for 5 minutes and the supernatants removed and discarded. Pellets were resuspended in water and normalized to an OD\(_{600nm}\) of 1.0 or resuspended in an appropriate volume. Each sample was sonicated (Ultrasonic Processor XL (Misonix)) for 1 minute (3 X 20 seconds at 95% \(s^{-1}\) pulse) and kept on ice during treatment. The sonicated suspensions were centrifuged for 10 minutes at 13,000 x g at 4°C, the supernatant removed and stored at -20°C. The pellets were resuspended in an appropriate volume of buffer or fractionated as described in section 2.6.3.
2.7.2 Total Protein Quantification

Total protein was quantified using the BCA™ Total Protein Assay Kit (Pierce) in a 96-well microtitre format as per manufacturer’s guidelines. Bovine serum albumin (BSA) (Gibco BRL) standards ranging from 0 µg ml\(^{-1}\) to 2000 µg ml\(^{-1}\) (0, 25, 125, 250, 500, 750, 1000, 1500, 2000 µg ml\(^{-1}\)) were used to quantify protein concentration from sonicated whole cell lysate samples by interpolation from the corresponding curve. The 0 µg ml\(^{-1}\) protein standard was used as a blank for both standard and sample readings. Briefly, 25µl of standard or sample was pipetted into a microtitre well, 200 µl of Working Reagent (50 parts Solution A: 1 part Solution B) was added to each well and mixed with gentle shaking for 30 seconds, covered and incubated at 37°C for 30 minutes. The plate was allowed to cool to room temperature (approximately 10 minutes) and the OD\(_{560}\) read on the µ-Quant Spectrophotometer (Bio-Tek Instruments). All standards and samples were run in duplicate. The average standard absorbances were plotted against standard concentration to obtain the Standard Calibration Curve.

2.7.3 Cell Fractionation

Cells were fractionated as described by Carlone et al. (1986). A broth culture was centrifuged 4000 x g for 10 minutes and the supernatant removed. The cell pellet was resuspended in 1.5ml ice cold 10 mM HEPES buffer (10mM N-2-Hydroxyethylpiperazine-N-2-ethanesulfonic acid, pH 7.4), transferred to a clean microfuge tube, washed in HEPES and centrifuged at 13,000 rpm for 5 minutes. The pellets were resuspended in 1ml 10 mM HEPES buffer and sonicated on ice. Sonicated samples were centrifuged at 13,000 rpm for 10 minutes in the microfuge. Soluble proteins within the supernatant were removed and saved at -20°C. The pellet was resuspended in 0.2 ml 10 mM HEPES buffer containing 1% w/v sodium N-lauroyl sarcosinate solution (Sarkosyl). The suspension was incubated at RT for 30 minutes to solubilise the inner membrane. The solution was centrifuged at 13,000 x rpm for 10 minutes in the microfuge. Inner membrane proteins located within the supernatant were removed and stored at -20°C. The outer membrane protein pellet was again washed in HEPES buffer without resuspending the pellet, centrifuged at 13,000 rpm in the microfuge then resuspended in 50 µl HEPES buffer and stored at minus 20°C.
2.7.4 NuPAGE Novex Bis-Tris Mini Gel - Protein Gel

Sonicated protein samples were mixed 1:1 with protease inhibitors and DNase-1 (1 mg ml\(^{-1}\) Benzamidine in ethanol, 0.5 mg ml\(^{-1}\) Phenylmethylsulfonyl fluoride (PMSF) in ethanol and 10 µl DNase-1 (5 U µl\(^{-1}\))). Samples were mixed, 65 µl: 25 µl of loading dye (4X) : 10 µl DTT and the mixture boiled for 10 minutes in a water bath. The chamber and gel plate were prepared according to manufacturer's instructions and the chamber loaded with 1X NuPAGE\(^{TM}\) MOPS Running Buffer (Invitrogen). 25 µl of prepared sample was added to the gel well. The gel was electrophoresed at 200V for 35-50 minutes. Gels were stained in SimplyBlue SafeStain (Invitrogen) and de-stained in water. A Fermentas or Sigma protein ladder was added to one lane of each gel.

2.7.5 Coomassie Blue Staining of Proteins

Following SDS-PAGE gel electrophoresis, gels were rinsed in dH\(_2\)O and stained with Coomassie Blue stain (100 ml L\(^{-1}\) Acetic acid, 400 ml L\(^{-1}\) Methanol, 2.5 g L\(^{-1}\) R-250 Coomassie Blue (PhastGel\(^{TM}\) Blue R) (Amersham Pharmacia Biotech) for 1-2 hours with shaking. The stain was removed and the gels rinsed 2-3 times in distilled water before destaining (80 ml L\(^{-1}\) Glacial Acetic Acid, 250 ml L\(^{-1}\) Ethanol, 670 ml L\(^{-1}\) distilled water). The gels were left in destain solution with shaking until protein bands were visible and the background staining removed.

2.8. Inhibition of Transcription and Translation

Inhibition of RNA synthesis was performed by the incubation of cultures with rifampicin at 100 mg mL\(^{-1}\) for 30 min before the addition of iron. Inhibition of new protein synthesis was achieved by the incubation of cultures with chloramphenicol at 35 mg mL\(^{-1}\) for 30 min before the addition of iron.

2.9. Chromazurol S (CAS) for Siderophores

CAS shuttle solution was made as described by Schwyn & Neilands (1987). Briefly, the ternary complex (chromazurol S / iron III /hexadecyltrimethylammonium bromide (HDTMA)) serves as a colour indicator for the presence of siderophores. When a strong chelator removes the iron from the dye, its colour turns from blue to orange.

\[
\text{FeDye + Siderophore} \rightarrow \text{FeSiderophore + Dye}
\]
The solution was prepared by adding 6 ml (10 mM) in water HDTMA diluted to 20 ml with water, 1.5 ml (1mM in 10 mM HCl) FeCl$_3$, and 7.5 ml 2 mM CAS to the above volumes while stirring. To this 36.25 ml piperazine solution (4.307 g anhydrous piperazine in 30 ml 50% NaOH, pH adjusted to 5.6 with 12 M HCl added dropwise) was added with 10 ml (0.2 M) of a 5-sulfosalicylic acid solution (in water). The volume was adjusted to 100 ml and the solution (blue-violet in colour) was stored in the dark. The CAS shuttle solution was mixed 1:1 with culture supernatant and incubated for 30 minutes at RT before reading by spectrophotometer at OD$_{630\text{nm}}$. An un-inoculated CAS shuttle solution is used as a blank. The relative abundance of siderophore is determined by

\[
\text{Relative abundance of siderophore} = \text{OD}_{630\text{nm}} \text{ Blank} - \text{OD}_{630\text{nm}} \text{ sample}
\]

### 2.10. Motility Assays

Motility agar plates were used in a modified method originally described by Tittsler and Sandholzer (1936). Bacteria were assessed for their ability move through soft agar. Bacteria displaying swimming motility have fully assembled flagella that rotate and permit the bacteria to swim through the liquid of the media. Swarming motility on the other hand, is flagella mediated translocation over the surface of solid media.

#### 2.10.1 Swimming

Media used for the motility assay was LB broth, RPMI 1640 or RPMI 1640 + 10µM FeCl$_3$ that contained 0.3% (w/v) Eiken agar. Swim plates were inoculated with bacteria from an overnight culture in RPMI + 10 µM FeCl$_3$ at 37°C with a sterile pipette tip. The plates were incubated at 30°C or 37°C for 12–14 h. The diameter of the zone travelled measured in mm was recorded.

#### 2.10.2 Swarming

Media used for the swarming assay consisted of 0.6% (w/v) Eiken agar with 20 g L$^{-1}$ LB, to which 5 g L$^{-1}$ glucose was added. Swarm plates were typically allowed to dry at room temperature overnight before being used. Bacterial culture (10 µl) from an overnight culture in RPMI + 10 µM FeCl$_3$ at 37°C was dispensed onto swarm plates and incubated at 37°C for 12–14 h. The diameter of the zone travelled measured in mm was recorded.
2.11. Visualisation of Bacterial Cells and Cellulose

2.11.1 Fluorescent Staining

Fluorescent dyes 4',6-diamidino-2-phenylindole (DAPI) (1 μg ml\(^{-1}\)) and N-(3-triethylammoniumpropyl)-4-(p-diethylaminophenyl-hexatrienyl)-pyridinium-diromide (FM 4-64) (1 μg ml\(^{-1}\)) were added to the inside edge of a clean microfuge tube, then 1 ml of culture was pipetted into the tube and immediately drawn up again to mix with the dyes. The portion of the mixture was immediately transferred to a slide and cover-slipped and allowed to settle in a dark moisture box until ready to use.

2.11.2 Live/Dead Staining

Staining for viable cells was done using the Invitrogen Viability / Cytotoxicity Kit. One µl of each stain was added to 1 ml of culture and incubated for one minute before microscopic examination. Propidium iodide stained dead cells red and Syto 9 stained live cells green.

2.11.3 Calcofluor White Staining

Calcofluor White (Fluka) is a non-specific fluorochrome that binds to cellulose and chitin in cell walls of fungi and other organisms. Evans blue present in the stain acts as a counter stain to diminish the background fluorescence of tissues and cells when using blue excitation (not UV). A range of 300 to 440 nm is used for emission wave length and excitation occurs at 355 nm. One drop of stain was mixed with one drop of culture, covered with a glass coverslip and left for one minute before microscopic examination.

2.11.4 Crystal Violet Staining for Biomass

Crystal violet was used to visualize biomass of adherent cultures on glass. Culture was grown on glass until the desired time point. Planktonic cells were discarded and the tube and adherent biomass rinsed with water twice. A 0.1% solution of crystal violet was added to the tube and allowed to complex for 15 minutes. The dye was then discarded and the tubes rinsed with water until purple colour no longer seeped from the biomass. Adherence of biomass was for visual purposes only.
2.11.5 Confocal Laser Scanning Microscopy

Biofilm morphology and antibiotic penetration were visualising using CLSM. To this end, 1 µl of Syto9 (exc, 480 nm; em, 500 nm; 3.34 mM in DMSO) and 1 µl of Propidium iodide (exc, 490 nm; em, 635 nm; 20 mM in DMSO) (LIVE/DEAD BacLight bacterial viability kit L7012, Invitrogen) were added to the culture (planktonic or biofilm). Stain and culture were incubated for 15 minutes at RT and visualised using the Leica TCS SP2 confocal laser scanning microscope.

2.11.6 Cryoscanning Electron Microscopy

Biofilm and planktonic cell samples were examined using a Gattan Alto 2500 Cryo unit, a SiLi (Lithium drifted) detector and a Philips XL30S FEG (Field Emission Gun) (Netherlands) scanning electron microscope. Briefly, samples were transferred under liquid nitrogen to a sample holder for examining. The holder was placed on the cold stage at 100°K in the pre-pump chamber. The pressure was reduced and the holder moved to the variable-temperature cold stage in the adjacent metal-coating chamber and warmed to 183°K for approximately 60 minutes to remove frozen moisture and to develop topography by differential sublimation of the exposed surface of the sample. The stage was cooled to 100°K and the sample surface was sputter coated with gold. The holder was moved through the gate valve to the cold stage in the specimen chamber of the microscope for imaging.

2.12. Extracellular DNA

Samples for extracellular DNA testing were harvested according to the method described by Alleson-Holm et al. 2006. One culture was grown to Rmax and another culture was grown in RPMI supplemented with 10 µl FeCl₃. The supernatant was extracted by centrifugation at 8000 x g for 3 min and prepared as described in section 2.6.4. One microliter of φDNA was added to 10 µl of supernatant from each time point and incubated for 1, 2 and 3 hours at 37°C. At each hourly time point an agarose gel was prepare, electrophoresed and stained with ethidium bromide. A 1µl of φDNA + 2 µl of DNase I sample (in a total volume of 10 µlTE Buffer) and an aliquot of 3 hour supernatant without DNA were also incubated and visualise on the gel as controls.
2.13. Carbohydrates

2.13.1 Cellulose Recovery

To ensure adequate quantities of cellulose were recovered for at least three assays, one litre of culture was grown in RPMI or RPMI with 10 µM FeCl₃, to the desired time point. Bacterial cells were pelleted at 3220 x g for 10 min. The supernatant was discarded and the cell pellet washed twice in cold sterile water at full volume. The pellet was then washed in approximately 25 ml 100 % absolute ethanol with vigorous shaking and centrifuged again. The ethanol was removed and the pellet washed in approximately 25 ml methanol, shaken vigorously and the cells pelleted by centrifugation. The methanol was discarded and approximately 20 ml of N-pentane was added to the pellet and mixed with a polypropylene pipette tip to ensure maximum contact with the pellet. Most of the N-pentane was removed carefully using a pipette and the sample left in the laminar flow hood overnight to evaporate the residual N-pentane. Cellulose chips were stored in airtight glass vials until prepared for Gas Chromatography.

2.13.2 Non-Cellulosic Neutral Sugars and Cellulose Content by Sulphuric Acid Hydrolysis

This method was carried out as described by Melton and Smith (2001), although the initial step of cellulose recovery was modified for bacterial polysaccharide production as described in section 2.12.1.

1. Hydrolysis of Polysaccharides

Exact weights of cellulose chips were recorded and dispensed to clean borosilicate glass Kimax tubes. To each sample tube and one control (no sample) 250 µl of 72% (v/v) sulphuric acid was added, the tube head space flushed with N₂ gas and vortexed gently to mix. The sample was hydrolyzed for 3 hr at 30°C in a dry bath with gentle mixing approximately every 30 minutes. After cooling to TR 2.75 ml of MQ water was added to the mixture which was again flushed with N₂ gas and incubated for 3 hr at 100°C.

After cooling 600 µl of 15 M ammonia was added to neutralize the solution. Myo-inositol was used as an internal standard and 50 µl of 20 mg ml⁻¹ was added to each sample and mixed. The solution was centrifuged at 2000 x g for 2 min to pellet any remaining particulate matter. 200 µl of each hydrolysate was transferred to clean Kimax tubes.
2. Reduction of Monosaccharides to Corresponding Alditols

Two clean tubes were added to the test set as controls for the 13-sugar standard and the water blank. To each tube, 20 µl of 15 M ammonia and 1 ml of 0.5 M sodium borohydride was added. Tubes were then capped, mixed and incubated for 90 min at 40ºC in a heating block, after which 100 µl of 18 M acetic acid was added and mixed with gentle vortexing.

3. Acetylation of the Alditols

In the fume hood 200 µl of RT 1-methylimidazole and 2 ml acetic anhydride was added to each tube and vortexed to mix. The mixture was incubated at RT for 10 min before the addition of 5 ml MQ water to destroy the excess acetic anhydride and left at RT for a further 10 min or until cool. A 1 ml volume of dichloromethane (DCM) was added to extract the alditol acetates, vortexed gently and allowed to rest while the phases separated. The lower DCM phase was transferred to a clean borosilicate Kimax tube using glass Pasteur pipettes ensuring that all of the lower phase was removed. Another 1 ml volume of DCM was added to the original solution and the extraction process repeated and the lower DCM phase combined with the previous DCM extract. DCM extracts were washed with MQ water 4 times with the water being removed with a glass pipette each time. The DCM was evaporated completely in a stream of N₂ gas. The sample was resuspended in 0.5–2 ml DCM. Glass vial caps were sealed with Teflon tape or parafilm and stored at minus 20°C until analyzed by GC (within 1 week).

4. Gas Chromatography Analysis

The alditol acetates were separated and quantified using gas chromatography (Model HP 6890, Hewlett Packard), fitted with a flame ionization detector and a dedicated cool on-column capillary inlet (set on oven track mode) on a BPX-70 open tubular fused silica column (25 m long, 0.33 i.d. and 0.25 µm film thickness). The initial oven temperature was set to 38ºC and maintained for 30 s and then increased to 170ºC at 50ºC min⁻¹ and increased further to 230ºC at 2ºC min⁻¹ and held at 230ºC for 5 min. The detector temperature is held at 250ºC and helium is used as the carrier gas at a column head pressure of 40 kPa. Standard and control volumes of 0.5 µl were injected into the sampling port where the standard reference alditol acetates elute from the BPC-70 column in the following order: erythritol triacetate, 2-deoxyribitol tetraacetate, rhamnitol pentaacetate, fucitol pentaacetate, ribitol pentaacetate, arabinitol pentaacetate, xylitol pentaacetate, 2-deoxyglucitol hexaacetate, allitol
hexaacetate, annitol hexaacetate, galactitol hexaacetate, glucitol hexaacetate, and myo-inositol hexaacetate. Sample alditol acetates are identified by the retention times relative to the standards in the 13-sugar mix. The amount of each neutral monosaccharide in the samples was calculated relative to the myo-inositol internal standard.

2.13.3 Non-Cellulosic Neutral Sugars by Trifluoroacetic Acid (TFA) Hydrolysis

This method was carried out as described by Melton and Smith (2001), although the initial step of cellulose recovery was modified for bacterial polysaccharide production as described in section 2.12.1. Briefly, hydrolysis of dehydrated matrix samples (approximately 10 mg) was carried out using 0.5 ml of 2 M TFA for 60 min at 121°C. The sample was allowed to cool before the addition of the internal standard and the hydrolysate was filtered using a Swinney stainless steel 13-mm filter unit and a 0.22 μm PTFE filter. The sample was then evaporated to dryness and the reduction of monosaccharides to corresponding alditols, acetylation of alditols and gas chromatography was carried out as described in section 2.2.13 for determination of non-cellulosic neutral sugars and cellulose content by sulphuric acid hydrolysis.

2.13.4 Uronic Acids

The quantification of uronic acid production was based on the method described by van den Hoogen et al. (1998). Bacterial culture was separated to planktonic and aggregated populations by centrifuging at 610 x g for 2 min. Cell pellets were resuspended in 500 μl water and sonicated (Ultrasonic Processor XL (Misonix)) for 1 min (2 X 30 s.) at a pulse of 95% s⁻¹. Samples were centrifuged at 13,000 rpm in the microfuge and the supernatants were subjected to uronic acid quantification. The experiment was performed in the fumehood. A volume of 150μl of 120 mM sodium tetraborate in concentrated 18 M sulphuric acid was pipetted into each well of a 96-well microtitre plate, and 50μl of cell supernatant was added to the mixture and mixed by gentle pipetting. The microtitre plate was incubated for 1 hour at 80°C in a hybridisation oven (PersonalHyb (Stratagene)). The samples were allowed to cool to RT for 10 min before the first absorbance reading was taken at OD₅₄₀nm in the plate reader (μ-Quant Spectrophotometer). A 40 μl aliquot of detection reagent (100 μl of 100 mg ml⁻¹ 3-phenylphenol, dissolved in DMSO added to 4.9 ml 80% (v/v) sulphuric acid prepared immediately prior to use) was mixed with the samples in each well. The second OD₅₄₀nm absorbance reading was taken 15 min later in a plate reader. To remove background
absorbance, the first reading was subtracted from the second. D-glucuronic acid standards ranging from 0.1 µg ml\(^{-1}\) to 4.0 µg ml\(^{-1}\) (0.1, 0.5, .8, 1.0, 1.5, 2.5, 3.0, 3.5, and 4.0 µg ml\(^{-1}\)) were used to quantify the results obtained and the uronic acid levels interpolated from the corresponding reference curve. All samples were assayed in triplicate.

2.13.5 Poly-\(\beta\)-1,6-N-acetyl-glucosamine (PNAG)

PNAG was quantified according to the method described by Tote et al. 2007, up-scaled to allow analysis of 5 ml volumes in 50 ml tubes. Briefly, 5 ml of bacterial culture was grown and harvested at desired time points. The cells were centrifuged at 3228 x g for 10 min and the cell pellet washed twice with PBS. Ten millilitres of DMMB working solution (section 2.2.3) was added to each tube and incubated for 30 min at RT protected from light. The sample was centrifuged at 2800 x g for 20 min and the dye solution discarded. The cell pellet was washed once with water after which 12.5 ml of Deco solution (section 2.2.3 #5) was added and mixed well. The sample was incubated for a further 30 min protected from light after which the OD\(_{650nm}\) was read. The absorbance is determined to be proportional to the amount of PNAG in the EPS matrix.

2.14. HPLC for c-di-GMP

The determination of cyclic diguanylic acid (c-di-GMP) was carried out as described by Stocchi et al. 1985 and Antoniani et al. 2010. Bacterial cells were centrifuged at 3224 x g for 10 min, the supernatant removed and the pellet wet weight measured. The cell pellet was resuspended at a ratio of 45 mg cell pellet to 350 \(\mu\)l in 0.4 M perchloric acid and mixed carefully with the pipette tip. Samples were placed in the sonication bath for 15 min and centrifuged to remove cellular debris. The supernatant was transferred to a clean microfuge tube, neutralized with 1 ml of 0.16 M \(K_2CO_3\) and mixed well. Samples were left in the freezer overnight to allow the precipitate to fall out. Thawed samples were centrifuged in a microfuge at 13,000 rpm for 3 min and the supernatant was filtered through a 0.22 \(\mu\)m Millipore syringe filter prior to injection into the HPLC machine (Agilent HPLC system equipped with a diode-array detector and fitted with a Zarbox Eclipse XDB-C18 (4.6 × 150 mm) and 5 \(\mu\)m particle size reverse phase column). The mobile phase consists of: 0.1 M \(KH_2PO_4\) solution, pH 6.0 (Buffer A) and a 0.1 M \(KH_2PO_4\) solution, pH 6.0 containing 20% (v/v) methanol (Buffer B). All buffer solutions after preparation and pH adjustments, as well
as standards and samples, were filtered through a 0.22 µm Millipore filter. The chromatograph elution conditions were 9 min at 100% buffer A followed by 25%, 90% and 100% of Buffer B at 15, 17.5 and 19.5 minutes respectively. Buffer B was run at 100% at 25 min to 0% at 30 min. A flow rate of 1.3 ml min\(^{-1}\) was used. The c-di-GMP elution peak was identified by co-elution and UV absorption spectra comparison with a c-di-GMP standard (Biolog, Bermen, Germany). The c-di-GMP concentration was calculated based on an extinction coefficient (\(\varepsilon\)) of 23,700 at 254 nm (Hayakawa et al. 2003).

### 2.15. Antibiotic Outgrowth Assay

To determine the ability of the biofilm to protect bacteria from antibiotic bacteriostatic effects an antibiotic outgrowth assay was developed. To show that Gentamicin was able to "kill" and reduce growth, an antibiotic outgrowth assay was first performed on planktonic cells. A 5 ml culture of UPEC 536 in LB broth (LB prevents aggregate formation) was grown to \(OD_{600nm} = 0.1\). The culture was diluted 1:50 with fresh LB to four new tubes ("treated" and "untreated" in duplicate). A sample of planktonic cells was taken from each tube for enumeration of viable cells (Section 2.2.2) (T= minus 2 hr). Gentamicin was added to the "treatment" tubes and all tubes incubated for 2 hours at 37°C with shaking. After treatment, all tubes were centrifuged at 3226 \(X g\) for 2 min. The cell pellets were washed twice in fresh LB to remove any residual antibiotic. The cells were resuspended to full volume with LB broth, a sample taken for cell enumeration, incubated at 37°C with shaking and enumerated for viable cells after 2 and 3 hours (T2 and T3, respectively).

To determine the sensitivity of aggregates to antibiotic treatment, an overnight culture of UPEC 536 (RF) was diluted 1:100 to fresh RPMI (RPMI induces aggregates) to "treated" and "untreated" labelled tubes (in duplicate for each time point, T-2, T0, T2, T3) and the culture grown to Rmax. At Rmax, the T-2 tubes were centrifuged at 610 \(X g\) for 2 min and a sample of planktonic cells taken for enumeration of viable cells before treatment. Gentamicin was added to the "treatment" tubes and all samples incubated At T0, planktonic cells were separated from the aggregates by centrifugation at 610 \(X g\) for 2 min and the planktonic cells discarded. Aggregates were washed twice in fresh LB to remove residual antibiotic and then resuspended in LB broth. The culture was centrifuged at 610 \(X g\) for 2 min and a sample of the planktonic portion removed for enumeration of viable cells. All tubes were incubated at 37°C with shaking and planktonic cells sampled again at 2 hours and 3 hours incubation to
determine outgrowth from surviving cells. At each time point the tubes were spun at 610 x g for 2 min and a sample taken from the upper portion of the culture. The number of viable cells versus pre and post incubation with antibiotic was plotted over time. See method overview Chapter 7.1 Figure 74.

An aggregate control was used to ensure cells within the aggregate could be killed. This was performed by heat kill. This was based on the time and the temperature required to kill all cells within an aggregate. This was performed by plating a series of inoculums of treated aggregates from different time points on to Nutrient agar plates and incubating overnight. The time and temperature until no growth was detected was considered the kill time.

2.16. *Galleria mellonella* Larvae Infection Assay

The "time-to-death" infection assay is based on the method described by Brennan *et al.* 2002. The greater wax moth *Galleria mellonella* was used to investigate the contribution of the cellulose matrix to virulence of UPEC 536. Strains of cellulose producing (UPEC 536), cellulose negative (UPEC\(_{\Delta bcsA}\)) and \(\Delta hj\) cellulose (UPEC\(_{\Delta yhjK}\)) were grown overnight in RPMI 1640 supplemented with 10\(\mu\)M FeCl\(_3\). The culture was centrifuged and the pellets washed in PBS three times. The cultures were resuspended in a full volume of PBS and a dilutions series prepared for inoculation.

The *Galleria mellonella* larvae were supplied by Living Foods Direct (http://www.ak.planet.gen.nz/~bio/index.html) and housed in a plastic container at 15°C. Caterpillars are placed at RT for at least 10 minutes before use as this allows them to warm up and be less rigid. The larvae are approximately 3cm x 0.5 cm in size and weigh approximately 250 mg, although this may vary. All experimentation groups contained 15 larvae and each experiment was repeated using larvae from a different batch. In all experiments there were two negative control groups; one that underwent no manipulation, while the other group (uninfected control) was injected with PBS only to control for the impact of physical trauma and the inoculum diluent. During the experiment the larvae were stored in 85 mm petri dishes in the dark at 37°C for a maximum of 72 hours. Larvae were inspected every 24 hours and considered dead if they did not respond to touch.
Caterpillars were infected by injecting 10 µl of washed bacteria into the body cavity (haemocoel) via the lower left pro-leg on the larvae's underside with a 27 gauge insulin syringe. The pro-leg is the thinnest part of the larvae's outer layer (cuticle) and therefore, minimizes damage to the insect. Injection requires holding the larvae in one hand and pressing firmly between the thumb and index finger to keep the larvae rigid and to expose the pro-leg. After injection each group were placed into a petri dish for incubation.

**Safety** Injection requires holding the larvae in one hand and pressing firmly to keep the larvae ridged and to expose the pro-leg allowing accurate injection. This presents an inherent risk to accidental injection of the finger. To reduce the risk of injury all bacterial work should be performed in a class II safety cabinet. Latex gloves should be worn for greater dexterity while holding the larvae body. Hold the body of the syringe to insert the needle into the haemocoel and inject the inoculum once the hand holding the larvae is free.

**Measurement of bacterial loads** - Hemolymph was collected from five untreated, PBS treated, UPEC 536 living UPECΔbcsA living and five dead larvae (pooled) and combined in an Eppendorf microfuge tube. The total volume was diluted to 250 µl with LB broth. Twenty five µl was plated on to a BAP and a MAC agar plate.

### 2.17. Statistical Methods

A Student’s T-test (Excel) was used to determine significant differences between Aggregation and Dispersion at the Aggregation Index end-point (generally 7 h culture). To determine significant differences between the results in the PNAG, Uronic Acid and Motility assays, an analysis was carried out using Prism software with the assistance of Dr. S. Wiles to calculate p-values using a one-way ANOVA with Bonferroni post hoc tests or a one-tailed Mann-Whitney test. All data are presented as the mean ± 1 standard error where appropriate. Assays were performed with 3-8 replicates unless otherwise stated. P-values calculated for the Aggregation Index and the *G. mellonella* assay were carried out using a Student’s T-test (Excel). A p-value <0.05 was considered to be significant.
Chapter 3. Characterization of the Biofilm Matrix

3.1. Introduction

Life for bacteria within a biofilm may provide protection from a harsh environment and assaults from factors that may exist within that environment such as biocides, predators or immune defences. The protective factors lie within the matrix and the unique niches produced within the structure, although the composition of the matrix is often dependent on the bacterial strain and the conditions of the environment in which the biofilm grows. Bacterial produced extracellular polymeric substances and proteins have been shown to be major components of the matrix (Sutherland 2001; Stewart and Costerton 2001). Other minor macromolecules may play a role in strengthening the matrix to re-enforce the scaffolding of the major matrix components (Whitchurch et al. 2002). The bacterially produced carbohydrate-rich extracellular polymers are classified as capsular polysaccharides (cell associated) and exopolysaccharides (those that remain in the supernatant) (Branda et al. 2005). The consideration of polymers that are formed to produce a biofilm matrix may not offer a clear distinction between the two polymers.

Previous work on this project (Rowe 2006; Rowe et al. 2010) determined that an Aggregation Index for UPEC 536 aggregates could be obtained using low speed centrifugation and optical density if a 0.3 NaCl wash was used to disperse the aggregates (Figure 12.) The Aggregation Index with the salt wash is an easy and reproducible method for determining the aggregation dynamics within a UPEC 536 culture for the first seven hours of growth. After this time, the Aggregation Index is less sensitive due to the aggregates becoming more difficult to disperse in the salt wash (Rowe 2006).

This section will investigate and identify the major polymers contributing to the UPEC 536 matrix. The temporal changes of the matrix will be investigated in both iron restricted and iron replete conditions and the contribution of other polymers supporting the biofilm matrix will be investigated utilizing biochemical and enzymatic assays in conjunction with the Aggregation Index as a standard analytical tool. Signals, both environmental and biological, that may induce dispersion will be tested and qualified by the Aggregation Index. Qualitative and semi-quantitative analysis of exopolysaccharides produced within the matrix was performed using biochemical assays, staining and microscopy.
3.1.1 The Aggregation Index

Enumeration of cells within an aggregating culture poses a difficulty due to the heterogeneous nature of the culture. Observation of UPEC 536 culture grown in RPMI shows the production of small threads that form into small aggregates and then large flocs at approximately 240 minutes. This point, referred to as Rmax, shows large white flocs that settle quickly to the bottom of the tube on standing and the broth appears clear (Figure 11, A-C). The aggregates persist beyond 24 hours. Cultures are grown in RPMI and supplemented with 10 μM FeCl₃ grow and produce aggregates earlier than when grown in RPMI without supplementation. Large white aggregates form for approximately three to four hours and then begin to disperse. The broth becomes cloudy, indicative of planktonic cells, and the aggregates become smaller over time (Figure 11 E-G). At 24 hours, small aggregates remained in the cultures which are easily dispersed on vortexing (Figure 11, G). E.coli strain MG1655 was used as a negative control (Figure 11, D).

Cultures grown to maximum aggregation in RPMI and then supplemented with iron began to disperse within 30 minutes. At approximately 8 hours incubation most of the remaining aggregates were small and were dispersed with vortexing.

![Figure 11. UPEC 536 Aggregates in RPMI 1640 and RPMI 1640 + 10 μM FeCl₃.](image)

UPEC 536 grown in RPMI produces long fibrous ribbons (A circle) that evolve into large spherical aggregates (B) which persist up to 24 hours (C). A non-aggregating E.coli strain, MG1655, grown in RPMI 1640 produces no aggregates (D). UPEC culture grown in RPMI supplemented with iron produces transient aggregates that disperse within 24 hours (E-G).
Conventional methods used to determine relative proportions of cells involved in aggregation depend on a planktonic cell OD measurement and vortexing to disperse the aggregates and measure the optical density of the total culture. UPEC 536 aggregates do not disperse by vortexing; in fact aggregation appeared enhanced with vortexing. To overcome this problem a modified method combining a 0.3M NaCl wash of total cells was incorporated (see section 2.4). This modification allowed for a uniform suspension and allowed a total optical density measurement to be recorded and the relative ratio of cells involved in aggregation to be calculated. This method is effective up to approximately 7 hours incubation, after which the aggregates become difficult to disrupt. The Aggregation Index provides a means to graphically monitor the involvement of cells in aggregation and dispersion over time (Figure 12).

![Figure 12. The Aggregation Index.](image)

The relative aggregation was determined by using low speed centrifugation, a NaCl wash and measurement of optical density. The relative value of aggregation was determined by using the Aggregation Index equation OD600nm (total) \( \div \) OD600nm (planktonic) / OD600nm (total). Lines represent cultures in RPMI (◊); RPMI and FeCl₃ (□) and RPMI supplemented with FeCl₃ to 10µM at ↓ (△). Data points are an average of at least 10 independent assays. Error bars represent ± 1SE. The p-value between the aggregated and dispersed culture three hours after Rmax is < 0.05 (p=1.4 x 10⁻¹¹) (Student's T-test) indicating the magnitude of the significant difference between the two phenotypes.

### 3.1.2 The Matrix is Composed of Exopolysaccharide

The role of outer membrane proteins, pili and exopolymeric substances in adhesion is important in the establishment of infection (reviewed in Mulvey 2002). To determine whether cell-to-cell interactions and the major matrix components of UPEC 536 were linked by
proteinaceous structures such as pili or by exopolymeric substances, cryo scanning electron microscopy (CSEM) was used to investigate the microscopic structure of the matrix. It was proposed that if cells were connected by outer membrane organelles the matrix would most likely be proteinaceous in nature. If the cells were bound by amorphous material then the matrix would be polysaccharide in nature.

CSEM suggested bacteria encased within a polymeric matrix (Figure 13). The overall structure of the aggregates is composed of polymeric sheets which form into layers with bacteria embedded within the sheet (Figure 13, A - C). Structured perpendicular sheet arrangements that showed bacteria embedded within and on the polymeric sheet. Fibers of EPS interconnect bacteria to each other (Figure 13, D).

Figure 13. Cryoscanning Electron Microscopy of UPEC 536 at Maximum Aggregation. The overall structure of the aggregates is composed of polymeric sheets which form into layers with bacteria embedded within. Scale bar = 50 μm, magnification 1000 X (A). Bacteria are seen within the polymeric sheet (circles), scale bar = 20 μm, magnification 2,500 X (B). Bacteria are embedded and attached to the polymeric matrix (arrows), scale bar =10 μm, magnification 6,500 X (C). Intercellular polymeric substance is produced by the bacteria linking cells together (D) scale bar = 2 μm, magnification 15,000 X.
3.1.3 Cellulose is the Major Component of the Matrix

CSEM visualization of the aggregates strongly suggests an exopolymeric matrix. A review of the literature identified cellulose, colanic acid, poly-β-1,6-N-acetyl-d-glucosamine and extracellular DNA as candidates. It was hypothesised that aggregates would not form in the presence of exogenous enzymes able to degrade the polymer forming the aggregate matrix and that the enzyme would disperse aggregates in the absence of iron.

Cellulase from *Aspergillus niger* was added to the initial RPMI growth media and monitored using the Aggregation Index. Cellulase at 1 mg ml⁻¹, (1,4-(1,3:1,4)-β-D-glucan-4-glucanohydrolase) prevented the formation of aggregates when UPEC 536 was grown in RPMI 1640 and all cells remained planktonic (verified by wet mount, data not shown). Cellulase was added to UPEC 536 aggregates grown to Rmax. Cellulase dispersed aggregates at a faster rate than disaggregation induced by iron alone (Figure 14). Amylase (1 mg ml⁻¹), a negative control enzyme which cleaves 1,4-β-glucosidic linkages, had no effect on aggregate formation or dispersal.

![Figure 14. Exogenous Cellulase Degrades Formed Aggregates.](image)

*Figure 14. Exogenous Cellulase Degrades Formed Aggregates. Rmax cultures containing aggregates was supplemented with cellulase, iron or cellulase plus iron. Cellulase rapidly degrades UPEC 536 aggregates. Graphs are based on an average of at least three independent assays. p = <0.05(Student’s T-test) between the aggregated and the dissociated phenotypes at 3 h growth after the addition of cellulase.*
3.1.4 Visualization of Cellulose

Aggregate formation and structure is compromised by exogenous cellulase suggesting cellulose is the major component of the aggregate. A differential stain was used to visualize the presence of cellulose in UPEC 536 culture. Congo Red, (benzidinedazo-bis-1-naphtylamine-4-sulfonic acid) a secondary diazo dye was added to M9 Minimal agar to a final concentration of 0.001% (w/v). Ten µl aliquots were taken from exponentially growing cells, dropped on to the plate and incubated overnight. The Congo Red dye has a strong affinity for cellulose and curli, producing a red colony and providing evidence of cellulose synthesis by the bacteria (Figure 15 A). The negative control strain, MG1655 does not produce cellulose (Da Re and Ghigo 2006) and the colony remains white (Figure 15, B). An aliquot of UPEC 536 grown to Rmax in RPMI was stained with Calcofluor White and imaged by light microscopy (Figure 15, C), and fluorescent microscopy (Figure 15, D) indicating the presence of cellulose localised to cell aggregates.

![Figure 15. Cellulose in UPEC 536. Cultures were grown in RPMI 1640 for 4 h and inoculated to M9 minimal media plates supplemented with 0.001% (w/v) Congo Red Dye. Plates were incubated for 24 h at 37°C. Cellulose produced by UPEC 536 stains the colony red (A) whereas MG1655, a non cellulose producing strain, remains white (B). Aggregates were stained with Calcofluor White and imaged under light microscopy (C) and UV light and indicate the presence of cellulose (D).](image-url)
3.1.5 The UPEC 536 Aggregate Contains Live and Dead Cells

The UPEC 536 aggregate is a cluster of cells held together within a cellulose matrix. Cells were examined for viability, using Live / Dead staining (section 2.10.2) and CLSM. Images show that most cells within the aggregate are live with some dead cells present (Figure 16, A) distributed throughout the cellulose matrix (Figure 16, B).

![Live / Dead Staining of the UPEC 536 Aggregate](image)

**Figure 16. Live / Dead Staining of the UPEC 536 Aggregate.** Confocal microscopy images display a random distribution of both live (green) and dead (red) cells (A) within the cellulose matrix (blue) (B). Scale bar = 50 µm.

3.2. Dispersion

3.2.1 Introduction

For bacteria to re-seed and re-infect they must leave the biofilm. Initiation of dispersion can be induced by a number of signals such as changes in nutrient availability (Sauer et al. 2004; Gjermansen et al. 2005), fluctuations in oxygen concentrations (Thormann et al. 2005) or nitric oxide (Barraud et al. 2006), quorum sensing (Pukas et al. 1997) and probably many more environmental signals that have yet to be elucidated. Dispersal from the biofilm matrix requires the expression of enzymes to degrade the EPS matrix that surrounds the cells, such as the alginate lyase produced by *P. aeruginosa* (Boyd and Chakrabarty 1994) or the enzyme specific for hydrolysis of glycosidic poly-β-1-N-acetylglucosamine linkages produced by the oral pathogen *Aggregatibacter actinomycetemcomitans* (Kaplan et al. 2004; Wang et al. 2004).
It is accepted that iron is an essential nutrient for bacterial growth. Many genes involved in iron uptake and metabolism are regulated by Fur, an iron responsive DNA binding repressor (Hantke 1981). When iron is abundant in the environment, a Fe^{2+}-Fur complex binds to target DNA sequences in the promoter region of certain genes and inhibits transcription. When iron levels are low, Fe^{2+} dissociates from Fur, enabling transcription of Fur regulated genes (Buchanan 2005). The uptake of iron from the environment in Gram-negative organisms is dependent on the interaction of iron-bound siderophores, outer membrane receptors, ancillary transport proteins and an integral inner membrane TonB protein complex (Noinaj et al. 2010). Control of Fur-regulated genes is not totally dependent on iron. In vitro binding by other divalent metals such as zinc (Althaus et al. 1999), manganese (Adrait et al. 1999; Escolar et al. 1999), cobalt (Adrait et al. 1999) and nickel (Ahn et al. 2006) have been shown to associate with Fur. There is abundant diversity in metal selectivity and biological function within the Fur family of proteins. The mechanism of metal sensing by the Fur family proteins is still controversial (reviewed in Lee and Helmann 2007).

Interbacterial quorum sensing molecules (Miller and Bassler 2001) and host expressed neurotransmitter signals (Lyte 2004) have been proposed as signals for *E.coli*. Although *E.coli* does not produce specific N-acylhomoserine lactones (AHLs) quorum sensing molecules, both *E.coli* K12 strain and *Salmonella* Typhimurium have been shown to respond to AHLs from other species (Michael et al. 2001). In this section a range of potential inducers, ranging from a selection of cations and biological iron sources to quorums sensing molecules were investigated as inducers of dispersion. The Aggregation Index was used to quantify the sources analyzed.

3.2.2 Inhibition of Transcription and Translation

Dispersion of aggregates is induced upon the addition of iron. To gain insight into the mechanism of dispersion within an iron-enriched culture, inhibitors of transcription and translation were used within the culture media. Rifampicin inhibits DNA-dependent RNA polymerase within bacterial cells by binding to the beta-subunit and preventing transcription. Rifampicin was added to an aggregating culture grown in RPMI 1640 at approximately 300 minutes incubation, and allowed to incubate for a further 30 minutes before the addition of iron. Dispersion of aggregates was determined by the Aggregation Index (Figure 17). Dispersion did not occur when rifampicin was present indicating that new gene transcription is required for dispersion of aggregates to occur with iron. To determine if translation was
required, chloramphenicol was used to inhibit protein synthesis. Chloramphenicol is a bacteriostatic antibiotic that inhibits proteins synthesis by inhibiting protein chain elongation. The antibiotic was added to culture grown in RPMI 1640 at approximately 300 minutes, allowed to incubate for a further 30 minutes before the addition of iron (Figure 19). Dispersion did not occur in the presence of chloramphenicol indicating that new protein synthesis is required for dispersion to occur.

![Figure 17. Inhibition of Transcription](image1)

Rifampicin was used to inhibit transcription of RNA. Iron was added to a UPEC 536 culture to induce dispersion of aggregates. Data points are an average of at least three independent assays.

![Figure 18. Inhibition of Translation](image2)

Chloramphenicol was used to inhibit new protein synthesis. Iron was added to a UPEC 536 culture to induce dispersion of aggregates. Data points are an average of at least three independent assays.
3.2.3 Ferric Chloride as a Standard for Dispersion

UPEC 536 grown to Rmax displays clear broth and large aggregates (Figure 11, A-C, Figure 20, A). The Aggregation Index was measured using 10µM FeCl₃ as an inducer of dispersion where UPEC 536 aggregates disperse within 4 hours of supplementation with iron (Figure 11, E-G) and disruption of remaining aggregates is enhanced by vortexing. Within approximately 30 minutes after the addition of FeCl₃ changes in the culture were visible (Figure 19) as cells were released into the bulk medium and the broth became cloudy (Figure 19, B & C) and aggregates become smaller (Figure 19, D). Further investigation of inducers of dispersal were tested and compared to the effects of ferric chloride dispersion and monitored by the Aggregation Index.

![UPEC 536 Aggregates](image1)

**Figure 19. Induction of Dispersion of UPEC 536 Aggregates by FeCl₃.** Aggregates at Rmax (A) display large flocs and clear broth in RPMI media. One hour after the addition of iron (B) the aggregates begin to disperse and planktonic cells are visible within the broth (seen as cloudy). At 2.5 and 4 hours (C and D) only small aggregates remain and most are easily dispersed with vortexing. Images were taken in 5.5 cm petri plates using the BioSys Biocount.

3.2.4 Induction of Dispersion by Divalent Metals

Iron is not the only metal ion to play an important role in bacterial function (Hantke 2005; Papp-Wallace & Mcguire 2006; Rink & Hasse 2007; Sabri et al. 2009). Other divalent metals were tested to determine if they could replace iron as an inducer of aggregate dispersal. Manganese (MnSO₄), copper (CuCl₂), zinc (ZnSO₄) and nickel (NiCl₂) ions were added (to a final concentration of 10 µM) to an aggregated UPEC 536 culture grown to Rmax to determine if other divalent metals could replace iron as an inducer of aggregate dispersion. Manganese and zinc dispersed the aggregates in a manner similar to FeCl₃ (Figure 20). Zinc initially dispersed aggregates at a slower rate but after 3 hours incubation the aggregates...
dispersed to a similar extent as those in iron and manganese. Nickel and copper had no effect on the aggregates as the aggregates remained tightly packed and the broth clear.

![Figure 20. Dispersion of Aggregates by Divalent Metals.](image)

**Figure 20. Dispersion of Aggregates by Divalent Metals.** UPEC 536 Aggregates were grown to Rmax and dispersion was induced by iron, manganese and zinc but not by copper or nickel. Graphs are based on an average of at least three independent assays. Aggregated and dispersed cultures indicate $p = < 0.05$ (Student’s T-test) three hours after the addition of the metal ion.

### 3.2.5 Titration of Metal Ions for Dispersion

To determine the lowest concentration of metal ion required to induce dispersion, a dilution series for each of the inducing metal supplements was prepared and added to Rmax aggregates. Dispersion was monitored using the Aggregation Index. Iron and manganese induced dispersion with concentrations as low as 0.5 µM (Figure 21, A & B). Maximal dispersion was seen for zinc at concentrations of 1.0 µM and above (Figure 21, C). For iron, manganese and zinc some dispersion was seen at concentrations as low as 0.1 µM. These results indicate that iron and two other bio-relevant metal ions, Mn$^{2+}$ and Zn$^{2+}$ can act as a signal for dispersion from the UPEC 536 aggregate. Table 8 provides a summary of the Relative AI Reduction of each culture at each metal concentration.
Table 8. Summary of the Relative AI Reduction in Various Metal Ion Concentrations

<table>
<thead>
<tr>
<th>Divalent Ion Source</th>
<th>10μM</th>
<th>1 μM</th>
<th>0.5 μM</th>
<th>0.1 μM</th>
<th>0.01 μM</th>
<th>0 μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>FeCl₃</td>
<td>47</td>
<td>32</td>
<td>30</td>
<td>10</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>ZnSO₄</td>
<td>41</td>
<td>41</td>
<td>32</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>MnSO₄</td>
<td>31</td>
<td>31</td>
<td>25</td>
<td>19</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*Relative AI Reduction is calculated as described in Section 2.4

3.2.6 Dispersion by Bio-relevant Iron Sources

The acquisition of iron from the host is an important component of microbial pathogenesis (Fischbach et al. 2006). Iron availability for bacterial growth is dependent upon the efficiency of the bacterial iron uptake systems to remove iron from the host iron-binding proteins (Carpenter et al. 2009). To determine if UPEC 536 is able to utilize iron from human iron binding proteins to induce dispersion, Rmax aggregates were supplemented with 10 μM haemin, 10 μM haemoglobin, 0.5 μM ferritin, 0.6 μM and holo-lactoferrin, 0.6 μM holo-transferrin. Apo-transferrin, (0.6μM) was used as a control for a non-iron carrying protein. Dispersion was quantified using the Aggregation Index. Haemin, haemoglobin and ferritin induced dispersal in a manner similar to that observed for ferric chloride (Figure 22, A-C). Dispersion of aggregates was delayed when lactoferrin and transferrin were used as an iron source but dispersed to the same extent as haemin, haemoglobin and ferritin after three hours of incubation (Figure 22, D & E). The apo-transferrin negative control showed no effect on dispersion (Figure 22, E). These results indicate that protein bound bio-relevant iron sources are able to induce dispersion of UPEC 536 aggregates. Table 9 summarises the overall relative reduction in the Aggregation Index for each observed iron source after three hours incubation.
Figure 21. The Effect of Bio-Relevant Iron on Dispersal. Cultures supplemented with bio-relevant iron sources (10 µM haemin (A), 10 µM haemoglobin (B) and 0.5 µM ferritin (C) 0.6 µM lactoferrin (D)and holo-transferrin (E) display a dispersion trend similar to ferric chloride and apo-transferrin (E) (negative control) shows no effect. RPMI=RPMI 1640, RF=RPMI 1640 + 10 µM FeCl₃, Ferr=ferritin, Lf=lactoferrin, Tf=transferrin. Each graph represents an average from a minimum of three separate assays.
Table 9. Summary of the Relative Reduction by Bio-relevant Iron

<table>
<thead>
<tr>
<th>Iron Source</th>
<th>Relative AI Reduction (% / 3 hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 µM FeCl₃</td>
<td>56 ± 0.09</td>
</tr>
<tr>
<td>10 µM Haemin</td>
<td>53 ± 0.09</td>
</tr>
<tr>
<td>10 µM Haemoglobin</td>
<td>70 ± 0.12</td>
</tr>
<tr>
<td>0.5 µM Ferritin</td>
<td>58 ± 0.06</td>
</tr>
<tr>
<td>0.6 µM Lactoferrin</td>
<td>54 ± 0.08</td>
</tr>
<tr>
<td>0.6 µM Transferrin (iron loaded)</td>
<td>53 ± 0.07</td>
</tr>
<tr>
<td>0.6 µM Apo-transferrin (no iron)</td>
<td>0</td>
</tr>
</tbody>
</table>

*Relative AI Reduction is calculated as described in Section 2.4

3.2.7 Adrenaline / Noradrenaline and Quorum Sensing Molecules

Dispersion is the result of an environmental cue that influences a change in gene expression to produce the enzymes and other organelles required to leave the biofilm and re-infect (Costerton et al. 1999). Bacteria-bacteria (Miller and Bassler 2001) and bacteria-host communication (Sperandio et al. 2003) is an accepted phenomenon in various environments. The effect of host signalling molecules and their interaction with bacteria has been observed for various pathogens (Powell et al. 1983; Sonnex 1998; Woods et al. 1993). These observations have led to the concept of microbial endocrinology which proposes that infectious organisms utilize hormones present within the host as environmental cues to initiate growth and pathogenic processes (Freestone et al. 1995; Freestone et al. 1999; Burton et al. 2002; Lyte 2003). Endogenous signals such as those produced by quorum sensing can also invoke such an effect. Quorum sensing influences a number of bacterial phenotypic traits but importantly it enhances biofilm formation (Dunne 2002) and may enhance (Davies et al. 1998; Hammer and Bassler 2003; Sakuragi and Kolter 2007) or reduce (Hammer and Bassler 2003; Heithoff and Mahan 2004) EPS production. *E.coli* does not produce quorum sensing molecules but is able to detect and respond to homoserine lactones produced by other species (Michael et al. 2001). The interplay between bacteria and the host during infection is a complex process and difficult to emulate in a laboratory. It was hypothesised that the presence of quorum sensing or neuroendocrine hormone signals may influence the production of EPS and aggregate formation and/or dispersal.
The initial RPMI 1640 culture media or was added to aggregates grown to Rmax. The culture was supplemented with $N$-acyl homoserine lactone or neuroendocrine hormone at two concentrations. Aggregation and dispersion were quantified by the Aggregation Index. Quorum sensing molecules, $N$-butyryl-$L$-homoserine-lactone (1 & 10 µM) and $N$-3-oxo-dodecanoyl-$L$-homoserine lactone and neuroendocrine hormones, adrenaline and noradrenaline (5 & 10 µM) had no effect on the formation of aggregates when grown in either RPMI 1640 or on the dispersal of preformed aggregates (Table 9). These results suggest that aggregation and dispersion may not be quorum sensing, or neuroendocrine hormone regulated processes.

Table 10. The Effect of Quorum Sensing Molecules and Neuroendocrine Hormones

<table>
<thead>
<tr>
<th>Transient Aggregate formation*</th>
<th>Aggregate Dispersion**</th>
</tr>
</thead>
<tbody>
<tr>
<td>FeCl$_3$ (10 µM)</td>
<td>Aggregate formation</td>
</tr>
<tr>
<td>Cellulase (13.8 U ml$^{-1}$)</td>
<td>No aggregate formation</td>
</tr>
<tr>
<td>$N$-butyryl-$L$-homoserine-lactone (1 &amp; 10 µM)</td>
<td>Aggregate formation</td>
</tr>
<tr>
<td>$N$-3-oxo-dodecanoyl-$L$-homoserine lactone (1 &amp; 10 µM)</td>
<td>Aggregate formation</td>
</tr>
<tr>
<td>Adrenaline (5 &amp; 10 µM)</td>
<td>Aggregate formation</td>
</tr>
<tr>
<td>Noradrenaline (5 &amp; 10 µM)</td>
<td>Aggregate formation</td>
</tr>
</tbody>
</table>

*The supplement is added to the initial culture in RPMI
** The supplement is added to the culture at Rmax

3.3. Other Components of the Biofilm Matrix

3.3.1 Introduction

It is generally accepted that the biofilm matrix is composed of both the macromolecules available from the environment and those produced by the bacteria. It is a varied and complex mixture of extracellular polysaccharides, proteins and nucleic acids that provides a physical barrier of protection to the bacteria but also reflects the specific differences in components and structure of the biofilm. Polysaccharides common to *E.coli* biofilms include cellulose (*Zogaj et al. 2001*), colonic acid (*Grant et al. 1969*), poly-$N$-acetyl-glucosamine (*Wang et al.*
2004) and other less significant lipopolysaccharide and capsular polysaccharides. In this section the presence of polymeric substances other than cellulose were investigated.

### 3.3.2 Extracellular DNA

Biofilms are held together by a complex mixture of macromolecules including exopolysaccharides, proteins and DNA (reviewed in Flemming and Wingender 2010). Previously, the presence of DNA in the biofilm matrix was presumed to be derived from lysed cells and not considered an important component of the biofilm structure. However, some organisms such as *Pseudomonas aeruginosa* produce substantial quantities of exDNA by a mechanism that is thought to be independent of cell lysis and appears to involve the release of small vesicles from the outer membrane (Kadurugamuwa and Beveridge 1995; Muto and Goto 1986). The role of exDNA in biofilms remains unclear but current opinions include enhancing gene transfer (Molin and Tolker-Nielsen 2003), provide nutrition during oligotrophic conditions (Finkel and Kolter 2001) and provide stability to the biofilm structure (Whitchurch *et al.* 2002).

The culture supernatant and a UPEC 536 bacterial cell surface wash were examined to determine the presence of exDNA within UPEC 536 cultures. If exDNA is present in significant quantity, it may have a role in the structure and stability of the aggregate matrix. It is hypothesised that if exDNA is produced in the bacterial culture during aggregation then the addition of DNase I would inhibit the formation of the exDNA polymer and aggregation would be weakened or would not occur.

Bacteria produce a number of endonucleases (Madigan *et al.* 2002) that cleave phosphodiester bonds within a polynucleotide chain. To explore whether UPEC 536 produces endonucleases that enhance the dispersion process, the presence of DNase activity in culture supernatants and washed cell pellets was investigated. The regulation of DNase activity by iron is hypothesised if exDNA was an important feature of the UPEC 536 aggregate.

**DNA is Present in the Culture Supernatant and on the Bacterial Cell**

The exDNA was recovered from aggregated cultures grown in RPMI 1640 and dispersing cultures grown in RPMI 1640 supplemented with iron using the method developed by Alleson-Holm *et al.* 2006. The DNA was extracted, purified and precipitated. The concentration of exDNA in the supernatant and the washed pellet solution were quantified.
using the Nanodrop. Extracellular DNA was present in higher concentrations in dispersing cultures than in aggregating cultures (Table 10).

Table 10. ExDNA Recovery from Aggregated and Dispersed Supernatant and Washed Pellets

<table>
<thead>
<tr>
<th>RPMI Culture Supernatant (ng/μl)</th>
<th>RPMI Washed Pellet (ng/μl)</th>
<th>RPMI +Fe Culture Supernatant (ng/μl)</th>
<th>RPMI +Fe Washed Pellet (ng/μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 hour culture&lt;sup&gt;a&lt;/sup&gt;</td>
<td>21.7 ± 0.3</td>
<td>92.9 ± 1.0</td>
<td>71.1 ± 1.2</td>
</tr>
<tr>
<td>5 hour culture&lt;sup&gt;a&lt;/sup&gt;</td>
<td>41.8 ± 1.4</td>
<td>143.1 ± 3.8</td>
<td>89.1 ±3.4</td>
</tr>
</tbody>
</table>

Quantification of exDNA is an average of three results from at least three independent assays.
<sup>a</sup> 3 hour and 5 hour culture grown in RPMI and RF. 3 hour = Rmax, 5 hour= 5 hour from incubation.

Extracellular DNA is Not a Major Component of the Biofilm Matrix

The presence of exDNA in the matrix of *Pseudomonas aeruginosa* has been demonstrated to add to biofilm stability (Whitchurch *et al.* 2002). To investigate if the extracted exDNA was contributing to the scaffold of the UPEC 536 biofilm, cultures grown in RPMI 1640 were supplemented with DNase I enzyme (90, 135 and 180 U ml<sup>-1</sup>) and quantified by the Aggregation Index. Culture media supplemented with DNase I had no effect on the formation of aggregates (Figure 22). Cultures grown in RPMI 1640 to Rmax and supplemented with either 10 µM FeCl<sub>3</sub>, or DNase I or both showed that DNase I alone, had no effect on preformed aggregates and that dispersion is due to the effect of iron (Figure 23).

![Figure 22. The Effect of DNase I on Aggregation.](image)

Figure 22. The Effect of DNase I on Aggregation. Three different concentrations of DNase I (90 U ml<sup>-1</sup>, 135 U ml<sup>-1</sup>, and 180 U ml<sup>-1</sup>) were added to the initial RPMI culture media and grown for two hours. All concentrations of DNase I supplementation indicate that DNA does not affect the production of aggregates within the culture. Each graph represents an average from a minimum of three separate assays.
Chapter 3                                               Characterisation and the Biofilm Matrix

Figure 23. The Effect of DNase I on Dispersion. UPEC 536 was grown RPMI or RPMI+Fe and supplemented with DNase I (135 U ml\(^{-1}\)). The effect on dispersion was measured using the Aggregation Index. Each graph represents an average from a minimum of three separate assays.

Bacterial DNase Activity is Higher in Dispersing Culture

To further investigate bacterial DNase activity in dispersing culture, DNA was extracted from bacterial culture or washed bacterial pellets purified, precipitated and quantified using the Nanodrop, to determine the difference in concentration of DNA attached to bacterial cells and that secreted into the supernatant (Figure 24). The concentration of DNA is greatest in iron enriched RPMI 1640 culture supernatant. During dispersion more DNA is associated with the pellet and later released into supernatant (Figure 24, Table 11).

Figure 24. Extracellular DNA is Abundant in Dispersing Cultures. Samples were collected over a time course and each sample was precipitated, purified and measured on the nanodrop for DNA concentration. DNA is greater in iron enriched culture and more DNA is associated with the pellet during dispersion. Results represent an average from at least three separate assays.
To assess exonuclease activity in dispersing culture, UPEC 536 was grown in RPMI to Rmax and sampled directly before and 1, 2 and 3 hours after the addition of FeCl₃ to 10 µM. Independent aliquots of each supernatant collected were incubated with Lambda HindIII DNA at a final concentration of 50 ng µl⁻¹. After 1, 2 and 3 hours incubation 10 µl samples were analysed by agarose gel electrophoresis and ethidium bromide staining (Figure 25). Lambda DNA degradation is apparent in the supernatant from the Rmax culture and the dispersing culture 1 hour post FeCl₃ addition. DNase activity in the supernatant of the dispersing culture declines 2 and 3 hours after FeCl₃. Supernatant incubated against genomic DNA showed degradation of DNA albeit at a slower rate (data not shown).

![Figure 25. UPEC 536 Culture has DNase Activity.](image)

**Figure 25. UPEC 536 Culture has DNase Activity.** Lambda HindIII DNA (A-C) was incubated with supernatants from Rmax culture (Lane 1), and Rmax culture dispersed with FeCl₃ at 10 µM for 1 hour (Lane 2); 2 hours (Lane 3) and 3 hours (Lane 4). Lane 5 is blank. Lane 6 is lambda HindIII DNA alone. Supernatant was incubated with lambda HindIII DNA for 1 hour (A); 2 hours (B) and 3 hours (C).

### 3.3.3 Uronic Acid

Colanic acid, a form of uronic acid, is up-regulated in E.coli K12 biofilms (Priget-Combaret et al. 1999; Danese et al. 2000) and is not produced by planktonic cells or in rich media at 37°C (Beloin et al. 2008). Uronic acid is a negatively charged polymer of glucose, galactose, fucose and glucuronic acid that forms a protective capsule around the bacterial cell surface (Hanna et al. 2003). It has been suggested that colanic acid inhibits adhesion to polyvinyl chloride (Danese et al. 2000) and further studies indicate that colanic acid blocks the establishment of specific binding interactions between bacteria and inert substrates in early stages of biofilm development (Hanna et al. 2003).

UPEC 536 aggregates produced in RPMI 1640 are soluble in 0.3 M NaCl for approximately the first 7 hours of incubation after which time the aggregates become increasingly resistant to the salt wash. This creates difficulty in obtaining a homogenous suspension for use in the
aggregation index measurement (Figure 26, A). Aggregates at Rmax (Figure 26, B), are soluble in 0.3 M NaCl and appear as a loose fibrous network when stained with Calcafluor White (Figure 26, C). Aggregates older than 7 hours are more resistant to degradation in a salt wash and iron induced dispersion. After 7 hours, the cellulose fibres of the remaining aggregates appear tightly packed and some areas show a smooth surface (Figure 26, D circle).

Due to the changes in UPEC 536 aggregate stability over time it was hypothesised that another polysaccharide, such as colanic acid may be involved in the biofilm matrix rendering it more resistant to dispersion by FeCl₃. Quantitative measurement of total uronic acid is commonly performed using a colorimetric method after first hydrolyzing the polysaccharides in sulphuric acid (Ahmed and Labavitch 1978; Selvendran et al. 1979). An uronic acid assay described by van den Hoogen et al. (1998) (section 2.13.4) was used to determine the presence of colanic acid at three time points; Rmax, R-24 hours and RF-24 hours (Figure 27).

---

**Figure 26. Phenotypic Changes in the Aggregate Structure Over Time.** UPEC 536 Aggregates at Rmax (A and B) are easily dispersed with 0.3M NaCl and appear microscopically as loose fibrous networks with a rough surface texture (C). At greater than 7 hours incubation the aggregate becomes tightly packed with smooth surfaces in some areas. (D). Scale bar = 50µm.
Uronic acid was detected in Rmax and R-24 aggregates and in cells from RF-24 cultures. A comparison of uronic acid concentrations between R-24 and Rmax; and R-24 and RF-24 indicated no significant difference in uronic acid production (Figure 27). Results suggest that differences in aggregation and aggregate stability observed between the three cultures are not due to changes in colanic acid concentration.

![Figure 27. Uronic Acid is Present in RPMI Grown Biofilm.](image)

**Figure 27. Uronic Acid is Present in RPMI Grown Biofilm.** Uronic acid was determined by acid hydrolysis and the addition of 3-phenylphenol colour detection reagent. P-values were calculated using a one-way ANOVA with Bonferroni post hoc tests. Each point represents an independent assay.

### 3.3.4 Poly-β-1,6-linked-N-acetylglucosamine (PNAG)

The relative concentrations of PNAG within the UPEC 536 matrix at Rmax, R-24 and RF-24 were measured using the Dimethyl Methylene Blue (DMMB) assay (section 2.13.5). The absorbance at OD_{650nm} is determined to be proportional to PNAG concentration. The polymer is present in the UPEC 536 matrix (Figure 33) with higher concentrations in Rmax and RF-24 (Figure 28). There is a significant difference (p<0.05) in PNAG concentration between the samples from the three conditions tested. As RF-24 has the greatest level of PNAG it is unlikely that aggregate stability in Rmax and R-24 cultures is conferred by the production of this polymer.
Figure 28. Poly-β-1,6-linked-N-acetylglucosamine Within the Biofilm Matrix. Dimethyl Methylene Blue was used to determine the relative amount of poly-β-1,6-linked-N-acetylglucosamine within the biofilm matrix at Rmax, R-24 and RF-24. PNAG is present in actively growing culture, especially in the presence of iron. P values were calculated using a one-way ANOVA with Bonferroni post hoc tests.

To further investigate the role of PNAG in the UPEC 536 matrix, Dispersin B was used to hydrolyze specific β-1,6-glucosidic linkages. Two concentrations (5 µM and 20 µM) of Dispersin B were added to initial growth medium and the culture monitored for changes in aggregate formation using the Aggregation Index (data not shown). Dispersin B had no effect on the formation of aggregates or on the disruption of preformed aggregates when added to Rmax culture (Figure 29). These results further indicate that PNAG is not a component of the UPEC 536 biofilm matrix involved in formation or stability of the aggregate.

Figure 29. The Effect of Dispersin B on Formed Aggregates. UPEC 536 cultures were grown to Rmax, and treated with Dispersin B at two different concentrations or with iron as a control. Dispersin B was unable to disrupt the aggregate. Changes in dispersion were assayed using the Aggregation Index. Each point on the graph represents one AI result.
3.3.5 The Effect of Combined Dispersants

To determine if a synergic effect using combined dispersants would be able to disrupt R-24 aggregates a series of combinations of dispersants was prepared. Enzymes and/or iron were incubated with R-24 aggregates for 4 hours. If the aggregate scaffold required disruption of two or more polymers, aggregates would disperse and a difference would be detected in the culture and reflected in an Aggregation Index. None of the combinations used was able to disrupt the aggregates indicating that bonding within the matrix was outside of our range of our testing. Table 12 summarises the results of combined dispersants.

Table 12. The Effect of Combined Dispersants on 24 hour Aggregates

<table>
<thead>
<tr>
<th>24 hour Aggregates</th>
<th>Remain Aggregated&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cellulase&lt;sup&gt;a&lt;/sup&gt;</td>
<td>yes</td>
</tr>
<tr>
<td>Amylase&lt;sup&gt;a&lt;/sup&gt;</td>
<td>yes</td>
</tr>
<tr>
<td>DNase I&lt;sup&gt;a&lt;/sup&gt;</td>
<td>yes</td>
</tr>
<tr>
<td>Dispersin B</td>
<td>yes</td>
</tr>
<tr>
<td>FeCl&lt;sub&gt;3&lt;/sub&gt;&lt;sup&gt;a&lt;/sup&gt;</td>
<td>yes</td>
</tr>
<tr>
<td>Amylase + Cellulase</td>
<td>yes</td>
</tr>
<tr>
<td>DNase I + Cellulase</td>
<td>yes</td>
</tr>
<tr>
<td>Dispersin B + Cellulase</td>
<td>yes</td>
</tr>
<tr>
<td>FeCl&lt;sub&gt;3&lt;/sub&gt; + Cellulase</td>
<td>yes</td>
</tr>
<tr>
<td>Cellulase, Amylase, DNase I, Dispersin B, FeCl&lt;sub&gt;3&lt;/sub&gt;</td>
<td>yes</td>
</tr>
<tr>
<td>Cellulase, Amylase, DNase I, Dispersin B</td>
<td>yes</td>
</tr>
<tr>
<td>Amylase, DNase I, Dispersin B, FeCl&lt;sub&gt;3&lt;/sub&gt;</td>
<td>yes</td>
</tr>
</tbody>
</table>

<sup>a</sup>The concentration of each dispersant was 13.8 U ml<sup>-1</sup> cellulase, 1600 U ml<sup>-1</sup> amylase, 90 U ml<sup>-1</sup> DNAse I, 20 µM Dispersin B and 10µM FeCl<sub>3</sub>.  
<sup>b</sup>aggregate dispersal was monitored for a change in broth turbidity, and aggregate dispersion.

3.3.6 Detection of Cellulose and Other Polysaccharides Within the Matrix

Analysis of polysaccharides in a biofilm presents challenges. The paramount problem is obtaining sufficient exopolysaccharide (EPS) which is truly biofilm derived for study (reviewed in Flemming and Wingender 2010). Some bacteria are capable of producing
several different polysaccharides under differing growth conditions. The effect of this may be that bacteria within a biofilm will produce varying proportions of polysaccharide due to stress related responses over time (Sutherland 2001).

The aims of this section were, 1) confirm cellulose as the major polymeric substance and; 2) To identify other monomers that could represent other known polymers that may be produced at different time periods.

Samples for exopolysaccharide analysis were recovered at Rmax, R-24 hours and RF-24 hours. These samples were used for the semi-quantitative analysis of cellulose by H2SO4 hydrolysis and investigation of other polymers that may be complexing with the cellulose was performed using Trifluoracetic Acid hydrolysis (section 2.13.3). Samples undergo acid hydrolysis, reduction and acetylation and were then analysed by gas chromatography (Figure 30). As the aggregates did not adhere to polypropylene tube walls we assumed the entire EPS portion to be contained within the sample. Culture was grown in either RPMI 1640 or RPMI 1640 supplemented with 10 µM FeCl3 and the cells pelleted by centrifugation and processed (section 2.13.1). On average, the yield of EPS from 1 litre of culture was 0.1 g of dehydrated sample.

![Chemical Processes for the Determination of Cellulose Monomers](image)

**Figure 30. Overview of the Chemical Processes for the Determination of Cellulose Monomers.** A sample of dehydrated bacterial culture was hydrolyzed to its monosaccharide using a strong acid (concentrated sulphuric acid or trifluoroacetic acid). The monosaccharide was then reduced to alditol acetate which then undergoes acetylation of its hydroxyl group. The resultant alditol acetates are volatile and can be identified and quantified by gas chromatography. (Adapted from Melton and Smith 2001)

Myo-inositol was chosen as an internal standard for cellulose analysis for its close retention time to glucose. The RPMI growth media which contains 35 mg l⁻¹ myo-inositol was checked for its additive effect on the concentration of the internal standard and was found to contribute less than 1±g mg⁻¹ of sugar. The 13-Sugar Standard verifies the retention time and
location of the myo-inositol internal standard (Figure 31, red circle) and the adjacent glucitol hexaacetate (glucose monomer).

Gas Chromatography results have been reported as relative amounts of the total sugar to provide an indication of the proportion of monosaccharide within a total sample. An absolute value would not be representative of the variation within sample batches nor take into account the biological variations under which the cultures were grown.

Our initial findings that cellulose is the major EPS polymer by enzymatic degradation (section 3.1.) are supported by sulphuric acid hydrolysis that indicate that cellulose composed approximately 60% of the Rmax aggregate (Table 13). H₂SO₄ hydrolysis is required for the breakdown of cellulose fibres, but may also degrade other polysaccharides within the matrix. To detect polysaccharides besides cellulose, hydrolysis was carried out using trifluoroacetic acid and gas chromatography and was performed on portions of the EPS preparations used for sulphuric acid hydrolysis. Results indicate that EPS from RPMI grown cultures contain more total sugar than EPS from RPMI + iron cultures. All samples showed the presence of ribose, mannose, galactose and glucose in varying proportions. Each sample was set up in duplicate and sampling at specific time points was carried out at least twice.
Table 13. Cellulose and Other Sugar Monosaccharides of the UPEC 536 Matrix.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Method</th>
<th>Total Sugar (μg/mg)</th>
<th>Neutral monosaccharide (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>ribose</td>
<td>mannose</td>
</tr>
<tr>
<td>Rmax</td>
<td>TFA</td>
<td>64.6 ± 41.7*</td>
<td>34.7 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>Sulphur</td>
<td>28.9 ± 3.1</td>
<td>-</td>
</tr>
<tr>
<td>R-24</td>
<td>TFA</td>
<td>26.0 ± 7.2</td>
<td>38.3 ± 2.5</td>
</tr>
<tr>
<td></td>
<td>Sulphur</td>
<td>30.0 ± 0.1</td>
<td>-</td>
</tr>
<tr>
<td>RF-24</td>
<td>TFA</td>
<td>3.8 ± 1.5</td>
<td>16.2 ± 3.3</td>
</tr>
<tr>
<td></td>
<td>Sulphur</td>
<td>0.8 ± 0.1</td>
<td>-</td>
</tr>
</tbody>
</table>

TFA hydrolysis was for the relative abundance of polysaccharide, not including cellulose. H$_2$SO$_4$ hydrolysis was used to measure cellulose only.

*Indicates 3 separate batches (batch variation)

The RF-24 sample indicates the least amount of total sugar, suggesting greater metabolism and utilization of carbon sources. The RPMI grown aggregates were composed of approximately 60% (Rmax) and 45% (RPMI-24) cellulose and the dispersed RF-24 sample approximately 25%. The RF-24 sample had the greatest proportion of galactose per total sugar and all three samples contained ribose, mannose, glucose and galactose in varying proportions. These results indicate that the polymers produced by the bacteria are consistent and UPEC 536 does not produce differing polymers over time.

3.4. Discussion

UPEC 536 forms aggregates when grown in RPMI. The aggregates disperse when iron is provided. The objectives of this section were 1) to identify the major exopolymeric substances of the biofilm matrix and 2) the extent of inducers of dispersion. This was achieved through the identification of the components of the matrix using a combination of enzymatic, biochemical, and microscopic analysis, and where possible, quantified using the Aggregation Index. Iron is an inducer of dispersion. Other inducers of aggregate dispersion were investigated; specifically bio-relevant metals and physiological iron sources as well as bacterially recognised signals that may be produced by other bacteria or the host and recognized by UPEC 536. In addition, an investigation was carried out into other polymers that may contribute to the production and the stability of the UPEC 536 biofilm matrix over time.
UPEC 536 grown in RPMI 1640 tissue culture media forms large flocs that resemble biofilms. The urinary tract is a complex environment with constant changes in osmolarity, pH, nutrients and substrate availability and where the conditions are not defined or simple, and change considerably during infection due to inflammation, tissue breakdown and dissemination of the pathogen (Smith 1998). These conditions differ greatly from laboratory growth conditions where bacteria are grown in rich nutrient media with no requirement for virulence factors to withstand and overcome host defence mechanisms. Our choice to use a tissue culture medium, RPMI 1640 as a growth media, was to introduce the bacteria into an iron-restricted environment that would mimic the host environment, where iron is tightly sequestered by binding proteins with limited availability to bacteria. Growth in tissue culture media allowed us to observe the bacteria in “survival” mode and investigate the development of its survival strategies, mainly through biofilm development. Further, UPEC 536 biofilm grown in polypropylene tubes produce self aggregating flocs with no adherence to the tube surface therefore eliminating surface cell considerations. In addition the UPEC 536 model provides a unique system in which to investigate dispersion of biofilms, an area of biofilm development that is not easily studied nor well understood.

The challenges in studying dispersion of aggregates lies in separating aggregates from planktonic cells, separating cells from an aggregate and enumeration of cells within an aggregate. Further difficulties arise from the fact that aggregates are not uniform in size, that not all cells within an aggregate may be viable and that an aggregate contains a large number of cells. Numerous methods have been used to quantify bacterial cells within an aggregate such as manual microscopic counts, live / dead staining with fluorescent dyes and micrographs coupled to computer software to determine frequency and size of aggregates, flow cytometry coupled to dyes (Monier and Lindow 2005) or mathematical modelling (Bos et al. 1999). Optical density is one of the commonest ways to determine the number of cells involved in aggregation with a culture. This method calculates a relative ratio using optical density of planktonic cells and optical density of the total culture (Malik et al. 2003) and the resultant ratio is assumed to be cells involved in aggregation. More precise quantification of cells can relate the total optical density to the number of colony forming units per ml.

Bacterial growth preference is to live in close association with surfaces or reside at air-water interfaces as multi-cellular aggregates or biofilms (Watnick 2000). It is generally accepted that biofilm architecture may take on a number of different forms depending on where the bacteria take up residence. Surface attached biofilms often form characteristic pillar and
mushroom-like structures separated by water-filled channels (Sutherland 2001) whereas floating aggregates or pellicles in static culture develop at liquid-air interfaces as mats (Friedman and Kolter 2004; Branda et al. 2005). Floc-forming bacteria grow primarily as clumps of large numbers of cells which settle out of suspension on cessation of agitation and the growth medium remains clear (Friedman et al. 1969) such as those observed by UPEC 536 grown in RPMI (Figure 11). Aggregation has been noted in *E.coli* K12 strains expressing Type 1 pili which can be inhibited by the addition of 2% α-mannose to culture media resulting in the abolishment of biofilm formation (Pratt and Kolter 1998). UPEC 536 RPMI culture was supplemented with 2% mannose and observed for aggregation. Aggregation was not inhibited by the addition of mannose and cells grew in a manner similar to those grown without mannose supplementation (data not shown), suggesting Type 1 pili are not involved in aggregate interactions.

UPEC 536 produces persistent aggregates when grown in RPMI 1640 and transient aggregates when grown in RPMI 1640 supplemented with iron. Iron is an essential nutrient for growth in *vivo* and in *vitro* (Carpenter et al. 2009). Identification of bacterial behaviour in a host is difficult to mimic in *vitro*, but by providing conditions that are similar to that found in a host, some correlations may be found. RPMI, a buffered, iron limited defined tissue culture media was used to mimic the environment that an uropathogen may inhabit. The formation of urine produces a changing environment that is dependent upon diet and the proper functioning of body organs, yet ultimately urine is an iron-limited buffered solution. The relevance of identified nutrients or macromolecules added to a defined medium is useful in identifying how bacteria respond to a change in their environment. Specifically, how do bacteria respond in the absence of an essential nutrient such as iron?

Iron induced signalling, regulated by Fur is a possible explanation for the observations seen in this study. An example of the complexity of iron induced intracellular signalling has been demonstrated by Garcia-Herrero and Vogel (2005) using NMR analysis and crystallography. The binding of iron-bound siderophore to the outer membrane receptor initiates a signalling cascade, forming interactions between the receptor’s TonB box, the N-domain of the outer membrane receptor to an inner membrane regulator which interacts with a cytoplasmic sigma factor. The result is transport of iron into the cytoplasm and the induction of iron transport genes (*fecABCDE*) (Garcia-Herrero and Vogel 2005).
To ascertain the effects iron has on a uropathogen, UPEC 536 was grown in a defined medium with and without iron to determine the response of the bacteria to the nutrient. The absence of iron induces bacteria to produce a robust cellulose-based matrix that can persist for greater than 24 hours (Figure 11). Transient aggregates are also produced in the presence of iron, although after approximately four hours of growth they begin to disperse (Figure 11 & 12). Formed aggregates can be induced to disperse upon supplementation with iron, manganese or zinc in a concentration dependent manner (Figure 20 & Table 8). Manganese and zinc are found in the host in trace concentrations in both serum and faeces and play an important role in human physiology (Rink and Hasse 2007). Urine concentrations of iron, manganese and zinc are approximately, 100-300 µg / day, 0 î 0.2 µg l\(^{-1}\) and 15-120 µg dl\(^{-1}\) respectively, (ARUP, National Reference Laboratory, Utah, USA), (http://www.aruplab.com/guides/ug/tests). Host iron sources are sequestered by a variety of high-affinity iron-binding proteins, limiting availability for iron use. Pathogenic bacteria have developed high affinity iron-acquisition mechanisms to extract and utilize iron and alternative nutrients from the host. Host bio-relevant iron sources, were also able to induce dispersal of UPEC 536 aggregates (Figure 21) suggesting that an iron-rich environment induces bacterial virulence strategies that encourage growth and colonization (infection). The ability to induce dispersal with iron, manganese and zinc suggests that in an iron-restricted environment, there may be metal nutrient redundancy.

The concept of stress related-illness is generally accepted to be a consequence of the depression of the host immune system but the possibility that hormones released by the host may directly influence bacterial growth and the production of virulence factors associated with pathogenesis is also worthy of consideration. Studies conducted by Lyte (Lyte and Ernst 1992; Lyte and Ernst 1993; Lyte et al. 1996; Lyte et al. 1997) demonstrated that exposure of Gram-negative bacteria to noradrenaline resulted in increased growth (up to five log orders) in a medium enriched with iron from serum, and increased expression of virulence-associated factors such as adhesins and toxins. Neither adrenaline nor noradrnaline showed an effect on aggregation or dispersion in UPEC 536 grown in RPMI 1640 (Table 10).

In addition, bacterial intercellular communication or quorum sensing permits bacteria within a population to behave with coordinated activity in a multi-cellular organism fashion (Bassler and Losick 2006). The ability for bacteria to act collectively provides a survival advantage such as migration to more favourable environments, adaption to different modes of growth such as sporulation or biofilm formation, providing protection from harsh environments.
Quorum sensing is the regulation of gene expression in response to fluctuations in bacterial cell population density (Miller and Bassler 2001) where bacteria produce and release chemical signal molecules (autoinducers) that increase in concentration as a function of cell density. When threshold concentrations of autoinducer are reached, quorum sensing cells respond and modulate their behaviour in concert with other cells within the population.

The effects of quorum sensing are highly variable and dependent on the bacterial species and the experimental conditions (Parsek and Greenberg 2005). *E.coli* does not produce acylhomoserine lactones but is able to detect and respond to AHLs produced by other species (Michael *et al.* 2001). Specifically, a shared signal from both Gram positive and negative bacteria involves autoinducer-2 (AI-2), a metabolic by-product of metabolism, detected via LuxS (Surette *et al.* 1999; Xavier and Bassler 2005). LuxS is conserved in many bacterial species and found in spent supernatants, suggesting a role for AI-2 signaling for interspecies communication (Schauder *et al.* 2001). UPEC 536 grown in RPMI supplemented with iron produces transient aggregates that begin to disperse at approximately 240 minutes incubation. This observation reflects quorum sensing behaviour. However, neither of the AHLs tested in this section induced a change in aggregation or dispersion when UPEC 536 was grown in RPMI 1640 or iron supplemented RPMI. This does not infer that quorum sensing is not involved in aggregation or dispersion, as other AHLs and autoinducers were not tested (Table 10).

Enteropathogenic *Escherichia coli* (EPEC) carries a plasmid encoded Type IV bundle forming pilus (Bfp), involved in cell-to-cell interactions and microcolony formation (Giron *et al.* 1991). The bundle forming pilus gene cluster forms proteins involved in piliation, local adherence and twitching motility as well as a role in dispersion from autoaggregation (Bieber *et al.* 1998). The bundle forming pili undergo a transition form thick to thin pili bundles releasing aggregated cells to the medium (Knutton *et al.* 1999) upon adherence to epithelial cells (Cleary *et al.* 2004). Outer membrane proteins such as Antigen 43 found on many of the Enterobacteriaceae confer clumping of cells and promote bacterial autoaggregation, microcolony and biofilm formation (Schembri *et al.* 2003, Danese *et al.* 2000). Other strains of *E.coli*, specifically, *E.coli* O157:H7 exhibit strong binding to alfalfa sprouts growing in water through interactions of bacterial lipopolysaccharide, capsular polysaccharide and exopolysaccharide, specifically cellulose, colanic acid and poly-β-1,6-N-acetylglucosamine (Matthysse *et al.* 2008).
CSEM of the UPEC 536 matrix was useful in distinguishing the polymeric substance from proteinaceous material. The images clearly show bacterial cells embedded within the matrix and cells forming intracellular connections (Figure 13, A-D). The polymer forming the matrix is cellulose (Figure 14 and 15). An important feature of bacterial cellulose is its chemical purity. Initial production of cellulose nascent chains, begin to aggregate and form subfibrils (Kudlicka 1989). The subfibrils are crystallized into microfibrils (Jonas and Farah 1998) and these form into bundles and then into ribbons (Yamanaka et al. 2000). Cellulose is a polymer of un-branched linked β-D-glucose monomers. The linkage occurs when water is removed by combining the ßOH group from one monomer and the hydrogen from another. The polymer is difficult to degrade mechanically and is largely dependent on specific endoglucanases or strong acids to reduce the polymer to its neutral sugar (Bielecki et al. 2010). UPEC 536 aggregation begins with the production of small strands or ribbons in the culture that become entangled and form large flocs (Figure 11). Aggregates older than 7 hours and especially 24 hour aggregates are very difficult to disperse (Table 12) suggesting a structural change in the cellulose polymer. The change in structure is supported by the finding that Rmax and R-24 aggregates have similar monosaccharide composition (no ðnewð polymers).

Our observations showed that the RPMI aggregate becomes less soluble over time (Figure 12). Biochemical analysis of the matrix indicates that colanic acid and poly-N-acetylglucosamine are also present in the matrix (Figure 27 & 28). PNAG is more abundant in RF-24 than R-24 culture suggesting the polymer is not involved in structural stability of the aggregate. Uronic acid is detectable in Rmax, R-24 and Rf-24 aggregates but no statistical significance exists between concentrations of each sample suggesting differences in aggregation and aggregate stability between the three cultures is not due to the colanic acid changes. Together these results may suggest that PNAG is expressed during active growth but not as a structural polymer, and uronic acid is produced in exponential phase of growth. Unfortunately, specific hydrolysis of uronic acid was not achieved as the fucoside hydrolase (Verhoef et al. 2005) enzyme was not available.

Other polymers that may be contributing to the biofilm matrix or providing temporal stability to the matrix were analysed using acid hydrolysis and gas chromatography on EPS samples at two time periods (Rmax and R-24) and two conditions (R and RF). The analysis provided a semi-quantitative value to define the amount of cellulose by H2SO4 hydrolysis, produced at
teach time period (Table 13) and other polymers that may be present by TFA. The chromatograph indicated the presence of ribose, mannose, galactose and glucose as monomers of other polysaccharides (or even those analysed in this study), other than cellulose, but further investigation into their role was not undertaken.

The development and stability of biofilms has been shown to be enhanced by extracellular DNA (Whitchurch et al. 2002, Alleson-Holm et al. 2006). Investigation of exDNA indicated that it was produced and secreted into the culture medium but did not contribute to the biofilm scaffold (Figure 22 & 23). During exponential growth, DNase activity was detected in the culture supernatant (Figure 24 & 25) but played no major role in inhibiting aggregate formation or dispersion of the aggregate confirming exDNA as a component of the UPEC 536 biofilm matrix but not key to its structure or stability.

Overall, the Aggregation Index has been an effective and reproducible tool for the investigation of aggregate formation and aggregate dispersion for a variety of aspects tested in this section. The UPEC 536 matrix is composed mainly of cellulose but exDNA, colanic acid and poly-β-1,6-linked-N-acetylglucosamine were detected at different time periods under iron-deficient and iron-replete conditions. Biochemical and enzymatic analysis of the matrix have directed the study to a genetic investigation into the production and degradation of cellulose from UPEC 536 that may also help to gain insight into possibilities for regulatory mechanisms controlling these processes.
Chapter 4. Construction of Mutant Strains and Complementation Vectors

4.1. Introduction

The phenotypic differences in aggregation and dispersion of UPEC 536 that were described in Chapter 3 have directed this study to investigate the differences at a genetic level. Genetic mutations were created in candidate genes and construction of vectors for complementation and over expression studies were carried out.

4.2. Candidate Genes

The ability to produce cellulose is a common trait amongst *Escherichia coli* strains (Da Re and Ghigo 2006) and is the major component of the UPEC 536 biofilm (section 3.1.3). To confirm that cellulose is the primary polysaccharide of the UPEC 536, gene insertions and deletions were constructed within the cellulose operon of UPEC 536. Two genes of the bacterial cellulose synthase operon (*bcs*) were targeted; the cellulose synthase catalytic subunit *bcsA* and *bcsZ* a 1,4-β-endoglucanase.

Iron is a key element in the physiological response of bacteria to their environment. Generally the abundance of iron is sensed as a "favourable" environment where iron binds to the Fur protein and represses the transcription of genes associated with iron uptake systems and virulence factors. The presence of iron also induces the activation of genes involved in defence against oxidative molecules (Wosten *et al.* 2000). Whereas, an iron restricted environment de-represses the iron regulator and bacteria express a number of iron uptake mechanisms and associated virulence factors. Regulation of iron acquisition is controlled through the ferric uptake regulator (Fur). To determine if the iron-mediated aggregation and dispersion phenotype was regulated by Fur, the *fur* gene was deleted.

Cyclic-di-GMP nucleotides are second messenger signalling molecules and associated with biofilm characteristics such as cellulose production, curli expression and aggregation (Romling 2005). The regulatory gene *yaiC* of UPEC 536 carries a GGDEF motif within its protein structure and is annotated as a putative guanylate cyclase involved in the production of c-di-GMP (Hochhut *et al.* 2006). Increased concentrations of c-di-GMP induce bacterial
characteristics involved in biofilm formation, including the production of exopolysaccharides (Romling 2005). The \textit{yaiC} gene was mutated to determine its effect on cellulose production and the formation of the aggregates displayed by UPEC 536 in RPMI media.

There is growing evidence that the transition from sessility to motility is reliant on the intracellular balance of c-di-GMP and the c-di-GMP degrading phosphodiesterase (Janel 2004; Bobrov \textit{et al.} 2005; Romling 2005). Phosphodiesterases carry an EAL or HD-HYL motif domain and are associated with the breakdown of c-di-GMP, thus biofilm dispersion. Immediately downstream the cellulose biosynthesis operon is a gene annotated on the BLAST database (ID 4191265), \((yhjK)\) a putative phosphodiesterase. Gene deletion of \(yhjK\) was performed to determine its influence on dispersion of UPEC 536 aggregates. Candidate genes for insertion mutagenesis and deletion are summarized in Table 13.

Table 14. Candidate Genes for Mutation Analysis.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Position in Genome 5’ – 3’ ORF (bp)</th>
<th>Insertion fragment size(bp)</th>
<th>Size of protein (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>fur</td>
<td>745310-745856</td>
<td>447</td>
<td>374</td>
</tr>
<tr>
<td>bcsA</td>
<td>3806997-3809615</td>
<td>2618</td>
<td>335</td>
</tr>
<tr>
<td>bcsZ</td>
<td>3803534-3804640</td>
<td>1106</td>
<td>397</td>
</tr>
<tr>
<td>yaiC</td>
<td>471173-472289</td>
<td>1116</td>
<td>406</td>
</tr>
<tr>
<td>yhjK</td>
<td>3798009-3799964</td>
<td>1955</td>
<td>485</td>
</tr>
</tbody>
</table>

4.3. Insertional Mutagenesis of Candidate Genes

4.3.1 Strategy for Single Cross-Over Insertion Mutants

Insertion mutants were constructed as described previously by Milton \textit{et al.} 1996. For an internal gene fragment DNA was amplified by PCR. This fragment was cloned into the suicide vector pNQ705 introduced into UPEC 536 by conjugation. Transconjugants were selected on M9 minimal media agar containing chloramphenicol. Correct insertions of the transconjugants were verified by PCR and sequencing.
4.3.2 Overview of Conjugation Cloning Method

Insertion fragments were produced by PCR, and restricted with SacI and either XhoI or XbaI and ligated into vector pNQ705. The construct was electroporated into *E. coli* strain CC118 ∅ *pir*. Selection of clones containing the plasmid was on LB<sub>cm</sub> agar plates and verified by PCR using *cat*R and the Forward or Reverse primer for the specific gene. Purified plasmid DNA was transformed into *E.coli* strain S17-1 ∅ *pir* and mated with UPEC 536 (Figure 34). Selection of transconjugants was carried out on M9 Minimal Media plates supplemented with chloramphenicol. Minimal media was used for selection as UPEC 536 will grow on minimal media and *E.coli* S17-1 ∅ *pir* will not. Verification was performed by using PCR amplification of the integration site using the forward chloramphenicol primer and one internal and one external to the cross-over site Figure 33, B). A PCR product of the correct size will be produced if a single crossover has occurred.

![Figure 32. Overview of Conjugation.](image)

Constructs of each candidate gene were confirmed and used for conjugation. The selected construct was electroporated into *E.coli* strain S17-1 ∅ *pir* and conjugated with UPEC 536. Selection of transconjugants was carried out on M9 Minimal Media plates supplemented with chloramphenicol.

4.3.3 Overview of Single Cross-Over Strategy

Conjugation of UPEC 536 and *E.coli* S17-1 ∅ *pir* (pNQ705(insert)) transfers pNQ705 to UPEC 536. Homologous recombination between the target gene on the genome and the fragment will insert the plasmid into the UPEC 536 chromosome creating two truncated
forms of the target gene (Figure 33, A). Verification was performed by PCR amplification across the insertion site and sequencing.

Figure 33. Overview of Single Cross-Over Insertional Mutation. A simplified overview, where (A) indicates the basic steps of PCR amplification of the gene area of interest, insertion of the PCR product into the vector, and the integration of the plasmid into the bacterial chromosome (adapted from Clow 2002). Integration of the plasmid into the chromosome was verified by PCR using the plasmid Cm primer and the external reverse primer of the gene of interest (B).

4.3.4 Preparation of Cloning Vector pNQ705

Insertional mutagenesis was performed using pNQ705 which was reconstructed from pDM4 (Milton et al. 1996) by deleting a PstI fragment containing the sacB (Figure 36). The pNQ705 vector carries an origin of replication (oriR6K) which is derived from the naturally occurring plasmid R6K and requires the R6K pir gene for replication which is absent from this vector. The laboratory *E. coli* strains CC118 *pir* and S17-1 *pir* used to supply the Pir
protein for replication. The transfer origin that directs conjugal transfer of the vector from one bacterial strain to another is mobRP4, from the naturally occurring plasmid RP4. This allows the conjugal transfer of pNQ705 to recipient bacterial strains. The vector carries the cat gene which encodes for chloramphenicol acetyltransferase and confers resistance to the antibacterial chloramphenicol allowing for selection of transconjugants. The vector also carries a multiple cloning site (MCS) into which PCR produced inserts were cloned for introduction into a recipient bacterial cell (Turner et al. 2005).

Figure 34. Reconstruction of pNQ705. pDM4 was Digested with PstI for removal of the sacB gene (red X) to reconstruct pNQ705.

4.3.5 Verification of pNQ705 with Insert

A UPEC 536 genomic template was used to PCR an internal fragment of each gene of interest (Table 14). The PCR product was restricted with XhoI and SacI or XbaI and SacI, purified, ligated into pNQ705 and dialyzed before being electroporated into E.coli CC118 pir. Verification of the plasmid insert was performed by EcoRI and BamHI restriction digest of extracted pNQ705(insert) plasmid (Figure 35, A & B). All gene insertion fragments (bcsA, bcsZ, yaiC, fur, yhjK) are approximately 500 bp in size. Plasmid digests revealed an increase in size from 0.8 to 1.1 kb for EcoRI and 3.0 to 3.5 kb for BamHIII digests.
Figure 37. Verification of insertion of PCR fragments in pNQ705 by EcoRI Restriction Digest. Plasmids were extracted from presumptive clones and digested with EcoRI (A) and BamHI (B) and a portion of the restriction digest was electrophoresed and visualized on a 0.8% agarose gel. Clones carrying the gene insert show an increase of approximately 500 bp within the multi-cloning site segment. (A) Lane 1, uncut pNQ705, Lane 2, EcoRI digested pNQ705, Lane 3, a bcsA clone (0.9 kb, 1.1 kb and 2.5 kb band), Lane 4, a clone lacking an insert (0.8 kb and 0.9 kb band), Lane 5, 100 bp ladder (Invitrogen), Lane 6, HindIII ladder. (B) Lane 1, uncut pNQ705, Lane 2, BamHI digested pNQ705, Lane 3, a bcsA clone (1.5 kb and 3.0 kb band), Lane 4, a clone lacking an insert (1.5 kb and 3.0 kb band), Lane 5, 100 bp ladder (Invitrogen), Lane 6, HindIII ladder.

4.3.6 Verification of Single Cross Over Mutation

Colony PCR was first performed on potential clones using the chloramphenicol reverse primer and the external reverse primer for the selected target gene. Strains that produced an expected size band by colony PCR were grown overnight and genomic DNA extraction performed. The PCR was repeated on the genomic DNA and bands that produced the expected band size (Figure 36, A & B) were verified by sequencing.
Figure 36. Verification of Single Cross Over Mutations. Colony PCR was first performed on candidate clones. Strains that produced the expected size band on an agarose gel by colony PCR were PCR again from genomic DNA and verified by sequencing. Expected band sizes were Lane 1 bcsZ 1 kb, Lane 2 blank, Lane 3 yhjK 1.8 kb, Lane 4 1kb ladder, Lane 5 yaiC, 1.2 kb, Lane 6 bcsA 2.0 kb and Lane 7 1 kb ladder (Invitrogen).

The deletions mutants were screened for phenotypic changes by growth in RPMI and RPMI supplemented with 10 µM FeCl₃. As insertional mutation strategies may produce polar and effect the expression of downstream genes (Datsenko and Wanner 2000), mutants that displayed changes in aggregation and dispersion from the parental strain were selected for double cross over deletion.

4.4. Deletion Mutants

This aspect of cloning was undertaken using the homologous recombination system of bacteriophage λ. The Red· system consists of three proteins that induce a bacterium into a hyper-rec· state permitting genetic exchange during its latter phase of growth. The genes encoding these proteins are exo (reda), β (redβ) and γ. The exo gene product is a exonuclease that progressively degrades the 5’-ended strand of dsDNA, generating 3’ overhangs (Poteete 2001) and the β protein promotes renaturation of complementary DNA strands and mediating strand annealing and exchange reactions in vitro (Li et al. 1998). The β protein forms complexes with exonuclease and modulates both nucleolytic and recombination-promoting activities. The γ protein forms a dimer which binds to the host RecBCD protein and inhibits all of its known activities (Murphy 1991).

Red-mediated recombination in the bacterium proceeds via a number of different pathways. A number of methods have been developed that differ in the mechanism by which DNA with homologous regions to the chromosome is delivered to the cell (Lee et al. 2009, Datsenko and Wanner, 2000). The method used in this study, produced a linear fragment of DNA with extended homologous regions (1 kb) to promote recombination. Briefly, transformants
carrying a \(\lambda\)Red helper plasmid (pACBSCE) or (pKD46) were grown in 5 ml SOB media with L-arabinose and the appropriate antibiotic at 30°C to an OD\(_{600nm}\) of \(~0.6\) and then made electrocompetent as described in section 2.5.10. DNA for gene deletions was prepared with 1 kb flanking gene regions over a Flippase Recognition Target (FRT)-flanked kanamycin resistance gene using A and C deletion primers and genomic DNA from a corresponding BW25113 knock out strain (Baba et al. 2006) from the Keio collection as a template. PCR products were purified and dialysed against water and the linear DNA was electroporated into UPEC 356 using the the GenePulser Xcell. If efficiency was low or no colonies grew within 24 hours, the remainder of the recovered culture was spread to LB\(_{km}\) plates after standing overnight at RT. After primary selection, mutants were subcultured to a fresh LB\(_{km}\) plate and colonies displaying strong growth were verified by PCR amplification using K1 and A primers or K2 and A primers (Table 7d). Verified mutants were colony purified once, non-selectively at 37°C and then tested for chloramphenicol sensitivity to test for loss of the helper plasmid (pACBSCE only). If the helper plasmid was not lost, then a verified mutant colony was inoculated to LB broth, incubated at 37°C with shaking for 2, 4, and 24 hours and plated for colony isolation. From the isolation plate colonies were restreaked onto LB\(_{km}\) and LB\(_{cm}\) grid plates to verify loss of the helper plasmid. Clones that had lost the helper plasmid were stored in 25% glycerol at minus 80°C.

### 4.4.1 Gene Deletion Strategy for UPEC 536

A helper plasmid pACBSCE or pKD46 (Figure 37, A) carrying \(\lambda\)Red recombinases, chloramphenicol resistance and a temperature resistant origin was transformed into UPEC 536 and transformants selected on LB\(_{cm}\) plates. Electrocompetent cells were prepared of the UPEC 536 strain expressing \(\lambda\)recombinases (Figure 37, B) and linear DNA with 1 kb extended homologous regions to the gene of interest and a kanamycin resistance cassette were electroporated into UPEC 536 / \(\lambda\)recombinase expressing cells (Figure 37, C) and transformants selected on LB\(_{km}\) plates (Figure 37, D). (Lee et al. 2009). Verification of gene deletions was performed using A and K1 primers. The deletion method used in this study created mutants that are initially non-polar as downstream genes can be expressed from the kanamycin resistance gene promoter (Baba et al. 2006).
Figure 37. PCR Gene Replacement Strategy in UPEC 536. A helper plasmid (A) carrying λ-Red recombinases (pACGSCE or pKD46) CmR, and an arabinose inducible promoter and a temperature sensitive origin was transformed into UPEC 536 and transformants selected on LB<sub>cm</sub> plates. (B) Electrocompetent cells expressing λ-red proteins were prepared and transformed with linear DNA. Transformants were selected on LB<sub>km</sub> plates (Lee et al. 2009).

4.4.2 Verification of Deletion Mutants

Colony PCR was performed on candidate clones selected from LB<sub>km</sub> plates using the upstream A and kanamycin K1 primers (Figure 38, A). DNA was visualized on an agarose gel and clones with an expected band size of 1.5 kb (Figure 38, B & C) were selected as deletion mutants. Gene deletions and replacement with the Kanamycin /FRT cassette in UPEC 536<sub>Δfur</sub>, 536<sub>ΔyhjK</sub>, and 536<sub>ΔcsA</sub> and 536<sub>ΔcsZ</sub> produce the same band size as the equivalent BW25113 mutant (Figure 38, B & C).
Figure 38. Verification of Deletion Mutants by PCR using A and K1 Primers. Strains with gene deletions were verified using the A forward primer for the gene of interest and the K1 reverse kanamycin primer (A) to verify deletion of the target gene and replacement with the Kanamycin cassette. PCR products were visualized on a 0.8% agarose gel (B & C) and clones with the appropriate size band were selected as deletion mutants. Lane 1, UPEC ∆fur, Lane 2, UPEC ∆yhjK, Lane 3, UPEC ∆bcsA, Lane 4, BW25113 mutant template, Lane 5, 1 kb ladder, Lane 6, UPEC ∆bcsZ, Lane 7, BW25113 mutant template, Lane 8, 1 kb ladder.

4.4.3 Complementation Vector pTrc99A

The pTrc99A plasmid vector (Figure 39.) is derived from the pKK233-2 and pKK240-11 vectors (Amann and Brosius 1985). These vectors were constructed for regulated expression of genes in E. coli. The advantages of the pTrc series of vectors are that they have a small plasmid size, a high plasmid copy number, a lacI allele for auto-repression, and a pUC18 polylinker region. The plasmid also carries a NcoI site 8 bp downstream from the lacZ ribosomal binding site (RBS) which provides an ATG codon that can be exposed for direct fusion by digestion with NcoI (Amann et al. 1988).
Each of the candidate genes was amplified by PCR, verified on an agarose gel for the appropriate gene size (Figure 40) and restricted with NcoI and HindIII, except for bcsA which had an EcoRI and HindIII restriction site due to a NcoI site within the gene. Each PCR fragment was then ligated into pTrc99A. The ligated plasmid was dialyzed and electroporated into E.coli T10 and transformants selected on LB_Amp plates. Candidate clones were subcultured into LB_Amp grown overnight and a plasmid extraction performed. The extracted plasmid was digested with NcoI and HindIII or NcoI and EcoRI (bcsA) and a portion of the digest mixture was visualized on an 0.8% agarose gel to verify the size of the gene insert (Figure 41).

![Image](image.png)

Figure 39. The pTrc 99A Plasmid Expression Vector. (Taken from Amann et al. 1988)

Figure 40. Verification of Gene PCR Inserts. Each gene was amplified by PCR and visualized on a 0.8% agarose gel for the expected molecular size prior to restriction and ligation into pTrc99A. The expected size of each gene PCR product was as shown on the gel. Lane 1, yhjKalt 2.0 kb, Lane 2, 1 kb ladder, Lane 3, yaiC 1.1 kb, lane 4, bcsZ 1.1 kb, Lane 5, bcsA 2.6 kb, Lane 6, fur 0.5 kb, Lane 7, yhjK 2.0 kb and Lane 8, 1 kb ladder.
Figure 41. Verification of the Gene Insert into pTrc99A. Verification of gene inserts in the pTrc99A complementation vector was performed by restriction digest with HindIII and NcoI and EcoRI and Hind III (bcsA). Plasmid preparations were performed and the plasmid digested in the appropriate restriction enzymes. Digests were visualized on a 0.8% agarose gel for the appropriate band size correlating to the gene.

4.4.4 The Start Site of YhjK

The yhjK gene is approximately 2.0 kb in length and annotations of the genome sequence for the gene indicates two possible start sites (Figure 42, A). When this project began the gene start site was denoted as ttg which resides 30 base pairs upstream of an atg start site that is now specified. For the construction of complementation vectors, we prepared two forward primers; one using the ttg start site designated as yhjKF, and the other with the atg start site designated as yhjKaltF (alternative) to determine if a difference in complementation would be apparent. DNA for this region was amplified by PCR and both produced a product of the same size Figure 42, B). The ttg start site was converted to an atg site in the cloning process by using an NcoI restriction site (Figure 42, C). All gene inserts were amplified by PCR and verified by sequencing. Expression vectors, pTrc99A(bcsA), pTrc99A(bcsZ), pTrc99A(fur), pTrc99A(yhjK), pTrc99A(yhjK)alt were transformed into E.coli T10 and grown in LB_Amp overnight and subcultured to fresh LB_Amp supplemented with 100 µM IPTG and grown for 4 hours. Bacterial cells were harvested and the protein pellet prepared and examined by SDS PAGE electrophoresis and stained with Coomassie Blue. Only the pTrc(yhjK) vector expresses visible 73 kDa protein product expected for the phosphodiesterase, YhjK (Figure 43).
Figure 42. The \( p_{Trc}yjhK \) Complementation Vectors. The \( yjhK \) gene has two potential start sites, a ttg and an atg are underlined and highlighted in bold. A potential ribosome binding site sequence \( ggag \) upstream of the potential \( ttg \) start site is also underlined and in bold (A). Two gene products were amplified by PCR, one using a forward primer incorporating an \( atg \) start site where the potential \( ttg \) site is, and the other using the \( atg \) site now designated in GenBank. Both sets of primers produced a DNA product that was inserted into the \( p_{Trc}99A \) expression vector as \( NcoI/HindIII \) clonings (B). In the cloning process the \( ttg \) site was converted to an \( atg \) site by the creation of an \( NcoI \) restriction site. The recognition sequence of \( NcoI \) (CCATGG) is underlined. The sequences (C) of potential YhjK proteins is given for TTG start on i) the genome, ii) cloned into \( p_{Trc}99A \) and ATG start site on iii) the genome and iv) cloned into \( p_{Trc}99A \). In all cases nucleotides in UPPER CASE indicate those changed in the cloning process. \( ttg \) and \( atg \) codons are given in bold, as are sequences with homology to the 5\(^{\prime}\)ACCUCC-3\(^{\prime}\)16S rRNA sequence widely recognised as the ribosome binding site consensus sequence.
4.4.5 Expression of Protein from pTrc 99A Complementation Vectors

To ensure that the pTrc-complementation vectors were expressing the appropriate protein product, strains containing the vectors were grown in LB<sub>Amp</sub> supplemented with 100 µM IPTG for 4 hours and the bacterial pellet harvested. The pellet was prepared as described in section 2.7.4 electrophoresed by SDS-PAGE and visualized by Coomassie stain (Figure 45). The Fur protein* (16.8 kDa) and YhjK(ttg) * (73 kDa) protein were present but BcsZ (41.2 kDa), YhjKalt (73 kDa) and BcsA (100 kDa) were not.

Figure 43. Expression of Protein by pTrc99A Complementation Vectors. Strains carrying the designated gene were grown in LBamp + 100µM IPTG for 4 hours. Protein was electrophoresed by SDS-PAGE and stained with Coomassie Blue stain. Protein product was visible for Fur * (16.8 kDa) and YhjK (ttg) * (73 kDa) but not YhjK (atg) (73 kDa) BcsZ (41.7 kDa) or BcsA (100kDa).
Chapter 5. A Genetic Approach to Study the Role of Cellulose in UPEC 536

5.1. Introduction

The understanding of the molecular mechanisms that induce cellulose production and the signals that regulate its degradation are now emerging for a number of different species (Jenal 2004; Jenal and Malone 2006; Hengge 2009). To further develop our analysis of UPEC cellulose aggregates that form under iron-limited conditions and disperse in iron-replete conditions, insertional and deletion mutants were created. A strategy was adopted where insertional mutations were initially made to determine which genes were involved in aggregation and/or dispersion. If phenotypic differences were found in either aggregation or dispersion that differed from the parent strain then deletion mutants were constructed.

Two genes encoding structural components of the cellulose operon were targeted, the cellulose synthase (bcsA) and the endoglucanase (bcsZ) (Figure 47, A). It is hypothesized that if either bcsA, encoding the catalytic subunit or bcsZ, an endoglucanase involved in cellulose biosynthesis is deleted cellulose would not be produced and aggregation would not occur.

Aggregation and dispersion describes the biofilm transition between motility and sessility, which has been linked to the production of the bacterial cell signaling molecule c-di-GMP (DeArgenio and Miller 2004; Jenal 2004; Jenal and Malone 2006). To determine if regulation of cellulose production and ultimately aggregation in UPEC 536 was induced through c-di-GMP signaling an insertion was created in yaiC a diguanylate cyclase homologous to adrA of S. Typhimurium (Romling 2007; Zogaj et al. 2001). A hypothesis that mutation of the yaiC gene would inhibit aggregation of UPEC 536 grown in RPMI 1640 through a lowering of the intracellular concentration of c-di-GMP was tested.

The degradation of c-di-GMP and the transition from sessility to motility is dependent upon the action of phosphodiesterases. Analysis of the UPEC 536 genome sequence database identified a putative phosphodiesterase, yhjK immediately downstream of the cellulose operon. It is hypothesised that a mutation in yhjK would result in an accumulation of intracellular c-di-GMP and increase aggregate formation and that a decrease in phosphodiesterase activity would alter dispersion upon supplementation of iron (Chapter 6).
Iron is a key element in the physiological response of bacteria to their environment (Carpenter et al. 2009). To determine if iron-regulated aggregation and dispersal are Fur regulated processes the fur gene was chosen for mutation to test the hypothesis that a disruption in fur would alter the process of aggregation and dispersion observed using the Aggregation Index.

5.2. Fitness of Parental and Knock Out Strains

To ensure that all strains used in this study of equally fit a growth curve was performed (Figure 44). Strains were grown in LB broth to avoid the formation of aggregates. All strains grew at a similar rate to UPEC 536.

Figure 44. Fitness of Wild Type and Mutant Strains. Growth in LB of UPEC 536 ◊, UPECΔfur ▲, UPECΔyhjK ●, UPECΔbcsA ▪, and UPECΔbcsZ □, was monitored by absorbance at 600nm over time. Graph is representative of two independent assays.

5.3. Disruption of bcsA Inhibits Aggregation and Cellulose Production

An insertional mutation in bcsA was constructed. The mutant did not aggregate in RPMI 1640 media therefore a deletion mutant of bcsA was constructed. The UPECΔbcsA deletion mutant did not produce flocs in RPMI media (Figure 45, B & E), produced white colonies on Congo Red agar (Figure 45, H) and bacterial cells did not stain with Calcofluor White (Figure 45, J & K), indicating loss of cellulose production. The finding that inhibition of cellulose relates to loss of aggregation supports the finding that cellulose is the main component of the UPEC 536 matrix (Chapter 3 section 3.1.3).
To demonstrate the loss of adherent biomass due to loss of cellulose production, crystal violet staining was used. UPEC 536 culture grown in 1 cm diameter glass tubes was stained with 0.1% crystal violet and demonstrates a fibrous network of cellulose and bacterial cells (biomass) that are strongly adherent to glass (Figure 45, L) and absent in UPECΔbcsA (Figure 45, M, arrow).

**E. coli Cellulose Biosynthesis Operon**

![E. coli Cellulose Biosynthesis Operon diagram]

**UPEC 536 at Rmax**

**UPEC 536ΔbcsA at Rmax**

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**UPEC 536**

**MG 1655**

**UPECΔbcsA**

**UPEC 536**

**MG 1655**

**UPECΔbcsA**

**UPEC 536**

**UPECΔbcsA**

**UPECΔbcsA**

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Figure 4. Inhibition of Aggregation Correlates to Loss of Cellulose. (A) Indicates a schematic diagram depicting the cellulose biosynthesis operon and an indication of the mutation site. The wild type strain shows large aggregates in clear broth and none in the UPECΔbcsA strain (B & E) at Rmax. Rmax is the time taken for UPEC 536 to reach maximal aggregation. For non-aggregating strains Rmax0 indicates incubation for the time taken for UPEC 536 to reach Rmax. Congo Red agar plates for the detection of cellulose indicate loss of cellulose from the UPECΔbcsA strain (H) and MG1655 (G) but not UPEC 536 (F). Calcofluor White stain confirms loss of cellulose in UPECΔbcsA under UV light (K). Crystal violet staining indicates the UPEC 536 fibrous network of cellulose and bacterial cells (biomass) that are strongly adherent to glass (L) and absent in UPECΔbcsA (M and arrow). Scale bar = 50 µm.

5.4. Disruption of bcsZ Inhibits Aggregation

An insertional mutation in bcsZ was constructed that did not aggregate in RPMI. A deletion mutant of bcsZ was therefore constructed. The UPECΔbcsZ did not form flocs in RPMI media (Figure 46, B & E), produced white colonies when grown on Congo Red agar (Figure 46, H) or stain with Calcofluor White (Figure 46, J and K), indicating loss of cellulose production. UPECΔbcsZ and MG1655 cultures grown in glass tubes did not stain strongly with crystal violet (Figure 46, M & N) indicating a loss of adherent biomass. The finding that inhibition of cellulose relates to loss of aggregation confirms the role of bcsZ in cellulose biosynthesis. Clearly, if cellulose synthesis is inhibited any role the endoglucanase activity of BcsZ may have in dispersal cannot be investigated.
Figure 46. Loss of Cellulose and Aggregation in UPECΔbcsZ. (A) Indicates a schematic diagram depicting the cellulose biosynthesis operon and an indication of the mutation site. UPEC 536 displays large aggregates in clear broth at Rmax and UPECΔbcsZ does not (B, and E). Non-aggregating strains were examined by light microscopically throughout the course of the Aggregation Index. Congo Red agar plates for the detection of cellulose indicate loss of cellulose from the UPECΔbcsZ strain (H) and MG1655 (G) but not UPEC 536 (F). Calcofluor White stain indicates the presence of cellulose in the UPEC 536 aggregate (I) but not UPECΔbcsZ planktonic cells under light microscopy (J) or under UV light with Calcafluor stain (K). Crystal violet staining indicates the UPEC 536 strongly adherent cellulose and bacterial cells (biomass) to glass tubes (L) and absent in MG1655 (M) and UPECΔbcsZ (N and arrow). Scale bar = 50 µm.
Complementation of the \textit{bcsA} and the \textit{bcsZ} mutant was not possible with either a \textit{bcsA} / \textit{bcsZ} gene under the control of an IPTG inducible pTrc99A promoter, or the full operon. The result is consistent with other research findings in this area, where no published complementation of the \textit{bcs} cellulose biosynthesis operon mutant has been reported (Kai-Larsen \textit{et al.} 2010).

5.5. \textbf{Disruption of yaiC Has No Affect Cellulose Production or Aggregation}

An insertional mutation in \textit{yaiC} was constructed. The mutant aggregated in RPMI 1640 and dispersed when supplemented with iron (Figure 47, A). The \textit{yaiC} insertion mutant produced aggregates similar to that observed for UPEC 536 (Figure 47, D), produced red colonies on Congo Red agar (Figure 47, G) and stained with Calcofluor White (Figure 47, I & J) indicating cellulose production. Crystal violet staining of biomass adherent to glass tubes was similar to that seen in UPEC 536 (Figure 47, M). UPEC\textit{yaiC} formed aggregates in RPMI that dispersed when supplemented with iron in a similar manner to that of UPEC 536 and therefore, a deletion mutant was not constructed. These findings suggest that the contribution of c-di-GMP from the YaiC protein was not sufficient to induce a measurable change detectable by the screening methods used in this study, or that the intracellular levels of c-di-GMP were sufficient to induce biofilm formation when UPEC\textit{yaiC} was grown in RPMI.
Figure 47. Aggregation and Cellulose in UPECyaiC. An AI performed on UPECyaiC, displays a similar aggregation and dispersion trend as the wild type, throughout the assay (A, B and D) although the UPECyaiC strain displays smaller aggregates that may be due to the selection antibiotic. Congo Red dye agar indicates the presence of cellulose (G), Calcofluor White stain indicates the presence of cellulose in the UPEC 536 aggregate (H) and UPECyaiC, and crystal violet staining shows UPEC 536 and UPECyaiC, biomass (K & M) adherent to the glass tube (biomass), whereas, MG 1655 (L) does not. Cultures were imaged with the Biocount 5000. Scale bar = 50 µm.
5.6. Deletion of fur Does Not Effect Aggregation or Dispersion

It was not possible to construct a stable insertional mutation of fur using the pNQ705 based methodology chosen, but a deletion mutant was constructed. The aggregation and dispersion profiles for UPEC536Δfur were indistinguishable from that of the parent strain (Figure 48, A). The UPECΔfur mutant produced red colonies on Congo Red agar (Figure 48, D) and the aggregates stained with Calcofluor White (Figure 48, G) indicating the presence of cellulose and that aggregation is cellulose-mediated.

Figure 48. Aggregation of UPECΔfur. An AI performed on UPECΔfur displays a similar aggregation and dispersion trend as UPEC 536, throughout the assay. UPECΔfur produces red colonies on Congo Red agar (D) and aggregates stain with Calcofluor White under UV light (G).
5.7. The Fur Mutant Displays Loss of Fur Regulation

To confirm that the *fur* mutant was not involved in iron-mediated dispersal evidence was sought to verify that UPEC*fur* behaved according to the well characterized phenotype of *E.coli fur* mutants. UPEC*fur* and UPEC*fur* complemented with pTrc*fur*, were grown in RPMI 1640 with and without 10 µM FeCl₃ and cells harvested for a protein preparation. The outer membrane proteins were analyzed by SDS-PAGE and visualized by Coomassie stain. The gel showed the expression of iron-regulated proteins when grown in iron-enriched medium which indicate that UPEC*fur* has lost Fur regulation (data not shown). Consistent with the literature the *fur* mutant outer membrane proteins produced in RPMI were only repressed in the UPEC*fur* strain complemented with pTrc*fur* (Bagg and Neilands 1987; Noinaj *et al.* 2010).

The Chromazurol S (CAS) assay (Figure 49) was performed to evaluate siderophore production. When strains were grown in RPMI 1640 siderophores (strong chelators) remove iron from the Chromazurol S dye solution and produce a colour change in the Chromazurol S solution (blue → yellow / orange). UPEC*fur* and UPEC*fur*(p*fur*) and UPEC 536(pTrc) were grown in RPMI 1640 with and without provision of 10 µM FeCl₃. When UPEC 536(pTrc) was grown in RPMI 1640 supplemented with iron, a decrease in absorbance was observed as would be expected with Fur regulation. UPEC*fur*(pTrc) grown in iron supplemented media displays an increased absorbance due to loss of Fur regulation.

![Figure 49](image_url)

**Figure 49.** UPEC*fur* Displays a Loss of Regulated Siderophore Production. The Chromazurol S assay was used to determine siderophore production. UPEC*fur* produces siderophores in both iron-restricted and iron-replete conditions due to its loss of Fur regulation.
5.8. Deletion \textit{yhjK} Alters Dispersion but not Aggregation

The \textit{yhjK} gene lies outside of the recognised cellulose biosynthesis operon (Figure 50, A). It is annotated as a putative phosphodiesterase in the NCBI Genbank. The protein carries an EAL domain motif which is found in diverse bacterial signaling proteins and it is hypothesized that EAL domain proteins degrade c-di-GMP and therefore, favours motility over sessility. The EAL domain has been shown to substitute for the full-length protein as a negative regulator of biofilm formation and purified EAL domain exhibits phosphodiesterase activity as demonstrated in the HmsP protein of \textit{Yersinia pestis} (Bobrov \textit{et al.} 2005). The HmsP protein displays activity in the presence of manganese. The protein also carries a GGDEF domain, a HAMP domain and a domain of unknown function (http://www.genome.jp/kegg/pathway.html) (Figure 50, B). The UPEC\textit{yhjK} insertion strain formed aggregates in RPMI media so a deletion mutant was constructed. The deletion strain formed flocs in a manner similar to UPEC 536 at Rmax (Figure 50, C), stained red on Congo Red agar (Figure 50, F) and aggregates stained white with Calcofluor White (Figure 50, I) indicating the presence of cellulose.

\begin{center}
\textbf{E. coli Cellulose Biosynthesis Operon}
\end{center}

\begin{center}
\textbf{A}
\end{center}

\begin{center}
\textbf{B}
\end{center}

\begin{center}
\textbf{C}
\end{center}
Figure 50. The YhjK Protein. A schematic diagram of the cellulose biosynthesis operon and location of the mutation relative to the operon (A). The domain architecture of the YhjK protein (B) (http://www.genome.jp/kegg/pathway.html). UPECΔyhjK formed aggregates at Rmax (C), stain red on Congo Red agar (F) and indicate cellulose in the matrix when stained with Calcofluro White (I). Scale bar = 50 µm.

5.9. The Aggregation Index is Not Sensitive Enough for UPECΔyhjK

The UPECyhjK insertion mutant aggregated but did not completely disperse upon the addition of iron to RPMI media. A deletion mutation was therefore constructed. An Aggregation Index was performed and showed that UPECΔyhjK aggregated in a manner similar to that of the parental strain (Figure 51, A). A qualitative observation of iron-induced dispersion following the addition of 10 µM FeCl₃ to Rmax cultures indicated dispersal was impaired. Aggregates at Rmax are heavily aggregated and the broth appears clear (Figure 51, B and E). At 1 hour dispersion with iron the UPEC 536 aggregates in the culture are dispersing and the broth appears cloudy, whereas UPECΔyhjK aggregates still remain heavily packed (Figure 51, C and F). By 3 hours most of the UPEC 536 aggregates have dispersed (p = < 0.05) yet many UPECΔyhjK aggregates still remain (p = <0.05) in the culture and can not be dispersed with 0.3 M NaCl (Figure 51, D and G).
Figure 5.1. Aggregation Index of the UPEC 536ΔyhjK Mutant. An Aggregation Index performed on UPECΔyhjK displays aggregation that occurs in a manner similar to that of UPEC 536 strain in RPMI but fails to fully dissociate on the addition of FeCl₃. (A and B & E). One hour after the addition of iron, UPEC 536 aggregates begin to disperse and the culture broth becomes cloudy, whereas UPECΔyhjK culture is still heavily aggregated with some cloudiness (C & F). After 3 hours UPEC 536 aggregates have dispersed yet some UPECΔyhjK aggregates persist (D & G). Images show bacterial culture in 55 mm petri dishes and imaged using the Biocount 5000.

Observations show that UPECΔyhjK aggregates are more stable in dispersing culture and do not breakdown in the 0.3M NaCl wash. A quantitative analysis of the dispersion process failed to fully represent the observations as the NaCl wash did not completely disperse preformed aggregates. The AI = (OD_{Total} – OD_{planktonic}) / OD_{Total} so, if the Total OD remains
decreased due to incomplete dispersion of the aggregates, then the AI will remain elevated. A visual comparison of UPEC 536 (Figure 52, A-C) and UPECΔyhjK (Figure 52, D-F) after iron induced dispersion and a 0.3 M NaCl wash, clearly shows aggregates that remain in the broth and also a cloudy portion of the culture (planktonic cells) indicating some dispersion of aggregates occurs in the mutant. UPEC 536 aggregates disperse readily up to approximately 7 hours incubation, after which time the aggregates become less soluble.

![UPEC 536 at Rmax](image1)

![UPECΔyhjK at Rmax](image2)

**Figure 52. Incomplete Dispersion of UPECΔyhjK with 0.3 M NaCl.** Aggregates of UPEC 536 and UPECΔyhjK appear similar at Rmax (A&D). Samples were induced to disperse with iron, washed in 0.3 M NaCl, and vortexed before a total OD reading. UPEC 536 forms a homogenous solution (B & C), whereas, UPECΔyhjK aggregates are more resistant to disruption by 0.3 M NaCl (E & F). Images show salt washed culture in 55 mm petri dishes and imaged using the Biocount 5000.

### 5.10. Deletion of yhjK Alters the Appearance of Cellulose

A microscopic analysis of RPMI-grown aggregates from UPEC 536 and UPECΔyhjK cultures at Rmax (Figure 53, A-D) using Calcofluor White showed that the UPECΔyhjK mutant produced aggregates with cellulose morphology different to that of UPEC 536. Although macroscopically the aggregates of both strains appear similar in the culture broth at Rmax, comparison staining of the aggregates with Calcofluor White at Rmax show UPEC
536 aggregates with loose and amorphous fibres which appear diffuse with a rough surface (Figure 53, A and B, arrows), whereas UPEC\textit{\textgreek{y}hjK} aggregates show dense cellulose fibres that are tightly packed and form smooth surfaces (Figure 53, C and D, circles) although loose and more diffuse areas are also present in some aggregates (Figure 53, D white arrows).

![Image](image1.png)

**Figure 53. Deletion of the yhjK gene Alters the Appearance of the Cellulose.** Macroscopically, aggregates from the wild type and mutant appear similar at Rmax but Calcofluor White staining displays differences in the cellulose appearance. UPEC 536 appears to have a loose, diffuse, fibrous network of cellulose at Rmax (A-B). The cellulose fibers of UPEC\textit{\textgreek{y}hjK} appear dense, tightly bound and appear to have a smooth surface in many areas of the aggregate (C-D).

Three hours after iron induced dispersion, some UPEC 536 aggregates are reduced in size and show a loose fibrous network that with cloudiness around the edges (Figure 54, A-B, black arrows) whereas; UPEC\textit{\textgreek{y}hjK} aggregates remain dense in structure (Figure 54, C, circle) and retain tightly packed areas with a smooth ice-cube\textcircled{c} like surface (Figure 54 D, circle).
Figure 54. **UPECΔyhjK Cellulose Aggregates Remain Tightly Packed.** Three hours after the addition of iron, aggregates disperse. UPEC 536 aggregates are smaller and appear cloudy on the edges (A-B). The cellulose aggregates of UPECΔyhjK remain dense, tightly packed (C) and retain smooth surfaces on many areas of the aggregate (D).

R-24 aggregates of UPEC 536 and UPECΔyhjK share similar features. Most UPEC 536 aggregates appear as densely packed fibres (Figure 55, A-B). UPECΔyhjK culture still contains many large aggregates that show collapsed and tight, dense fibre networks (Figure 55, C-D) with large smooth surfaces.
Figure 5.11. Discussion

The objectives of this section were to examine the processes of aggregation and dispersion using a genetic approach with the insertion mutant UPEC\textit{yaiC} and deletion mutants UPEC\textit{bcsA}, UPEC\textit{bcsZ}, UPEC\textit{fur}, and UPEC\textit{yhjK} constructed in Chapter 4. Each mutant was screened using an Aggregation Index for aggregation and dispersion trends, Congo Red agar and cellulose specific staining using Calcafluor White for cellulose and biomass adhesion to glass using crystal violet staining. Mutants that displayed different phenotypes to that of the parent strain were investigated further. Before examination of the mutant strains, it was important to ensure the insertion and deletion strains were of equal fitness and would not aggregate in LB broth. A growth curve was performed and indicated all
strains grew in a similar manner (Figure 44) and no cellulose or aggregation was detected in any of the mutated or wild type strains when grown in LB broth.

The major component of the UPEC 536 biofilm matrix is cellulose (see section 3.1.4). Cellulose synthesis in bacteria, specifically *S. enterica* Typhimurium (Romling 1998; Romling 2005; Brown et al. 2007) and *Gluconacetobacter xylinus* (Ross et al. 1991; Williams and Cannon 1989; Nguyen et al. 2008) has been well documented. The *E.coli* K12 (MG1655) strain contains a cellulose biosynthesis operon which consists of *yhjQ*, two structural genes (*bcsA* and *bcsB*), an endoglucanase (*bcsZ*) and *bcsC*, encoding a protein believed to be involved in protein-protein interactions (Romling 2007). This operon is also present in the UPEC 536 genome sequence (ECP_3630-3634, Hochhut et al. 2006). The deletion of any of these genes inhibits cellulose production. Specifically, deletion of *bcsA* displays no aggregate formation when grown in RPMI 1640 (Figure 45, B and C and F), produces a white colony on Congo Red agar (Figure 45, I), shows no cellulose when culture is stained with Calcofluor White (Figure 45, L) and displays no adhesion when grown on glass and stained with crystal violet (Figure 45, N).

Deletion of *bcsZ*, displays no aggregate formation when grown in RPMI 1640 (Figure 46, B and C and F), produces a white colony on Congo Red agar (Figure 46, I), shows no of cellulose when culture was stained with Calcofluor White (Figure 46, L) and displays no adhesion when grown on glass and stained with crystal violet (Figure 46, O). Together these results confirm that the cellulose biosynthesis operon is directly responsible for the production of cellulose and aggregation of UPEC 536 grown in RPMI.

Complementation of either *bcsA* or *bcsZ* (or any gene within the cellulose biosynthesis operon) has thus far proven elusive. A recent study (Kai-Larsen et al. 2010) overcame this problem by uncoupling the normal regulation of the *bcs* operon and placing it under tight control of the Tet promoter. However, the strategy used does not allow study of individual genes in the operon.

Cellulose and aggregation is detected in cells grown in RPMI 1640 supplemented with iron but aggregates do not persist. The involvement of iron in the control of dispersion suggests a role for genes regulated via Fur (Pennella & Giedroc, 2005). To investigate if this was the case, a deletion was constructed in *fur*. The Aggregation Index showed that aggregation and dispersion proceeded in a manner similar to that of the parent strain (Figure 48, A). Congo Red agar displayed red colonies (Figure 48, D) and aggregates stained with Calcofluor White
To ensure UPEC\text{\textcopyright}fur displayed phenotypic characteristics that indicate loss of Fur regulation, further examination was undertaken and showed that iron-regulated protein expression and the production of siderophores in iron-enriched media (Figure 49) were lost in the mutant strain. The UPEC\text{\textcopyright}fur(pTrc) complemented strain confirming the deletion of \textit{fur} and that dispersion is not a Fur regulated process.

Regulation of aggregation through the production of c-di-GMP was also a consideration. Biofilm characteristics such as sessility and the production of exopolymeric substances are regulated by the production of the internal second messenger, c-di-GMP (Romling et al. 2005). Information in the literature indicates that AdrA in \textit{S. Typhimurium} (Romling 2006) is involved in c-di-GMP production and is homologous to YaiC in \textit{E.coli} (Zogaj et al. 2001, Garcia et al. 2004). YaiC is a diguanate cyclase responsible for the production of c-di-GMP (Romling and Amikan 2006; Galperin and Gormelsky 2006) and has been shown to bind to the PilZ domain of \textit{bcsA} and induce cellulose synthesis in \textit{E.coli} strain MG1655 (Ryjenkov et al. 2006). UPEC\text{\textcopyright}yaiC was constructed to determine if the c-di-GMP produced by YaiC was responsible for the aggregation seen in UPEC 536 grown in RPMI 1640. An Aggregation Index showed that UPEC\text{\textcopyright}yaiC aggregated and dispersed in a manner similar to that of UPEC 536 (Figure 47, A and D), produced red colonies on Congo Red agar (Figure 47, G) and aggregates stained with Calcofluor White (Figure 47, J). Biomass adhesion to glass (Figure 47, M) appeared similar to that of the parental strain. The contribution of c-di-GMP from YaiC did not appear to play a role in the aggregation seen in UPEC 536 in RPMI 1640. From this study it is difficult to determine if intracellular concentrations of c-di-GMP are high enough to induce aggregate formation or if redundant pathways are able to compensate for the loss of one protein.

The YhjK protein of UPEC 536 is a multi-domain protein that lies downstream of the cellulose biosynthesis operon (Figure 50, A), has phosphodiesterase activity and carries sensory domains (Figure 50, B) (Hulko et al. 2006) and has high similarity to the EAL carrying protein HmsP of \textit{Yersinia pestis} (>45%) (Bobrov et al. 2005), the PDEA1 phosphodiesterase of \textit{G. xylinus} (20%) (Tal et al. 1998), and the VieA phosphodiesterase of \textit{Vibrio cholerae} (16%) (Tischler and Camalli 2005). Construction of UPEC\text{\textcopyright}yjhK was hypothesised to effect dispersion of the aggregate. Results indicate that aggregate formation is macroscopically similar to that of UPEC 536 (Figure 50, C), colonies stained red on Congo Red agar (Figure 50, F) and are cellulose based as determined by Calcofluor White staining (Figure 50, I). The Aggregation Index revealed that iron-induced aggregate
dispersion (Figure 51, A and E-G) and disruption by 0.3 M NaCl (Figure 52, D-F) were ineffective in forming the homogenous solution required for accurate and reproducible measurements with the Aggregation Index. Microscopic examination of the Calcofluor White stained aggregates revealed that aggregates at Rmax appeared different from those seen in UPEC 536 (Figure 53) where the cellulose matrix was dense tightly packed and had smooth surfaces, as opposed to the light, amorphous fibres seen in the parent type. Many of the aggregates show similar features as those seen in UPEC 536 in addition to the smooth, dense areas (Figure 53, C-D, white arrows). Aggregates were stained and microscopically examined 3 hours after iron-dispersion and showed that most UPEC 536 aggregates had reduced in size and showed a cloudiness around the edges suggesting dissolution (Figure 54, A-B) whereas, the UPEC\textit{Δ}yhjK aggregates were larger, remained densely packed and had smooth surfaces and collapsed appearance (Figure 54, C-D). This morphology is more apparent after 24 hours in aggregates grown in RPMI (Figure 55, C-D). The images clearly indicate that the loss of \textit{yhjK} impacts iron-induced dispersion of the aggregates, probably due to a change in the characteristics of the cellulose.

Taken together the phenotypes displayed by the deletion strains indicate that cellulose is the major polymeric substance involved in aggregate formation and that both \textit{bcsA} and \textit{bcsZ} are essential for cellulose biosynthesis, which is consistent with other studies. The \textit{yaiC} insertional mutant gave no indication into the regulation of cellulose production as a c-di-GMP-dependent process under the growth conditions used in this study. Dispersion is not a Fur regulated process. Most interestingly, the deletion of \textit{yhjK}, showed an effect on the morphology of the cellulose and iron-induced dispersion suggesting the gene may play an important role in maintaining the integrity of the cellulose produced as the biofilm matrix.
Chapter 6. Characterisation of UPECΔyhjK

6.1. Introduction

Preliminary examination of UPECΔyhjK showed that the mutant did not disperse in a manner similar to that of the parent strain when induced with iron, nor were the aggregates able to be made into an homogenous solution when washed in 0.3 M NaCl. The inability to disrupt the aggregates and form a homogenous solution in the salt wash, implies the Aggregation Index is not sensitive enough to be used to quantify the aggregation/dispersion dynamics within the culture of UPECΔyhjK in a reproducible manner. The robust nature of the aggregate, compared to UPEC 536 clearly indicates a phenotypic change in the mutant strain. To explain the increased stability of the UPECΔyhjK aggregates, two hypotheses were tested: 1) the aggregate has a "stronger" cellulose matrix or 2) the aggregate matrix is strengthened by the presence of another polymer.

6.2. Polysaccharide Monomers in the UPECΔyhjK Matrix

To determine the polysaccharide composition of UPECΔyhjK matrix, aggregates grown in RPMI 1640 for 24 hours were collected and underwent sulphuric acid hydrolysis followed by gas chromatography to determine the relative amount of cellulose present. The hydrolysis of polysaccharide by sulphuric acid was used for the detection of monomers from cellulose hydrolysis only, as other polysaccharides may be consumed by the sulphuric acid hydrolysis and be underestimated. Equivalent samples were analyzed for TFA hydrolysis to determine polysaccharides other than cellulose that may be present in the biofilm matrix. No striking differences were observed in the monosaccharide distribution of polymers from UPECΔyhjK grown in R-24. The relative contributions of polysaccharides to the UPECΔyhjK matrix are summarized in Table 15. The aggregates of UPECΔyhjK grown in RPMI for 24 hours appear to have similar proportions of total sugar and monomers of ribose, mannose, galactose and glucose similar to that of UPEC 536 R-24. This suggests that there are no "new" polysaccharides being produced in the R-24 aggregate based on the monomers tested.
Table 15. Relative Contribution of Polysaccharides to the UPECΔyhjK Biofilm Matrix

<table>
<thead>
<tr>
<th>Sample</th>
<th>Method</th>
<th>Total Sugar (µg/mg)</th>
<th>Neutral monosaccharide (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>ribose</td>
<td>mannose</td>
</tr>
<tr>
<td>UPEC 536</td>
<td>TFA</td>
<td>26.0 ± 7.2</td>
<td>38.3 ± 2.5</td>
</tr>
<tr>
<td></td>
<td>H₂SO₄</td>
<td>30.0 ± 0.1</td>
<td>-</td>
</tr>
<tr>
<td>UPECΔyhjK</td>
<td>TFA</td>
<td>44.3 ± 0.5</td>
<td>32.3 ± 0.6</td>
</tr>
<tr>
<td>R-24</td>
<td>H₂SO₄</td>
<td>36.9 ± 2.5</td>
<td>-</td>
</tr>
</tbody>
</table>

6.3. Colanic Acid is Increased in the UPECΔyhjK Matrix

The role of capsular polysaccharides has been well studied (Costerton et al. 1981; An and Friedman 1998; Pratt and Kolter 1998) yet how these surface molecules interact in initial surface binding and intermolecular interactions within the biofilm matrix are not well understood. The E.coli matrix contains a number of polysaccharide components including colanic acid or M-antigen (Meredith et al. 2007; Grant et al. 1969). A method described by van den Hoogen et al. (1998) was utilized to quantify and compare the abundance of colanic acid in the UPEC 536 and UPECΔyhjK biofilm matrix at Rmax, R-24 and RF-24. There is a significant difference between UPEC 536 colanic acid content and UPECΔyhjK at Rmax and at RF-24 but not at R-24 (Figure 56). These results suggest that colanic acid is produced early in aggregate development and may contribute to formation of the aggregate. During dispersion colanic acid in the UPECΔyhjK mutant is not degraded.
Figure 56. Colanic Acid in the UPECΔyhjK Biofilm Matrix. The concentration of colanic acid in the UPECΔyhjK biofilm is significantly different from UPEC 536 at Rmax and RF-24 but not at R-24, suggesting colanic acid is produced during exponential growth. P-values were calculated using a non-parametric Kruskal Wallis with Dunns post-hoc test. Each graph point represents a value from three replicates taken from three independent assays.

6.4. PNAG is a Minor Component of the UPECΔyhjK Biofilm Matrix

PNAG has been extensively studied in coagulase negative *Staphylococcus epidermidis* which synthesizes polysaccharide intercellular adhesin (PIA) and coagulase positive *Staphylococcus aureus* which synthesizes poly-β-1,6-N-acetyl-glucosamine (PNAG). PNAG has also been isolated from Gram negative organisms such as *E.coli*, *Y. pestis* and *Actinobacillus* spp. (reviewed in Pamp *et al.* 2009). Comparative genome sequence analysis has revealed that homologues of the PNAG biosynthesis genes are present in many bacterial species. In *Staphylococcus* PNAG/PIA has been found to be the most important biofilm matrix component (Foster and Hook 1998; Gotz 2002). *E.coli* K-12 strain is able to synthesize a PNAG/PIA-like polysaccharide poly-N-acetyl-glucosamine (PGA) which has been shown to enhance biofilm formation (Blattner *et al.* 1997, Wang *et al.* 2004). The polymer is composed of long unbranched chains of D-glucosamine linked by Ú-1,6 glucosidic bonds. To determine if PNAG is a component of the UPECΔyhjK biofilm a colorimetric method described by Tote *et al.* 2007 was used. Cells were incubated with Dimethyl Methylene Blue reagent, rinsed and the PNAG-bound dye extracted. The absorbance at 650nm is deemed to be proportional to the amount of PNAG in the matrix. PNAG was detected in the biofilm of both UPEC 536 and
UPEC\(\Delta yhjK\) (Figure 57). Statistical comparisons between the strains indicate that no significant differences are present between the samples and PNAG remains increased in RF-24 cultures, suggesting the polymer does not contribute to aggregate formation.

Figure 57. PNAG is a Minor Component of the UPEC\(\Delta yhjK\) Biofilm Matrix. PNAG is increased in dispersing culture suggesting PNAG does not play a role in aggregate formation. Data was analysed with a one way ANOVA with Bonferroni post test. Each point represents a value taken from three independent assays.

6.5. The Effect of Dispersin B on UPEC\(yhjK\) Aggregates

Specific enzymatic hydrolysis was performed using Dispersin B, a 42 kDa glycosidic enzyme produced by Aggregatibacter actinomycetemcomitans (Kaplan et al. 2003) that catalyzes the hydrolysis of \(\alpha\)-1,6-poly-N-acetyl-glucosamine (Ramasubba et al. 2005). The enzyme was added to initial cultures of UPEC 536 and UPEC\(\Delta yhjK\), which were incubated to maximum aggregation and examined for the presence of aggregates. No change was detected in aggregate formation between UPEC 536 and UPEC\(\Delta yhjK\) or cultures grown without treatment (data not shown). Dispersin B was added to preformed aggregates at Rmax, and no change was detected in aggregate dispersion between UPEC 536 and UPEC\(\Delta yhjK\) cultures or cultures grown without treatment (data not shown). These observations together with data presented in section 3.3.2 indicate that PNAG is produced by UPEC 536 and UPEC\(\Delta yhjK\) and is incorporated into the matrix but plays no significant role in the structural scaffold of the matrix of UPEC 536 or UPEC\(\Delta yhjK\).
6.6. The YhjK Mutant Displays Varied Dispersion in Divalent Metals

Cultures of UPEC 536 and UPECΔyhjK were grown in RPMI 1640 to maximum aggregation and divalent metal ions added to the cultures to a final concentration of 10 µM. Iron (FeCl₃) and manganese (MnSO₄) induce dispersal of UPECΔyhjK* but not zinc (ZnSO₄) (Figure 58). Culture induced with zinc remained clear and the aggregates intact. Culture induced with iron and manganese became cloudy within one hour and some of the aggregates reduced in size. Although many aggregates that remain in the culture do not degrade in prolonged iron/manganese induced dispersion (> 3 hours) or when washed in 0.3 M NaCl and the aggregates settle on standing. The Aggregation Index measurements denoted with * indicate incomplete dispersion and a falsely elevated AI as described in section 5.9.

Figure 58. Iron and Manganese Induce Dispersion in UPECΔyhjK. An Aggregation Index was used to determine divalent metal induced dispersion in the UPECΔyhjK. Iron and manganese induce partial dispersion, and zinc has no effect. * Denotes the aggregates are not fully dispersed by the Aggregation Index resulting in higher AI values. The graph represents an average value from three independent assays.

6.7. Does Disruption of the Phosphodiesterase Affect Motility

Second messenger signalling by c-di-GMP is known to regulate the transition from biofilm to planktonic lifestyle (Romling et al. 2005). An important feature of this event is non-motile sessile cells regaining motility. The turnover of c-di-GMP is regulated through the action of diguanylate cyclases and c-di-GMP phosphodiesterases. It is hypothesised that the loss of phosphodiesterase activity in UPECΔyhjK will decrease motility due to increased levels of c-di-GMP. Motility was investigated on LB-based or RPMI-based swimming (0.3% w/v) or swarming (0.6% w/v) Eiken agar. Interestingly, swimming motility was significantly
increased in UPECΔyhjK when grown on LB swimming agar (Figure 59). Motility on RPMI based swimming agar plates showed no effect. No change was detected in swarming motility on LB or RPMI based swarming agar (data not shown). This result differs from other data in the literature that indicate phosphodiesterase mutants have decreased motility (Ryjenkov et al. 2006).

![Figure 59. Motility is Increased in UPECΔyhjK. LB-based swimming motility agar indicates increased motility in the UPECΔyhjK strain and no change when tested on RPMI-based swimming agar. Data was analysed using a one-tailed Mann-Whitney test. Each point denotes one replicate from at least two independent assays.](image.png)

6.8. Complementation and Over Expression of YhjK

Increased c-di-GMP enhances biofilm formation, the adhesive characteristics of biofilm lifestyles and the production of exopolymeric substances (Pamp et al. 2009). Conversely, increased phosphodiesterase activity has been shown to increase motility and loss of adhesive abilities (Romling et al. 2005). It is hypothesised that over-expression of the YhjK phosphodiesterase will inhibit cellulose production and aggregate formation in UPEC 536. Further, complementation of UPECΔyhjK with pyhjK or pyhjK(alt) will decrease cellulose production and aggregation. To determine the effect of increased phosphodiesterase activity on adhesion, an over-expression strain UPEC(pyhjK) and UPEC(pyhjK)(alt) were constructed. Complementation strains UPECΔyhjK(pyhjK) and UPECΔyhjK(pyhjK)(alt) were constructed. Crystal violet staining was used to visualize the biomass of each strain (Figure 60). No adhesion was observed for MG1655 (Figure 60, A) as opposed to UPEC 536 which shows strong biomass attachment to glass tubes (Figure 60, B). UPECΔyhjK biomass
adhesion (Figure 60, C) is similar to that of UPEC 536. Over-expression of pyhjK(alt) (Figure 60, D) indicates no change in biomass adhesion compared to UPEC 536 and UPECΔpyhjK whereas over-expression with pyhjK carrying the ttg start site abolished aggregate formation and biomass adhesion (Figure 60, E). Complementation of UPECΔpyhjK with the pyhjK(alt) has no effect on biomass adhesion (Figure 60, F) whereas; complementation with the pyhjK carrying the ttg start site abolished aggregate formation and biomass adhesion (Figure 60, G). These results suggest the start site for the yhjK gene is ttg. These results also indicate that the presence of the putative phosphodiesterase YhjK enhances the characteristics associated with planktonic life.

![Figure 60. Phenotypes of YhjK Over-expression and Complementation Strains.](image_url)

The effects of over-expression and complementation were investigated through An Aggregation Index (Table 16 and 17). Over-expression strain UPEC536(pyhjK) (alt) showed a high Aggregation Index at Rmax whereas strain UPEC536(pyhjK) showed no aggregation. Complementation strain UPECΔpyhjK(pyhjK)(alt) did not alter aggregation, although complementation strain UPECΔpyhjK(pyhjK) produced weak aggregates that were easily dispersed by vortexing. These findings support the ttg as a start site and are consistent with biomass adhesion results shown in Figure 63 that show the expressed protein of pTrc_yhjK is required to degrade aggregates.
Table 16. Over-Expression of YhjK

<table>
<thead>
<tr>
<th>Strain</th>
<th>Aggregation Index - Rmax</th>
</tr>
</thead>
<tbody>
<tr>
<td>UPEC 536</td>
<td>0.85 ± 0.01</td>
</tr>
<tr>
<td>UPEC536(pTrc)</td>
<td>0.79 ± 0.02</td>
</tr>
<tr>
<td>UPEC536( pyhjK)(alt) (atg)</td>
<td>0.86 ± 0.01</td>
</tr>
<tr>
<td>UPEC536(pyhjK) (ttg)</td>
<td>No aggregation</td>
</tr>
</tbody>
</table>

Table 17. Complementation with YhjK

<table>
<thead>
<tr>
<th>Strain</th>
<th>Aggregation Index - Rmax</th>
</tr>
</thead>
<tbody>
<tr>
<td>UPECΔyhjK</td>
<td>0.94 ± 0.05</td>
</tr>
<tr>
<td>UPECΔyhjK(pTrc)</td>
<td>0.95 ± 0.04</td>
</tr>
<tr>
<td>UPECΔyhjK(pvyhjK)(alt) (atg)</td>
<td>0.95 ± 0.01</td>
</tr>
<tr>
<td>UPECΔyhjK(pvyhjK) (ttg)</td>
<td>Weak aggs*</td>
</tr>
</tbody>
</table>

*Weak aggs denotes aggregates that disperse with vortexing

6.9. Viability of Cells within the UPECΔyhjK Biofilm Matrix

The aggregates of UPECΔyhjK appear to have greater stability than those of UPEC536. Fluorescent microscopy using live/dead and Calcofluor White stain was used to determine if the robust exopolymeric substance influenced bacterial cell viability within the matrix (Figure 61). Aggregates were examined at Rmax and R-24. The images show both live and dead cells evenly distributed throughout the cellulose matrix (Figure 61, A-B and D-E). The tightly packed smooth surfaces of the cellulose matrix (Figure 61, C and F, black arrows) encases an abundance of both live and dead cells, whereas the lighter diffuse areas appear to have less cells associated with the matrix (Figure 61, C white circle). At R-24 many of the lighter areas of the matrix contain more dead cells (Figure 61, F, white circles and E white arrows) suggesting dispersion from the area or that cells encased in the area die over time.
Figure 6.1. Cell Viability Within the UPECΔyhjK Matrix. Fluorescent microscopy, live/dead staining and Calcofluor White were used to estimate the distribution of live/ dead cells within the matrix. Rmax aggregates are evenly distributed throughout the matrix (A-B, D-E), especially in the dense, smooth cellulose areas of the matrix (black arrows). Cellulose that appears lighter and less dense also appears to have fewer cells associated with it (white circles). R-24 aggregates show more dead cells in the lighter areas of the matrix (white arrows).

6.10. Cyclic-di-GMP is Present in UPEC 536 at Rmax

Signalling by second messenger molecules has been shown to be involved in biofilm characteristics such as adhesiveness and production of polymeric substances (Simm et al. 2004; Jenal 2004; Jenal and Malone 2006; Romling 2007). Strains mutated in c-di-GMP biosynthesis do not produce biofilms. A methodology for high throughput screening using HPLC was used to determine c-di-GMP formation in UPEC 536 at Rmax. Cultures were prepared as outlined in section 2.14. A series of dinucleotide standards (10 µM of NAD, NADP, NADPH, NADPH₂ and c-di-GMP) were used to locate the NAD peak which was used as a reference point and to locate the approximate area of c-di-GMP retention (Figure 62, A). Culture was grown to Rmax and the area of c-di-GMP located (Figure 62 B) relative to the spiked sample. An amplification of the area of interest indicates a definite peak at the c-di-GMP location (Figure 62, C). These results indicate the method is suitable for the
detection of c-di-GMP. Further investigation into c-di-GMP concentration and the effect of the loss of yhjK requires further study.

**Figure 6.2. C-di-GMP.** A series of standards was used to locate the NAD peak which is consistently found within the bacterial supernatant (A) and using a 10 µM c-di-GMP spiked sample precise location of c-di-GMP(A). Rmax culture was used to assess the presence of c-di-GMP (B). An amplification of the area of interest (B, red circle) indicates a definite peak at the c-di-GMP location (C).
6.11. Discussion

In this section UPEC\textsuperscript{\textDelta}yhjK aggregates were investigated to gain insight into the increased stability of the matrix. UPEC\textsuperscript{\textDelta}yhjK aggregates were examined at various time periods and compared to those of UPEC 536 by the application of biochemical and enzymatic methods and microscopic methods. The primary questions asked were; does the cellulose produced by UPEC\textsuperscript{\textDelta}yhjK make a "stronger" fibre? Or, are other polymers present that integrate into the UPEC\textsuperscript{\textDelta}yhjK aggregate matrix to provide greater resistance to degradation?

Levels of intracellular c-di-GMP are influenced by the opposing action of two enzymes. The first, a diguanylate cyclase (DGC) catalyzes the formation of c-di-GMP synthesis. These enzymes are identified by conserved amino acid GGDEF motifs in the protein domain. The second, a phosphodiesterase catalyzes the degradation of c-di-GMP and carries a conserved amino acid EAL or HD-GYP motif in the protein domain (Ryan et al. 2006, Simm et al. 2004). Hydrolysis of c-di-GMP to phosphoguanyl guanosine (pGpG) is dependent on the presence of Mg\textsuperscript{2+} or Mn\textsuperscript{2+} which then slowly converts back to GMP (Schmidt et al. 2005). YhjK is a putative phosphodiesterase immediately downstream of the cellulose biosynthesis operon. It is not considered part of the cellulose biosynthesis operon and its role in cellulose biosynthesis is not known. Based on the close association between sessility-associated GGDEF domain proteins and motility-associated EAL or HD-GYP domain proteins, investigation of yhjK seemed an important aspect of understanding the aggregation and dispersion processes observed in UPEC 536 grown in RPMI 1640. The deletion mutant aggregated at a manner similar to that of UPEC 536 and produced aggregates that were macroscopically similar in appearance. From the Aggregation Index it was apparent that dispersion differed from the wild type in that a NaCl wash could not break down the aggregates (Figures 51 and Figure 52). The aggregates appeared more robust, yet like the wild type they had a cellulose-based matrix (Figure 61), but that UPEC\textsuperscript{\textDelta}yhjK displayed a smooth surfaced, "ice-cube" like morphology in some areas of the aggregate. These features are also present in the UPEC 536 R-24 aggregate.

The GGDEF and EAL / HD-GYP motifs are generally found in multi-domain proteins and a GGDEF and EAL / HD-GYP domain may also occur in the same protein as in the case of YhjK. Within the protein, single or multiple sensory domains may also be present (Ryan et al. 2006). The complexities of this signalling pathway are further highlighted by the wide range of sensory domains that may exist and also the sequence variation in the EAL motif.
and GGDEF motif of the various GGDEF and EAL domains (Romling and Gromelsky 2005; Ryan et al. 2006). The YhjK protein carries a GGDEF domain and an EAL domain, a HAMP domain (Histidine kinase, Adenylyl cyclase, Methyl-accepting protein, and Phosphatase) which connects extracellular sensory with intracellular signaling domains (Hulko et al. 2006) and a domain of unknown function (Figure 50, B).

The challenge in investigations of signal transduction are determining the correct biochemical connection between a sensor and the proteins it regulates, as all sensor GGDEF and EAL / HD-GYP domain proteins use the same signaling molecule, c-di-GMP (Solano et al. 2009). The specific action of c-di-GMP may require selected temporal expression of GGDEF- and EAL / HD-GYP domain proteins, co-localization of proteins with their targets and / or activation of these enzymes under specific environmental or intracellular stimuli (Romling and Gomelsky, 2005). Phosphodiesterase deficient mutants in S. Typhimurium show decreased motility (Ryjenkov et al. 2006) which is an expected result as increases in c-di-GMP favour sessility (non-motile) phenotypes. This also suggests a signalling pathway between the phosphodiesterase and the flagella operon. Our investigation into whether or not signalling by YhjK was linked to motility was examined using motility assays. RPMI-based swimming agar detected no difference between UPEC 536 and UPEC ΔyhjK motility, an expected result as RPMI induces aggregation (non-motile). Swimming motility on LB-based agar showed that the UPEC ΔyhjK strain was significantly more motile than UPEC 536 (Figure 59). Initial results would suggest that the YhjK phosphodiesterase activity is not linked to motility and its effects are localised to cellulose ï formation and / or dispersion through the degradation of c-di-GMP. Further investigation into motility in UPEC ΔyhjK and the inducers of phosphodiesterase activity would be necessary to elucidate the any transduction pathway and a connection to its increased motility.

Investigation into the composition of the polysaccharides in the matrix was carried out using H₂SO₄ and TFA hydrolysis and gas chromatography on EPS recovered from R-24 culture. The results indicate the presence of the same monomers found in UPEC 536 at R-24, specifically, ribose, mannose, galactose and glucose in approximately the same proportion and that the matrix was approximately 50% cellulose (Table 15). This indicated that more than likely, the same polymers were produced in the UPEC ΔyhjK matrix as in UPEC 536 R-24 matrix. However, the total sugar present in the mutant strain was higher by almost 50% than that of UPEC 536 R-24, suggesting more of the extracellular material is produced.
PNAG was not quantified by gas chromatography, instead staining with DMMB and sensitivity/resistance to Dispersin B were used to investigate this exopolymeric substance. PNAG is present in similar concentrations to that of the parent strain at Rmax, R-24 and RF-24 (Figure 57) and does not appear to play a structural role in the formation of the matrix as aggregates are insensitive to disruption by Dispersin B (section 6.5). Colanic acid has been shown to be up-regulated in stress-related conditions and in *E.coli* K12 architecture (Danese *et al.* 2000). The wild type strain forms pillars of cells that show cell bodies within microcolonies that do not physically interact but appear to be suspended over the surface. Cells deficient in colonic acid production form densely packed structures with extensive cell-surface and cell-cell interactions and display no depth as seen in the parental strain biofilm. Colanic acid is synthesized by the *cps* gene cluster (Stevenson *et al.* 1996) and is tightly regulated by a complex signal transduction cascade regulated by the *rcs* (regulator of capsule synthesis) phosphorelay system (Gottesman 1995).

Colanic acid was detected in both UPEC 536 and UPEC*Δ*yhjK* matrices in significantly higher concentration in the *yhjK* mutant (Figure 56) at the three culture conditions (Rmax, R-24 and RF-24) tested. Specific hydrolysis of colanic acid is difficult and was first achieved using phage induced enzyme hydrolysis (Sutherland 1971). An enzyme isolated from *Streptomyces* was able to partially degrade colanic acid (van Speybroeck *et al.* 1996) and a new enzyme, β-fucoside hydrolase which causes complete degradation of colanic acid to its corresponding hexasaccharide is not commercially available (Verhoef *et al.* 2005). Therefore, enzymatic hydrolysis of colanic acid was not undertaken. Unfortunately, this limits our understanding on the structural role colanic acid may play in the formation of aggregates and the matrix stability of UPEC 536 and UPEC*Δ*yhjK*.

There is close similarity with YhjK and the HmsP phosphodiesterase of *Y. pestis*. HmsP phosphodiesterase has been shown to be strictly dependent upon the presence of manganese and negligible activity with magnesium, nickel, cobalt, calcium and zinc (Bobrov *et al.* 2005), although iron was not tested. Phosphodiesterase activity is often dependent on divalent metals (Galperin *et al.* 1998; Vogel *et al.* 2002). Further, specificity of activity for HmsP was dependent on the presence of one amino acid (E506) suggesting specificity for metal binding (Bobrov *et al.* 2005). UPEC 536 dispersion was induced by iron, manganese and zinc but UPEC*Δ*yhjK* was induced by iron and manganese albeit incompletely but not zinc (Figure 58). This may lead speculation to redundancy of phosphodiesterase proteins or that perhaps zinc was the metal required for full activity.
Complementation and over-expression studies show that the production of YhjK by UPEC 536 inhibits cellulose production, which is reflected in the absence of robust aggregates. It is likely that this is due to uncontrolled phosphodiesterase activity reducing c-di-GMP levels required to activate cellulose synthesis (Figure 60 and Table 16 and 17).

Cellulose is regulated by the production of the internal second messenger signal, c-di-GMP (Romling et al. 2005). The detection of c-di-GMP is often difficult and involves laborious extraction methods and HPLC coupled to Mass Spectroscopy (Simm et al. 2004, Simm et al. 2009). Methodologies are being developed to utilize high-throughput strategies for the detection of c-di-GMP to test enzymes involved in its biosynthesis with antimicrobial or anti-biofilm agents (Antoniani et al. 2010). One such method was used in this study to detect c-di-GMP in UPEC 536 Rmax samples. A c-di-GMP standard was used to determine the retention time of the c-di-GMP and a sample peak was verified with a c-di-GMP-spike (Figure 62, A-C). Although the peak indicating the presence of c-di-GMP was small, further optimisation of the samples and growth conditions with this method may be useful to monitor c-di-GMP concentrations under various conditions and determine if c-di-GMP plays a regulatory role in aggregation of UPEC 536 grown in RPMI 1640. The HPLC method detects gross levels of intracellular c-di-GMP although localised changes in concentration may be more important (Hengge 2009).

To summarise, YhjK is hypothesised to be a phosphodiesterase that can reduce c-di-GMP levels and de-activate cellulose production, thus leading to dispersal. The mechanism by which iron (manganese and zinc) act to induce dispersion is not clear, as phosphodiesterase mutants form stronger aggregates, and iron and manganese promote incomplete dispersion, suggesting there may be other proteins involved. The increased strength of UPECΔyhjK correlates to that of R-24 aggregates and appears not to be due to large changes in the composition of the matrix but may reflect structural changes in the cellulose as observed in P. fluorescens where acetylation of the cellulose enhances the strength and adhesion of the biofilm (Spiers et al. 2003).
Chapter 7. Infection and Immunity

7.1. Introduction

Biofilms, or aggregates of bacteria encased in a polymeric matrix, are inherently more resistant to antimicrobial agents than planktonic cells (reviewed in Donlan 2002). The UPEC 536 biofilm matrix is composed mainly of cellulose with other minor polymers integrated within it (Chapter 3). This section investigates protection provided by the matrix against antibiotic killing using an outgrowth assay, protection from host innate immune responses using a caterpillar infection model and the relevance of cellulose-based aggregation during infection by clinical isolates.

There are no standard methods for determining the resistance of biofilm cells to antimicrobial agents. Biofilm cells are more difficult to analyze due to fact that they are aggregates of many cells and many phenotypes may be represented due to the unique environments that may exist within the matrix. Some methods monitor the resistance of biofilm cells by physically removing the biofilm from a surface and enumerating viable cells, assessing growth and / or biomass production (Ceri et al. 1999) or monitoring cells by microscopy, before and after treatment with the antimicrobial agent or a combination of a variety of methods. Regardless of the methodology used to assess viability of biofilm cells after treatment with antimicrobials exact enumeration will always carry some degree of uncertainty.

7.2. Antibiotic Outgrowth Assay

It is hypothesised that treatment with antibiotic will substantially reduce the number of planktonic cells but cells within cellulose aggregates will be protected from antibiotic killing. Gentamicin was chosen as an antimicrobial agent to test the resistance of UPEC 536 biofilms and chosen for this assay as this drug is used in the clinical setting for the treatment of persistent urinary tract infection (Pohl 2007). To investigate the resistance of UPEC 536 to gentamicin an Outgrowth Assay was developed. Initial testing was done to determine the "Kill Time" for all cells within the aggregates as described in Chapter 2 section 2.15 ensuring
that cells within the aggregate could be killed. The "Kill Time" by heat, for cells within the aggregates to die was determined to be 10 minutes at 72°C.

To show that Gentamicin was able to reduce growth, an antibiotic outgrowth assay was first performed on planktonic cells. A 5 ml culture of UPEC 536 in LB broth (LB prevents aggregate formation) was grown to OD$_{600}$nm=0.1. The culture was diluted 1:50 with fresh LB to four new tubes (treated and untreated in duplicate). A sample of planktonic cells was taken from each tube for enumeration of viable cells (section 2.2.4) representing T= minus 2 hr. Gentamicin was added to the treatment tubes and all tubes incubated for 2 hours at 37°C with shaking. Optimisation of this assay indicated that two hours was the minimum time required to show an effect in cell reduction. After treatment, all tubes were centrifuged and washed twice in fresh LB to remove any residual antibiotic. The cells were resuspended to full volume with LB broth, a sample taken for enumeration (T=0), incubated at 37°C with shaking and enumerated for viable cells after 2 and 3 hours (T=2 and T=3, respectively). Gentamicin treatment reduces cells by approximately 2.5 log values after two hours of treatment (Figure 63). Outgrowth indicates that cell growth begins to climb significantly after two hours. Cells undergoing antibiotic and no antibiotic treatment were visually monitored using live/dead staining (Figure 64, A-L). Culture undergoing no treatment was visualised at T0 and at T3 and shows an increase in cells over time (Figure 64, A-F). Planktonic cells treated with Gentamicin show reduced numbers at T0 and an increase after three hours (Figure 64, F-L) indicating efficacy of Gentamicin to reduce the number of planktonic cells.

**Figure 63. Gentamicin Kills Planktonic Cells.** UPEC 536 cells grown in LB broth and treated with Gentamycin decrease approximately cells by approximately 2.5 log values. Outgrowth of the remaining viable cells reaches that of untreated cells after greater than 3 hours incubation in fresh LB media.
Figure 6.4. Planktonic Cells are Reduced by Gentamicin Treatment. Planktonic cells undergoing no treatment (A-F) Live cells stained with Syto9, and (B) dead cells stained with Propidium iodide. C) Merged images at T0 (C) and three hours out-growth (F). Images (G-I) show cells after 2 hours Gentamicin treatment and resuspended in fresh LB broth (I) and three hours outgrowth of planktonic cells (T3) (L). Cells were pelleted and resuspended 1:10. Scale bar = 50 µm.

To determine the sensitivity of aggregates to antibiotic treatment, an overnight culture of UPEC 536 (RF) was diluted 1:100 to fresh RPMI (RPMI induces aggregates) to treated and untreated labelled tubes (in duplicate for each time point, T-2, T0, T2, T3) and the culture
grown to Rmax. At Rmax, the T minus 2 hour tubes were centrifuged at 610 x g for 2 min and a sample of planktonic cells taken for enumeration of viable cells before treatment. Gentamicin was added to the treatment tubes and all samples incubated at 37°C with shaking for 2 hours. At T0, planktonic cells were separated from the aggregates by centrifugation at 610 x g for 2 min and the planktonic cells discarded. Aggregates were washed twice in fresh LB to remove residual antibiotic and then resuspended in LB broth. The culture was centrifuged at 610 x g for 2 min and a sample of the planktonic portion removed for enumeration of viable cells. All tubes were incubated at 37°C with shaking and planktonic cells sampled again at 2 hours and 3 hours incubation to determine outgrowth from surviving planktonic cells. At each time point the tubes were spun at 610 x g for 2 min and a sample taken from the upper portion of the culture (Figure 65). The number of viable cells versus pre and post incubation with antibiotic was plotted over time.

![Figure 65. Overview of Aggregate Outgrowth Assay.](image)

Results indicate that the outgrowth of cells from treated aggregates matches that of untreated aggregates suggesting cells may have protection from Gentamicin treatment (Figure 66, A). Microscopic examination of aggregates without Gentamicin treatment shows (Figure 66, B and D) many cells both live and dead throughout the matrix. Gentamicin treated (2 hour)
aggregates show some areas of the aggregate to have a high concentration of cells, both live and dead (Figure 66, E-G).

![Graph showing CFU/ml over time for antibiotic treatments.](Image)

**Figure 66. Cellulose Provides Protection against Gentamicin.** Gentamicin treated aggregates were dispersed in LB broth. Aggregates treated with antibiotic appear to release viable cells in a manner similar to untreated aggregates (A) suggesting the cellulose matrix may provide protection against Gentamicin. Aggregates stained with live/dead stains and Calcafluor White show untreated aggregates with many live and dead cells distributed throughout the matrix (B-D) and treated aggregates show areas of high cell density but contain both live and dead cells (E-F). Scale bar = 50 µm.
7.3. The Effect of Gentamicin on UPECΔyhjK Aggregates

The matrix of UPECΔyhjK contains substances that are extremely difficult to disrupt specifically colanic acid (Figure 59). It is hypothesised that the increased colanic acid may provide more protection to the cells within the matrix against the effects of Gentamicin. Live/dead staining and CLSM were used to examine the aggregates before and after Gentamicin treatment. The UPEC 536 and UPECΔyhjK aggregates appear to contain equivalent proportions of both live and dead cells in both treated and untreated samples (Figure 67, A & B). The addition of Calcofluor White reveals that some UPECΔyhjK aggregates have a non-uniform distribution of cellulose of the matrix and a higher proportion of dead cells within the cellulose deficient area. (Figure 67, C & D). A Z-stack was performed on the imaged aggregates to determine antibiotic penetration into the aggregate. Results of the Z-stack showed no difference between the internal and external structure (data not shown).

**Figure 67. UPECΔyhjK displays a Non-uniform Cellulose Matrix.** Gentamycin treated aggregates were dispersed in LB broth. Aggregates of UPEC 536 and UPECΔyhjK show many live cells (green) and dead cells (red) (A and B). Gentamicin treatment of UPEC 536 aggregates does not increase the proportion of dead cells (C) but UPECΔyhjK aggregates appear to have a non-uniform cellulose matrix where dead cells appear to be increased relative to other areas of the aggregate (D). Scale bar = 75 µm.
7.4. Antibiotic Penetration of the UPEC<sup>Δ</sup>yhjK Matrix

The penetrance of antibiotic into the biofilm matrix was investigated in UPEC<sup>Δ</sup>yhjK. As UPEC<sup>Δ</sup>yhjK aggregates have a higher concentration of colanic acid it was hypothesised that the polymer may affect the penetrance of Gentamicin and/or affect only cells on the periphery of the aggregate. UPEC<sup>Δ</sup>yhjK aggregates treated with Gentamicin show predominately live cells in the centre the aggregate (Figure 68, A) and dead cells appeared increased around the periphery of the aggregate (Figure 68, B). The merged image (Figure 68, D) shows the boundaries of the matrix where on the periphery fewer cells are present and of these more cells are dead suggesting the matrix of the YhjK mutant may contain some areas that make bacterial cells more sensitive to gentamicin treatment.
Figure 68. Penetration of Gentamicin in the UPECΔyhjK Matrix. Gentamicin treated aggregates were examined 2 hours after treatment using Live/Dead and Calcofluor White stain. Aggregates of UPECΔyhjK show large amounts of live cells in the centre of the aggregate and dead cells around the periphery (A and B). The cellulose matrix (C) shows the boundaries of the aggregate and the merged image shows fewer cells associated with the aggregate on the outside edges, most of which are dead (D). Scale bar = 50 µm.
7.5. *Galleria mellonella* as an Infection Model for UPEC 536

The use of insects as infection models is gaining popularity for study of innate immune responses. Mammals and insects share many features; specifically the insect hemocytes and the mammalian neutrophils function similarly by engulfing, phagocytosing and killing pathogens (Kavenagh and Reeves 2007). Upon infection, immune factors are stimulated and the invading bacterium are encapsulated within melanin (melanization) (Figure 72) which produces free radical by-products during the formation of the capsule which is thought to enhance killing of bacteria (Cerenius and Soderhall 2004; Kavenagh and Reeves 2007).

*G. mellonella* was used as a host to examine the effect of cellulose during UPEC 536 infection using the cellulose+ parental strain, the UPECΔ*bcsA* cellulose- strain, and the UPECΔ*yhjK* modified cellulose strain. It is hypothesised that cellulose will provide protection to the bacteria against the larvae immune defense, decreasing survival of the larvae.

![PBS Treatment Control](image1) ![Treated G. mellonella](image2)

**Figure 69. Galleria mellonella Larvae.** The treatment control sample larvae are alive, creamy white in colour and produce silk. Larvae treated with UPEC 536 10⁷ cfu ml⁻¹ die within 24 hours from infection and turn black due to melanization.

The experimental conditions affecting UPEC killing in *G. mellonella* were optimized by making a series of bacterial concentrations and treating a set of larvae with a different concentration and determining the dose giving the best dose response. The concentration used was 10⁷ cfu/ml. Caterpillars were placed at RT for at least 10 minutes to allow them to warm up and be less rigid. The larvae are approximately 2-3 cm x 0.5 cm in size and weigh
approximately 250 mg, although this may vary. All experimentation groups contained 15 larvae and each experiment was repeated using larvae from a different batch. In all experiments there were two negative control groups; one that underwent no manipulation, while the other group (uninfected control) was injected with PBS only, to control for the impact of physical trauma and the inoculum diluent. During the experiment the larvae were stored in 85 mm petri dishes in the dark at 37ºC for a maximum of 72 hours. Larvae were inspected every 24 hours and considered dead if they did not respond to touch.

Caterpillars were infected by injecting 10 µl of washed bacteria into the body cavity (haemocoel) via the lower left pro-leg on the larvae’s underside, with a 27 guage insulin syringe. The pro-leg is the thinnest part of the larvae’s outer layer (cuticle) and therefore, minimizes damage to the insect. Injection requires holding the larvae in one hand and pressing firmly between the thumb and index finger to keep the larva rigid and to expose the pro-leg. After injection each group were placed into a petri dish for incubation.

The larvae were injected with bacteria at a concentration of 2 x 10⁵ cfu per caterpillar and observed daily for survival. The percentage of G. mellonella survival was plotted against time (Figure 70.) The data indicates increased survival of caterpillars infected with UPEC∆bcsA (p<0.05) and the UPEC∆yhjK strains (p>0.05) suggesting the aggregating strains exhibit more virulence than the cellulose deficient strain.

**Figure 70.** UPEC 536 Virulence in G. mellonella. Larvae were injected with 2x10⁵ cfu ml⁻¹ and monitored daily for survival. Results are an average of four assays and indicate greater survival in the UPEC∆bcsA and UPEC∆yhjK strain than UPEC 536 after 3 days. The data shown is the mean of four independent experiments (n = 15 insects / experiment). Data represents a significant change for UPEC∆bcsA but not UPEC∆yhjK. (Δ represents UPEC 536, ■ represents UPEC∆bcsA, ▲ represents UPEC∆yhjK and ◊ represents PBS)
Verification of infection was carried out on the third day after infection. Hemolymph was recovered from five untreated, PBS treated, UPEC 536 living, UPECΔbcsA living and five dead larvae (pooled) and combined in an Eppendorf microfuge tube. The total volume was diluted to 250 µl with LB broth. Twenty five µl was plated on to a BAP and a MAC agar plate and the remainder held overnight as an enrichment broth. Controls for treatment (untreated) in injection (PBS) indicate that no coliforms were present in the larvae (Table 18). UPECΔbcsA live samples indicate an infection by coliforms (>1000 cfu/plate) and the larvae appeared dark in colour. Bacteria recovered from the "dead larvae pool" indicate an infection by coliforms (>1000 cfu/plate) and all dead larvae had melanised. The UPEC 536 coliform recovery was low (<20 cfu/plate) indicating these larvae were able to overcome the infection and eliminate the bacteria.

<table>
<thead>
<tr>
<th>Sample (pool of 5 larvae haemolymph)</th>
<th>Coliform Recovery*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>0</td>
</tr>
<tr>
<td>Phosphate Buffered Saline</td>
<td>&lt; 10</td>
</tr>
<tr>
<td>UPECΔbcsA (10⁷) live</td>
<td>&gt; 1000</td>
</tr>
<tr>
<td>UPECΔyhjK (10⁷) live</td>
<td>Not done</td>
</tr>
<tr>
<td>UPEC 536 live</td>
<td>&lt; 20</td>
</tr>
<tr>
<td>Dead Larvae (pool)</td>
<td>&gt; 1000</td>
</tr>
</tbody>
</table>

* coliforms were recovered on a blood agar and a MacConkey agar plate

7.6. Clinical Isolates

Laboratory bacterial strains and clinical strains may differ in their pathogenesis and may respond differently to laboratory based testing (Smith 2000). In vivo bacterial growth encourages bacteria to adapt to a rapidly changing environment and utilize strategies to evade immune defenses. Laboratory strains that have been sub-cultured for decades may have lost important patho-physiological characteristics due to the accumulation of genetic changes. Therefore, laboratory-adapted reference strains may not reflect "real world" pathogenesis (Fux et al. 2005, Stover et al. 2000). Importantly, standard laboratory growth conditions may fail to simulate in vivo adaptive strategies used by pathogens.

With these considerations in mind, a small sample of twelve fresh clinical isolates were acquired from Auckland Hospital and tested for growth in RPMI, aggregation and dispersion.
and the production of cellulose during aggregate formation. As most uropathogens fit into a small family of serotypes, the twelve isolates were also serotyped for O antigen.

Seven of the isolates formed aggregates (Table 18) and in each case, aggregates of bacterial cells, identified by phase contrast microscopy and showed staining of cellulosic material (Figure 71, A & B). Five isolates that did not aggregate, which included two isolates from asymptomatic patients, did not stain with Calcofluor White indicating cellulose was not produced (Table 18).

The aggregating clinical isolates from patients with UTIs were tested for iron-induced dispersal, and aggregation and dispersal in the presence of exogenous cellulase (Table 19). Each strain dispersed upon the provision of 10 µM FeCl₃. The addition of cellulase disrupted preformed aggregates and also inhibited aggregation if added to the initial culture. The aggregates of two isolates, OF 5409 and OF 6636, showed partial dispersal from preformed aggregates upon the addition of cellulase, suggesting that in some cases, the matrix of the aggregate may contain other polymers.

Table 19. Characteristics of Uropathogens

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relative AI Reduction/ 3 hours (FeCl₃)</th>
<th>Relative AI Reduction/ 3 hours (cellulase)</th>
<th>Calcofluor White Stain</th>
<th>O Antigen</th>
</tr>
</thead>
<tbody>
<tr>
<td>UPEC 536</td>
<td>60</td>
<td>65</td>
<td>pos</td>
<td>O6</td>
</tr>
<tr>
<td>MG 1655</td>
<td>NA</td>
<td>NA</td>
<td>neg</td>
<td>OR</td>
</tr>
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<td>OF 5409-C</td>
<td>56</td>
<td>18</td>
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<td>NI</td>
</tr>
<tr>
<td>OF 6636-C</td>
<td>51</td>
<td>22</td>
<td>pos</td>
<td>NI</td>
</tr>
<tr>
<td>OF 5862-P</td>
<td>61</td>
<td>66</td>
<td>pos</td>
<td>saline</td>
</tr>
<tr>
<td>OF 6020-C</td>
<td>66</td>
<td>30</td>
<td>pos</td>
<td>NI</td>
</tr>
<tr>
<td>OF 6786-C</td>
<td>41</td>
<td>63</td>
<td>pos</td>
<td>NI</td>
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<td>OF 6860-P</td>
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<td>67</td>
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<td>OF 6762-C</td>
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<td>OF 6703-C</td>
<td>NA</td>
<td>-</td>
<td>neg</td>
<td>NI</td>
</tr>
<tr>
<td>OF 5179-C</td>
<td>NA</td>
<td>-</td>
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<td>NI</td>
</tr>
<tr>
<td>OF 5625-AB</td>
<td>NA</td>
<td>-</td>
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</tr>
<tr>
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</tr>
<tr>
<td>OF 6869-C</td>
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<td>NI</td>
</tr>
<tr>
<td>CFT073</td>
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<td>-</td>
<td>-</td>
<td>O6</td>
</tr>
<tr>
<td>UTI89</td>
<td>NA</td>
<td>-</td>
<td>-</td>
<td>018</td>
</tr>
</tbody>
</table>

* denotes cystitis isolate, † pyelonephritis isolate, ‡ asymptomatic, bacteruria, § model lab strains, ¶ uropathogens, NA= No Aggregation, NI = not identified, saline agglutination in saline. Each result is based on an average of three independent experiments except for serotyping, except serotyping and Calcofluor White staining.
Figure 7.1. Clinical Isolates Produce Cellulose. An example of a clinical isolate strain that formed aggregates when grown in RPMI. Two populations of bacteria are present in culture – planktonic cells and those involved in aggregation (A). Aggregated cells produce cellulose (B). Scale bar= 50 µm.

7.7. Discussion

The objectives of this section were to demonstrate the protective nature of the cellulose matrix of UPEC 536. Protection from the effects antibiotic treatment was achieved using an antibiotic outgrowth assay and protection from innate immune responses was demonstrated using a G. mellonella infection model. The viability of bacterial cells within a matrix after assault from either biocides or immune molecules is not easily demonstrated. The degree of difficulty lies in the aggregate. It is difficult to enumerate bacteria within an aggregate and to assess viability of cells as the number of cells within an aggregate is often too high to distinguish specific differences. As lab strains do not always display the mechanisms used by clinical isolates during infection, hospital acquired isolates were tested for cellulose production and aggregation in RPMI 1640 and dispersion upon the supplementation of iron. Are the characteristics observed in UPEC 536 conserved in other UPEC strains?

The small sample of clinical isolates tested indicates that the phenotype displayed by UPEC 536 is conserved within a larger subpopulation of clinical strains (Table 19). Seven of the twelve isolates formed aggregates when grown in RPMI, and in each case showed cellulose by Calcofluor White stain (Figure 7.1) and dispersal when supplemented with cellulase.
or 10µM FeCl₃. Five of the samples did not aggregate nor was cellulose present in the culture. Two isolates showed only partial dispersion from preformed aggregates upon the addition of cellulase, suggesting that in some cases, the matrix of the aggregate may contain other polymers.

In a urinary tract infection, attachment and aggregation of bacteria are the initial steps in colonization of the urinary tract (Mulvey 2002). The recurrence and re-infection also rely on the ability of bacteria to aggregate and form microcolonies (Hunstad and Justice 2010). Colonization is therefore an important aspect of urinary tract infection to understand and its inhibition a candidate for control. Treatment of urinary tract infection relies on the use of antibiotics to kill planktonic cells but conventional treatments may not be adequate for the treatment of biofilm cells in chronic infection. Gentamicin is an aminoglycoside antibiotic for persistent urinary tract infection, that exerts its mode of action through the inhibition of protein synthesis by binding to the 30S ribosomal subunit of the bacteria (Harvey and Champ 2009) indicating that a decrease in cell division (rather than cell lysis) would be the expected outcome. The outgrowth assay was able to demonstrate the effectiveness of Gentamicin treatment on planktonic cells (Figure 63) with a 2.5 fold decrease in cell numbers after two hours of treatment (Figure 64). Aggregates treated with Gentamicin showed an outgrowth that was similar to untreated aggregates, suggesting protection (Figure 66, A). Unfortunately, the difficulties with testing aggregates may also effect the interpretation of this assay. The seed inoculum released from the aggregate in LB broth may in fact be too large to accurately assess differences in high numbers of bacteria in UPEC 536 (Figure 66, B-G). Although outgrowth is useful to assess antibiotic treatment of planktonic cells there may be limitations on how this test can be used to assess cells within aggregates.

Aggregates of UPECΔyhjK show a modified cellulose matrix. These aggregates were treated with Gentamicin and assessed for cell viability by live/dead fluorescent staining, using epi-fluorescence microscopy and CLSM. Images of gentamicin treated aggregates show areas of the matrix that have a higher proportion of dead cells (Figure 67), although Z-stack analysis did not show any changes in cell viability between the external and internal structure of the aggregate. Some aggregates of UPECΔyhjK show distinct differences in live/dead areas of the matrix where many dead cells lie on the periphery of the matrix (Figure 68, B). This investigation was unable to demonstrate definitive effects of antibiotic treatment on aggregates but did indicate that differences in cell viability are present in gentamicin treated
UPEC\textsuperscript{∆}yhj\textsubscript{K} cells but would require more study to draw any conclusion about the role of the UPEC\textsuperscript{∆}yhj\textsubscript{K} matrix in protection from antibiotic treatment.

\textit{Galleria mellonella} is a useful model to study innate immune responses as many aspects of human innate responses are similar. Hemocytes and neutrophils utilize the same biochemical substances to kill pathogens, making insects a useful and cost-effective alternative in the screening of pathogen innate immunity interactions. Insect immunity involves both cellular and humoral defenses consisting of the process of melanization, hemolymph clotting and the production of a number of potent antimicrobial peptides (Kavenagh and Reeves 2007). Melanization (Figure 69) is the formation of a black pigment (a polymer of dihydroxyindole carboxylic acids) and is triggered by the presence of minute amounts of bacterial molecules such as \(\beta\)-1,3 glucans, lipopolysaccharides and peptidoglycan (Cerenius and Soderhall 2004). The humoral component of the insect immune response involves the production of antimicrobial peptides that damage the bacterial membrane. The cellular response involves synthesis and mobilization of immune cells called hemocytes that act similar to human neutrophils by engulfing invading organisms and releasing a burst of reactive oxygen species (ROS) (Bergin \textit{et al.} 2005). Bacterial virulence tested in \textit{G. mellonella} correlates with virulence tested in BALB/c mice (inbred mice used in cancer and immunology research) (Brennen \textit{et al.} 2002) highlighting the versatility of the insect model. \textit{G. mellonella} have been used as infection models for other human bacterial pathogens such as \textit{Pseudomonas aeruginosa} (Miyata \textit{et al.} 2003), \textit{Bacillus cereus} (Fedhila \textit{et al.} 2006), \textit{Proteus mirabilis} (Morton \textit{et al.} 1987) and \textit{Francisella tularensis} (Aperis \textit{et al.} 2007). The use of \textit{G. mellonella} is a useful alternative to provide comparable data from mouse models but in a more cost-effective, less laborious and more ethically acceptable manner. A glimpse into what role cellulose may play in bacterial virulence was tested, and suggests increased virulence during infection by the parental strain (Figure 70) than either the UPEC\textsuperscript{∆}bcs\textsubscript{A} or UPEC\textsuperscript{∆}yhj\textsubscript{K} strain. A broader examination must be carried out, to draw a definitive conclusion about the protective role of cellulose, perhaps in a mouse UTI model to encounter the effects of both innate and adaptive immunity.

Overall, the results of this section were useful to show that cellulose provides a protective barrier to biofilm cells. Cellulose production and aggregation are conserved phenotypes seen in clinical uropathogens. Assessment of bacterial virulence in alternative infection models
and the effects of antibiotic treatment are useful assays to assess potential treatment strategies. Current antibiotic test have been developed for planktonic cell suspensions. Aggregates of cells do not translate well to this mode of testing, but are probably the more relevant mode of growth to test for treatment of chronic infection. Alternative criteria for the diagnosis of chronic infection have lead to a new diagnostic criterion to describe biofilm infections that takes into consideration biofilm characteristics. The presence of bacterial aggregates, matrix and viable (culture negative) cells, infection focus, recalcitrance to antibiotic therapy and localized inflammation may help diagnose and treat biofilm-related infections more effectively (Parsek and Singh 2003) although specialized assays to carry out these analysis may not always be available.
Chapter 8. General Discussion

Urinary tract infections are the most common bacterial infections in humans (Foxman 2003) and uropathogenic *Escherichia coli* (UPEC) are the leading cause of these infections (Foxman and Brown 2003). Infections of the urinary tract are approximately fifty times more common in women than men and recurrent infection rates are estimated to exceed greater than 50% (Hunstad and Justice 2010) within one month of initial infection (Ikaheimo *et al.* 1996; Foxman 2002). Infections in children are associated with significantly greater long-term sequale than UTIs in adults and chronic and persistent infections have a negative impact on the quality of life for many women. The costs of treatment of urinary tract infection therefore impose considerable cost to healthcare funding.

Uropathogens persist within the urinary tract through the formation of intracellular bacterial communities that may disperse and reinfect, or reside as quiescent reservoirs and reseed later infections (Justice *et al.* 2004). Intracellular bacterial communities and biofilms produce a matrix to withstand the damaging effects of biocides and immune attack. The matrix is composed of bacterially produced substances that provide a barrier from the environment. In this study it has been shown that UPEC 536 grown in RPMI 1640 media produces a matrix that is composed mainly of cellulose, which provides structure and stability. This has been demonstrated using specific cellulose stains and epifluorescence microscopy, biochemical assays and the construction of deletion mutants unable to produce cellulose. Other components of the UPEC 536 matrix detected were DNA, PNAG and colanic acid although these polymers did not participate in the initial formation of the matrix structure or provide stability to the biofilm.

The dominant polymeric substances produced by a bacterium are often strain specific and dependant on the conditions of growth (Reisner *et al.* 2006; Flemming and Wingender 2010). The EPS matrix of *E.coli* is generally accepted to be composed of cellulose, poly-N-acetyl-glucosamine and/or colanic acid. Each of these polymers can be detected in differing concentrations depending on the media and the conditions in which the bacteria are grown (Reisner *et al.* 2006). In other species of bacteria the EPS has also been studied in detail. *Pseudomonas fluorescens* SBW25 produces cellulose (Spiers *et al.* 2003) as the major component of its EPS. Cellulose in this bacterium is acetylated and provides increased
attachment to air-liquid interfaces and increased strength of the biofilm matrix. *P. aeruginosa* produces alginate as its major EPS component, although extracellular DNA has also been shown to play an important ancillary role in biofilm stability in mature biofilms, but not in early biofilms (Whitchurch *et al.* 2002), suggesting that the expression of EPS components may be temporally regulated. The expression of EPS is often associated with different phenotypes; increased alginate in *P. aeruginosa* displays mucoid phenotypes, whereas the expression of cellulose in *P. fluorescens* on agar plates appears as dry wrinkled colonies, a phenotype also displayed by *S. Typhimurium* and *S. enteritidis* due to cellulose expression (Zogaj *et al.* 2001; Solano *et al.* 2002). In these cases the consequence of EPS production is a biofilm matrix that may protect embedded bacteria from desiccation (Quinones *et al.* 2005), immunity (Kai-Larsen *et al.* 2010) and antibiotics (Leid *et al.* 2005). The understanding that biofilm bacteria are substantially better protected has led to treatment strategies that include specific enzymes to degrade the biofilm matrix (Donelli *et al.* 2007; Lamppa *et al.* 2011). In the case of UPEC, the findings in this thesis suggest that a treatment strategy including a cellulase, delivered to the site of bacterial infection may assist in disrupting biofilm matrix components and allowing antibiotics access to the bacteria. As humans do not produce cellulose, the use of an enzyme specific for a bacterially produced product would be of benefit.

Ideally, the growth conditions used for studying biofilms should mimic the environment in which the bacterium causes infection (Smith 1998). To fully understand UPEC infection and recurrence an understanding of the signals controlling biofilm formation and dispersal is needed. RPMI as a growth medium provides defined essential nutrients for bacterial growth but not nutrients that the bacteria must compete with the host for in order to survive. The iron-restricted environment induces a UPEC 536 phenotype that produces copious amounts of cellulose that appear as flocs in liquid culture when grown at 37ºC. Interestingly, Reisner *et al.* (2006) found no correlation between *E. coli* pathogenicity and the ability to form biofilms on plastic. The findings presented in this thesis support this study. Strains used in the Reisner study were propagated through LB broth as opposed to strains used here that were propagated through RF media (10µM FeCl₃). Our observations were that propagation through iron-enriched media diminished aggregate formation when sub cultured to RPMI, highlighting the importance of maintaining pathogenic characteristics by using growth media that reflects “host” conditions.
There is a transition in phenotype from flocs to planktonic cells upon the supplementation of RPMI with iron. Induction of dispersal of the matrix by iron, manganese and zinc and the biological iron sources tested may provide important insight into the pathogenesis of urinary tract infection. All of the dispersants tested are available from the host from either tissue secretions, urine or faeces and the close proximity of dispersants to biofilm bacteria provides an opportunity for bacteria to re-colonize the urinary tract and progress to an acute infection.

The correlation between urinary tract infection and iron availability through menstruation and/or recent sexual activity has been studied. UTIs occur at a higher rate in younger sexually active women (Hooten et al. 1996), but UTIs do not correlate with menstruation (Scholes et al. 2000). This thesis showed that lactoferrin, an iron binding protein found in vaginal secretions, is able to induce biofilm dispersal. One could speculate as to a link with lactoferrin in vaginal secretions during sexual activity and the onset of recurrent urinary tract infection.

Alternative therapies using iron as a biofilm dispersion inducer, rather than cellulase during drug treatment delivery may be a consideration. Planktonic cells are killed by antibiotics, however, the supplementation of iron in this study showed an increase in bacterial growth concomitant with the production of cellulose and transient aggregates. This approach may have a negative impact on health problems other than the targeted approach using specific ancillary therapies such as cellulase.

Inhibition of biofilm formation is an important aspect to treatment for chronic biofilm related illnesses. Understanding the mechanisms that drive biofilm formation is important in understanding and implementing ways in which to inhibit them. The relationship between c-di-GMP and phosphodiesterase activity has been described in numerous other studies and investigated in this thesis. We have shown that over-expression of phosphodiesterase inhibits aggregation and the production of cellulose in UPEC 536, although deletion of YaiC, the diguanylate cyclase, had no effect on reduction of biofilm formation. YaiC is homologous to AdrA, the regulator of c-di-GMP in S. Typhimurium cellulose biosynthesis (Romling 2005).

Investigation into the GGDEF domain proteins of S. Typhimurium indicate that most GGDEF proteins are functionally related, probably by controlling the levels of cyclic di-GMP, which include among its regulatory targets cellulose production and biofilm formation (Garcia et al. 2004). Lack of effect through interruption of this gene may suggest YaiC does not directly regulate c-di-GMP production for cellulose biosynthesis in UPEC 536. It may
also reflect on complexities associated with the growth conditions used in this study that induce copious amount of cellulose, the intracellular concentrations of c-di-GMP or redundancy in GGDEF proteins for the induction of cellulose biosynthesis. Further investigation into the role of c-di-GMP and YaiC is necessary to draw any conclusions. As a regulator of biofilm formation, interference with c-di-GMP production is an important consideration for drug treatment for chronic and persistent infections (Karaolis et al. 2005) as an inhibitor of c-di-GMP binding would control initial stages of biofilm formation.

This thesis only touched on the possibilities of quorum sensing as a regulator for dispersion. Although the molecules tested indicate homoserine lactone is not associated with dispersion, quorum sensing signaling cannot be ruled out. The consistent time for RF grown bacteria to disperse (approximately 3.5 hours) and the formation of transient aggregates suggest a possibility of quorum sensing involvement in dispersion.

In this study we were able to prevent dispersal by pre-treatment of aggregates with antibiotics that prevent new transcription and translation. Our conclusion is that new gene expression is required to effect the phenotypic changes induced by the transition to an iron-enriched environment. Our results suggest that the production of an endoglucanase or a modifying activity that affects the strength of the cellulose matrix is required to effect dispersal. In *E. coli*, endoglucanase activity resides in BcsZ, which is part of the cellulose operon (Romling 2002), but is not thought to be secreted. In *P. fluorescens*, the beneficial characteristics of the matrix, mainly increased strength and adhesion, require acetylation of the cellulose polymer and the presence of lipopolysaccharide and protein factors (Spiers et al. 2003; Spiers and Rainey 2005). Perhaps a role for YhjK may lay in regulating the maintenance of cellulose integrity for rapid dispersal in favourable environments, in concert with phosphodiesterase activity.

Interestingly, dispersal is not regulated by Fur but the impact of other metal ions on dispersion suggests the involvement of an additional regulatory mechanism. The sensory domains of YhjK may play a role in metal-sensing and induce activity in the presence of specific metal ions similar to the HmsP phosphodiesterase of *Y. pestis* (Bobrov et al. 2005). Growth of UPEC 536 in RPMI with iron induces aggregates within the first few hours of growth, suggesting there may be regulatory input in later stages of growth. Likewise, the expression of GGDEF/EAL proteins occurs in late exponential phase (Somerfelt et al. 2009) as are AHLs and autoinducers associated with quorum sensing (Bassler 2006). During
infection, where UPEC are successful in the acquisition of iron, the results in this thesis support the initiation for dispersion from the cellulose matrix. Further, the release of glucose from the degradation of cellulose can be used as an energy source which may also affect subsequent gene expression mediated by cAMP-CAP (Weyand et al. 2001).

It is important to consider the implications of cellulose in the biofilms matrix of the urinary tract and within urinary catheters. Iron starvation of UPEC induces the production of a cellulose matrix and that overtime, acquires greater stability. Further, the production of cellulose and the formation of aggregates in iron-restricted media is a conserved characteristic in the sampling of clinical isolates tested, and is in agreement with other findings that many pathogenic isolates form cellulose at 37°C (Bokranz et al. 2005; Da Re and Ghigo 2006; Monteiro et al. 2009). Further study is required to make an association between the ability to produce cellulose aggregates and pathogenicity with statistical confidence. To do this the primary results seen using G. mellonella as a model organism may be used to justify experiments in mice, using biofilm producing UPEC and biophotonic imaging (Wiles et al. 2006; Lane et al. 2007)
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