Understanding the Evolution of Extra-Intestinal Pathogenic *Escherichia coli* (ExPEC) 
Via Genetic Analysis of Capsular Prevalence Among Clinical Isolates 
and the Role of Sialic Acid in ExPEC Niche Specificity

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Dissertation submitted in partial fulfillment of 
the requirements for the degree of Doctor of Philosophy in the Department of 
Molecular Genetics and Microbiology in the Graduate School of Duke University 

2013
ABSTRACT

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Abstract

Our purpose was to gain knowledge of the bacterial demographic using recently isolated strains causing uncomplicated UTI; we also sought to understand how capsular type has changed over time. Using multiplex PCR, data show Group 2 encapsulated strains have significantly increased in uncomplicated UTI in the past twenty years, suggesting an expansion of previously under-represented capsule types. Additionally, we find K1 encapsulated ExPEC are significantly associated with symptomatic UTI.

K1 ExPEC are able to catabolize sialic acid for energy (glycolysis), and membrane production (LPS and peptidoglycan), as well as synthesize sialic acid for capsule expression. NanR is an ExPEC global regulator of known virulence factors, however regulation via NanR is controlled by sialic acid. Using an established murine model, fluorescent microscopy, high performance liquid chromatography, and tissue culture methods, we find K1 ExPEC use sialic acid as a signaling molecule to determine location within the host.

Capsule and Type-1 pili (T1P) are important for ExPEC pathogenesis, both in the urinary tract and in more disseminated disease; coordinated regulation of these two virulence factors has been implicated. Using a tissue culture model, Western blot analysis, T1P agglutination and reporter assays, we found capsule and T1P are counter-regulated, and that this mechanism is conserved in non-pathogenic E. coli.
Dedication

To my parents, Vallen and Rebecca Prest, who always believed I could do anything, and were there regardless of rain, sleet, snow, distance, and time. To my sister Anya, thank you for teaching me the true meaning of perserverance. To my grandparents both alive and gone, you never stopped cheering. To the Gunter Estate Girls, both past and present: Katie DeConto, Alaina Kleinbeck, Sarah Howell; for endless hours of laughter, band practices that turned into jam sessions that became youtube videos, Saturday morning coffee, Netflix, HIMYM, and every single night of sanity. To my old lab spouse, Stacey Batemen, you are the reason I never gave up. Only with Christ was this even possible.

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1. Introduction

*Escherichia coli* (*E. coli*) are among the most abundant organisms in humans (Tenaillon et al., 2010); these Gram-negative bacteria are known to be both commensal and pathogenic in nature (Bingen et al., 1998; Johnson et al., 2001; Duriez et al., 2001; Rasko et al., 2008). Pathogenic strains fall into two major groups: diarrheagenic strains including enteropathogenic *E. coli*, enterotoxigenic *E. coli*, and the like, as well as extraintestinal pathogenic (ExPEC; urinary tract, meningitis, blood etc) strains (Roberts 1996). Although each group has genetic markers that typically distinguish them from each other as well as commensal isolates, *E. coli* has considerable genetic heterogeneity within and between groups; markers associated with pathogenesis can be found in strains genotypically categorized as commensal, and vice versa, implicating that host-bacterial interactions are multi-faceted, dependent upon factors such as bacterial virulence, location within a host, and host susceptibility, among others (Bingen et al., 1998; Johnson et al., 2001; Duriez et al., 2001; Rasko et al., 2008). Genetic trees rooted in the closest relative of *E. coli*, *E. fergansii*, show ExPEC are most closely related to the ancestral organism as compared to *E. coli* associated with gastrointestinal disease, and commensals alike (Gordon et al., 2008). Evidence suggests that ExPEC resides at the base of the phylogenetic tree, implicating recent divergence of commensal strains (Bergthorsson and Ochman, 1998; Lecointre et al., 1998; Gordon et al., 2008). This, in
turn, suggests that: (1) a reduction in genomic size has occurred, potentially due to a loss of virulence factors. Consistent with this idea, strains categorized as pathogenic have 0.5-1.0 Mb of additional DNA as compared to commensal strains (Bergthorsson and Ochman 1998); and/or (2) a change in genetic regulation via acquisition of global regulators through horizontal transfer, or evolutionary shaping of genetic regulation via ancestral regulators may have occurred.

1.1 The Epidemiology of UTI

Urinary tract infections (UTI) are among the most common human infections, second only to respiratory infections (Foxman 2010). However, while respiratory infections have numerous infectious etiologies, including viral, bacterial, and fungal pathogens, by comparison 80% of community-acquired UTI are associated with ExPEC (Foxman 2010). Twenty percent of women 15-29 years of age will acquire a single UTI (Foxman & Brown, 2003); of these women, 24% will experience at least one recurrence within 6 months (Foxman et al., 2000). The annual cost for UTI has been estimated at about $2.3 billion USD in 2010 (Foxman 2010); cost estimates are a combination of both direct cost (health care) and indirect costs (loss of productivity at work).

UTI are diagnosed using a combination of clinical symptoms, signs, urinalysis, and culture-based methods. Asymptomatic bacteriuria (ASB) is diagnosed in the context of a positive urine culture and absence of the symptoms and signs of UTI (Scholes et al.,
2000; Vraneš et al., 2003; Hooten et al., 2000). Infection of the bladder, also called cystitis, is described as the host having both symptoms and positive urine culture (Scholes et al., 2000; Hooten et al., 2000). For purposes of this body of work, we delineate a difference between lower tract UTI (cystitis) and upper tract, ascending UTI associated with the kidney (pyelonephritis). UTI may disseminate to the bloodstream and central nervous system under conditions with a susceptible host and an ExPEC strain carrying a combination of virulence factors that cooperate to allow the organism to successfully carry out the stages of infection. Indeed, certain ExPEC strains, such as those encapsulated in sialic acid (K1), have been closely associated with neonatal meningitis (Robbins et al., 1974; Mccracken et al., 1974; Kim et al., 1992; Ørskov et al., 1979; Bonacorsi et al., 2000; Cross et al., 1984; Bingen et al., 1998).

1.2 Phylogenetic Typing

Comparative molecular epidemiology studies of UTI and commensal E. coli isolates aides in identifying the virulence factors that may instigate and perpetuate infections while elucidating novel ways to combat UTI and more disseminated disease. E. coli evolution is observed via analysis of common genetic patterns, through PFGE, MLST, or gene/fragment directed PCR (Gordon et al., 2008; Ochman and Selander 1984; Selander et al., 1986; Herzer et al., 1990; Duriez et al., 2001; Clermont et al., 2000; Bingen et al., 1998). Subsequent sub-divisions enables E. coli to be classified, uncovering clonal
group associations while tracing the evolutionary tree (Ochman and Selander, 1984; Bingen et al., 1998; Gordon et al., 2008; Clermont et al., 2000).

Phylogenetic typing – or phylotyping – is now a standard methodology for approximating the evolutionary relationships between E. coli strains; phylotyping of E. coli was initially performed by pulse-field gel electrophoresis (PFGE) and multi-locus enzyme electrophoresis (MLEE; Ochman and Selander, 1984). It was later simplified as a multiplex PCR (Clermont et al., 2000), relying on the amplification of two genes, chuA and yjaA, and a DNA fragment, TspE4. As described by Clermont et al., certain combinations of PCR amplicon patterns categorize E. coli into different phylotypes; initial use of the multiplex PCR assay identified 4 phylotypes, A, B1, B2, and D (Clermont et al., 2000). Further validation of the Clermont method for phylotyping has arguably identified a fifth phylotype – type E – where none of the genomic markers are amplified, identified by the absence of all amplicons (Gordon et al., 2008). Further evaluation of phylotype E using PFGE demonstrated it is actually a combination of phylotypes A, B1, B2, and D (Gordon et al., 2008). For purposes of our research here, we categorized those strains PCR negative for phylotyping as a separate group and did not draw any further conclusions from this cohort.

It has been observed that certain phylotypes are associated with clinical syndromes or commensalism, though no method for sub-typing E. coli is without
exceptions. In general terms, phylotype A has been commonly associated with commensal strains isolated from the gut of mammals (Ochman and Selander, 1984; Duriez et al., 2001; Bingen et al., 1998). These strains are less likely to cause disease, but are still able to elicit infection in compromised hosts (Johnson et al., 2001; Duriez et al., 2001; Bingen et al., 1998). Phylotype B1 is also associated mostly found among commensals, though B1 has been observed at a lower frequency than phylotype, A, within the gut microbiome (Duriez et al., 2001; Bingen et al., 1998). As with phylotype A, B1 strains are able to elicit infection though at a relatively low percentage (Johnson et al., 2001). ExPEC are comprised mostly of phylotype B2 (Bingen et al., 1998; Ochman and Selander 1984); as mentioned earlier, ExPEC of the B2 phylotype are believed to be the most anciently derived phylotype when compared to phylotypes A, B1 and D (Gordon et al., 2008; Bingen et al., 1998). Strains phylotyped as B2 have been shown to harbor more genes associated with virulence than strains in either phylotype A, B1, or D; phylotype B2 is rarely associated with commensal gut flora (Ochman and Selander, 1984; Lecointre et al., 1998; Gordon et al., 2008; Duriez et al., 2001; Bingen et al., 1998). The fourth phylotype, D, is also associated with ExPEC pathogenesis, though is found less frequently than B2 (Duriez et al., 2001).
1.3 ExPEC Pathogenesis during UTI

The molecular pathogenesis of ExPEC during UTI has been studied in depth within the past 20 years, and it is now known that multiple surface factors are important for ExPEC pathogenesis. These include: adhesins such as type-1 pili (T1P), P pili, and sfa/foc fimbriae; iron acquisition mechanisms (siderophores) – iroN, and fyuA among others; capsule – Group 2 and to a lesser extent Group III types; and toxins – cytotoxic-necrotizing factor I (cnfI), and hemolysin (hlyA), to name a few (Anderson et al., 2004; Arthur et al., 1989; Che et al., 2011; Cross et al., 1984; Elo et al., 1985; Falzano et al., 1993; Gander et al., 1985; Hacker et al., 1993; Huang et al., 2001; Johnson et al., 1988; Roberts 1996; Hunstad and Justice 2010).

Community-acquired UTI begins after bacteria gain access to the urinary tract, though there are multiple theories as to how this may occur; it is generally accepted that ExPEC reside within the intestinal tract, and migrate from the peri-anal region to peri-urethral region; in many cases, factors considered necessary for virulence are also factors that promote colonization of the gastrointestinal tract and migration to the peri-urethral area (Tenaillon et al., 2010; Duriez et al., 2001; Gall et al., 2007; Dobrindt et al., 2003; Ochman and Selander, 1984; Chang et al., 2004; Almagro-Moreno and Boyd, 2009). Once established in the peri-urethral area, ExPEC ascend through the urethra to the bladder; adhesins and flagella are important for residence within the intestine, as well as
movement to the bladder, providing a mechanism for both adherence and motility
(Justice et al., 2004; Hunstad and Justice 2010). Capsule is also important during
pathogenesis, as a mechanism to protect from host innate immune defenses
(phagocytosis, complement-mediated bacteriolysis to name a few), as well as foster
biofilms, post epithelial cell invasion (Marques et al., 1992; Vimr and Lichtensteiger,
2002, Harvey et al., 2001; Wright et al., 2007; Roberts, 1996).

Within the bladder, ExPEC takes up initial residence via adherence to urothelial
cells (Justice et al., 2004). Without adherence, bacteria are flushed out of the bladder by
micturition and are unable to invade uroepithelial cells, obtaining protection from the
extra-cellular host immune response (Abraham et al., 1988; Mulvey et al., 1998; Hunstad
and Justice, 2010). T1P are the major adhesin necessary for adherence to and invasion of
bladder epithelia; T1P null mutants have a severe fitness defect in vivo (Abraham et al.,
1988; Schilling et al., 2003; Wright et al., 2007; Zhou et al., 2001). The lectin tip of T1P has
binds mannosylated uroplakins (Zhou et al., 2001); T1P mediates urothelial invasion
through multiple mechanisms, including invasion of urothelial cells via caveolae,
clatherin, and by uroplakins binding, stimulating uptake into Rab27b-positive fusiform
vesicles (Bishop et al., 2007). Due to association with fusiform vesicles, which are used to
regulate the surface area of the bladder, many invading ExPEC are also expelled from
the epithelial cell by the same mechanism as uptake (Bishop et al., 2007). Indeed,
bacterial adherence does not guarantee an invasive event, as invasion is known to be at least a relatively rare event (Wright et al., 2007; Hunstad and Justice 2010). Those ExPEC that are not expelled into the lumen escape into the cytoplasm of the host cell in a yet unknown fashion and are the initial seed for intracellular bacterial communities (IBCs; Inset (1); Figure 1; Mulvey et al., 1998; Justice et al., 2004).

IBCs are clonal intracellular biofilm-like communities that are (Justice et al., 2004; Wright et al., 2007; Rosen et al., 2007) closely associated to the nucleus within the host epithelial cytoplasm (Inset (2); Figure 1; Wright et al., 2007; Mulvey et al., 1998; Bishop et al., 2007). Initially IBCs are comprised of bacillary ExPEC; as the bacterial cells divide, IBCs grow in size, and daughter cells obtain a coccoid shape characteristic of biofilm formation (Justice et al., 2004). This change in morphology enables a greater number of ExPEC to fill a smaller space; therefore, mid to late IBCs are packed with approximately $10^5$-$10^6$ ExPEC per urothelial cell (Anderson et al., 2003; Justice et al., 2010). Both T1P and capsule are expressed within an IBC (Inset (2-4); Figure 1; Justice et al., 2004; Wright et al., 2007; Anderson et al., 2010), and mutants unable to express T1P or capsule are unable to survive in vivo, suggesting that these extracellular factors are necessary for IBC formation and survival (Wright et al., 2007; Anderson et al., 2010). Occupying the majority of the epithelial cell after 24 hours, peripheral ExPEC within late IBCs become
motile and either bacillary or filamentous in shape, exiting the host cell to infect again (Justice et al., 2004; Hunstad and Justice, 2010; Justice et al., 2008; Hovarth et al., 2010).

Figure 1: The Intracellular Life-cycle of ExPEC

ExPEC are introduced into the luminal space of the bladder, triggering the innate immune system and promotes influx of neutrophils (neutrophils 🍊), which cleave sialic acid from epithelial cells (sialic acid 🌅). Inset: T1P mediated adherence and invasion occurs. Inset: (1) ExPEC invade uroepithelial cells through several mechanisms, many of which are contained in Rab27b positive vesicles. (2) Intracellular bacterial communities begin to form; bacteria within early IBCs are clonal populations of rod-shaped cells. (3) Intracellular bacterial communities continue to mature and grow in size; bacteria within mid-late IBCs are clonal populations of cocci. (4) Intracellular bacterial communities enlarge, and bacteria begin to filament and flux out of the IBC and host cell by 24 hours. Subsequent invasion occurs, though IBCs mature at a slower rate.
ExPEC adherence and invasion by T1P activates host immunity (Schilling et al., 2003; Hunstad et al., 2005; Shahin et al., 1987). In an uncompromised host, neutrophil activation is one of the initial responses of the innate immune system (Shahin et al., 1987). To some extent, certain ExPEC strains are able to suppress the epithelial cytokine response as compared to commensal E. coli; however, the binding of T1P and activation of a TLR4 response via LPS is able to stimulate the release of cytokines from the bacterially bound host epithelial cells, mediating neutrophil chemotaxis (Schilling et al., 2003; Hunstad et al., 2005). Translocation of sialidases to the surface of neutrophils cleaves sialic acid from the bladder epithelium (Figure 1; Sakarya et al., 2004; Shahin et al., 1987). Additionally, an inflammatory response stimulates host exfoliation of the superficial epithelial cells, and eventual loss reveals the underlying transitional epithelial layer (Schilling et al., 2003; Hunstad and Justice, 2010).

Bacillary ExPEC that have exited host cells before exfoliation and/or micturition are most likely engulfed by localized neutrophils and macrophages; however, ExPEC also exit the host cell in filamentous form, and due to the increased length of the cell morphology, are able to avoid the innate immune system (Figure 1; Inset (4); Hovarth et al., 2011; Justice et al., 2004; 2006; 2008). Non-synchronized re-infection and deeper invasion is mediated by ExPEC, and due to exfoliation of the bladder epithelia, ExPEC now have access to deeper host tissue (Hovarth et al., 2011; Justice, 2006). Depending
upon the invasiveness of the ExPEC strain and the susceptibility of the host, ascension to
the kidneys or dissemination into the bloodstream may occur, where a myriad of pili,
toxins, and other invasive factors, including T1P and capsule, are required for survival
(Mulvey et al., 1998; Justice et al., 2004; Wright et al., 2007).

1.4 Polysaccharide Capsules

The classical virulence factor capsule, K-antigen, is comprised of acidic
polysaccharides (reviewed in Jann and Jann, 1987) and is integral for invasive disease.
The broadest definition of capsule includes both excreted exo-polysaccharides as well as
cellular attached extra-cellular polysaccharides (Roberts, 1996). We prefer a stricter
definition of capsule, which only includes membrane-attached extra-cellular
polysaccharides. Capsule is a multi-faceted virulence factor, protecting the bacterial cell
from the environment it may encounter (Roberts, 1996). This includes both intra- and
extra-host environments, protecting the bacterium from dessication in harsh innate
environments, as well as the host immune system (Roberts, 1996; Whitfield, 2006).

Capsule enables bacterial protection from the host immune system in various
ways. Many of the surface factors required for virulence are also antigenic, potentially
leading to a host response that may result in clearance of the infection (Schembri et al.,
2004; Roberts 1996). Expressing necessary virulence factors, while limiting the host
immune response, is partially reconciled by masking surface antigens with capsule
(Schembri et al., 2004; Roberts 1996). This allows bacteria to have antigenic virulence factors at the ready without over-stimulation of the host immune response and/or host recognition (Schembri et al., 2004; Whitfield, 2006). In addition to masking the bacterial cell surface from the host immune system, certain capsular types are composed of sugars mimicking host cells (Harvey et al., 2001), enabling bacterial avoidance of the host immune system, since bacterial cells will be more likely recognized as “self” by the host.

Capsules are also able to interact with the host immune system in other ways. Not only are most capsules anti-phagocytic, they also inhibit antibody deposition and complement activation (Marques et al., 1992; Vimr and Lichtensteiger, 2002). Recently, it has been observed that certain capsules, those anionic in nature, are able to act as a type of sink, interacting with positively charged defensins, and inhibiting their ability to reach the bacterial surface, rendering them inactive (Campos et al., 2004; Llobet et al., 2008). This means that even if a bacterium is recognized as an intruder to the host, capsule has the ability to inhibit the bacterium’s removal.

1.5 Escherichia coli Capsule

Capsule is commonly expressed in E. coli (Corbett and Roberts, 2008). The approximate 80 different K-antigens in E. coli, are subdivided into 4 different groups, largely based on biochemical and genetic data (Corbett and Roberts 2008; Whitfield and Roberts, 1999; Roberts 1996). Capsules have a large impact on pathogenesis, with
different capsule types associated with certain disease manifestations (King et al., 2007; Corbett and Roberts 2008). Because they have a role in colonization, persistence, systemic spread, and are ubiquitous amongst *E. coli*, a critical role for capsules during ExPEC UTI might be anticipated (King et al., 2007; Corbett and Roberts 2008; Whitfield and Roberts, 1999; Roberts 1996). Extracellular polysaccharides that are polymerized and exported out of the cell to compose capsule are termed K-antigens; while all K-antigens are capsules, not all capsules are comprised of K-antigens, as proteinaceous capsules are also possible; for the purposes of this body of work, we will focus only on capsules comprised of K-antigens (Whitfield and Roberts, 1999).

Group 1 capsules are comprised of high-molecular-weight capsular K antigens, able to mask O-antigen and constitutively expressed at all growth temperatures (Whitfield and Roberts, 1999; Roberts 1996). This group is comprised of what was initially characterized as Group 1a, though will be referred to throughout this body of work as Group 1 (Roberts 1996; Whitfield and Roberts 1999). In general, Group 1 capsules are mostly considered lipid-A linked, and unlike Groups 2-4 are unable to be co-expressed with colanic-acid (Whitfield and Roberts 1999). They are encoded at the genetic locus that also contains the O-antigen synthesis genes and are only expressed with a limited number of O-antigens, O8 and O9 (Whitfield and Roberts, 1999; Whitfield, 2006).
Group 4 capsules include what was formerly known as Group 1b “O-antigen capsules;” conventional labeling has shifted to replace this antiquated categorization and moved toward including these capsular types as a separate group (Whitfield and Roberts, 1999). Categorization of Group 4 capsules is also considered O-antigen in type as these capsules are lipid-A linked (K\textsubscript{LPS}). They are assembled in a similar fashion as Group 1 capsules, using similar capsule machinery, and can be expressed at all temperatures; however, unlike Group 1, Group 4 capsules can be co-expressed with colanic acid (Whitfield and Roberts, 1999).

Bacteria encoding for Group 2 capsule are generally facultative animal ExPEC (Russo and Johns, 2003; Moulin-Schouleur et al, 2006; King et al., 2007). Group 2 capsule genes are neatly organized into three genetically linked regions, and the locus is conserved across all Group 2 encapsulated \textit{E. coli} (Roberts et al., 1996; Whitfield and Roberts, 1999). Though we focus here on Group 2 capsules in \textit{E. coli}, it is important to note that these are both structurally and functionally related to capsules expressed by other pathogenic bacteria, including \textit{Neisseria}, \textit{Haemophilus}, \textit{Salmonella}, and \textit{Klebsiella} (reviewed in Jann and Jann 1987; Kroll et al., 1990; Frosch et al., 1991; Hashimoto et al., 1993)
1.6 Group 2 Capsule

Group 2 capsule loci are generally situated near the serA locus (Orskov and Nyman 1974; Jann and Jann, 1987). This locus is divided into three regions that encode for both biosynthetic and transport genes (Bliss and Silver, 1996; Vimr et al., 1995; Whitfield and Roberts, 1999; Boulnois et al., 1987; Vimr et al., 1989; Roberts et al., 1986). Regions 1 and 3 are homologous in Group 2 encapsulated strains, though the capsule polymer – or serotype – synthesized in Region 2 differs; the genetic organization of the capsule polysaccharide (kps) locus is conserved (Boulnois et al., 1987; Boulnois and Jann, 1989; Petit 1995; Roberts, 1996; Whitfield, 2006). Group 2 capsule genes are transcribed from two convergent temperature-regulated promoters; PR1 is upstream of Region 1, and controls transcription of assembly/export genes, while PR2 lies upstream of Reg 3/2, and transcriptionally controls expression of capsule export and synthesis genes (Simpson et al., 1996; Stevens et al., 1997).

First cloned by Silver and colleagues (Silver et al., 1981), Region I encodes for an 8kb polycistronic message (Roberts 2000), containing 6 genes, kpsFEDUIC. Interestingly, compared to the genetically similar meningococcal locus, E. coli contains “extra” Region 1 genes: kpsF, kpsD, and kpsU (Tzeng et al., 2002; Bliss and Silver 1996). Region 3 encodes the ABC-transporter (kpsMT) necessary for polysaccharide translocation to the periplasmic space (Higgins et al., 1990; Higgins 1992; Pigeon and Silver, 1994; Bliss et al.,
1996). Mutations in most Region 1 or Region 3 genes result in an acapsular phenotype, with certain genetic mutations varying in amounts of intracellular polysaccharide pools, called lacunae (Vimr et al., 2004).

Figure 2: The Group 2 Capsule Genetic Locus

Regions 1 and 3 are highly conserved among Group 2 capsule loci. Region 2 determines capsular (K) type, and is highly variable. Two representative Region 2 operons are shown. Sialic acid encapsulated K1 strains carry six genes within Region 2, while K5 encapsulated ExPEC carry four.

Genetic orientation and homology of the flanking Regions 1 and 3 across bacterial isolates suggest recombination events most likely occurred, to give rise to different Group 2 K-antigens (Boulnois and Jann, 1989). Capsule serotype (K-antigen) is
dependent upon genes encoded by Region 2; protein products of this region aid in polysaccharide synthesis, polymerization, and are indirectly involved in transport of the polymer to the cell surface (Bliss and Silver, 1996; Vimr et al., 1995; Whitfield and Roberts, 1999). Deletion of one or all parts of Region 2 results in an acapsular phenotype, though no lacunae.

Thermoregulation of transcription is a hallmark of Group 2 capsules. Though it is believed that Regions 2 & 3 have some degree of constitutive expression, they are still controlled by certain regulatory mechanisms (Rowe et al., 2000; Simpson et al., 1996; Cieslewicz and Vimr, 1996). Transcription of Regions 2 & 3 via PR2 is dependent upon both cis-acting factors (JUMPStart sequence – similar in mechanism to rho-antitermination), and trans-acting factors (anti-termination factor RfaH), to drive DNA polymerases through the end of Reg 2 (Hobbs and Reeves, 1994; Stevens et al., 1997; Stevens et al., 1994). Region 1, however, is thermo-regulated via the global regulator H-NS, IHF and SlyA (Rowe et al., 2000; Corbett et al., 2007; Cieslewicz and Vimr, 1997); additionally, the ribosome-binding GTPase, BipA, has also been shown to be involved in gene regulation of the kps gene cluster, though most likely through an indirect effect (Rowe et al., 2000; Grant et al., 2003). With few exceptions, Group 2 capsule is not expressed below 20° C (Bortolussi et al., 1983; Jann and Jann, 1987), and it takes newly synthesized capsule polysaccharide 25-30 minutes to appear extracellularly post shift in
temperature to $37^\circ$C, demonstrating not only thermoregulation of capsule but also that capsule is \textit{de novo} synthesized in contrast to being assembled from pre-stored oligosaccharides (Jann and Jann, 1987).

\textbf{1.7 Capsule Polymerization}

Capsule polymerization most likely occurs in a protected compartment at the cytoplasmic face of the inner membrane, and polymerization does not appear to be coupled to transport and translocation (Bliss et al., 1996; Silver et al., 1987; Steenbergen and Vimr 2008; Whitfield and Roberts, 1999). ExPEC Region 2 gene products are able to utilize UDP-GlcNAc to synthesize capsule; additionally, ExPEC can acquire extracellular, environmental sugars to incorporate into the capsule (Zapata et al., 1992; Roberts, 1996; Whitfield and Roberts, 1999). The first irreversible step of \textit{E. coli} Group 2 capsule production begins with the attachment of CMP to a capsular monosaccharide (Zapata et al., 1989). A phosphosugar precursor is then linked, in a heat labile fashion, to a phosphatidic acid and subsequent polymerization occurs; because polysaccharide is synthesized by extension at the non-reducing end, without the initial lipidation of the phosphosugar (and potential addition of KDO), further capsular polymerization is unable to occur (Whitfield and Roberts, 1999; Jann and Jann 1987; Schmidt and Jann, 1982; Gotschlich et al., 1981; Finke et al., 1989; 1991). Finally, passage of the polymer across the periplasm is aided via Region 1 and 3 gene products (Higgins et al., 1990).
1.8 The Hetero-oligomeric Group 2 Capsule Export Complex

Either immediately following polymerization or simultaneously with polymerization, the capsular polysaccharide is exported to the bacterial surface through the capsule machinery complex, comprised of Region 1 and 3 gene products (Boulnois et al., 1987; Boulnois and Jann, 1989). Identification and characterization of Group 2 capsule in *E. coli* has elucidated many of the protein functions, and some of the protein-protein interactions necessary to form the large hetero-oligomeric capsule machinery complex (Figure 3; Silver et al., 1987; McNulty et al., 2006). Assembly of the complex is located at the poles of the cell, without preference for new or old poles, spanning the bacterial membranes from the cytoplasmic face of the inner membrane, to the apical side of the outer membrane (McNulty et al., 2006; Jann and Jann, 1987). It has been suggested that pole localization of the capsule complex is due to region stability, and is possibly assembled where the inner- and outer-membranes come closer together, Bayer’s adhesion sites (Bayer et al., 1982; McNulty et al., 2006; de Pedro et al., 1997; 2004).
The Group 2 capsule machinery complex is highly conserved amongst all Group 2 encapsulated organisms. Genes necessary for capsule assembly and export are encoded by Regions 1 and 3 of the capsule locus. The capsule complex is guided to the bacterial pole by KpsS (7), where the complex assembles. The arabinose 5-phosphate isomerase, KpsF (6), is located in the cytoplasm. Synthesized capsule monomers are activated and lipidated (8); CMP-KDO is additionally added to the growing capsule polymer (11) by KpsU (10). The capsule synthase (9), encoded by Region 2 (ex: K1 – NeuS) interacts with the KpsC (5) dimer enabling stabilization and anchoring to the membrane. KpsT (4) and KpsM (3) form the ATP-binding cassette transporter; the polymer moves through the inner-membrane pore, most likely created via KpsM dimers, and moves through the periplasm and through the outer pore via KpsE (2) and KpsD (1). Capsule polymer is then integrated into the outer membrane (12). IM: Inner-membrane; OM: Outer-membrane; Adapted from Vimr and Steenbergen, 2009
The last gene to be characterized, *kpsF* (Figure 3) encodes for an arabinose 5-phosphate isomerase (Tzeng et al., 2002). Though it is the first gene in Region 1, KpsF is neither essential for capsule production (Cieslewicz and Vimr, 1996) nor required for thermoregulation (Cieslewicz and Vimr, 1996). Based on sequence similarity, KpsF appears to be functionally redundant to GutQ, to which it has 49% identity (Cieslewicz et al., 1993). Indeed, a nonpolar mutation in *kpsF* does not render the bacteria acapsular, but results in only a 10-fold reduction of capsule translocation to the surface, causing an accumulation of lipidated polysaccharide intracellularly (Cieslewicz and Vimr 1996).

Additionally, other Group 2 encapsulated bacteria, such as *Neisseria meningitidis*, do not encode for *kpsF*, further supporting the redundancy hypothesis (Pazzani et al., 1993).

KpsS (Figure 3) is encoded at the end of Region 1, and is processed post-transcriptionally, yielding a separate 1.3kb transcript. Differential regulation from the rest of the polycistron suggests an important role for KpsS in capsule production. It is intrinsically localized to the poles of the cell within the bacterial cytoplasm (McNulty et al., 2006), and like *kpsC*, KpsS is homologous to Group B meningococcus lipidation proteins (LipB) as well as lipid metabolism enzymes in other bacteria (Vimr et al., 1995; Rigg et al., 1998); deletion of *kpsS* behaves similarly to a *kpsC* deletion, forming lacunae - intracellular pools of polysaccharide (Bronner et al., 1993). Chemical crosslinking and co-purification with histidine tagged KpsS identified interactions between the ATP-binding
cassette proteins (KpsM, KpsT) and the periplasmic outer membrane proteins (KpsE, KpsD), in addition to interacting with the Region 2 sugar transferase; however, no interaction was found with the protein possibly responsible for initial monomeric lipidation (KpsC; McNulty et al., 2006). Despite its association with many of the capsule assembly and export proteins, the role of KpsS during polysaccharide transport to the periplasmic space has yet to be fully elucidated. kpsS null strains have minimal to no effect on polymerization and do not affect the function of the monosaccharide transferase if bacteria are supplied with exogenous polysaccharide (Bronner et al., 1993). Additionally, KpsS is not required for lipidation of capsular polysaccharide (Bronner et al., 1993; Cieslewicz and Vimr 1996). Therefore, it is believed that KpsS most likely directs polysaccharide production to the poles, and possibly acts in concert with KpsC as a polysaccharide “chaperone” within the cytoplasm, localizing polysaccharide to the intracellular membrane (Vimr et al., 1995; Rigg et al., 1998; Bronner et al., 1993).

Two-hybrid analysis has shown that synthetic KpsC (Figure 3) is able to interact with itself, the periplasmic spanning protein (KpsE), and polysaccharide polymerase (NeuS), suggesting the possibility of both homo-dimerization and docking of the polysaccharide polymerase (NeuS) to the cytoplasmic face of the inner membrane by a KpsC-KpsE interaction (Steenbergen and Vimr 2008). kpsC is believed to have arisen from a gene duplication event, with the N- (residues 60-270) and C- (residues 387-598)
terminal domains having 58% nucleotide identity and 39.2% amino acid similarity (Rigg et al., 1998). Because kpsC is homologous to lipA, a protein involved in lipidation of capsular polysaccharide in the group B Neisseria meningitidis, it was believed that KpsC was important for lipidation of the capsule polymer; however, in K1 E. coli, capsular polysaccharide lipidation is not dependent upon KpsC (Vimr et al., 1995; Cieslewicz and Vimr, 1996). It has also, been shown in a K1:K12 hybrid strain that polymerization and production of the capsule polysaccharide is not affected in kpsC null strains (Rigg et al., 1998; Bronner et al., 1993). Instead, lacunae form intracellularly, adding more credence to the hypothesis that KpsC is required for localization of the capsule polymer to the inner-membrane (Rigg et al., 1998; Bronner et al., 1993).

kpsU (Figure 3), found solely within the bacterial cytoplasm, encodes a 27kDa CMP-ketodeoxyoctonate (KDO) synthetase used for capsule production; it is similar in function to KdsB, which is employed for LPS generation (Bronner et al., 1993; Rosenow et al., 1995; Tzeng et al., 2002; Pazzani et al., 1993; Goldman and Kohlbrenner; 1985). Despite their association with different synthetic pathways, their functions are somewhat redundant; KdsB is able to partially restore a kpsU null mutant, enabling 20% of bacteria to express extracellular polysaccharide (Bronner et al., 1993; Rosenow et al., 1995).
As previously mentioned, Region 3 encodes for \textit{kpsM} and \textit{kpsT}. These gene products work in concert to form an ABC-transporter (Figure 3), creating a pore in the inner membrane, and generating energy for capsule translocation via ATP hydrolysis (Pigeon and Silver 1994; Bliss et al., 1996; Hyde et al., 1990; Higgins et al., 1990). ABC-transporters of Group 2 encapsulated strains are unlike single gene ABC-transporters; Group 2 capsules encode the integral membrane protein (KpsM) and an ATP-binding component (KpsT) separately (Higgins et al., 1990; Pigeon and Silver, 1994; Bliss et al., 1996). The termination sequence of \textit{kpsM}, however, overlaps with the initiation sequence of \textit{kpsT}, suggesting both proteins are expressed in equimolar amounts (Pavelka et al., 1991).

Based on consensus ATP-binding motifs – walker domains – KpsT is responsible for ATP hydrolysis (Walker et al., 1982; Bliss et al., 1996; Pavelka et al., 1994); it is peripherally associated with the cytoplasmic side of the inner membrane, coupling ATP hydrolysis to transport, via KpsM (Higgins et al., 1986; 1990). KpsM, a hydrophobic integral membrane protein has 6-transmembrane spanning helices and is found as a homodimer in the inner membrane (Higgins et al., 1986; Pigeon and Silver, 1994). Mutations in \textit{kpsM} or \textit{kpsT} cause polysaccharide to accumulate in the cytoplasm (Kroncke et al., 1990; Pavelka et al., 1991; Wunder et al., 1994). Based on these data, a model of KpsM and KpsT involvement in encapsulation was derived: KpsT binds and
hydrolyzes ATP, creating a KpsMT complex that is able to transport polysaccharide to the periplasmic space (Bliss et al., 1996). If KpsT is unable to hydrolyze ATP, the apparatus freezes, and polysaccharide pools cytoplasmically, unable to be transported, and thus capsule formation is aborted (Bliss et al., 1996).

KpsE is located in both periplasmic and inner membrane fractions and is thought to connect the ABC transporter to the outer membrane (Figure 3), via interaction with KpsD (Rosenow et al., 1995; McNulty et al., 2006; Arrecubieta et al., 2001). Based on hydropathy plots, KpsE contains a hydrophobic α-helical region comparable to BexC and CitrB of H. influenzae and N. meningitidis, respectively (Pazzani et al., 1993; Kroll et al., 1990; Frosch et al., 1991). Spheroplast degradation with proteinase K suggests KpsE is largely exposed within the periplasm, with the N-terminus exposed on the cytoplasmic side of the inner membrane (Rosenow et al., 1995), and the C-terminus potentially functioning as an inner membrane anchor, though quite possibly in a transient manner (Rosenow et al., 1995; Phoenix et al., 2001). In a K1:K12 hybrid strain, kpsE null mutants accumulate polysaccharide within the periplasm (Bronner et al., 1993).

Approximately 60.5 kDa in size, kpsD was identified by Western blot in virtually all E. coli strains expressing Group 2 capsule, thus far studied; these include the invasive serotypes: K1, K2, K5, K7, K12, K13, and K92 (Silver et al., 1987). KpsD dimerizes, and is localized to both the new and old poles in the presence of the capsule assembly complex
(Figure 3; McNulty et al., 2006). Identification of an amino terminal signal peptide suggests the role of KpsD within the periplasmic or extracellular space (Silver et al., 1987), and fusion of the predicted signal peptide of KpsD to alkaline phosphatase allowed for transport of the enzyme to the periplasmic space (Silver et al., 1987). Indeed, a kpsD null mutant accumulates polysaccharide in the periplasmic space, suggesting this protein’s involvement in translocation of the capsule polymer to the extracellular space (Bronner et al., 1993; Silver et al., 1987). Further characterization of KpsD in E. coli has revealed protein surface exposure, with the C-terminus potentially located in the periplasm, suggests KpsD potentially functions as an uncharacteristic outer membrane pore, possibly acting in concert with other proteins (McNulty et al., 2006). Despite the lack of typical translocation protein characteristics, such as heat resistant dimerization and acylation of the protein itself, it is believed that KpsD is the main extracellular pore of the capsule machinery (McNulty et al., 2006; Whitfield and Roberts, 2002). A double deletion of both kpsE and kpsD in a K1:K12 hybrid strain drastically decreases capsule polymerization and translocation; this suggests removal of both periplasmic and outermembrane capsule proteins may produce a feedback mechanism affecting capsule production, though this has yet to be elucidated (Bronner et al., 1993).

As previously mentioned, Regions 1 and 3 are highly conserved among E. coli; therefore, one of the major conundrums of Group 2 capsule export is how homologous
proteins from Regions 1 and 3 are be able to export different capsular polysaccharides. The use of an export signal in the form of a phosphatidic acid esterified to the reducing end of the polysaccharide, KDO, phospholipid, or both phospholipid and KDO, has been conjectured (Bronner et al., 1993; Pazzani et al., 1993; Frosch and Muller, 1993; Gotschlich et al., 1981). While there have been some data suggesting capsule composition may affect the method of lipidation and export, the relationship has yet to be fully elucidated (Cieslewicz and Vimr, 1996).

1.9 The Association of Capsule Types With Specific ExPEC Infections

Region 2 of the capsule locus differs among Group 2 encapsulated strains (Whitfield, 2006). This region encodes for all genes necessary to synthesize and polymerize capsule (Whitfield, 2006). Additionally, sugars from the extra-cellular environment can be scavanged and incorporated into the bacterial capsule (Roberts, 1996). Much of Group 2 capsule studies have been done on what have historically been considered the two most predominate capsular types found associated with UTI: K1 (Region 2 – neu genes) and K5 (Region 2 – kfi genes; Robbins et al., 1974; Mccracken et al., 1974; Kim et al., 1992; Ørskov et al., 1979; Bonacorsi et al., 2000; Cross et al., 1984, Corbett and Roberts, 2008). Purified K1 and K5 capsules elicit poor host responses due to antigenic similarity of bacterial and host glycosylation motifs (reviewed by Jann and Jann, 1987). K5 polymer is comprised of hyaluronic acid and is associated with both
urinary tract and sepsis isolates (Corbett and Roberts, 2008). E. coli strains expressing the K5 capsule have increased virulence due to the fact that K5 capsule is identical to the first polymeric intermediate in the biosynthesis of heparin (Corbett and Roberts, 2008).

Bacterial K1 capsules are comprised of repeating units of sialic acid with 2→8 linkage, commonly found on animal tissue, glycoproteins, and gangliosides; it is closely associated with neonatal meningitis and was the prevalent capsule type of urinary tract infections during the late 1980’s and early 1990’s (Robbins et al., 1974; Mccracken et al., 1974; Kim et al., 1992; Ørskov et al., 1979; Bonacorsi et al., 2000; Cross et al., 1984; Bingen et al., 1998).

1.10 De Novo Synthesis of Sialic Acid

Genes requisite for biosynthesis of sialic acid (N-acetyl-neuraminic acid; Neu5Ac; NANA) are encoded by K1 encapsulated ExPEC (Vimr et al., 1995). Encoded within Region 2 of the K1 capsule operon, are six genes necessary for sialic acid synthesis in E. coli, neuDBACES (Figure 4B; Vimr et al., 1995). Bacterial strains, including N. meningitides and E. coli K1, synthesize Neu5Ac de novo and incorporate sugars into cell surface glycoproteins via capsule or sialidated LPS (Vimr et al., 1995). Because Region 2 is under the control of PR2 and is simultaneously transcribed with Region 3 (kpsMT), biosynthesis of neu-genes is somewhat constitutive within K1 ExPEC at 37°C (Bortolussi et al., 1983; Cieslewicz and Vimr, 1996).
K1 ExPEC sialic acid biosynthesis begins with UDP-N-acetylglucosamine (GlcNAc) 2-epimerase (NeuC) catalyzing UDP-GlcNAc to N-acetylmannosamine (ManNAc); this is the first irreversible step in bacterial sialic acid biosynthesis (Figure 4A; Ringenberg, et al., 2003). While NeuC is required for sialic acid biosynthesis, exogenously added Neu5Ac rescues capsule production in neuC null strains, suggesting that imported sialic acid can be used as raw substrates for both capsule production and catabolism (Zapata et al., 1992).

Figure 4: The Sialic Acid Biosynthesis Pathway in K1 Encapsulated E. coli

Genes required for sialic acid (A) biosynthesis in K1 encapsulated organisms are encoded within (B) Region 2 of the capsule locus (neuDBACES).

The biosynthesis pathway next requires ManNAc interaction with the sialic acid synthase, NeuB, to create Neu5Ac. This reaction requires the cellular addition of phosphoenolpyruvate (PEP) to the newly epimerized ManNAc in the presence of NeuB,
an obligatory step for Neu5Ac biosynthesis de novo (Ringenberg et al., 2001). From here, the nascent neu monomer may either interact with the CMP-sialic acid synthase, NeuA, and be activated for polymerization, or be modified by the O-acetyltransferase, NeuD (Steenbergen et al., 2006; Deszo et al., 2005; Bergfeld et al., 2007); acetylation of monomeric Neu5Ac via NeuD does not inhibit subsequent NeuA activation (Steenbergen et al., 2006; Deszo et al., 2005; Bergfeld et al., 2007).

The process of ExPEC Neu5Ac biosynthesis is markedly different than eukaryotic biosynthesis, in that CMP-Neu5Ac production is not tightly regulated by allosteric inhibition (Steenbergen and Vimr, 1990; Vimr and Troy, 1985). Additionally, E. coli lack a CMP-Neu5Ac hydrolase (Vimr and Troy, 1985); therefore addition of CMP to Neu5Ac via NeuA, is an irreversible step, activating the Neu5Ac monomer for polysaccharide capsule production (Edwards et al., 1994).

NeuE is proposed to add activated Neu5Ac to an as of yet unidentified initiator molecule, potentially undecaprenyl phosphate or another membrane associated lipid (Figure 3; Figure 4A; Vimr et al., 2004), and subsequent activated Neu5Ac monomers are added to oligosialic acid receptors via the polysialytransferase, NeuS; newly synthesized polysialic acid capsule is exported via the capsule machinery (Vimr et al., 2004).

Whereas NeuD interacts with sialic acid monomers, creating sialic acid variants prior to polymerization, some K1 strains encode neuO at an unlinked loci; this O-
acetyltransferase acetylates polymerized sialic acid, and in addition to NeuD, is yet another way some ExPEC are able to create capsule variants modulated by phase variation (Deszo et al., 2005; Bergfeld et al., 2007).

1.11 Sialic Acid Importation and Catabolism

Bacteria are known to co-opt host sialic acid for the purposes of adhesion, invasion, and propagation within a host (Karlsson, 1998). While sialic acid biosynthesis is only able to occur in select few ExPEC strains (K1, K92), sialic acid catabolism is ubiquitous among E. coli; genes required for sialic acid catabolism are encoded between argG and rpoN at an unlinked Group 2 capsule locus, and are transcriptionally under the control of the global regulator, NanR (Vimr and Troy, 1985). Additionally, sialic acid catabolism in some pathogenic bacteria, such as V. cholera or E. coli, confers a niche dependent advantage (Almagro-Moreno and Boyd, 2009), and both nanA and nanT are required for E. coli colonization of the mouse colon in vivo (Chang et al., 2004). This suggests the importance of sialic acid catabolism in vivo.

Sialic acid is imported across the outer-membrane of E. coli via OmpC, OmpF, or the sialic acid specific porin, NanC (Condemine et al., 2005). Though sialic acid can be transported across the outer-membrane by multiple porins, NanT is the only known importer of sialic acid across the inner membrane of E. coli (Martinez et al., 1995). This approximately 54 kDa permease is encoded 135bp downstream of the first gene in the
*nan* operon, *nanA* (Martinez et al., 1995). Hydrophobic in nature, NanT is unique to other monosaccharide transporters; in addition to the 14 transmembrane segments spanning the inner membrane of *E. coli*, NanT has an amphiphilic alpha-helix and two additional membrane spanning segments that are potentially important for sialic acid importation (Martinez et al., 1995).

![Diagram](image.png)

**Figure 5: Sialic Acid Catabolism and UDP-GlcNAc Synthesis**

Proteins requisite for sialic acid importation and catabolism are encoded by *nanATEK* under the control of NanR regulation. Imported sialic acid is broken down into ManNAc by the NanA aldolase and phosphorylated on the carbon 6 by the kinase NanK. ManNAc-6P is then epimerized by NanE to GlcNAc-6p, and UTP added to form UDP-GlcNAc by GlmU. UDP-GlcNAc is the main building block for LPS, peptidoglycan, and capsule synthesis.

Once sialic acid is imported, it is broken down into carbon and nitrogen to create fuel for energy generating reactions (Figure 5; Kalivoda et al., 2003). The first enzyme required for sialic acid degradation is the aldolase, NanA; sialic acid is converted to N-acetylmannosamine (ManNAc) and pyruvate by NanA (Ringenberg et al., 2001). This aldolase is only involved in sialic acid catabolism, and does not appear to have
overlapping function in biosynthetic sialic acid pathways (Ringenberg et al., 2001).

Deletion of nanA, however, causes imported sialic acid to be shunted toward the biosynthetic pathway, and activated by NeuA (Ringenberg et al., 2001), suggesting imported sialic acid is kinetically separated from capsule production via NanA, and that imported sialic acid can be incorporated into the sialic acid capsule, though not preferentially.

Pyruvate released from sialic acid degradation is subsequently converted to energy by either the citric acid cycle (during aerobic respiration), or is used for fermentation (during anaerobic respiration), while the resulting ManNAc is phosphorylated via the sialic acid kinase NanK on carbon 6, producing ManNAc-6-P (Figure 5; Plumbridge and Vimr, 1999; Ringenberg et al., 2003). Unlike the mammalian counterpart, which is a bifunctional kinase/epimerase enzyme, E. coli has a genetically and functionally separate epimerase protein, NeuC, as previously discussed (Ringenberg, et al., 2003; Zapata et al., 2002). Interestingly, work done by Ringenberg et al., (2003) showed redundancy of genes from the biosynthetic neu pathway, with those in the sialic acid catabolism pathway; for example, ExPEC encode for a separate epimerase for nan catabolism, NanE, supporting the idea of horizontal acquisition of sialic acid biosynthetic genes (Ringenberg et al., 2003). Epimerization of ManNAc-6-P into N-acetylglucosamine (GlcNAc-6-P) creates a product that can be utilized in multiple
ways within the bacterial cell (Plumbridge and Vimr, 1999; Ringenberg et al., 2003). The last gene in the operon, \textit{yhcH}, has an as of yet undefined function (Plumbridge and Vimr, 1999).

This presents a paradox when considering K1 encapsulated strains are able to synthesize and catabolize sialic acid. While it appears NeuA does not compete with NanA for imported Neu5Ac substrate within a K1:K12 hybrid strain (Ringenberg et al., 2001), NanA has the ability to regulate the biosynthetic pathway when free Neu5Ac concentrations are permitted to rise, such as in a \textit{ΔneuA} strain (Ringenberg et al., 2001). In the presence of NeuA, however, a \textit{neuS} null strain accumulates activated sialic acid (CMP-Neu5Ac) to high intracellular levels, implying loss of capsule polymerization neither shuts down biosynthesis of sialic acid, nor inhibits activation of nascent monomers for polymerization (Vimr and Troy, 1985; Steenbergen and Vimr, 1990; Ringenberg et al., 2001). Thus, synthesized Neu5Ac is preferentially polymerized, preventing catabolism of newly synthesized sialic acid.

\textbf{1.12 Utilization of Sialic Acid in ExPEC}

Amino sugars have many functions within the bacterial cell, both in generating energy and structural function. For example, GlcNAc can be used for bacterial cell usage, cell wall formation, etc. (Morrison and Ryan, 1992), and in many cases, is the raw material for capsule synthesis (Hodson et al., 2000). Also, GlcNAc is both a carbon and
nitrogen source and able to generate bacterial energy via the N-acetylglucosamine utilization pathway \( (\text{nag}; \text{Plumbridge, 1995}). \)

The \textit{nag} pathway further breaks down GlcNAc-6-P for glycolysis via \textit{nagBA} \cite{Plumbridge1991, Plumbridge1999}. NagA (GlcNAc deacetylase) removes the acetyl group, producing glucosamine-6-P (GlcN-6-P) and acetate \cite{Plumbridge1991, Plumbridge1999}. NagB, next removes the amine group from glucosamine, generating the initial glycolysis substrate, fructose-6P, and ammonia as a nitrogen substrate \cite{Plumbridge1999}. Transcription of the \textit{nag} pathway \((\text{nagBA})\) is repressed by NagC; \textit{nagA} expression is increased 40-fold in a \textit{nagC} mutant \cite{Plumbridge1991}. Therefore, much like NanR, in the presence of high imported sialic acid \((\text{nan})\), NagC is displaced from the \textit{nagBA} promoter, yielding transcription; this mechanism is through the intermediate, GlcNAc-6-P \cite{Plumbridge1992, Plumbridge1995}.

As previously mentioned, amino sugars are extremely important to bacterial survival, necessary for LPS biosynthesis in addition to a metabolic energy source \cite{Plumbridge1991, Plumbridge1999, Tzeng2002}. In the absence of exogenous amino sugars, \textit{E. coli} are able to synthesize these important precursors via the \textit{glm} pathway \cite{Plumbridge1991, Plumbridge1992, Plumbridge1995}. However, because ExPEC have the ability to synthesize and degrade GlcNAc-6-P, biosynthesis and degradation of this molecule are coordinately regulated in order to inhibit a futile cycle; the presence of amino sugars
induces catabolic enzymes and decreases glucosamine synthase (Plumbridge, 1995).

GlmS (glucosamine synthase) is able to \textit{de novo} synthesize amino sugars in \textit{E. coli}; GlmS produces GlcN-6-P from fruc-6P and glutamine, which is the end result of the \textit{nag} utilization pathway (Plumbridge, 1995). Next, the phosphoglucomutase isomerizes GlcN-6-P (Mengin-Lecreux and Heijenoort, 1996), and GlmU converts GlcN-6-P to UDP-GlcNAc, the first dedicated precursor of the cell wall, peptidoglycan, and capsule synthesis (Mengin-Lecreux and Heijenoort, 1994).

\textbf{1.13 Regulation of Sialic Acid Catabolism}

Similar to many catabolic systems, sialic acid is a regulator of its catabolism; transcription of the \textit{nan} operon is induced by an increase in cytoplasmic free sialic acid monomers (Kalivoda et al., 2003). The monomer interacts with the bound form of NanR, inducing a conformational change in the regulator, and release of the DNA on which it sits (Kalivoda et al., 2003). This enables the main polymerase access to the operon for transcription. In the absence of sialic acid, NanR binds the promoter region of the \textit{nan} operon, \textit{nanATEK}, repressing transcription (Plumbridge and Vimr, 1999).
Sialic acid catabolism is also under control of the transcriptional activator responsible for catabolite repression: catabolite activator protein (CAP), also known as cyclic AMP receptor protein (CRP; Figure 6; Kalivoda et al., 2003). In the presence of glucose, CAP is found unbound as a homodimer (Kalivoda et al., 2003). Cyclic AMP (cAMP) levels rise when glucose is not readily accessible. This increase in cAMP induces the probability of cAMP interacting with CAP. Post cAMP-CAP interaction, CAP’s affinity for DNA increases, enhancing transcription of alternate catabolite operons; in this case, when glucose is not readily accessible to ExPEC, but sialic acid is, cAMP levels increase and interact with CAP, enhancing transcription of the nan operon (Kalivoda et al., 2003). Therefore, induction of high levels of sialic acid catabolite transcription is a two-step process: (1) glucose must be in low supply, leading to an accumulation of cytoplasmic cAMP, resulting in increased DNA-bound CAP protein and (2) sialic acid

Figure 6: Genetic Locus for Sialic Acid Catabolism in E. coli.
must be at a high concentration, interacting with DNA-bound NanR, and de-repressing nan transcription (Kalivoda et al., 2003).

**Figure 7: nanC and fimB are coordinately regulated by NagC and NanR**

In the DNA bound form, NanR and NagC activate expression of fimB. In the presence of free sialic acid and GlcNAc, NanR and NagC release DNA, derepressing nanC transcription, and down-regulating fimB transcription.

Sialic acid and the amino sugar derivative of glucose, GlcNAc, are also able to indirectly affect TIP transcription (Condemine et al., 2005; Plumbridge 1992). nanC, the outer membrane sialic acid transporter, is divergently transcribed from the fimB (Figure 7); therefore, this shared promoter region regulates nanC and fimB expression in a reciprocal fashion (Condemine et al., 2005). In a high sialic acid, high GlcNAc state, NanR releases the divergent nanC/fimB promoter region, enhancing nanC transcription, and decreasing fimB expression, indirectly affecting TIP expression (Condemine et al.,
2005). It has been postulated that in a high sialic acid state, T1P expression would be severely inhibited (Sohanpal et al., 2004; Plumbridge and Vimr, 1999).

### 1.14 Type-1 Pili

Type-1 pili (T1P) have been studied extensively in both commensal and pathogenic organisms (Abraham et al., 1988; Schilling et al., 2003; Wright et al., 2007; Zhou et al., 2001). Though T1P are pro-inflammatory, they are requisite for ExPEC adherence to and invasion of the bladder epithelia, in addition to survival post-invasion (Abraham et al., 1988; Schilling et al., 2003; Wright et al., 2007; Zhou et al., 2001). It has been proposed that T1P and capsule are coordinately regulated, enabling bacteria to reduce inflammation by capsular masking of surface antigens and low T1P expression, and down-regulating capsule once T1P have made contact with their receptor (Schwan et al., 2005). Additionally, the T1P operon is phase variable; recombinases are able to control the genetic orientation of the T1P promoter transcription of the operon, via homologous mediated inversion, orienting the promoter into either the ON position (T1P are transcribed) or the OFF position (T1P expression is repressed; Wolf and Arkin, 2002).

T1P recombinases FimB and FimE are ubiquitous among ExPEC (Wolf and Arkin, 2002; Abraham et al., 1988; Chen et al., 2006). FimE, which has the ability to turn T1p OFF is encoded immediately upstream of fimS, and is thought to be
transcriptionally controlled via \textit{fimS} orientation (Wolf and Arkin, 2002). FimB is bidirectional in its functionality, able to turn T1p both OFF and ON; this recombinase is encoded just upstream of FimE, and is under NagC and NanR control (Wolf and Arkin, 2002; Plumbridge and Vimr, 1999). Finally, UTI89 also encodes for a third recombinase at an unlinked loci, FimX. FimX is unidirectional, much like FimE, but in the opposite orientation; FimX turns T1p ON (Bateman and Seed, 2011).

\textbf{1.15 Genetic Organization and Protein Structure of T1P}

The T1p operon consists of seven genes which organized in a stoichiometrically sensible way; \textit{fimAICDFGH} are located just downstream of two of the major recombinases which control expression, \textit{fimB} and \textit{fimE}, separated by the T1p promoter (\textit{fimS}; Figure 8; Orndorff and Falkow, 1984; Wolf and Arkin, 2002). To express T1p, ExPEC use the chaperone/usher pathway, and therefore the operon does not contain genes necessary for inner-membrane export (Orndorff and Falkow, 1984; Wolf and Arkin, 2002). Post transcription, the polycistron is processed and individual pilus subunits translated (Orndorff and Falkow, 1984; Wolf and Arkin, 2002). These are then exported out of the cytoplasm and into the periplasm using the Sec system (Orndorff and Falkow, 1984; Wolf and Arkin, 2002).

FimD, the extracellular pilus pore is folded within the periplasm and inserted into the outer membrane (Orndorff and Falkow, 1984; Wolf and Arkin, 2002). Pilus
subunits, the major subunit FimA, minor units FimF and FimG, and major mannose
binding tip lectin FimH, are transcribed and translocated to the periplasm where they
are additionally folded and protected by the FimC chaperone (Orndorff and Falkow,
1984; Wolf and Arkin, 2002). Once the pore is in place in the outer membrane, it interacts
with the FimC chaperone, and the pilus subunits are loaded and secreted in sequential
form – FimH, FimG, FimF, and multiple FimA subunits – until the pilus is at a certain
length (Orndorff and Falkow, 1984; Wolf and Arkin, 2002). The mechanism for
determining pilus length and termination has not yet been elucidated. Though this pilus
is ubiquitous among E. coli strains, it has the ability to change in binding specificity
based on polymorphisms within the tip lectin, FimH (Chen et al., 2006).

Figure 8: Type 1-Pilus locus

Type 1-Pili are encoded in one genetic locus, downstream of an invertible promoter
element, fimS. Promoter orientation (ON; OFF) is regulated by FimE and FimB, which
are encoded upstream of the TIP subunits. fimB and nanC are divergently transcribed,
but coordinately regulated by NagC (1) and NanR (2). In the absence of GlcNAc and
Neu5Ac, NagC and NanR are DNA bound, repressing transcription of nanC, aiding
transcription of fimB. In the presence of GlcNAc and Neu5Ac, NagC (GlcNAc) and
NanR (Neu5Ac) release DNA, enabling nanC transcription, and decreasing fimB
transcription.
1.16 Regulation of fimB Transcription

As previously discussed, both NanR (nan regulator) and NagC (nag utilization regulator) act as repressors of sialic acid and GlcNAc degradation pathways, in the absence of high concentrations of these molecules (Figure 8; Plumbridge and Vimr, 1999). However, these repressors are able to positively regulate gene transcription in their DNA-bound form (Plumbridge, 1991; 1993). This is the case for fimB, the bifunctional recombinase that controls T1p expression (Sohanpal et al., 2004; Plumbridge and Vimr, 1999). Both mutations in NanR and NagC decrease fimB expression, to varying degrees (Sohanpal et al., 2004; Plumbridge and Vimr, 1999), and free sialic acid inhibits expression of fimB/T1p (Sohanpal et al., 2004, 2007).

While NanR and NagC positively regulate fimB transcription, the effect each of these regulators has on transcription is different. As previously discussed, the substrate that interacts with NagC is GlcNAc, and increased concentrations of GlcNAc disrupt only NagC from the fimB promoter, leaving NanR DNA-bound (Sohanpal et al., 2004; Plumbridge and Vimr, 1999). However, the addition of sialic acid disrupts both NanR, through presumably direct interaction, and NagC, via sialic acid degradation to GlcNAc (Sohanpal et al., 2004; Plumbridge and Vimr, 1999). Though both sugars affect fimB regulation, either in tandem, or separately, disruption of NagC by GlcNAc only has a stronger effect on fimB expression than sialic acid (Sohanpal et al., 2004). It has been
suggested that perhaps NagC is only partially activated by sialic acid, however this has yet to be investigated further (Sohanpal et al., 2004; Plumbridge and Vimr, 1999).

Additionally, nanC – the outer membrane sialic acid importer – is located upstream of fimB (Condemine et al., 2005). nanC is divergently transcribed, and thus shares a promoter region with fimB (Condemine et al., 2005). Therefore, nanC and fimB are coordinately regulated by both NagC and NanR (Sohanpal et al., 2004); in the presence of high amino sugar concentration, nanC transcription is induced, and fimB transcription is reduced (Sohanpal et al., 2004; Plumbridge and Vimr, 1999).
2. Methods

2.1 Prospective Study Design

One hundred sixty nine women ages 18 – 40 presenting with acute uncomplicated UTI were enrolled at the University of Washington, Seattle between 2008 and 2012. Subjects were excluded from the study if they had a UTI in the prior 6 months or had evidence of upper tract infection including fever and flank pain. Upon initial and subsequent visits, the following samples were collected: midstream urine, peri-urethral, vaginal, and rectal. Samples were cultured on selective media, and the presence or absence of bacteria was scored. Bacterial CFUs above the cutoff threshold of $>10^3$ CFU were frozen in 1/3 glycerol:LB and stored at -80°C. A rectal sweep was also taken at each visit and immediately stocked in glycerol and stored at -80°C. Patients were followed for the next 3 months to total 4 visits post initial visit. Visits 1 and 2 (V1 and V2) were scheduled approximately 2 weeks and 4 weeks following the initial presentation, respectively. Visits 3 and 4 (V3 and V4) were scheduled approximately 2 months and 3 months following the initial acute presentation. Those patients presenting with symptomatic episodes between scheduled visits were scored as having a recurrent infection at which time additional midstream urine, peri-urethral, vaginal and rectal samples were taken and stocked if possible. Additionally, patients presenting with symptomatic episodes upon scheduled visits were also scored as having recurrent UTI.
Human subjects included were approved by protocol at the University of Washington. Investigators were blind to subject identification and all isolates were used were unidentified.

2.2 Bacterial Culture and Growth

2.2.1 Growth For Multiplex PCR

Frozen clinical isolates were inoculated via sterile pipet tip into 1 mL LB and grown overnight in 96-deep well trays with gentle aeration at 37°C. Overnight cultures were then replica stamped into 100 µl LB in 96-well trays and then stored at -80°C. Stamped trays were grown for 2 hours at 37° C with gentle aeration, and 1µl was used for PCR amplification.

2.2.2 Growth of Strains for GFP Analysis, Tissue Culture Infections, and Murine Infections

Frozen strains were inoculated via sterile pipet tip into 5mL LB and grown overnight at 37°C with aeration. Cultures were back-diluted 1:100 in minimal media supplemented with: 0.2% 1M MgSO₄; 0.01% 1M CaCl₂; 1mM NAD; and 0.2% of appropriate carbon source (Glycerol, Sialic Acid). Cultures were grown at 37°C with aeration overnight; Cultures were either back-diluted into 5 mL minimal media with supplements and appropriate carbon source or pooled urine filter-sterilized and grown overnight at 37°C. After final back-dilution, cultures were washed 1x with PBS and resuspended in PBS to OD₆₀₀ of 0.8.
2.3 PCR

2.3.1 Multiplex PCR

Capsule multiplex PCR reactions were completed using the following conditions: 1 cycle of 95°C for 3 minutes; 30 cycles: 95°C for 30s, 50°C for 45s, 72°C for 50s; 1 cycle of 72°C for 7 minutes. Phylotype multiplex PCR reactions were performed as previously described by Clermont et al., 2000. Virulence multiplex profiles were performed as previously described by Johnson et al., 1991; Johnson & Stell 2000. See Figure 10 for capsule multiplex primer design. PCR analysis was performed using 2.5% agarose gel in TBE.

2.3.2 Phase PCR

Phase PCR was performed as previously described (Bateman and Seed, 2011). Briefly, 1 µL of bacterial culture was used for PCR amplification using previously published phase primers. Reactions were completed using the following conditions: 1 cycle of 95°C for 3 minutes; 30 cycles: 95°C for 30s, 57°C for 45s, 72°C for 45s; 1 cycle of 72°C for 7 minutes. PCR analysis was performed using 2.0% agarose gel in TBE.

2.3.3 RNA preparation and qrt-PCR

qrt-PCR was performed using 1 µL of dilute cDNA prepared using Ambion Purelink RNA Mini kit. Miniopticon (BioRad) and Opticon Monitor 3 were used for PCR amplification using SYBR-Green. Reactions were completed using the following
conditions: 1 cycle of 95° for 1 minutes; 40 cycles: 95°C for 10s, 57°C for 30s, 72°C for 15s,
82°C for 2s – plate read; melting curves were performed every 0.5°C from 55-95°C.
Product analysis was performed using 2.0% agarose gel in TBE.

2.4 Preparation of Genomic DNA for Sequencing

Bacterial isolates were grown in 25 ml cultures of LB media overnight at 37°C with shaking. The bacteria were pelleted at 3,000 RPM in a clinical centrifuge for 20 min. The supernatant was discarded and the pellet was resuspended in 1 ml of 10 mM Tris, pH 8/1% SDS with the addition of 10 µl of 1 mg/ml Proteinase K. The resuspended bacteria were incubated for a 2-18 hr at 50°C until complete lysis was achieved. One hundred microliters of 3M sodium acetate (pH 5) was added to the lysate at room temperature followed by 800 µl of isopropanol. After gentle mixing and visualization of precipitated genomic DNA, the mixture was decanted into a disposable urine filter device (Fisher) attached to a vacuum source. The genomic DNA precipitant on the filter was washed once with 5 ml of 80% ethanol and allowed to dry on the filter. One ml of washed celite resin (1.5 g [Sigma] per 100 ml of 7M guanidium thiocyanate, pH 5) was added to each filter unit, and the genomic DNA was gently resuspended by pipetting. A vacuum was applied, and the resin was washed with 2 ml of wash buffer (200mM NaCl; 20mM Tris.HCl, pH 7.5; 5mM EDTA; 50% Ethanol). The resin was allowed to dry completely after which the column was placed in a 15 ml clinical centrifuge tube, 350 ml
of nuclease free water was applied to the resin, incubated at RT for 5 min, and spun at 1,500 RPM for 3 min in a clinical centrifuge to elute the genomic DNA. One microliter of Rnase (4mg/mL) added to each elution.

2.5 Capsule Release and Alcian Blue Staining

Capsule polysaccharides were prepared as follows: bacteria were inoculated from freezer stock into 5 mL of standard LB and grown, shaking, at 37°C overnight. Cultures were then spun at 3,000 rpm for 15 minutes to pellet bacteria. Supernatant was removed, and bacteria were resuspended in 500 µl of 50mM Tris-Acetate pH 5.0. These were then incubated, shaking, at 37°C for approximately 2 hrs. Cells were then pelleted in 1.5 mL microfuge tubes at 9,000 rpm for 3 minutes. The supernatant was then removed and concentrated in AcroPrep Advance 96-well filter plates (30K Omega) from Pall – Life Sciences, to approximately 1/10 the starting volume (~50ml). SDS-page dye + bme was added, and concentrated samples were run at 125V on 7.5% SDS-page gels for 2hrs.

SDS-page gels were rinsed 3x with ddH2O, and incubated in alcian blue dye (0.125% (w/v) Alcian blue, 40%EtOH, 5% acetic acid in ddH2O) at room temperature for 1 hour, shaking. Dye was removed, and gels were de-stained for approximately 15 minutes in (40% EtOH, 5% acetic acid). Gels were then imaged and dried.
2.7 GFP Fluorescence Analysis

Cultures were grown in minimal media as stated above (Section 2.2.2). Once cells were resuspended to the aforementioned optical density, 1.5 mL was removed and centrifuged at 8,000 rpm for 3 minutes. Cells were resuspended in 150 µl PBS. The sample was subsequently split in two, and 75 µl was added to 96-well round-bottom tray for relative fluorescent reading using FLX800. Readings of samples were performed in duplicate.

2.8 Murine Infections

2.8.1 Modeling UTI and Plating for CFU

The prototypic murine model was used as previously described (Mulvey et al., 1998). Briefly cultures were inoculated into 5mL LB and grown with aeration at 37°C overnight. Cultures were back diluted 1:100 and grown in 25 mL LB static at 37°C for 20 hours. ExPEC were washed 1x with PBS, and resuspended to an OD₆₀₀ of 0.8 in PBS. Mice were briefly anesthetized using isofluorane, and instilled with 100 µl of prepared samples via trans-urethral catheterization, and subsequently revived. Post sacrifice, bladders, kidneys, and spleens were harvested and homogenized in a total volume of 1mL PBS with 0.1% TritonX. Appropriate dilutions were performed and plated for CFU.
2.8.2 Measuring Sialic Acid During UTI by HPLC

Mice were inoculated as previously described above. Urine was then harvested from the mice every 4 hours post infection, using eppendorf tubes. Urine was then analyzed by HPLC against sialic acid standards, by DMB labeling (Takara) and a Thermo hypersil column with 5µM particle size.

2.9 Fluorescence Microscopy: ex vivo Staining of Mouse Bladders

Mice were infected as previously described in section 2.8.1. Six hours and 24 hours post infection, mice were sacrificed, and bladders harvested, embedded in OCT media, and flash frozen at -80°C. Cryosectioning was performed to yield 5 micron thick sections, and were fixed in the dark with 4% Paraformaldehyde for 1 hour at 4°C. Sections were washed 3x with PBS, and stained in the dark with TOPRO-3 at a concentration of 1000ng/mL for 20 minutes at room temperature.

2.10 Western Blot Analysis

Fifteen percent SDS-PAGE gels were used for Western blot analysis. Samples were boiled in SDS-Page loading dye with β-mercaptoethanol for 5 minutes. FimA western blot samples were first treated with 1M HCl post addition of loading dye. Samples were then boiled in acid for 5 minutes, and neutralized with 1:1 ratio of 1N NaOH to the amount of HCl added pre-boiling.
Western blot analyses were performed using standard conditions. Post semi-dry transfer to nitro-cellulose membrane, αT1P antibody donated from Scott Hultgren’s lab was used at a dilution of 1:10,000 and the blot was probed overnight. The nitrocellulose membrane was washed 2x with TBS + 0.1% Tween. Secondary αRabbit (Sigma A3687) conjugated to Alk/Phos was added at a dilution of 1:5,000, and probed for 45 minutes, before washing 4x with TBS + 0.1% Tween. Colorimetric development was performed using BCIP/NBP liquid substrate. The blots were dried and imaged.

2.11 Tissue Culture Infections

Bacterial cultures were inoculated into 5mL LB and grown with aeration at 37°C overnight. Bacteria were back-diluted 1:100 in 25 mL LB and grown statically at 37°C for 18-20 hours. Cultures were washed 1x with PBS, and resuspended to a final OD₆₀₀ of 0.8 in PBS. Tissue culture cells were inoculated with approximately 5 x 10⁶ bacteria, at a multiplicity of infection (MOI) of 10.

5637 bladder cells were seeded at 1 x 10⁴ into tissue culture treated 24-well plates and grown overnight at 37°C in RPMI and 5% FBS. Media was removed and cells were serum starved in RPMI alone for 16 hours. Media was removed either fresh media alone, with mannose, with sialic acid urine, was added for 30 minutes pre-treatment of 5637 cells. Pre-treatment media was removed, and media w/ or w/o mannose, sialic acid or urine was added for infections. Cells were infected and briefly spun at 1000 rpm for 5
minutes to initiate bacterial contact; infections proceeded for 30 minutes post inoculation. Media was removed, and RPMI with 5% FBS and Gentamicin at a final concentration of 100mg/mL was added to 5637 cells to assess intracellular bacteria. Gentamicin treatment proceeded for 2 hours.

Adherence/Invasion was assessed after 30 minutes by washing 3x with PBS and briefly trypsinizing (with 1% EDTA) the cells. Wells were resuspended in a final volume of 1mL in PBS with 0.01% TritonX-100, and appropriate dilutions were plated. Invasion was assessed 2 hours after Gentamicin treatment by washing 3x with PBS, briefly trypsinizing (with EDTA) the cells and resuspending in a final volume of 1mL in PBS with 0.01% TritonX-100. Appropriate dilutions were plated.

2.13 Thiobarbituric Acid Assay (TBA)

Thiobarbituric acid assay was performed as described by Warren, 1959, and amended by Aminoff, 1961, and Skoza and Mohos 1976. Briefly, the reagents used were: Solution 1: 0.2M sodium periodate in 50% phosphoric acid; Solution 2: 10% sodium arsenite, 0.5M sodium sulfate, 0.1N H2SO4; Solution 3: 0.6% thiobarbituric acid, 0.5M sodium sulfate.

Acid hydrolysis of samples was performed in 0.025-0.05M H2SO4 at 80°C for 60 minutes, to liberate sialic acid not in monomeric form in order to obtain total sialic acid levels. To obtain initial free monomeric sialic acid, samples were not hydrolysed.
Samples were then treated with 0.5 mL of Solution 1, and incubated at 37°C for 30 minutes. Solution 2 was added (0.5 mL) and mixed. Solution 3 was then added (0.1 mL), and samples were boiled for 7.5 minutes in a water bath at a rolling boil. Samples were cooled on ice for 5 minutes. One mL of cyclohexanone was added and mixed with sample immediately before taking the optical density at 540 nm.

**2.15 T1P Agglutination**

Bacterial cultures were prepared as described in section 2.2.2. After normalization of samples to an OD600 of 0.8 in PBS, 1.5 mL of sample was pelleted and resuspended in 150 µl PBS. 96-well plates were prepared with 25 µl of PBS per well. Resuspended samples were added at a 1:1 ratio (25 µl in first well), and 2-fold dilutions were performed. A solution of 1% bakers yeast was prepared in PBS, and added at a 1:3 final volume. Plates were gently tapped, and scored for agglutination after 30 minutes.
3. Genetic Analysis of the Prevalence of Capsule Types Among Clinical Isolates of Extra-Intestinal Pathogenic \textit{Escherichia coli} (ExPEC)

3.1 Introduction

Urinary tract infection (UTI) is one of the most common human infections, second only to respiratory infections. Of those UTI that are community-acquired, it is believed that 80-90\% are caused by extra-intestinal pathogenic \textit{Escherichia coli} (ExPEC) (as reviewed by Foxman \textit{et al.}, 2010). In order to combat the host response to infection, ExPEC have employed multiple virulence factors, including cell-associated polysaccharide capsule, of which Group 2 capsules are the most prevalent of ExPEC capsule types among strains isolated up to the mid 1990’s (Johnson \textit{et al.}, 2001).

Group 2 capsule type is determined via different monosaccharide combinations; it has been suggested that there are 80 different capsules, with K1 (poly-sialic acid) as the most prevalent type (Hooten \textit{et al.}, 1996; Johnson \textit{et al.}, 1987; Johnson \textit{et al.}, 1988; Johnson \textit{et al.}, 1991; Ørskov \textit{et al.}, 1977; Ørskov \textit{et al.}, 1982). The Group 2 capsule locus is organized into three distinct linked, genetic regions (Figure 2; Whitfield 2006). Region 1 and 3 are highly conserved across Group 2 encapsulated bacteria, and encode genes requisite for capsule assembly and export. Region 2 encodes the genes integral for derivitizing capsule types and polymerizing capsular monosaccharides before export to the bacterial surface.
Antibiotics are the first line of defense against UTI (Foxman, 2010). Though antibiotic treatment of UTIs can lead to more rapid resolution of symptoms, it can also adversely affect the gut and vaginal microbiota, removing commensals, opening up a niche for pathogens to move in, uncontested (Foxman, 2010; Foxman and Brown, 2003; Foxman et al., 2000). Additionally, over-use of antibiotics can aide in the spread of antibiotic resistant strains, selecting for resistant uropathogens and commensals alike (Foxman, 2010). As of 2010, we have seen up to 80% resistance of ExPEC to these first lines of defense, pointing out the necessity to design alternative therapeutics to treat this extremely common disease (Foxman, 2010). Over the past decade, ExPEC epidemiology has undergone a major shift due, in part, to an increasing frequency and emergence of multidrug resistant strains (Johnson et al., 2002; Johnson et al., 2009), with the majority of ExPEC strains being somewhat clonal in nature (Bingen et al., 1998; Ochman and Selander, 1984; Herzer et al., 1990), based on selective pressures, we hypothesized that capsular type has undergone major shifts as well, perhaps due to survival via positive selection of specific capsule types or inheritance associated with other beneficial factors. Further knowledge about the contemporary epidemiology of the ExPEC capsules will underpin ongoing development of anti-capsule anti-infectives and modern vaccines, particularly important as conventional antibiotics become depleted.
3.2 Results

3.2.1 Non-K1 and Non-K5 Capsule Types Predominate In a Contemporary Prospective Collection of Uncomplicated UTI ExPEC Isolates

We analyzed 289 bacterial isolates that were collected from 169 hosts. The purpose of our study was to analyze uncomplicated UTI of the lower bladder, therefore all women enrolled within this study initially presented with uncomplicated UTI of the bladder. Analysis of all midstream urine samples (V0-V4) demonstrated that ExPEC caused 84.4% of uncomplicated UTI (Figure 9B; Foxman et al., 2010; Ronald, 2002). Analysis of only the initial uncomplicated UTI (V0), showed 79% are associated with ExPEC (V0; Figure 9C). These data are similar to previous UTI epidemiological data, supporting the use of this cohort for further genetic analysis (Hooten et al., 1996; Johnson et al., 1987; Johnson et al., 1988; Johnson et al., 1991; Ørskov et al., 1977; Ørskov et al., 1982).
Figure 9: *E. coli* Remain the Major Cause of Uncomplicated Cystitis in the Study Population

(A) Timeline of clinical isolate collection, all women presented with acute cystitis at the initial visit; V0: Initial visit; V1: scheduled visit 2 wks from initial visit; V2: scheduled visit 1 month from initial visit; V3: scheduled visit 2 months from initial visit; V4: scheduled visit 3 months from initial visit. (B) All midstream urine samples collected, V0-V4. (C) All organisms isolated and identified only at the initial visit (V0). Bacteria here speciated using classical clinical microbiology tests such as differential media.

We next explored the epidemiology of major capsule types carried by the *E. coli* isolated from midstream urine; we designed a multiplex PCR to detect conserved genes associated with Group 2 capsule (Ochman and Selander, 1984; Ochman and Selander,
We assessed the presence of *kpsD* and *kpsM*, located in Regions 1 and 3 of the capsule locus, respectively (Figure 10A & B). *kpsD*, the pore forming protein, enables translocation of capsular polysaccharide to the extracellular environment. *kpsM* encodes part of the capsule ATP-binding cassette transporter, which supplies energy for exportation of capsule to the surface of the bacteria (Bliss *et al*., 1996; Bronner *et al*., 1993; Higgens *et al*., 1986; reviewed by Higgens *et al*., 1990; Hyde *et al*., 1990; McNulty *et al*., 2006; Pigeon & Silver, 1994; Pavelka *et al*., 1991; Silver *et al*., 1987).

In order to identify capsule type encoded by Region 2, PCR primers internal to the variable Region 2 were designed, and PCR amplicons specific for known Group 2 capsule types were assessed. Historically ExPEC have been predominately K1, K2, or K5 Group 2 encapsulated; therefore we specifically, chose to identify the following Region 2 genes specific for capsule type: *neuS* (K1), *kfiC* (K5), or *ksIC* (K2; Figure 10C). By default this also allowed us to identify those isolates that had an undefined capsule type; these isolates were PCR positive for Group 2 capsule machinery, but PCR negative for capsule type. Based on previous studies, we hypothesized that the dominant capsule types would be known capsule types K1, K2, and K5, with a small subset of isolates with an undefined capsule.
Figure 10: Genetic Identity and Organization of the Group 2 Capsule Locus

Help
Generate a Multiplex PCR Capsule Assay

(A) Nucleotide sequence percent similarity as compared to the K1 reference strain UTI89. Percent similarity was calculated using the SDSC workbench nucleotide sequence program align. (B) Group 2 capsule locus. Region 1 transcribed from promoter 1 (PR1). Region 3/2 are transcribed via promoter 2 (PR2). Region 2 is variable; the number and genes encoded here are dependent upon capsule type. K1 and K5 are representative of Region 2. (C) Controls for capsule multiplex PCR, assessing presence/absence of conserved capsule machinery proteins: kpsM and kpsD; determining capsule type K5 (kfiC), K1 (neuS), K2 (ksIC).
3.2.2 Major Capsule Types Have Changed Over the Past Two Decades

Capsule multiplex PCR data show Group 2 capsule are dominant among ExPEC isolates; 94 of 131 (72%) midstream urine *E. coli* isolates are *kpsD* and *kpsM* positive at the initial symptomatic visit (V0; Figure 11B). Of these Group 2 encoding isolates, data show approximately 1/3 of *E. coli* midstream urine isolates are not the known capsule types K1, K2, or K5 (29%), but encode for, what we have termed, Group 2 Undefined (IIUD): positive for *kpsM* and *kpsD*, but with an as of yet undefined Region 2 (Figure 11B). Seven strains (4.5%) generated *kpsD* PCR amplicons, but did not generate *kpsM* PCR amplicons; the absence of *kpsM* amplicons is inconclusive. The amplicon absence could be due to the absence of the *kpsM* gene or from polymorphisms in the primer binding sites. For purposes of our study, we excluded isolates that were only *kpsD* positive from Group IIUD. Additionally, all three K15 strains identified by capsule multiplex PCR were isolated from urine that was culture positive for other organisms. Although interesting, the number of K15 strains is small (n=3; 1.5%) and may not be representative of all K15 strains limiting any conclusions based on these data.

Identification of the collective IIUD in samples isolated from 2008-present led us to question whether IIUD strains were found in past UTI cohorts, hypothesizing that the IIUD collective has more recently expanded (Hooten et al., 1996; Johnson, 1991; Johnson et al., 2001; 2002; 1987; 1988; 2001). In order to address this hypothesis, capsule multiplex
PCR was performed on both past UTI samples. ExPEC isolates within cohorts usually represent clonal expansions prevalent within a community, therefore we compared past samples collected via similar methods from the same university, using only strains collected from women presenting with acute cystitis (Hooten et al., 1996; Johnson et al., 1987).

Data show 72% of present day isolates, as compared to 66% of past UTI isolates, encode Group 2 capsule machinery (p=0.059; Figure 12), suggesting there has been an expansion of Group 2 encapsulated ExPEC among acute UTI. We hypothesized that this increase in Group 2 ExPEC may be due to a clonal expansion in IIUD. Comparison of the classically studied and predominate capsule type of past cohorts, K1 (Hooten et al., 1996; Johnson et al., 1987; Johnson et al., 1988; Johnson et al., 1991; Ørskov et al., 1977), with our IIUD collective showed a significant decrease in UTI associated with K1 ExPEC in present day samples, and a reciprocal increase in IIUD isolates (p=0.002; Figure 12), supporting, our hypothesis that there has been an expansion of the IIUD collective over the past 20 years.
Capsule types of *E. coli* isolated from midstream urine of women presenting with acute cystitis at the University of Washington, Seattle from (A) 1987 – 1996, and (B) 2008 - Present

### 3.2.3 Group 2 Undefined Strains are Associated with Hosts

Capsule analysis performed on past and present isolates investigated strains associated with initial acute cystitis (V0). Unlike previously analyzed cohorts, our prospective study followed hosts for 3 months after the initial infection. We hypothesized that known capsule types were most associated with hosts that go on to have multiple episodic events. Evaluation of K1, K2, or K5 capsular types, or the collective group IIUD, isoleted from initial infection (V0) were separated by the
eventual host outcome; these included single acute cystitis (sUTI), recurrent UTI (rUTI), and asymptomatic bacteruria (ASB; Scholes et al., 2000; Vraneš et al., 2003; Hooten et al., 2000). Hosts that experienced a single symptomatic episode at V0 and were culture negative for the remaining three months were grouped as single UTI (sUTI; Figure 12B); hosts with one or more symptomatic episodes post V0 were grouped as recurrent UTI (rUTI; Figure 12C); and hosts with no symptomatic episodes post initial acute UTI but were culture positive post V0, were placed in the group for asymptomatic infection (ASB; Figure 12D). Data show the IIUD collective are the most prevalent among sUTI (22%; Figure 12B), and increase in prevalence when observing hosts that proceed to have multi-episodic infections (rUTI – 34%; ASB – 37%; Figure 12B-D). However, capsule prevalence at V0 did not correlate with eventual host outcome, suggesting that capsule types of strains isolated from initial infection are not associated with specific host outcome.
Figure 12: Capsule types of *E. coli* isolated at the initial visit

(A) Capsule type of all *E. coli* isolated from midstream urine at the initial visit (V0), presenting with acute cystitis. (B) Capsule types of *E. coli* from women that presented with acute cystitis upon initial visit (V0), and had no symptoms or organisms isolated post V0; single UTI. (K1 v. K5) *p* = 0.03 (C) *E. coli* midstream urine isolate capsule types at V0. Hosts were grouped recurrent based on having a symptomatic episode anytime after V0. (D) *E. coli* midstream urine isolate capsule types at V0. Hosts were grouped asymptomatic based on having no symptomatic episodes after V0. Statistics performed using Fisher’s Exact: probability of K5 to have a single episode compared to K1, *p* = 0.03; probability of K2 & K5 to cause a single UTI as compared to K1, *p* = 0.005; probability of K1, K2, & K5 to cause single UTI as compared to IIUD, *p* = 0.05
3.2.4 Group 2 Undefined Strains are Associated with Both Asymptomatic and Symptomatic Episodes as Compared to K1, K2, and K5 Combined

The IIUD collective is highly associated with hosts that proceed to have multiple episodes, either asymptomatic or symptomatic. However, analyses were based on isolates collected at V0. We next investigated whether IIUD isolates collected from 2 weeks (V1) to 3 months (V4) post acute cystitis were more associated with asymptomatic or symptomatic infection. Based on our V0 data, we hypothesized that IIUD strains would be associated with both asymptomatic and symptomatic infections, while known capsule types would be significantly associated with symptomatic infections.

Analysis of all samples collected from 2 weeks to 3 months post infection (V1-4) showed 39% of known Group 2 capsule types (K1, 2, and 5) and 32% of IIUD strains are associated with symptomatic disease (Figure 13A); 19% of known capsule types and 49% of IIUD strains are associated with asymptomatic disease (Figure 13B). Comparison of known Group 2 capsule types (K1, 2, and 5) with IIUD demonstrated that known capsule types are significantly associated with symptomatic episodes (p=0.0009; Figure 13C), while the IIUD collective is associated with both symptomatic and asymptomatic episodes, 57% and 43% respectively (Figure 13C).
Figure 13: Proportions of Capsule Types Amongst Symptomatic and Asymptomatic Midstream Urine Isolates of *E. coli*

(A) Capsule types of *E. coli* isolated from midstream urine from all (V0-V4; n=160) symptomatic episodes with. (B) Capsule types of *E. coli* isolated from midstream urine from all (V1-V4; n=48) asymptomatic episodes (C) Percentage of Group II known capsule types (K1, K2, K5) or undefined capsule types (IIUD) found in midstream urine that present with either symptomatic or asymptomatic infection. Parenthetical numbers represent (n) for each group; Probability of IIUD causing an asymptomatic infection; p = 0.0009; Calculated using Fisher’s Exact Test

### 3.2.5 Group 2 Undefined Capsule Types Are Predominantly Phylotypes B2 and D

Thus far, we can conclude that the IIUD collective has increased in percentage over the past 20 years, and that IIUD strains are associated with both asymptomatic and
symptomatic episodes. Based on these data, we hypothesized that an increase in IIUD is due to a clonal expansion of a few ExPEC strains associated with unidentified capsule types. Therefore, we began investigating genomic characteristics of strains within group IIUD.

Previous studies have been able to group *E. coli* into different sub-groups, called phylotypes, based on specific genome content (Ochman and Selander, 1984; Selander et al., 1986; Gordon et al., 2008; Clermont et al., 2000). Recently, a rapid mechanism for phylotyping was developed based on multiplex PCR for two genes (*chuA* and *yjaA*) and one DNA fragment (*TspE4C2*); phylogenetic PCR is able to classify *E. coli* strains into 4 different groups, with PCR product indicated as parenthetical +/-: A (---; -+), B1 (--+), B2 (++-; +++), and D (+-; +++; Clermont et al., 2000). Phylotype B2 (+++; ++-) is associated with most ancient derivatives of ancestral *E. coli* and phylotypes B1 and A are most recently derived (Gordon et al., 2008; Bingen et al., 1998; Escobar-Páramo et al., 2004; Gall et al., 2007). Pathogenic strains associated with UTI are phylotypes B2, and to a lesser extent D, while phlyotypes more commonly associated with commensal *E. coli* are A, and to a lesser extent B1 (Clermont et al., 2000; Ochman and Selander, 1984; 1984; Bingen et al., 1998; Gall et al., 2007). Phylogenetic typing of K1 and K5 control groups supported use of phylogenetic typing as a measure of ExPEC prevalence (Table 1). Our data support the hypothesis that the IIUD collective is comprised of strains associated
with ExPEC; IIUD are most associated with ExPEC phylotypes, B2 (76%) and D (20%; Table 1), suggesting that an increase in IIUD strains is via expansion of ExPEC.

**Table 1: IIUD Consists of Phylotypes Associated With ExPEC**

<table>
<thead>
<tr>
<th></th>
<th>A (+)</th>
<th>B1 (-+)</th>
<th>B2 (+++)</th>
<th>B2 (+++)</th>
<th>D(+-)</th>
<th>D(++)</th>
<th>None</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>K1</strong> (n=41)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>85% (35)</td>
<td>0</td>
<td>15% (6)</td>
<td>0</td>
</tr>
<tr>
<td><strong>K5</strong> (n=25)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>80% (20)</td>
<td>16% (4)</td>
<td>4% (1)</td>
<td>0</td>
</tr>
<tr>
<td><strong>IIUD</strong> (n=90)</td>
<td>1% (1)</td>
<td>1% (1)</td>
<td>3% (3)</td>
<td>73% (66)</td>
<td>20% (18)</td>
<td>0</td>
<td>1% (1)</td>
</tr>
<tr>
<td><strong>Non-Group II</strong> (n=68)</td>
<td>28% (19)</td>
<td>26% (18)</td>
<td>15% (10)</td>
<td>3% (2)</td>
<td>13% (9)</td>
<td>0</td>
<td>15% (10)</td>
</tr>
</tbody>
</table>

Phylogenetic typing based on Clermont et al., 2000. Individual E. coli capsule types analyzed were K1, K5, IIUD, and Non-group II. Vertical lines demarcate phylogenetic types; parenthetical indicators (+/-) represent amplicon banding patterns.

Those labeled “Non-group II” *E. coli* include the phylotypes most associated with commensal *E. coli*, phylotype A (28%) and B1 (26%), (Table 1; Dobrindt et al, 2003; Johnson et al., 2001; Ochman and Selander, 1984; Duriez et al., 2001). We also found 15% (10 strains) of Non-Group II *E. coli* were phylotype B2, with the specific banding pattern (++-). This particular pattern suggests those B2 (++-) ExPEC within Non-Group II *E. coli* are genetically different from B2 (++) found in IIUD, K1, and K5. Subgroup analysis revealed that Non-group II capsule ExPEC, B2 (++-), were Group 3 encapsulated *E. coli*.
(Table 2). Group 3 capsules have similar assembly and export mechanisms as Group 2 capsule, though genetic locus organization is dissimilar, explaining the characterization of Non-Group II *E. coli* (Russo *et al.*, 1998; Whitfield 2006). These strains present the largest set of presumably Group 3 samples isolated from UTI to date.

### 3.2.6 The IIUD Strains Have A Virulence Factor Profile Distinct From K1 Isolates

We further explored our hypothesis that IIUD strains are expansions of pathogenic ExPEC, using primers for specific classical virulence factors (Johnson & Stell, 2005; Johnson *et al.*, 2005; Johnson *et al.*, 2001). Amplicon generation for virulence associated genes *ibeA, traT, sfal/foc, papA, cnf1, fyuA*, and *hlyA*, were used to create virulence profiles for IIUD strains. We compared the virulence factor profile for all IIUD strains to virulence factor profiles for known capsule types K1 and K5 strains, as well as Non-group II strains. Our data show K1 and K5 are highly associated with symptomatic infections, while IIUD are associated with both asymptomatic and symptomatic infections. Therefore, we hypothesized that virulence factor profiles of K1, K5 and IIUD would be distinctly different.

phagocytic uptake (cnfl) show almost half of IIUD isolates carry cnfl and hlyA (Table 2); prevalence of cnfl encoded by K1 or IIUD as compared with Non-Group II E. coli supports the hypothesis that IIUD are comprised of ExPEC, as these two genes are significantly more associated with K1 and IIUD (p=0.057; p=0.0394). Additionally, our data show 72% of IIUD strains are PCR positive for traT, important for mobilization of genetic material within E. coli. traT was significantly more associated with K1, K5, or IIUD as compared to Non-Group 2 E. coli (p=0.0001; p=0.001; p=0.025; Table 2). We conclude IIUD strains are significantly more likely to encode genes associated with ExPEC (cnfl, hlyA, traT) when compared to Non-group 2 E. coli, sharing similarity to known ExPEC strains with capsule types K1 and K5.
We next examined the presence of genes associated with more invasive disease, pyelonephritis and meningitis, hypothesizing that IIUD would be distinctly different from K1 encapsulated ExPEC; P-pili are associated with pyelonephritis, while IbeA, S-pili, and K1 capsule have been associated with each other, and implicated in *E. coli* meningitis in newborns (Ott *et al.*, 1988; Che *et al.*, 2010; Elo *et al.*, 1985; Enerbäck *et al.*, 1987; Gander *et al.*, 1985). Our data show P-pili (*papA*) are most prevalent among K1 isolates as compared to IIUD (*p=0.005*). Analysis of IbeA (*ibeA*), which encodes for the invasion implicated in invasion of brain microvascular endothelial cells (Bonacorsi *et al.*, 2000; Che *et al.*, 2010; Hacker *et al.*, 2003; Huang *et al.*, 2000; Huang *et al.*, 2001; Johnson *et al.*, 2001; Kim, 2003; Ott *et al.*, 1988; Parkinnen *et al.*, 1988), suggests IbeA is also

<table>
<thead>
<tr>
<th>Capsule Type (All)</th>
<th>None</th>
<th><em>ibeA</em></th>
<th><em>kpsMT I</em></th>
<th><em>kpsMT III</em></th>
<th><em>traT</em></th>
<th><em>sfa/foc</em></th>
<th><em>papA</em></th>
<th><em>cnfI</em></th>
<th><em>fyuA</em></th>
<th><em>hlyA</em></th>
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<td>K1 (n=41)</td>
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<td>59%</td>
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<td>0</td>
<td>93%</td>
<td>17%</td>
<td>66%</td>
<td>44%</td>
<td>39%</td>
<td>37%</td>
</tr>
<tr>
<td></td>
<td>(24)</td>
<td>(41)</td>
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</tr>
<tr>
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<td>92%</td>
<td>28%</td>
<td>24%</td>
<td>24%</td>
<td>92%</td>
<td>12%</td>
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<td>(3)</td>
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<td>78%</td>
<td>1%</td>
<td>72%</td>
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<td>(38)</td>
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<td></td>
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<tr>
<td>Non-Group II (n=63)</td>
<td>32%</td>
<td>3%</td>
<td>6%</td>
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<td>56%</td>
<td>19%</td>
<td>21%</td>
<td>25%</td>
<td>52%</td>
<td>6%</td>
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<tr>
<td></td>
<td>(20)</td>
<td>(2)</td>
<td>(4)</td>
<td>(15)</td>
<td>(35)</td>
<td>(12)</td>
<td>(13)</td>
<td>(16)</td>
<td>(33)</td>
<td>(4)</td>
</tr>
</tbody>
</table>

Virulence factor profiles were determined for all midstream urine isolates. Factors analyzed were IbeA (*ibeA*); Group 2 capsule (*kpsMT I*); Group 3 capsule (*kpsMT III*); TraT (*traT*); S-fimbriae (*sfa/foc*); P-pili (*papA*); Cytotoxic-necrotizing factor I (*cnfI*); Iron acquisition (*fyuA*); and hemolysin (*hlyA*). Midstream urine isolates were grouped by capsule type.
significantly associated with K1 as compared to IIUD strains (p=0.00001; Table 2). However, our data support that S-fimbriae (sfa/foc), which encode for adhesive pili that bind sialidated receptors in the brain as well as epithelial cells, muscular layers, and connective tissue in the bladder (Hacker et al., 1993; Parkkinen et al., 1988; Virkola et al., 1988), are statistically more likely to be present in IIUD strains (p=0.02; Table 2). These data lead us to conclude that while IIUD strains carry specific factors associated with ExPEC, these strains have a different virulence factor profile than K1 ExPEC, supporting our hypothesis.

As part of our analysis, we compared the resolution and specificity of our Group 2 primers kpsD and kpsM to the resolution and specificity of previously published Group 2 or Group 3 capsule primers: kpsMT II, kpsMT III, respectively (Johnson & Stell, 2000; Johnson et al., 2001). It has been previously described that kpsMT II primers were able to confirm K1 and K5 encapsulated bacteria but were not able to confirm CFT073, a prototypic K2 strain, and others (Johnson & Stell 2000). We found the previously published kpsMT primers only confirmed 78% of those we found to be kpsD and kpsM positive (Table 2), indicating our Group 2 primers kpsD and kpsM have increased sensitivity to ExPEC carrying the Group 2 locus.
3.2.7 Group 2 Undefined Capsule Isolates have a Bimodal Distribution of Virulence Determinants Associated with Symptomatic and Asymptomatic Disease

Data showed that the IIUD collective is associated with both asymptomatic (43%) and symptomatic infection (57%), while known capsule types only significantly associated with symptomatic infections (80%; p=0.0009; Figure 13). These data led us to hypothesize that the IIUD collective is heterogenous in population, with at least 2 distinct populations. Analysis of individual virulence factors encoded in K1 and IIUD clinical isolates led to examination of total (aggregate) number of virulence factors (VF) encoded by the IIUD collective. Data revealed that IIUD strains fall into two distinct sub-populations based on aggregate VF (Figure 14), supporting the hypothesis that IIUD is heterogenous. Comparison of subgroup IIUD-1 (0-3 VF) and subgroup IIUD-2 (5-8 VF) demonstrated that IIUD-1 is more associated with asymptomatic infections; conversely, IIUD-2 is more associated with symptomatic infection (p = 0.05). In contrast, neither K1 isolates alone nor K1 combined with K5 isolates fell into distinct sub-populations based on statistical analysis of aggregate virulence factors.
Figure 14: Distribution of Virulence Factors in IIUD Compared to K1 and K5 Isolates

Virulence factor distribution of aggregate virulence factors for (A) group IIUD and (B) K1 and K5 combined. Bimodal distribution of virulence factors in the IIUD collective resulting in two populations, labeled here as population 1, and population 2. Aggregate virulence factors of all (V0-4) strains.

This led us to question whether IIUD sub-populations (Figure 14) have specific virulence factor profiles. Despite differences in total number of virulence factors between IIUD sub-populations, analysis of individual factors from asymptomatic (IIUD-
1; Figure 14) or symptomatic IIUD (IIUD-2; Figure 14) sub-populations were not statistically different (Table 3). Comparison of all K1 and IIUD symptomatic strains revealed *ibeA* (p=0.0001), *traT* (p=0.0034), and *papA* (p=0.02) were significantly less likely to be encoded by IIUD (Table 3), while comparison of all K1 and IIUD asymptomatic strains revealed asymptomatic K1 strains are not statistically different from IIUD in 8 of 9 virulence profile genes (Table 3). We conclude that IIUD is a heterogenous collective with at least 2 sub-populations associated with either symptomatic or asymptomatic infection. We found no significant difference in antibiotic resistance between K1, K5, IIUD bacteria isolated in the present cohort (Figure 15), suggesting an increase in IIUD strains is not associated with antibiotic resistance. Based on virulence factors assessed, as well as antibiotic resistance, we are unable to resolve differences between these two sub-populations, implicating the potential involvement of other virulence associated factors or genetic regulation of factors analyzed.
Figure 15: Antibiotic Susceptibilities of K1, K5, IIUD and Non-group II ExPEC

Antibiotic Susceptibilities of known capsule types (K1 & K5), Group 2 undefined (IIUD), and *E. coli* that are non-Group 2 encapsulated (other). AM – Ampicillin; AMC – Amoxicillin Clavulanate; CIP – Ciprofloxacin; TE – Tetracycline; FM – Fosfomicin; GENT – Gentamicin; AMK – Amikacin; TMP – Trimethoprim; SXT – Sulfamethoxazole/Trimethoprim; FOS – Cefoxitin
Table 3: Virulence Profiles for K1 and IIUD Symptomatic and Asymptomatic Associated Strains

<table>
<thead>
<tr>
<th>Capsule Type (symptomatic)</th>
<th>None</th>
<th>ibeA</th>
<th>kpsMT II</th>
<th>kpsMT III</th>
<th>traT</th>
<th>sfa/foc</th>
<th>papA</th>
<th>cnfI</th>
<th>fyuA</th>
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<td>59%</td>
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<td>0</td>
<td>93%</td>
<td>21%</td>
<td>69%</td>
<td>45%</td>
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<td>45%</td>
</tr>
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<td>(12)</td>
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<td>(13)</td>
</tr>
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<td>IIUD (n=52)</td>
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<td>79%</td>
<td>0</td>
<td>63%</td>
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<td>40%</td>
<td>44%</td>
<td>46%</td>
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<th>Capsule Type (Asymptomatic)</th>
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<th>kpsMT III</th>
<th>traT</th>
<th>sfa/foc</th>
<th>papA</th>
<th>cnfI</th>
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<tr>
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<td>79%</td>
<td>3%</td>
<td>79%</td>
<td>29%</td>
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<td>34%</td>
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<td>(13)</td>
<td>(15)</td>
<td>(13)</td>
<td>(13)</td>
</tr>
</tbody>
</table>

3.2.8 Group 2 Undefined Strains Express Acidic Capsules

While the presence of key Group 2 capsule genes indicates the potential for ExPEC isolates to produce capsules, we sought more direct evidence for the expression of capsule. Group 2 capsules are known to be acidic in nature and released from bacteria under mild acidic conditions (Reeves et al., 1996; Whitfield 2006). Taking this into account, we first extracted and concentrated extracellular capsule material in mild acid and determined if the extracted material was high molecular weight and acidic, consistent with a Group 2 type capsule, via staining with the cationic dye alcian blue (McKinney, 1953). We sampled 2/3 of Group IIUD at V0 and found 100% of IIUD strains sampled had extractable high molecular weight alcian blue stained material. Capsule release and alcian blue staining were collected in duplicate on two separate days.
Molecular weight banding patterns of extracted material stained with alcian blue was inconclusive for capsule type differentiation.

3.2.9 Whole Genome Sequencing Suggests the IIUD Collective is Comprised of a Limited Number of Region 2 Loci

In order to further elucidate the genetic organization of the heterogenous collective IIUD Group 2 capsule Region 2, we sequenced 37 representative genomes via Illumina HighSeq. Virulence factor profile and phylogenetic type analysis revealed at least 30 different permutations, thus we chose 30 representative IIUD strains with differing profiles on which to perform whole genome sequencing. Additionally, we sequenced 2 previously unsequenced control genomes, J96 (Non-group II) and DS17 (K5), 2 representative strains that were phylotype A, and 3 representative strains that were Non-group 2, B2 (++-). de novo sequence assembly was performed to generate working contigs for evaluation. Based on known clonality of *E. coli* strains associated with UTI and meningitis, we hypothesized that the heterogeneous collective IIUD would have a limited number of Region 2 loci, predicting that heterogeneity among virulence factors could be due to genome plasticity and horizontal gene transfer. We also hypothesized that those Non-group 2, B2(++-) would encode for Group 3 capsule.

Contig analysis showed 28 of 30 strains (93%) that were part of the IIUD collective contained the highly conserved Group 2 *kps* Regions 1 and 3. Analysis of Region 2 revealed 4 representative loci for Group 2 capsule (Figure 16). Contig analysis
of non-Group 2, B2 (++) strains confirmed these isolates as Group 3 encoding ExPEC, supporting PCR results (Table 2), representing a fifth Region 2 type capsule (Figure 16). Both phylogenetic type A strains (n=2; 100%) were PCR negative for the kps locus. Analysis of proteins encoded by Region 2 revealed genes coding for enzymes, including acetyltransferases, epimerases, hydrolases, dehydrogenases, glycosyltransferases, as well as putative membrane associated and capsular biosynthesis proteins. Blast analysis revealed 90-100% similarity for proteins encoded within identically organized Region 2 loci, suggesting similar, if not identical protein functions and subsequent capsules.
Figure 16: Whole Genome Sequencing Indicates at Least Five Uncharacterized Capsule Types are Part of Group 2 Undefined (IIUD)

Whole genome sequencing results found 5 capsule types among 37 strains sequenced. Each Region 2 is schematically represented here, in comparison to K1 encoding Region 2. Types labeled 1-4 were associated with Group 2 capsule. Type 5 was associated with Group 3 capsule.
3.3 Discussion

The epidemiology of ExPEC has undergone major shifts over the past decade, as evidenced by a significant rise in multidrug resistance. These changes are necessitating the development of innovative chemotherapeutics and vaccines. However, these developments are predicated on a contemporary understanding of ExPEC genetic and phenotypic characteristics. In our current study, we sought to determine the predominant capsule types among contemporary ExPEC isolates and their co-association with clinical phenotypes and bacterial virulence determinants.

Based on previously published epidemiological data, we hypothesized that K1 and K5 would be the major capsule types found in uncomplicated UTI (Johnson and Stell 2000; Hooten et al., 1996; Johnson, 1991; Johnson et al., 1987; 1988; 2001; 2002). Many of the studies that formed the basis for this hypothesis were conducted over 20 years ago; with increasing antibiotic resistance in ExPEC, shifts in the dominant capsule types and associated virulence factors, as well as clonal expansion is likely (Foxman et al., 2010). Our results show a trend towards increasing Group 2 capsule genetic markers among the contemporary ExPEC isolates suggesting expansion of pathogenic Escherichia coli. However, while K1 and K5 K-types still dominate known capsule types of ExPEC, they represent a smaller percentage of total isolates compared to the past samples (Johnson and Stell 2000; Hooten et al., 1996; Johnson, 1991; Johnson et al., 1987; 1988;
2001; 2002). In their place, there has been an emergence of non-K1/K2/K5 Group 2 capsule isolates, referred to in this work as IIUD.

Using molecular epidemiological tools, the IIUD cohort was distinguished from K1 and K5 isolates. Like K1, K2, and K5 isolates, ExPEC with the IIUD multiplex PCR pattern were also mostly phylotypes B2 (76%) and D (20%), phylotypes associated with ExPEC (Gall et al., 2007; Bingen et al., 1998). Further investigation of a defined set of virulence associated factors among IIUD isolates indicated that there are two different subgroups: those that cause symptomatic infections and have a higher number of virulence associated factors and those that cause asymptomatic infections with lower aggregate virulence associated genes. Expanding this further, we find that IIUD isolates overall are statistically more likely to cause an asymptomatic infection than all major known capsule types combined (K1, 2, and 5). Although still requiring experimental proof, these data are consistent with a hypothesis that IIUD strains are undergoing balanced evolution with possible genomic reduction of potential virulence determinants, producing more asymptomatic colonization rather than inflammatory interactions with the host, thus potentially promoting their persistence in a commensal-like way (Dobrindt et al., 2002; Johnson et al., 2001). Epidemiological data alone are insufficient to know if the different capsules of the IIUD also play a role in masking these isolates from a host response, again promoting asymptomatic colonization and persistence, or
whether they are in epistasis with other factors increasing their prevalence. Future research will be necessary to answer these questions and to discern the genomic and phenotypic heterogeneity of the IIUD group, particularly with regard to host interactions.

Our epidemiology does support the notion that the IIUD strains are genetically distinct from strains harboring capsule types such as K1 and K5, strains typically analyzed within the literature. Measurement of the prevalence of 2 major virulence associated factors (cnfl and hlyA) demonstrated that IIUD strains carry genetic markers highly associated with ExPEC, and were statistically different from Non-group II strains analyzed. Interestingly, analysis of more invasive factors (IbeA, S-fimbriae, and P-pili) revealed significant differences between K1 ExPEC and the IIUD collective. Specifically, data showed S-fimbriae, associated with neonatal meningitis and K1 capsule, was significantly more prevalent among IIUD strains, while P-pili and IbeA were significantly more associated with K1 ExPEC (Bonacorsi et al., 2000).

This increase of IIUD over the past 20 years does pose many interesting questions. We know, based on our study, that IIUD do make acidic capsules, and based on whole genome sequencing of 30 representative IIUD strains, there are 4 major Region 2 loci within the heterogeneous IIUD collective. Due to the fact that 4 capsule types were found within the IIUD collective, and IIUD strains are associated with both
asymptomatic and symptomatic infections leads to the question of whether these capsule types illicit a different cytokine response within the host, or if these capsules are genetic markers associated with other factors leading to more asymptomatic infection. Increased capsule prevalence presents many interesting questions for continuing efforts in understanding how to combat ExPEC induced UTI.
4. Sialic Acid as a Regulatory Molecule During ExPEC Pathogenesis

4.1 Introduction

Sialic acids, a family of 9-carbon α-keto acids (Tanner, 2005), are used to coat eukaryotic cellular surfaces, via attachment to the terminal end of cell-surface and secreted glycan molecules (Varki, 2007). It is believed that sialic acids have decorated cells for billions of years; despite this, the majority of sialic acid producing organisms are vertebrates (Varki, 2007). Proteins that interact with sialic acid play a large role in eukaryotic cell signalling, intercellular adhesion and other various biological processes important for survival (Tanner, 2005; Varki, 2007; Pilatte et al., 1993). Host cells are able to delineate invading pathogens from “self” cells, based on glycans the host cell is coated with, which in many cases sialic acid (Varki, 2007). Differentiation between invading pathogens and host cells is necessary for removal of disease-causing bacteria with minimal damage to self (Pilatte et al., 1993; Varki, 2007; Roberts, 1996; Whitfield, 2006).

In addition to being able to use sialic acid as a method for self-recognition, sialic acid is also used as a signaling molecule in vertebrates (Pilatte et al., 1993; Varki 2007; Tanner, 2005). Pathogens invading host epithelia have the ability to stimulate cytokine release from epithelial cells (Schilling et al., 2003; Hunstad et al., 2005). Cytokines, in turn, are able to recruit neutrophils, one of the first lines of host defense against invading pathogens (Cross et al., 2003; Schilling et al., 2003; Hunstad et al., 2005).
Neutrophils recruited to the site of infection are able to mediate cleavage of sialic acid from host epithelial cells via translocation of sialidases to the neutrophil surface (Cross et al., 2003); this enables the host immune system to hone in on sites of inflammation by activating and recruiting resting neutrophils in addition to other immune cells in vivo (Cross et al., 2003).

While the majority of sialic acid-producing organisms are vertebrates, there are a few pathogenic bacteria that are able to synthesize and utilize this sugar (Whitfield, 2006; Varki et al., 2007). These include more invasive bacteria, such as *Escherichia coli* K1 (ExPEC) and *Neisseria meningitidis*, able to cause meningitis, as well as *Campylobacter jejuni*, now known to be associated with gastroenteritis (Tanner, 2005; Steenbergen and Vimr, 2003; Kim, 2003; Korhonen et al., 1985). Though bacteria and eukaryotes synthesize sialic acid using parallel pathways, there are slight, yet important differences between the two (Tanner 2005; Varki, 2007). For example, both vertebrates and K1 producing ExPEC have the ability to activate sialic acid (Neu5Ac → CMP-Neu5Ac); however, *E. coli* is unable to perform the reverse hydrolysis reaction (Tanner 2005).

K1 ExPEC is able to synthesize and catabolize sialic acid (Plumbridge, 1995, 1999), though genes required for synthesis and catabolism are located at unlinked loci (Zapata et al., 1989, 1992; Whitfield and Roberts, 1999; Vimr et al., 1995; Vimr et al., 2004; Tanner 2005; Steenbergen and Vimr, 2008, 1990; Silver et al., 1981; Ringenberg et al., 1985).
In ExPEC, sialic acid catabolism genes are located in the \textit{nan} operon, encoded just downstream of the sialic acid regulator, NanR (Figure 16B; Kalivoda et al., 2003; Sohanpal, et al., 2004; Plumbridge and Vimr, 1999). NanR binds to accumulated sialic acid in the cytosol, eliciting de-repression of sialic acid catabolite genes (Kalivoda et al., 2003). NanR also regulates expression of other ExPEC genes, including \textit{fimB}, which encodes for a recombinase that controls T1p expression (Sohanpal, et al., 2004; Plumbridge and Vimr, 1999). Direct regulation of \textit{fimB} and indirect regulation of T1P, by NanR, is mechanistically opposite from sialic acid catabolism (Kalivoda et al., 2003; Sohanpal, et al., 2004). In the presence of free sialic acid, NanR interacts with the monomeric sialic acid ligand, releases DNA, and down-regulates \textit{fimB} transcription; this indirectly leads to a down regulation of T1P (Sohanpal, et al., 2004).

ExPEC infection stimulates neutrophils and activates sialidases within the urinary tract. The bacterial global regulator, NanR, responds to exogenous sialic acid, de-repressing genes required for sialic acid catabolism when sialic acid levels are high. In addition to sialic acid catabolism, K1 ExPEC are able to synthesize sialic acid \textit{de novo}. We hypothesized that increased urinary sialic acid post ExPEC infection and increased
de novo synthesized sialic acid are important for bacterial niche recognition, acting as regulatory signals for virulence factor coordination during ExPEC infection.

4.2 Results

4.2.1 Growth of ExPEC in Urine Results in De-repression of the NanR-Regulated Sialic Acid Catabolic Pathway In Vitro

We hypothesized that free host sialic acid is an important signaling molecule during ExPEC infection, and predicted infection of the urinary tract would result in increased sialic acid in the urine leading to a change in NanR-dependent target expression. We expected ExPEC infection to result in increased urinary sialic acid and a de-repression of NanR-controlled targets. To test this prediction, we monitored changes in the NanR-controlled sialic acid catabolism regulon, as well as levels of sialic acid in the urine, pre- and post-infection. Sialic acid levels were determined \textit{in vivo}, using a murine UTI model, and urine samples were collected from mice every 4 hours post infection (Mulvey et al., 1998). 1,2-diamino-4,5-methyleneoxybenzene (DMB) labeling, which has been shown to specifically label sialoglycoconjugates (Neu5Ac, NeuGc, KDN, and their O-acetyl derivatives; Klein, et al., 1997; Hara et al., 1989; Hara, et al., 1987). Samples were (DMB) labeled and analyzed by HPLC. Actual sialic acid levels were determined based on a standard curve using pure sialic acid. Data show sialic acid levels increase in urine over the course of ExPEC infection (Figure 16A).
Figure 17: Sialic Acid Increases In Urine During Infection

(A) Sialic acid increases in the urine during UTI. Each line represents individual infected mice, which had urine samples collected at specified time points following infection. Each sample is normalized to level of creatinine in the urine. (B) Urine significantly increases expression of sialic acid catabolism and NanR regulated operons. (C) Sialic acid catabolism operon, under the control of NanR; sialic acid operon promoter fused to gfp (nan:gfp). (D) Sialic acid import and catabolism. Student T-test: ** p < 0.005; *** p-value < 0.0005; OM: outer membrane; IM: inner membrane
To measure de-repression of a NanR-mediated regulon, a single copy chromosomal transcriptional reporter strain was created. Fusing the sialic acid catabolism operon promoter (nan) to gfp, the reporter nan:gfp was introduced into the genome of the prototypic K1 ExPEC strain, UTI89 (UTI89 nan:gfp; Figure 1C). To validate the ability of external sialic acid to activate NanR, we grew UTI89 nan:gfp in the presence or absence of sialic acid. These data demonstrate that the reporter fusion was sensitive to sialic acid in a dose dependent fashion (Figure 1B).

While the mice are commonly used to model and investigate events during human UTI, mouse urine is a limiting resource for large-scale experiments. Therefore, we collected and pooled urine from uninfected human donors, to establish the basal level of NanR controlled regulons in urine. It has been suggested that human urine from uninfected donors contains low levels (~250µM) of sialic acid (Wang and Brand-Miller, 2003); indeed our data show a significant increase in GFP expression when UTI89 nan:gfp are grown in urine as compared to growth in minimal media or minimal media with 500µM sialic acid added (Figure 1B) suggesting other factors within urine may affect NanR-mediated regulons, or that the basal level of sialic acid in urine is higher than expected.

We next wanted to mimic an increase in sialic acid as seen during the first hour post ExPEC infection. ExPEC were grown in urine with 500µM of sialic acid added. Data
show NanR repressed genes are activated significantly when additional sialic acid was added to urine as compared to urine alone. Fluorescent microscopy readings confirmed fluorescent plate readings (Figure 17 & 18). We conclude that NanR-mediated regulons are activated in urine, with and without the addition of sialic acid.

**Figure 18: UTI89 nan:gf Reporter Expression in vitro.**

Bacteria were grown in either urine or urine + 500 µM sialic acid and washed in PBS. Bacterial nuclei were stained with DAPI. Fluorescence microscopy was performed at 100x. Strains UTI89 nan:gf and UTI89 vector control (Vc) were analyzed, representative 5 slides with 20 fields of view each presented.
Figure 19: UTI89 Vector Control at 6 Hours Post Infection

Ex vivo staining of murine bladders 6 hours post infection with UTI89 vector control. 100x objective was used to image host cell nuclei (DAPI). Bacterial nuclei were also co-stained with Topro-3 in order to more easily identify bacterial cells. Representative of 5 slides with 20 fields of view each. 40x view with DAPI stain for reference.
4.2.2 NanR-Mediated Regulation is Induced in vivo

Based on our in vitro data, we hypothesized that expression of NanR-mediated regulons would be affected in vivo, during infection. We infected C3H/HeN mice with UTI89 nan:gfp at a concentration of $5 \times 10^9$ bacteria, and sacrificed mice at either 6 or 24 hours post infection. Bladders were harvested for cryogenic sectioning, ex vivo staining, and fluorescent microscopy adjusted for autofluorescence. Both host and bacterial cells were stained with TOPRO-3, as well as nuclei stained with DAPI. Microscopy data (Figure 17), confirmed expression of NanR repressed regulons in media alone is minimal. At 6 hours post infection, GFP positive UTI89 nan:gfp co-localized with TOPRO-3 staining (Figure 20), while vector control had minimal to no GFP expression (Figure 19). Analysis of infected bladders at 24 hours with UTI89 nan:gfp (Figure 21), further supports the hypothesis that NanR-mediated regulon expression changes during ExPEC infection.
Figure 20: UTI89 nan:gfp 6 Hours Post Infection

*Ex vivo* staining of murine bladders 6 hours post infection of UTI89 nan:gfp with Topro-3 and DAPI. 100x objective was used to image host cell nuclei (DAPI). Bacterial nuclei were also co-stained with Topro-3 in order to more easily identify bacterial cells. Representative of 5 slides with 20 fields of view each.
4.2.3 Non-native NanR expression is Associated With Enhanced Persistence During Acute UTI

NanR is a global regulator, controlling not only sialic acid catabolism, but also virulence factors, such as type 1 pili (T1P; Sohanpal, et al., 2004). Data show urinary sialic acid increases in concentration during ExPEC infection, and NanR-repressed regulons are also activated during ExPEC infection, indicative of ExPEC importation of host sialic acid. We hypothesized that ExPEC use sialic acid as a sensory molecule.

Figure 21: UTI89 nan:gfp 24 Hours Post Infection

Ex vivo staining of murine bladders 24 hours post infection with UTI89 nan:gfp. 100x objective was used to image host cell nuclei stained with DAPI. Bacterial nuclei were co-stained with Topro-3. Representative of 5 slides with 20 fields of view each.
potentially enabling the bacterium to distinguish location within the host, via expression changes in NanR-controlled regulons, affecting pathogenesis in vivo. We hypothesized deletion of nanR (UTI89ΔnanR) would significantly impair ExPEC in vivo, since bacteria would lose sialic acid-dependent niche sensing and thus be unable to respond with appropriate gene expression.

We constructed the deletion strain UTI89ΔnanR via P1 transduction and introduced tac-gfp on a plasmid, pCom-GFP into either UTI89 wt, or UTI89ΔnanR to investigate both wt and ΔnanR survival in vivo. IBCs are requisite for ExPEC survival in vivo and phenotypically distinct within the bladder epithelia (Anderson et al., 2004; Mulvey et al., 1998; Justice et al., 2004). Both T1P and capsule are required for IBC formation, and T1P expression is indirectly regulated via NanR. Therefore, we hypothesized UTI89ΔnanR would not be able to form IBCs in vivo. However, fixed bladders post 24 hour infection with UTI89 com:gfp or UTI89ΔnanR com:gfp were similar in IBC formation (unpublished data – Carlos Goller), suggesting factors necessary for IBC formation were not significantly affected by the absence of NanR.
Figure 22: Deletion and Constitutive Expression of *nanR* at 48 Hours Post Infection in TLR-4 Responsive C3H/HeN

Post 48-hour infection CFU counts C3H/HeN (A) bladder and (B) kidney homogenates. Constitutive expression of NanR significantly increases fitness as compared to the knockout. Numbers are representative of 3 experiments, 10-20 animals per group in total. Numbers represent 2 experiments, 10 animals per group. Dotted line represents level of detection.

NanR-repressed genes are transcriptionally activated 6 and 24 hours post infection; however there is a time lag between transcription and translation (Bortulussi, 1983). Additionally, the cycle of ExPEC infection includes multiple rounds of host cell invasion (Mulvey et al., 1998). Therefore, we hypothesized that sialic acid sensing is important for ExPEC niche specificity during later infection, potentially having a role in either emergence from epithelial cells or further host invasion. Infections were extended to either 48-hours or 2-weeks, and *UTI89ΔnanR* fitness was assessed *in vivo* via colony
counts from homogenized bladders and kidneys. Forty-eight hours post infection data suggest a trend towards loss of fitness in a ΔnanR strain as compared to wildtype.

Complementation via pBAC-nanR, a single copy plasmid with nanR expressed under the tac promoter and by default constitutively expressed significantly increases fitness in the bladder (p=0.02; Figure 22A) and kidneys (p=0.002; Figure 22B) in C3H/HeN mice as compared to the isogenic knockout, UTI89ΔnanR. Infections carried out to 2 weeks did not affect fitness (Figure 23A & B). We conclude constitutive expression of NanR increases fitness at 48 hours in vivo. Constitutive expression could affect positively regulated NanR targets, as they would be less likely to be down regulated despite increased free host sialic acid. Alternatively, NanR constitutive expression may exceed sialic acid levels resulting in repression of NanR targets, which may be beneficial to chronic infection.

Toll-like receptor 4 (TLR-4) is important for innate immune system activation during Gram-negative infection (Schilling et al., 2003). TLR-4 is known to recognize LPS, which activates a signaling cascade, resulting in chemokine production (Rosenstreich and Glode, 1975) necessary for neutrophil chemotaxis and activation (Shahin et al., 1987) leading to sialidase translocation to the neutrophil surface (Sakarya et al., 2004). Host cell de-sialidation perpetuates the activation of the immune system (Sakarya et al., 2004), presumably causing an increase in urinary sialic acid as we observed (Figure 17A). The
initial inflammatory response during UTI is significantly reduced in the absence of TLR-4 signaling (Shahin et al., 1987). Therefore the result might be decreased urinary sialic acid. We have used C3H/HeN mice thus far, which are TLR-4 responsive, having a primary neutrophil influx 24 hours post ascending UTI inoculation (Shahin et al., 1987).

Figure 23: Deletion and Constitutive Expression of nanR at 2 Weeks Post Infection in TLR-4 Responsive C3H/HeN

Bacterial counts 2 weeks post infection of C3H/HeN mice, both (A) bladder and (B) kidney homogenates. Constitutive expression of NanR significantly increases fitness as compared to the knockout. Numbers are representative of 3 experiments, 10-20 animals per group in total. Numbers represent 2 experiments, 10 animals per group. Dotted line represents level of detection.

C3H/HeJ mice have a mutation at the lps (lipopolysaccharide) locus, manifesting a poor response to lipopolysaccharide (endotoxin), rendering HeJ mice as TLR-4
nonresponsive (Rosenstreich and Glode, 1975; Coutinho, 1976; Shahin et al., 1987). Absence of a host immune response to LPS during UTI significantly affects the primary neutrophil influx at 24 hours and presumably decreases free urinary sialic acid (Shahin et al., 1987). Due to the lack of primary neutrophil influx, bacterial clearance is dampened at 24 hours (Sakarya et al., 2004). We hypothesized that the effect we witnessed in HeN mice was due to positive NanR mediated regulation, and that there would be no significant difference between ΔnanR, constitutive nanR, and wildtype strains when TLR-4 nonresponder mice (C3H/HeJ) were infected.

Figure 24: Deletion and Constitutive Expression of nanR at 48 Hours Post Infection in TLR-4 Non-responsive C3H/HeJ

Post 48-hour infection CFU counts C3H/HeJ (A) bladder and (B) kidney homogenates. Constitutive expression of NanR significantly increases fitness as compared to the knockout. Numbers are representative of 3 experiments, 10-20 animals per group in total. Numbers represent 2 experiments, 10 animals per group. Dotted line represents level of detection.
We tested fitness of UTI89ΔnanR, and the NanR constitutive expression strain *in vivo* infecting LPS non-responder mice, C3H/HeJ. Data show that strains constitutively expressing *nanR* are still significantly more invasive than UTI89ΔnanR for both bladders (p=0.0148; Figure 24A) and kidneys (p=0.0098; Figure 24B). However, wildtype invasion of HeJ mice is similar to a *nanR* constitutive strain in HeN (Figure 22A & 24A). We conclude that multiple factors are involved during UTI, potentially independent of sialic acid and NanR-mediated regulation. Additionally, these data suggest a low sialic-acid state within the bladder is favorable for K1-ExPEC survival, as both constitutive expression of *nanR* in a high sialic acid state (HeN), and wildtype in a low sialic acid (HeJ) are similar. These data support the hypothesis that NanR positively regulates genes necessary for increased pathogenesis and that constitutive expression of NanR potentially enables continued positive regulation of targets in a high sialic acid state, phenocopying gene regulation in a low sialic acid state.

### 4.2.4 A Low Cytoplasmic Sialic Acid State Benefits ExPEC *in vivo*

To further investigate the hypothesis that a low sialic acid state increases ExPEC fitness *in vivo*, we genetically deleted *nanT*, the sole importer of sialic acid across the inner-membrane (Figure 17C; Martinez et al., 1995). Deletion of *nanT* ablates sialic acid importation, and ExPEC will be in a continual low sialic acid state. Therefore we
predicted a \textit{nanT} mutant would phenocopy C3H/HeN mice infected with UTI89\textit{ΔnanR}/COMP, constitutively expressing levels of \textit{nanR} exceeding sialic acid amounts.

UTI89\textit{ΔnanT} was phenotypically tested via growth in minimal media with glycerol or sialic acid as the sole carbon source, and compared to wildtype. UTI89\textit{ΔnanT} grown in minimal media with sialic acid alone was unable to survive, confirming deletion of \textit{nanT} (Martinez et al., 1995). All strains grown in minimal media with glycerol exhibited similar growth. UTI89\textit{ΔnanT} fitness during UTI was tested in C3H/HeN mice and compared with isogenic wildtype infections at 48 hours and two weeks. UTI89\textit{ΔnanT} trended toward increased fitness in the bladder at both 48 hours and 2 weeks (Figure 23A & B), supporting the hypothesis that a low internal sialic acid state increases ExPEC fitness \textit{in vivo}.
Figure 25: Deletion of *nanT* at 48 Hours and 2 Weeks Post Infection in C3H/HeN

C3H/HeN mice were infected with either UTI89 wildtype or UTI89*DnanT* and bladders are harvested at (A) 48-hours post infection or (B) 2 weeks post infection. Thick black lines signify geometric mean. Statistics determined using a non-paired T-test of the geometric mean.

### 4.2.5 Sialic Acid Increases the Amount of Mannose Resistant Adherence

T1P expression is regulated via recombinase-mediated phase switching of the promoter region, *fimS*. Expression of *fimB*, the recombinase able to turn T1P both ON and OFF, is regulated via NanR and NagC (Plumbridge and Vimr, 1999). Both NanR and NagC have duplicative roles in gene regulation; the DNA bound form is able to both repress as well as activate transcription of genes (Plumbridge and Vimr, 1999). While NanR represses transcription of the sialic acid catabolism operon (*nan*), it positively regulates *fimB* transcription indirectly regulating T1P expression via FimB.
mediated fimS switching (Plumbridge and Vimr, 1999). NagC also positively controls fimB expression (Condemine et al., 2005). In the absence of available GlcNAc, NagC remains DNA bound, and transcription of fimB occurs, indirectly affecting T1P expression (Plumbridge and Vimr, 1999; Condemine et al., 2005).

fimB control via two regulators enables fine-tuning of T1P expression. fimB transcription occurs at the highest level when both NagC and NanR are DNA bound (Condemine et al., 2005). In the absence of both regulators, fimB transcription is repressed (Condemine et al., 2005). However, in the event there are high levels of cytosolic sialic acid and low levels of GlcNAc, and NagC is bound while NanR is not, fimB is expressed at moderate levels (Condemine et al., 2005). When NagC is unbound and NanR is bound, high cytoplasmic levels of GlcNAc and low levels of sialic acid, fimB is transcribed at low levels (Plumbridge and Vimr, 1999; Condemine et al., 2005). We hypothesized that an increase in exogenous sialic acid in minimal media may be affecting fimB expression, and that this could account for increased fitness in the absence of sialic acid. We investigated our predictions using in vitro tissue culture models.

UTI89 grown in minimal media with glycerol, and exogenously added sialic acid did not significantly decrease fimS ON orientation, as measured by phase PCR (Figure 26A), or affect T1P protein expression, as measured by T1P western (Figure 26B). Because expression of fimB has been shown to be moderately transcribed in the presence
of bound NagC (low GlcNAc) and unbound NanR (high sialic acid), we predicted that changes in T1P expression may be below our level of detection for both phase PCR and αT1P western.

**Figure 26: fimS Orientation, T1P Protein Expression, and T1P Mediated Adherence to 5637 Bladder Epithelial Cells**

(A) Orientation of the T1p promoter, fimS, in response to various media treatments. (B) T1p western, probing for the major pilus subunit, FimA. Representative samples prepared on separate days shown. (C) Thirty-minute adherence of UTI89 (K1) to 5637 bladder cells *in vitro* +/- 500 µM sialic acid in the absence or (D) presence of mannose in RPMI. Infections were performed at an MOI of 10. Bacteria were grown initially in LB overnight; back diluted 1:100 into M9 + 0.2% glycerol overnight; then back diluted into the respective medias. Media for (A) and (B) are as follows: (1) M9 (0.2% Glycerol); (2) M9 (Glycerol) + 250 µM sialic acid; (3) LB; (4) Pooled urine.
T1P mediate adherence to epithelial cells via binding of mannosylated receptors. Infection of confluent monolayers with UTI89 at a multiplicity of infection (MOI) of 10, in the presence or absence of sialic acid did not affect ExPEC adherence levels, correlating with both phase orientation and protein levels (Figure 24C). T1P are not the only factors able to bind epithelial cells (Elo et al., 1985; Gander et al., 1985; Hacker et al., 1993), and in the presence of exogenous mannose, T1P mediated adherence is ablated (Gander et al., 1985; Hacker et al., 1993). We hypothesized that adherence seen in the presence of sialic acid was not T1P-mediated, and therefore ExPEC in the presence of sialic acid would elicit mannose resistant adherence. To address this hypothesis, we grew ExPEC in the presence or absence of 500µM sialic acid and/or 1% mannose. Data show sialic acid causes a significant increase in mannose resistant adherence (Figure 26D).

4.2.6 Growth in Urine Increases Adherence of UTI89

Having explored the effects of sialic acid in minimal media on TIP expression, we next tested the effect sialic acid has on ExPEC grown in urine. As previously discussed, T1P are requisite for adherence and invasion of bladder epithelia as well as IBC formation post invasion of urothelial cells, thus expression of T1P during UTI is integral to ExPEC survival in vivo (Mulvey et al., 1998). Microarray data have shown ExPEC grown in urine genetically upregulate fimA (Snyder et al., 2004). Data suggest a
low cytoplasmic sialic acid state is favorable for ExPEC survival in vivo, and that sialic acid increases expression of mannose resistant adherent factors in vitro. We hypothesized that in the harsh environment of urine, exogenous sialic acid would decrease T1P expression, and increase mannose resistant adherence.

Initial analysis of UTI89 grown in urine showed a significant decrease in T1P protein expression by αT1P western (Figure 26B), suggesting possible strain differences in T1P expression when grown in urine (Snyder et al, 2004). Initial ExPEC infection would presumably be via bacteria that are urine naïve. Therefore, we predicted that exposing urine-naïve ExPEC to urine would increase adherence via T1P, while exposure of urine-naïve ExPEC to urine with sialic acid would increase mannose resistant adherent factors.

We investigated the effect additional sialic acid in urine had on bacterial adherence and invasion. Monolayers were pre-incubated with either RPMI (+/- sialic acid) or urine (+/- sialic acid) for 20 minutes, and corresponding fresh media was added immediately before infections. ExPEC grown in LB were added to monolayers and adherence and invasion were assessed. Because gentamicin is unable to permeate host cells, bacteria that are primarily extracellular are killed during gentamicin treatment, giving an estimate of intracellular bacteria, or invasion. Monolayers were treated with gentamicin for 2 hours after initial 30-minute adherence. ExPEC adherence (Figure 27A)
and invasion (Figure 27B) of urothelial cells was similar for all infections, regardless of media conditions during infection. These data suggest that despite a decrease in T1P expression when ExPEC are grown in urine, 30-minute exposure to either urine or sialic acid does not affect initial adherence and invasion.

**Figure 27**: Similar Levels of Adherence and Invasion of Naïve UTI89 Exposed to Urine and/or Sialic Acid for 30-minutes

(A) Adherence and (B) invasion of *UTI89* to 5637 cells. Bacteria were grown in LB and added at an MOI of 10 to 5637 monolayers that were either pre-incubated for 30 minutes in media or urine. Final infections were performed in fresh media that correlated to pre-incubation media. Invasion was determined after 2 hours of gentamicin treatment. Infections were repeated 3 times in triplicate.

ExPEC that have not succeeded in initial invasion, and have not been voided by the bladder, are exposed to the harsh environment of the urinary tract, presumably proliferating in urine. Factors necessary for LPS production, outermembrane porin
expression (OmpC, NanC), and genes necessary to combat stressful environments (pH, oxidative, etc.) are upregulated as seen by microarray data (Snyder et al., 2004). We hypothesized that growth in the harsh environment of urine would increase mannose resistant adherence and decrease mannose sensitive (T1P) mediated invasion.

Figure 28: Sialic Acid in Urine Increases the Amount of Mannose Resistant Adherence

(A) Adherence of UTI89 to bladder epithelial cells in the absence or (B) presence of mannose. Bacteria were grown in either M9, M9 + sialic acid, or Urine, Urine + sialic acid. Infections were performed at an MOI = 10, and repeated 3 times in triplicate. Statistics were performed by Students T-test: *p < 0.05

Wildtype UTI89 was grown in LB or urine for approximately 18 hours, and monolayers were pre-incubated with urine or RPMI for 20 minutes before infection. ExPEC grown in urine were significantly more adherent than bacteria grown in LB (Figure 28A), and this adherence was mannose resistant (Figure 28B). Microscopy of
ExPEC grown in urine revealed large bacterial aggregates as compared to ExPEC grown in LB. Additionally, UTI89 grown in sialic acid for pre-infection did not significantly affect total ExPEC adherence (Figure 28A) but did increase mannose resistant adherence to bladder epithelial cells (Figure 28B), similar to growth in M9 + sialic acid.

Additionally, we investigated whether increased adherence observed in urine was K1-specific. Data show the 10-fold increase in adherence observed with UTI89 (K1) is not observed for CFT073 (K2) or GR12 (K5; Figure 29). We conclude growth in urine decreases K1 T1P expression but potentially increases ExPEC/ExPEC interactions, suggesting increased adherence to urothelial cells *in vitro* is most likely via non-T1P mediated ExPEC/ExPEC interactions (Figure 28B) highlighting the relevance of *in vivo* study.

**Figure 29: Adherence of K1, K2, and K5 strains to 5637 Bladder Cells**

Adherence of UTI89 (K1), CFT073 (K2), and GR12 (K5) to bladder epithelial cells Bacteria were grown in either Minimal media supplemented with glycerol, or Urine. Infections were performed at an MOI = 10, and adherence determined as a percent of the initial inocula.
4.2.7 Growth in Urine Decreases ExPEC Invasion

Based on our prediction that ExPEC/ExPEC interactions might account for the increase in adherence despite decreased T1P expression, we hypothesized ExPEC grown in urine would have decreased invasion. Data showed that ExPEC grown in urine were able to invade at similar levels as ExPEC grown in LB, with a trend toward less invasive (Figure 30B), suggesting differences in T1P expression may be subtle, or less likely, occasional bacterial aggregates are taken up by urothelial cells.

![Bar chart showing adherence and invasion](image)

**Figure 30: Growth In Urine Increases Adherence; Addition of Sialic Acid Decreases Invasion**

(A) UTI89 adherence and (B) invasion of 5637 bladder epithelial cells. Bacteria were grown in LB or urine, and added at an MOI of 10 to monolayers pre-incubated with media or urine. Final infections were performed in media correlating with pre-incubation
Although the addition of sialic acid to urine did not affect bacterial adherence (Figure 30A), data demonstrate addition of sialic acid significantly increased mannose resistant adherence. We hypothesized infection in the presence of sialic acid would decrease invasion. Exogenous sialic acid in urine significantly inhibits invasion of bacteria grown in urine as compared to ExPEC grown in LB (Figure 30B). We conclude that the addition of sialic acid to urine significantly increases mannose resistant adherence, and decreases invasion in vitro.

4.2.8 K1 ExPEC Regulation of Sialic Acid: Capsule vs. Signaling

K1 ExPEC are able to both import and synthesize sialic acid. When capsule is synthesized but not assembled and exported, polysaccharides are pooled into vacuole-like lacunae, visible by TEM, and are presumably unable to affect NanR-mediated regulation (Cieslewicz and Vimr, 1996; 1997). It has been suggested that to maintain separation between sialic acid synthesis and catabolism/regulation, de novo synthesized sialic acid occurs in a protected compartment, thus inhibiting immediate degradation (Steenbergen and Vimr, 2008). However, deletion of NanR in a K1-capsule assembly mutant partially rescues ExPEC survival in vivo (Anderson et al., 2010). We hypothesized de novo synthesized sialic acid is involved in gene regulation via NanR in natively encoding K1 ExPEC strains.
4.2.9 Inhibition of Capsule Export Increases Intracellular Levels of Unpolymerized Sialic Acid

Because of the necessity to simultaneously express K1 capsule, regulate sialic acid controlled regulons, and catabolism of this sugar, we predicted that deletion of regI in our K1 strain, UTI89, would continue to synthesize sialic acid, but de novo synthesized sialic acid would be protected from degradation, and cytosolic levels of sialic acid would increase. Using previously published regI and regII mutants (Anderson et al., 2010) levels of free and total sialic acid were assessed via the thiobarbaturic acid assay (TBA; Warren, 1953). The TBA assay detects unpolymerized sialic acid colorimetrically. Because TBA does not react with polymerized sialic acid, we were able to delineate between mono- and poly-sialic acid. Monomeric/free sialic acid is oxidized for 30 minutes at 37° C, terminated, and thiobarbituric acid (chromophore) is added and developed by boiling for 7.5 minutes (Skoza and Mohos, 1976; Aminoff, 1961, Warren, 1953). Group 2 capsules are heat and acid labile, breaking down from oligosaccharides into monosaccharides; we assessed total amount of sialic acid by liberating polymerized sialic acid (sulfuric acid and heat) pre-TBA treatment. Acid/heat hydrolysis alone did not affect TBA readings (Figure 31A).
Figure 31: Amount of Internal Unpolymerized Sialic Acid Assessed by TBA and HPLC

(A) Acid treated TBA controls. (B) Intracellular free (unpolymerized) sialic acid levels of UTI89, UTI89ΔregI, and UTI89ΔregII. (C) Total sialic acid for UTI89, UTI89ΔregI, and UTI89ΔregII. (D) DMB labeled sialic acid HPLC results. TBA assay was measured at an optical density of 549 nm, and calculated as percent of wildtype (Y-axis). Light Units were measured by HPLC. Statistics calculated using Student T-Test; **p-value < 0.0001.

Levels of free sialic acid were significantly increased in an assembly mutant, compared to both wildtype and a synthesis mutant (Figure 31B). Total amount of sialic acid for UTI89 and UTI89ΔregI are significantly higher than a synthesis mutant (Figure 31C). Total levels of sialic acid were similar between wildtype and an assembly mutant (Figure 31C). These data support the hypothesis that an assembly mutant has increased
levels of free cytosolic sialic acid, and there is not a feedback mechanism on capsule synthesis genes in a capsule assembly mutant.

TBA is able to react with a few other molecules including deoxyribonucleic acid and lipopolysaccharide. DMB, however, has been shown to specifically label free (unpolymerized) sialic acid, and is easily detected by reverse phase HPLC. DMB-labeled sialic acid was used to confirm TBA results, and integrated free sialic acid standard curves were compared to HPLC results for UTI89, UTI89ΔregI, and UTI89ΔregII. HPLC data supported the TBA assay results (Figure 31D). These data support the hypothesis that cytosolic free sialic acid increases in a K1 mutant unable to assemble capsule, and is either not degraded or synthesized at a rate faster than degradation.

4.2.10 Synthesized Free Sialic Acid is Unable to Interact With NanR

An increase in cytosolic levels of free sialic acid led us to hypothesize that de novo synthesized sialic acid is able to interact with NanR. Using our nan:gfp reporter, wildtype, ΔregI, and ΔregII strains were grown overnight in rich LB media, and back-diluted 1:100 twice into minimal media with glycerol as the sole carbon source because LB contains trace amounts of sialic acid. GFP readings taken on the third day are presumably representative of the ability for de novo synthesized sialic acid to affect NanR–mediated regulons. Data show the level of NanR activation is significantly lower in an assembly mutant than wildtype or a synthesis mutant (Figure 32A). These data, in
conjunction with TBA and HPLC data suggest that the majority of free sialic acid does not de-repress NanR targets.

Figure 32: Internal free sialic acid is unable to derepress NanR-mediated regulons

(A) De-repression of NanR-mediated regulons post growth in M9 + glycerol. (B) De-repression of NanR-mediated regulons post growth in M9 + glycerol with 250µM of sialic acid. (C) Fold change of NanR-mediated regulons in wildtype UTI89, (D) a K1 capsule assembly mutant, and (E) a K1 synthesis mutant. Units are relative fold change as compared to respective vector controls. Statistics were performed using Students T-test: *p<0.05; **p<0.005; ***p<0.0001; ****p<0.00001
Though accumulated internal free sialic acid does not directly de-repress NanR-regulons in an assembly mutant, we hypothesized that internal free sialic acid mitigated ExPEC response to exogenous sialic acid. Data show de-repression of NanR-controlled regulons via addition of sialic acid to M9 + glycerol is independent of strain (Figure 32C, D, E). An assembly mutant has significantly lower NanR activation than either wildtype or a synthesis mutant (Figure 32B); however, calculations of the change in NanR de-repression from basal level (M9 + glycerol) to induced (M9 + glycerol and sialic acid), show NanR-controlled regulons are activated to similar levels in UTI89, UTI89ΔregI, and UTI89ΔregII (Figure 32B). These data suggest accumulated internal free sialic acid does not inhibit the K1 ExPEC response to exogenous sialic acid.

4.2.11 Increased de novo Synthesized Cytosolic Sialic Acid Correlates with Increased Transcription of NanR-controlled Regulons in Urine

Upon infection, ExPEC encounter a hostile environment within the urinary tract, stimulating changes in gene regulation (Snyder et al., 2004). Binding of adhesins is also able to stimulate a change in gene regulation, as is the case with binding of T1P to host mannosylated receptors, which leads to a decrease in expression of capsule assembly genes (Schwan et al., 2005). A capsule assembly mutant is unable to survive in vivo, however deletion of nanR in a capsule assembly mutant parially restores survival, implicating the possibility of cross-talk between binding of T1P, sialic acid capsule
synthesis, and sialic acid/NanR-mediated regulation (Schwan et al., 2005; Anderson et al., 2010). To investigate the interactions between these factors, we grew a capsule assembly mutant (ΔregI) in urine, and assessed NanR-mediated regulation. We hypothesized that NanR-mediated gene regulation is affected by a build-up of internal sialic acid, mitigating changes in sialic importation.

K1 ExPEC grown in urine significantly de-repressed NanR-controlled regulons, regardless of strain (Figure 33A, B, & C). These data support our previous conclusion that NanR-mediated genes are de-repressed when grown in human urine. Comparison of wildtype with UTI89ΔregI showed de-repression of NanR regulated genes was significantly increased when UTI89ΔregI was grown in urine (Figure 33D). In contrast, NanR activation was similar between wildtype and a synthesis mutant (Figure 33D). These data support the hypothesis that an internal excess of (presumably) de novo synthesized sialic acid possibly affects sialic acid importation. Additionally, these data suggest this effect is niche specific, as it was not seen when ExPEC were grown in minimal media with sialic acid.
Figure 33: Increased de novo synthesized cytosolic sialic acid correlates with increased transcription of NanR-controlled regulon in urine

(A) Wildtype UTI89, (B) a capsule assembly mutant, and (C) a K1 synthesis mutant, significantly increase NanR mediated signaling in the presence of urine. (D) UTI89ΔregI has significantly higher levels of NanR activation as compared to wildtype or a synthesis mutant in the presence of urine. Units are relative fold change as compared to respective vector controls. Statistics were performed using Students T-test: *p<0.05; **p<0.001; ***p<0.0001; ****p<0.00001
In this chapter, we observed the ability of urine alone to derepress NanR-controlled regulons in vitro (Figure 18), identified that NanR-mediated regulons are induced during UTI (Figure 20 & 21), and demonstrated that low levels of internal sialic acid increase fitness of K1 ExPEC during UTI (Figure 25). Increased levels of synthesized cytosolic sialic acid are unable to derepress NanR-controlled regulons, and had hypothesized that sialic acid import was affected by an increase of cytosolic synthesized free sialic acid. These data led to the hypothesis that NanR-mediated regulation is affected via a build-up of internal sialic acid, mitigating changes in sialic importation.

4.3 Discussion

Free urinary sialic acid increases during UTI. Presumably activated neutrophils cleave sialic acid from host cells during an inflammatory response (Cross et al., 2003; Sakarya et al., 2004), and de-sialidation stimulates neutrophil activation and signals for additional recruitment. Hosts with low neutrophil activation and translocation of sialidases are more prone to invasive disease as compared to hosts with an initial innate immune response (Sakarya et al., 2004; Coutinho, 1976; Schilling et al., 2003). ExPEC associated with UTI are able to import and catabolize sialic acid, as well as synthesize sialic acid in the case of K1 ExPEC (Andreishcheva and Vann, 2006; Condemine et al., 2005; Ganguli et al., 1994; Kalivoda et al., 2003; Kean, 1991). NanR, an ExPEC global regulator, is able to act as a genetic repressor and positive regulator controlling sialic
acid catabolism, T1P expression, and other ExPEC factors (Sohanpal et al., 2004). High cytoplasmic levels of sialic acid have been shown to repress fimB expression (Sohanpal et al., 2004, 2007). We hypothesized sialic acid is important for ExPEC recognition of sites within a host, enabling the bacteria to genetically regulate factors necessary for different niches.

Our data have shown the following: (1) both urine and sialic acid significantly affect NanR-controlled regulons; (2) a low sialic acid state trends toward increased ExPEC survival in vivo (3) urine increases adherence, most likely due to bacterial aggregation (4) T1P protein, mediated adherence and invasion are decreased when ExPEC are grown in urine; (5) free sialic acid significantly increases mannose resistant adherence and decreases invasion; (6) strains unable to assemble capsule have increased levels of cytosolic sialic acid; and (7) strains with increased cytosolic levels of sialic acid have increased NanR-mediated signaling in the presence of exogenous sialic acid (Figure 34).

Both uninfected urine and urine with additional sialic acid are able to stimulate NanR mediated regulation. Bacteria encounter harsh environments within the urinary tract during infection. We chose to focus our studies using urine as the primary media, as it is more relevant to study ExPEC. Growth in urine as well as 30 minute exposure to urine significantly increase mannose sensitive bacterial adherence, but decrease T1P
protein expression. Microscopy showed bacteria grown in urine were mostly found as aggregates, and we surmise that increased mannose sensitive adherence is indirect adherence due to increased bacterial aggregation. These data lead us to conjecture that ExPEC exposed to urine increase adherence, but growth in urine decreases T1P expression. ExPEC expressing T1P would still be able to mediate interaction with host cells, as well as form bacterial aggregates, and we conjecture this could permit non-T1P piliated bacteria to be intimately associated with host epithelial cells. We also surmise bacterial aggregation could hinder interaction between the innate immune system and T1P adherent cells.

**Figure 34: Model of Sialic Acid Effect on ExPEC During UTI**

1. Initial Infection
2. Neutrophil Influx
3. Urinary Sialic Acid
4. NanR-mediated de-repression
5. T1P expression
6. Mannose-resistant adherence
7. Bacterial aggregation

(1) ExPEC infect the urinary tract and trigger the innate immune response stimulating (2) neutrophil influx. (3) Urinary sialic acid increases and (4) NanR-mediated gene de-repression is induced. Exposure of ExPEC to urine and sialic acid decreases (5) T1P protein expression and T1P-mediated invasion, while increasing (6) mannose-resistant adherence, and (7) bacterial aggregation.
Overtime, genetic expression changes as ExPEC are grown in urine (Snyder et al., 2004). We infer ExPEC that do not initially invade urothelial cells, but are protected via aggregation grow and replicate within the harsh urinary tract environment, decreasing T1P expression as well as regulating other factors necessary for survival. Release of sialic acid at the site of infection via neutrophil sialidases increases non-T1P (mannose resistant) mediated adherence, decreasing the number of invasive events relative to adherence. Supporting this inference, data show a low sialic acid state increases survival at 48-hours during ExPEC infection.

A low sialic acid state suggests NanR-mediated positive regulation increases fitness in vivo, as seen by our NanR constitutive strain, as well as our nanT mutant. However, K1 ExPEC strains also synthesize sialic acid. We investigated the possibility of synthesized sialic acid to affect NanR-mediated regulation. Previous examination of capsule synthesis suggests capsule synthesis is either linked to capsule export or in a protected compartment, inhibiting degradation of newly created sialic acid via catabolism or de-regulating NanR-controlled genes (Steenbergen and Vimr, 2008). However, these studies were performed using EV36, a K1:K12 hybrid strain. Cross-talk between these pathways in a natively expressing K1 strain, as well as the affect loss capsule assembly has on NanR-mediated regulation has not been investigated until now.
TBA data show the loss of capsule assembly genes in a native K1 background, results in increased free sialic acid presumably within the bacterial cytoplasm. *de novo* synthesized sialic acid does not directly interact with NanR, suggesting K1 encapsulated ExPEC are able to differentiate between synthesized and imported sialic acid. NeuA activates sialic acid for polymerization by attaching a CMP to sialic acid monomers. Based on these data, we surmise that synthesized sialic acid is rendered inaccessible for degradation and direct involvement in genetic regulation by the addition and activation of sialic acid via CMP. We suspect that in the absence of capsule export, synthesized sialic acid monomers are marked as bacterially synthesized with the addition of CMP.

Binding of T1P to mannosylated receptors decreases capsule assembly genes (Schwan et al., 2004), and we speculate that this interaction only down-regulates transcription at Region 1, and not of Region 2 and 3, as genetic deletions within Region 1 do not feedback on synthesis of capsule monosaccharide. Though an increase in *de novo* synthesized sialic acid monomers does not directly increase NanR-mediated de-repression, the change in NanR-mediated de-repression is significantly increased when strains with an internal buildup of sialic acid are grown in urine. We extrapolate that by a yet unidentified mechanism, increased synthesized cytoplasmic sialic acid leads to an increase in exogenous sialic acid importation and signaling, and suggest synthesized internal sialic acid might indirectly affect NanR regulation. Because a synthesis mutant
behaves similarly to wildtype, this would imply increased NanR mediated gene
activation, is a result of de novo synthesized free sialic acid, presumably through sialic
acid import, and not the mere lack of extracellular capsule. This correlates with our in
vivo data, supporting a increased survival when ExPEC low cytoplasmic sialic acid state
within the bacteria.

We suggest sialic acid/NanR genetic regulation is an evolving mechanism the
bacteria is employing to regulate necessary genes in vivo. One can see how this is an
example of co-evolution between host and bacteria. Bacteria invade hosts, which in turn
evolve a signaling mechanism – cleavage of sialic acid. This may initially have inhibited
bacterial invasion and ablated infection. Over time bacteria have evolved genetic
regulation via sialic acid as a way to signal the environment inhabited, and possibly
prime the bacteria for transitional environments later during infection.
5. Co-regulation

5.1 Introduction

Capsule and T1P are important for the survival of ExPEC in vivo during UTI (Wright et al., 2007; Anderson et al., 2010). Capsule is integral for protection from host innate immune defenses within the lumen of the bladder (Roberts, 1996; Whitfield, 2006). However, capsule protection comes at a cost; bacterial surface factors potentially requisite for ExPEC survival may be masked or inversely regulated (Schembri et al., 2003). T1P are required for adherence and invasion of bladder epithelia (Wright et al., 2007). Binding of T1P to mannosylated receptors with shear force exertion decreases Region 1 gene expression within the first 2 hours of binding, as seen by relative levels of kpsD transcript (Schwann et al., 2005). We hypothesize capsule and T1P are co-regulated, limiting intimate interactions with host cells until initial binding of T1P has occurred (Schembri et al., 2004; Schwan et al., 2005). We predict T1P expression is increased when capsule is decreased, preparing the bacterium for the second phase of ExPEC pathogenesis, the intracellular IBC phase (Wright et al., 2007).

5.2 Results

T1p transcription is tightly controlled via promoter inversion, which orients the invertible region, fimS, in either a transcriptionally active state (ON) or inactive state (OFF; Wolf and Arkin, 2002). To facilitate studies of T1P and capsule co-regulation, we
created a single copy chromosomal transcriptional reporter with the T1P promoter (\textit{fimS}) fused to \textit{gfp}. The reporter was integrated at the lambda phage insertion site, which is unlinked from the T1P operon (Figure 35).

![fimS Transcriptional Reporter](image)

\textbf{Figure 35: fimS Transcriptional Reporter}

\subsection*{5.2.1 \textit{fimA} Transcription Is Increased In Genetic Capsule Mutants}

Previous literature has shown down-regulation of \textit{kpsD} transcription post T1P binding, implicating counter-regulation of capsule and T1P (Schwann et al., 2005). Antigenic T1P are not completely masked by capsule, enabling T1P-mediated bacterial binding to host cells and T1P interaction with the host immune system. We hypothesized antigenic T1P are not highly expressed when ExPEC are encapsulated, and down-regulation of capsule would increase pilus expression.

Strains with capsule mutations have a significant increase in T1P expression (Figure 36A). Levels of T1P transcript vary by capsular mutant, following trends previously observed in Chapter 4, measuring intracellular free sialic acid accumulation.
Region 1 capsule mutants have the most significant increase in T1P expression, followed by kpsC and kpsM mutants (Figure 36A). Relative levels of fimA transcription, as quantified by GFP expression, correlate with levels of fimA transcript, as assessed by qRT-PCR (Figure 36D & E).

Figure 36: fimA Transcription and Protein Levels in K1 ExPEC

(A) T1P expression measured in relative fluorescent units (RFU) via the transcriptional reporter strain UTI89 fimS:gfp. (B) T1P promoter (fimS) orientation measured by phase PCR of wt, Δreg1, and Δreg2 mutants. (C) αT1P Western blot showing FimA protein levels in wt, Δreg1, and Δreg2 mutants. (D) T1P transcription as measured by GFP fluorescence of an insertional Region 1 mutant, UTI89DkpsF and Region 1 complement UTI89DkpsF pSX50. (E) qRT-PCR data of fimA expression of insertional mutant as compared to complement; relative fold change as compared to vector control. Student T-Test: *p<0.003; **p<0.0001; FimA: major T1P subunit; SfaA: major S-fimbriae subunit; LC: Loading Control
T1P transcription is dependent on the orientation of the invertible promoter, and the activity of the promoter itself. Therefore, the observed increase of T1P transcription in the capsule mutants may have been due to increased promoter inversion to the ON orientation as well as increased promoter activity. We assessed promoter orientation by phase PCR of fimS, and assays were performed in triplicate. Data show an increase in the ON orientation of T1P in both Δreg1 and Δreg2 mutants (Figure 36B).

We hypothesized an increase in the ON orientation of fimS would produce increased T1P protein expression. Relative T1P protein levels were assessed using αT1P Western blot on whole cell preparations. Additionally, we used the preferential binding of T1P to mannosylated receptors on yeast as a surrogate to assess functional T1P surface expression. When mannose is present in free monosaccharide form, T1P preferentially bind the monosaccharide instead of mannosylated yeast cells, and a decrease in T1P-mediated yeast agglutination is observed, enabling differentiation between T1P and other pilus-mediated forms of yeast agglutination (mannose sensitive yeast agglutination; MSYA). Despite an observed increase in T1P transcription by both qRT-PCR and GFP fluorescence, data show no relative change in levels of surface piliation by yeast agglutination, MSYA and αT1P Western (Figure 36C).
5.2.2 K1 Capsule Mutants Have Increased Invasion *in vitro*

In Chapter 4, we saw a lack of sensitivity of phase assays and αT1P western in determining functional T1P expression. Tissue culture adherence/invasion experiments proved more sensitive to changes in levels of functional T1P in a K1 background. We hypothesized increased transcription of fimA would lead to an increase in mannose sensitive adherence and invasion of bladder epithelial cells in a K1 capsule mutant. As described previously, we used adherence and invasion of the bladder epithelial cell line, 5637, as a surrogate for functional T1P expression. Data show a UTI89 capsule mutant is significantly more invasive than wildtype (p=0.02; Figure 37A & B), suggesting that increased T1P transcription increases functional T1P expression in a K1 strain.

5.2.3 T1P-Capsule Counter-Regulation Is Conserved Amongst K1 and K5 Strains

Despite, an abundance of horizontal gene transfer within ExPEC, and increased genome plasticity (Whitfield, 2006) we hypothesized that counter-regulation of T1P and Group 2 capsule was conserved across ExPEC. Chapter 3 data show K5 encapsulated strains are prevalent among community acquired UTI. We next investigated T1P protein levels of the prototypic ExPEC K5-encapsulated strain, GR12, and an isogenic capsule-interrupted strain, GR12ΔkfiC. MSYA and T1P expression data show the basal level of wildtype GR12 T1P expression is significantly lower than wildtype UTI89. The isogenic K5-capsule mutant, however, has increased MSYA and T1P protein levels (Figure 37C &
D). Phase orientation of the T1P promoter region \((fimS)\) was not assessed for GR12, as the \(fimS\) region was unrecognizable to both phase primers and the asymmetric cut site. The K5-capsule mutant was also significantly more invasive than wildtype (Figure 37B), further supporting an increase in T1P expression in a K5 background. These data suggest that while basal levels of T1P expression may be strain specific, counter-regulation of Group 2 capsule and T1P expression are conserved amongst the Group 2 encapsulated strains tested.
Figure 37: Levels of Adherence, Invasion, and T1P piliation in UTI89, GR12, and GR12ΔkfiC

(A) 30 minute adherence to and (B) 2 Hour Invasion of 5637 Bladder Cells by UTI89 (K1), UTI89Dreg2 (K1 capsule mutant), GR12 (K5), and GR12DkfiC (K5 capsule mutant) (C) MSYA and (D) Western Blot for αT1P (FimA) for UTI89, UTI89Dreg2, GR12, GR12DkfiC. FimA: major T1P subunit; SfaA: major S-fimbriae subunit; LC: Loading Control; Student T-Test: *p=0.05; **p=0.02; ***p=0.03; Student T-Test: *p=0.02; **p=0.003; ***p<0.0002;

T1P are critical for ExPEC invasion of the bladder epithelium, and adherence and invasion via T1P are inhibited in the presence of free mannose (Abraham et al., 1988).

We hypothesized increased invasion was T1P mediated, and would therefore be inhibited in the presence of mannose. The addition of mannose to media significantly
inhibited increased GR12ΔkfiC invasion (Figure 38A), and comparison of UTI89, GR12, and GR12DkfiC invasion levels in the presence of mannose, are not statistically different. Additionally, data show K5 wildtype does not express T1P, but is still able to adhere to bladder epithelial cells similar to K1 levels. Non-T1P-mediated adherence, however, confers low-level invasion, and is not sustainable for prolonged infection. Together, data support the hypothesis that the loss of capsule leads to an increase in T1P expression. We presume that counter-regulation of capsule and T1P may be advantageous for the bacterium, facilitating an increase in invasion.

![Figure 38: Adherence and Invasion to urothelial cells by UTI89, GR12, and GR12
Capsule Mutant](image)

(A) Adherence of UTI89 (K1), GR12 (K5), and GR12DkfiC (K5 capsule mutant) to 5637 bladder epithelial cells in the presence or absence of mannose. Adherence is calculated as a percent of the inocula. Student T-test: *p = 0.0002; **p = 0.01; ***p = 0.034; (B) Invasion of 5637 bladder epithelial cells by UTI89 (K1), GR12 (K5), and GR12DkfiC (K5 capsule mutant), in the presence or absence mannose. Invasion is calculated as a percent of adherence in the absence of mannose. Student T-test: *p = 0.001; **p = 0.02; ***p = 0.04
5.2.4 Counter-Regulation of T1P and Capsule Is Inherent to the E. coli Core Genome

Though ExPEC encode for Group 2 capsule, T1P are ubiquitous amongst all E. coli. Commensal E. coli are recently diverged from ExPEC strains, believed to have lost significant amounts of the ancestral genome, reducing the bacterium to a core genetic backbone (Lecointre et al., 1998). Presumably, T1P and capsule counter-regulation has an important role during pathogenesis, and loss of Group 2 capsule and acquisition of other capsular types (non-pathogen-associated) may be accompanied by a loss of T1P/capsule and counter-regulation. We hypothesized counter-regulation of T1P/Group 2 capsule would not be conserved among commensal organisms.

In order to assess conservation of capsule-T1P co-regulation, we used a commensal hybrid strain that has the Group 2 capsule K1 locus introduced into a K12 background, strain EV36 (Cieslwicz and Vimr, 1996), and no known genes have been inserted into this commensal strain backbone. Phase assays showed an increase in the ON orientation of fimS in EV36 capsule mutants EV36Dreg1 and EV36DkpsT as compared to wildtype (Figure 39A). Data also show an increase in MSYA and T1P protein levels of the isogenic capsule mutants compared to wildtype (Figure 39B & C).
Figure 39: Phase Assay, MSYA, and $\alpha$T1P Western of EV36 Capsule Mutants

(A) T1p promoter orientation assessed by Phase-PCR in EV36 (K1:K12 hybrid), and subsequent EV36 capsule mutants. (B) MSYA of UTI89, EV36, EV36Dreg1, and EV36DkpsT. (C) aT1p (FimA) western blot in triplicate of UTI89, EV36, EV36Dreg1, and EV36DkpsT.

In vitro tissue culture studies provided similar results as were seen with UTI89, GR12, and the respective isogenic capsule mutants (Figure 40A & B; Figure 38A & B; Figure 37A & B). Similar to GR12, EV36 adherence is significantly higher than UTI89, though MSYA and Western data show lack of T1P-piliation. We conjecture that a different adhesive factor is present on hybrid strain surface, and this factor is not cross reactive with our $\alpha$T1P antibody or able to bind yeast cells. Invasion of EV36 capsule mutants also significantly increased as compared to wildtype, and was inhibited by mannose (Figure 39B). These data suggest expression of T1P increases in the absence of capsule, and that this function is conserved in both ExPEC and commensal organisms.
5.2.5 Chemical Inhibition of Capsule Expression Down Regulates T1P

Our lab has previously identified a chemical compound, 2-(4-phenylphenyl)benzo[g]quinolone-4-carboxylic acid (C_{26}H_{17}NO_{2}; C7), able to inhibit capsule production; lack of capsule was visualized by TEM and confirmed by growth in the presence of K1F phage (Goller and Seed, 2010). C7 treatment down-regulates Region 1 transcription, as assessed by an approximate 7-fold decrease in kpsD transcript levels via qRT-PCR as compared to vehicle treated UTI89 (unpublished Goller & Pilla). Use of thiobarbituric acid to indicate levels of total sialic acid showed UTI89 treated with C7 was similar to a synthesis capsule mutant, supporting the hypothesis that capsule synthesis is down-
regulated, and suggesting C7 affects capsule assembly, synthesis, and export genes (Figure 41B). We hypothesized counter-regulation of capsule and T1P expression would occur in C7 treated ExPEC similar to genetic capsule mutants. Data support the inverse: T1P and capsule are both down-regulated when ExPEC are treated with C7 (Figure 40A) suggesting T1P and capsule may be controlled directly or indirectly via a common regulator.

Figure 41: Level of Total Sialic Acid and T1P Expression Post C7 Treatment

(A) Relative fluorescence units (RFU) of UTI89 wt \textit{fim:gfp} and UTI89\textit{DkpsF: fim:gfp} treated with vehicle (1% DMSO) or C7 (100 mM C7 + 1% DMSO). UTI89 wt with vector control (VC; promoterless \textit{gfp}) is shown for comparison of ExPEC background fluorescence. (B) TBA results of whole cells treated with either vehicle (1% DMSO), C7 (100mM + 1% DMSO), or no treatment. Student T-Test: ***p<0.0001
5.3 Discussion

UTI is a highly orchestrated process in which ExPEC must coordinate virulence and metabolic programs. T1P and capsule are integral for survival *in vivo*, shielding antigens from the host, adherence, invasion, and formation of intracellular bacterial communities (IBC; Mulvey et al., 1998; Hunstad and Justice, 2010; Rosen et al., 2007; Justice et al., 2004). T1P lectin binding yielded down regulation of *kpsD* expression resulting in less capsule expression, demonstrating an interplay between T1P and capsule (Schwann et al., 2005). These observations led to the hypothesis that counter-regulation of T1P and capsule would occur, and in the absence of capsule a reciprocal increase in T1P expression would be observed.

Our data support the hypothesis of counter-regulation between capsule and T1P, and suggest that regulation is conserved amongst pathogenic and commensal strains. We observed similar effects of increased T1P expression via our transcriptional reporter, *fimS:gfp*, in a pathogenic K1 strain. Furthermore, increased GFP expression correlates with a shift towards the ON orientation of the T1P promoter orientation, and an increase in *fimA* transcript levels. Data indicate counter-regulation of T1P and capsule is at the transcriptional level, and correlated with an increase in T1P surface expression, as measured by MSYA, αT1P-Western (GR12, EV36), and mannose sensitive invasion (UTI89, GR12, EV36) in isogenic capsule mutants (Figure 42).
Our K1 capsule mutant exhibited a shift of the T1P invertible promoter towards the ON orientation, and increased T1P transcription. A significant increase in T1P protein expression, similar to the K5 or commensal hybrid strain, was not observed via MSYA or αT1P western. We surmise that while transcriptional counter-regulation of T1P and capsule is conserved independent of capsule type across E. coli, some strains may have additional translational or post-translational regulation of T1P, modifying the end phenotype. K1 encapsulated E. coli are the leading cause of neonatal meningitis, and it is possible that the acquisition or retention of certain regulatory mechanisms post transcription is important for life outside the urinary tract (Kim, 2003; Huang et al., 2000, 2001; Kim et al., 1992; Parkkinen et al., 1988; Che et al., 2011; Figure 42).

Pathogenic and commensal E. coli have the ability to express different adhesive surface factors, and these factors are accessible and able to confer adherence to epithelial cells (Korhonen et al., 1985; Parkkinen et al., 1988). Indeed, our data support these conclusions. Despite low levels of T1P expression in both GR12 and EV36, in vitro data show comparable (EV36) to increased (GR12) mannose resistant adherence. Increased adherence, however, does not facilitate increased invasion of cells in vitro, supporting the conclusion that while T1P are not necessary for adherence to urothelial cells, they are necessary for prolonged infection.
Figure 42: Model of T1P/Capsule Counter-regulation During UTI

(1) ExPEC infect the urinary tract and trigger the innate immune response stimulating neutrophil influx and urinary sialic acid increases. (2) Exposure of ExPEC to urine and sialic acid decreases T1P protein expression and T1P-mediated invasion, while increasing mannose-resistant adherence. (3) T1P binding down-regulates capsule and a reciprocal increase in T1P is observed, leading to increased mannose sensitive adherence and invasion. blue oval: capsule; black lines: T1P; burnt orange lines: mannose-resistant adherent factors; pink diamonds: free sialic acid

While more experiments need to be done in order to understand the underlying mechanism, preliminary data suggests C7 affects a global regulator important for both capsule and T1P expression. Chemical inhibition of capsule via C7 inhibits transcription at both capsule locus promoters. However, treatment with C7 also significantly decreases T1P transcription suggesting C7 may be inhibiting a regulator upstream of the T1P/capsule counter-regulation mechanism. Whether this regulator is directly inhibited at the protein level or at the transcriptional level is still a question in need of answering.
Investigation of capsule/T1P counter-regulation in pathogenic and commensal backgrounds has shown that a lack of exported capsule shifts all strains thus studied toward T1P expression, suggesting this may be a conserved mechanism by which *E. coli* are programmed to invade host cells in the presence of capsule machinery, and the absence of exported capsule. We conclude that ExPEC may be programmed to preferentially express T1P in the absence of capsule. One interpretation is that increased pilus expression in the absence of capsule is a remnant of ExPEC ancestral regulation, where decreased *E. coli* encapsulation, leads to increased T1P expression and subsequent cellular invasion. This could explain why a strain already producing a significant amount of T1P (UTI89) upregulates pilus expression with more subtlety.
6. Discussion

In this study, we examined clinical strains isolated from uncomplicated urinary tract infections (UTIs). Our purpose was to gain knowledge of the bacterial demographic using recently isolated strains associated with uncomplicated UTI and analyze change in capsular type over time. Data show Group 2 encapsulated strains have significantly increased in uncomplicated UTI in the past twenty years with a significant increase in ExPEC encoding for unidentified Group 2 capsule polysaccharides (IIUD).

Despite a shift in ExPEC capsule demographics, K1 ExPEC are the largest group of known encapsulated ExPEC associated with symptomatic disease. K1 strains are able to catabolize sialic acid for energy (glycolysis), and membrane production (LPS and peptidoglycan), as well as synthesize sialic acid for capsule expression (Plumbridge and Vimr, 1999). Our aim was to elucidate the role sialic acid has as a regulatory molecule during UTI.

Lastly, we investigated counter-regulation of two surface factors required for survival \textit{in vivo}, Type-1 pili (T1P) and Group 2 capsule. Previous data show shear force exertion on ExPEC bound via T1P to mannosylated beads down-regulates transcription of the Group 2 capsular pore, \textit{kpsD} (Schwan et al., 2005). We further investigated the interplay between T1P and capsule to understand counter-regulation during UTI \textit{in vivo}. 
6.1 Counter-Regulation of T1P and Capsule

T1P and capsule are required and expressed during UTI (REF). Previous data show shear forces exerted on bound T1P stimulates decreased expression of the capsule pore gene (*kpsD*; Schwan et al., 2005). These data, in addition to conclusions drawn in Chapter 5, suggest T1P and capsule are coordinately regulated during UTI *in vivo*.

Both capsule and T1P are necessary for UTI infection; T1P are required for adherence and invasion of bladder epithelia, and capsule is necessary for protection from the host immune system (Anderson et al., 2010; Wright et al., 2007). As we have discussed in Chapter 3, there are at least 80 different capsular types of *E. coli*, however, our data show that the predominant number of UTI isolates consist of K1 (sialic acid); K5 (hyaluronic acid); K2 (repeating units of glucose and galactose); and an expansion in IIUD encoding for 5 different Region 2 loci.

Capsule is thought to be a classical virulence factor, and while we do not deny that ExPEC capsule has the ability to mask bacterial antigens, mimic host cells, and protect the bacterium from host cell immune responses, we suggest coordinated regulation of T1P and capsule is necessary for reasons beyond classical factors, and that loss of capsule is important during initial steps of UTI (Anderson et al., 2010). *K. pneumoniae*, another cause of UTI, is unable to colonize the intestine of mice when lacking capsule, indicating colonization of the intestine with *K. pneumoniae* requires
capsule (Favre-Bonté, 1999). It is quite possible that capsule is necessary for colonization of the intestine, and surface factors masked by capsule are requisite for ExPEC’s extracellular lifestyle within the bladder (Chang et al., 2004).

The urinary tract is a drastically different niche than the intestine, and an environmental change of this magnitude likely requires different gene expression (Snyder et al., 2004). Many bacterial antigens are still masked after initial infection of the urinary tract, preventing interaction with the host (Schembri et al., 2004). T1P extend beyond the capsule and are able to interact with bladder epithelial cells (Schwan et al., 2004). T1P binding of mannosylated host receptors enables ExPEC to resist forces from urine flow, establishing an infection (Krogfelt et al., 1990; Sokurenko et al., 1994; Klemm et al., 2007).

Resistance of micturition by T1P binding, down-regulates capsule expression (Schwan et al., 2004). Capsule is known to reduce entrapment of S. pneumoniae in luminal mucus, promoting appropriate adherence and colonization (Nelson et al., 2007). Studies by Valle et al., demonstrated treatment with E. coli Group 2 capsule caused a significant reduction in both adhesion and biofilm formation on abiotic surfaces (Valle et al., 2006), suggesting capsule may have an anti-adhesive purpose within the urinary tract. It is possible that ExPEC down-regulate capsule once T1P have engaged their receptor, enabling more intimate contact with the host cell and shifting genetic
expression toward an intracellular lifestyle (Connell et al., 1996; Sokurenko et al., 1994; Wu et al., 1996; Schwan et al., 2004). T1P are known to enhance colonization in the murine UTI model, promoting invasion and biofilm formation (Connell et al., 1996; Klemm and Schembri (book), 2004; Mulvey et al., 1998). Binding of T1P to host cell receptors stimulates cytokine production, inflammation, and exfoliation of epithelial cells (Hedlund et al., 2001; Mulvey et al., 1998; Samuelsson et al., 2004; Wullt et al., 1998). We conjecture that increased accessibility of urinary sialic acid as a result of an inflammatory response leads to a high sialic acid state within ExPEC. We surmise a reciprocal increase in T1P expression when capsule is absent could counter-act the decrease in T1P observed when ExPEC are grown in urine.
In the presence of low sialic acid and low GlcNAc, both NagC and NanR are DNA bound, repressing nanC transcription, and increasing fimB expression. In the presence of high sialic acid and high GlcNAc, NagC interacts with GlcNAc, and NanR interacts with Neu5Ac, derepressing nanC transcription, and fimB expression is decreased. Activated sialic acid (CMP-Neu5Ac) and UDP-GlcNAc are unable to interact with NanR and NagC, enabling fimB expression.
Increased LPS synthesis uses the majority of CMP-KDO, which subsequently leads to an increase in CMP-Neu5Ac. Use of sialic acid for both capsule precursors and LPS production limits the availability of sialic acid to interact with NanR as a regulatory molecule; high levels of CMP-Neu5Ac do not interact with NanR; increased levels of UDP-GlcNAc, the raw material for LPS and capsule synthesis, is unable to interact with NagC. This in turn increases the amount of bound NanR and NagC upstream of fimB, drives expression of fimB, and other positively regulated virulence factors; increased FimB drives T1P expression. T1P increases the likelihood of ExPEC binding and invading host epithelia in subsequent rounds of invasion, potentially creating bacteria more likely to invade. Finally, post invasion, CMP-KDO is available, and waiting monomers of CMP-Neu5Ac are polymerized and exported to create capsule.

6.2 Sialic Acid As A Regulatory Molecule

ExPEC encode genes necessary to metabolize different sugars to gain energy, reinforce cell walls, and decorate their outer membrane. Additionally, sialic acid is able to interact with the global regulator, NanR, enabling NanR to act as both a repressor \((nan\) operon) and positive regulator \((fimB)\) of genes within the \(E.\ coli\) genome. K1 encapsulated ExPEC present us with very interesting questions: How does sialic acid signaling affect virulence \(in\ vivo\)? Are synthesized sialic acid molecules differentiated from sialic acid required for regulation, and if so, how? Since sialic acid biosynthesis is
considered constitutive, are sialic acid signaling and biosynthesis coordinated during pathogenesis?

Our data show an increase of sialic acid in urine during UTI when mice are infected with K1 ExPEC. *nan: gfp* reporter data showed an increase in *nanATEK* expression when bacteria were grown in urine *in vitro*, supporting previous K2 (CFT073) microarray data which showed *nanAT* expression was increased 2-4 fold (Snyder et al., 2004). Microarray data showed an increase in LPS and capsule transcription, as well as an increase in genes necessary for high osmolarity, including a 17 fold increase in the outermembrane pore, OmpC (Snyder et al., 2004), suggesting that ExPEC within the urinary tract experience outermembrane and capsular insult in an environment that is moderately oxygenated, with high osmolarity (Snyder et al., 2004).

Our *in vivo* data show a rise in free sialic acid in urine during UTI, supporting the idea that during infection, neutrophils are activated within the urinary tract, traffic to the sight of infection, and cleave sialic acid from uroplakins; increased sialic acid in the urine during UTI may be from cleavage of extracellular sugars from heavily sialidated host cells in response to ExPEC infection. Increased *nan: gfp* expression observed during ExPEC growth in uninflamed urine *in vitro* (Chapter 4), and increased expression of outermembrane (*ompC*) and innermembrane (*nanT*) pores requisite for sialic acid importation (Snyder et al., 2004), leads us to suggest uninflamed urine predisposes
ExPEC to upregulate genes necessary for sialic acid import, potentially readying the bacteria to utilize increased sialic acid post innate immune response. Additionally, we conclude that this mechanism is conserved across ExPEC strains, regardless of capsule type. We were able to stimulate nanAT expression above levels observed in urine alone, by adding exogenous sialic acid in vitro. Microscopy data showed increased GFP fluorescence in the lumen at 6 hours post infection, supporting increased sialic acid mediated regulation in ExPEC in vivo (Chapter 4).

Increased expression of sialic acid porins, and increased internal stimulation of sialic acid implies sialic acid has a role during infection. In a K1 encapsulated strain, imported sialic acid may traverse the following pathways: 1) catabolism (glycolysis) via nan operon gene products; 2) LPS, peptidoglycan, or capsule synthesis through the creation of the UDP-GlcNAc intermediate; 3) regulatory function, interacting with NanR, or another regulator. Our data, in conjunction with Snyder et al.’s microarray data, suggest sialic acid is most likely a regulatory molecule in vivo conserved among ExPEC strains regardless of capsule type.

Previous studies have attempted to understand the role of sialic acid during pathogenesis. Analysis of nanA deletions in E. coli have modeled the effect uncoupling sialic acid catabolism from sialic acid importation has on E. coli as nanA deletion strains are unable to catabolize sialic acid; deletion of nanA, shunted imported sialic acid
toward capsule synthesis in a K1:K12 hybrid strain. *nanA* mutants in a K1:K12 background are able to therefore utilize imported and synthesized sialic acid for capsule biogenesis. Despite the uncoupling of sialic acid importation and catabolism in *E. coli*, *nanA* mutants do not have a significant defect during infection *in vivo*. These data, in conjunction with increased expression of *nanT* and *ompC*, requisite for sialic acid importation, and increased sialic acid mediated stimulation *in vitro* and *in vivo*, suggests ExPEC do not utilize increased sialic acid solely for energy purposes.

To further investigate the necessity of sialic acid importation *in vivo*, we proceeded to genetically delete the sole sialic acid porin required for sialic acid importation across the inner-membrane, *nanT*, in a K1 background; a *nanT* mutant is unable to import sialic acid. However, in a K1 background, a *nanT* mutant is still able to synthesize sialic acid *de novo*. Mutations in the capsule export machinery suggest sialic acid generated via the biosynthetic pathway is unable to interact with the global regulator NanR (Chapter 4), suggesting synthesized sialic acid is only accessible for capsule production. These data are in agreement with previous findings suggesting that biosynthesis of sialic acid is in a “protected” compartment (Plumbridge et al., 2001); therefore, a *nanT* mutant is able to synthesize sialic acid, but unable to catabolize or utilize sialic acid as a regulatory molecule. *nanT* mutants trend toward being statistically more likely to survive *in vivo*, both at 48 hours and 2 weeks post infection. To assess
whether sialic acid was acting as a regulatory molecule via the global regulator NanR, or by an as of yet unidentified regulator, we proceeded to investigate the role of NanR during infection. We found constitutive expression of NanR phenocopied a nanT mutant during infection, significantly increasing survival \textit{in vivo}, and implicating sialic acid mediated genetic control is via NanR. Additionally, data support the inaccessibility of synthesized sialic acid to NanR. Therefore, we conclude imported free sialic acid decreases expression of genes important for survival, and suggest that this is a mechanism necessary for ExPEC to control growth rate to potentially escape detection in extremely high sialic acid niches within the host, such as during more disseminated infection like uro-sepsis and/or meningitis.

\textbf{6.3 Coordinated Sialic Acid Synthesis and Importation}

The idea of sialic acid as a regulatory molecule for ExPEC, presents us with an interesting conundrum; how does ExPEC control appropriate regulation via sialic acid while simultaneously synthesizing the same molecule. Previous studies have suggested synthesized sialic acid is protected from catabolism, hindering a futile energy cycle; protection is believed to be either via immediate polymerization of capsule polysaccharide or through a kinetic “compartment,” where capsule synthesis is linked to export. Transmission electron microscopy of capsule export mutants in the background of a K1:K12 hybrid strain, showed synthesized sialic acid polymerizes into
polysaccharide, forming lacunae, and implying accessibility of synthesized sialic acid is not possible due to immediate polymerization. Genetic studies looking at cross-talk between sialic acid synthesis and degradation in an artificial K1 background, K1:K12 hybrid strain EV36, may not be able to elucidate the fully how sialic acid is compartmentalized; therefore, we investigated how synthesized sialic acid is internally protected from sialic acid degradation in a K1 encapsulated ExPEC strain, and if sialic acid synthesis is coupled to NanR-mediated sialic acid signaling.

Genetic deletion of Region 1 genes in a K1 background resulted in an increase of internal sialic acid. However, our data show pools of increased sialic acid were not sequestered due to polymerization, as the majority of internal sialic acid was verified as free monomeric form by HPLC of DMB-labeled samples. Despite the increase in free sialic acid in a Region 1 mutant, our data do not show an increase in NanR activation, leading to the conclusion that a pathogenic K1 background strain behaves differently than a K1:K12 hybrid strain; sialic acid is not pooled internally as polysaccharide in a K1 strain. We conclude that synthesized sialic acid monomers are unable to interact with NanR. ExPEC strains have approximately 0.5-1.0 Mb of additional DNA, as compared to K12 strains, and these conclusions support unique sequestration and regulation of biosynthesized ExPEC sialic acid as compared to commensal.
Reconciliation of our data with previous findings, suggests sialic acid biosynthesis is protected from sialic acid degradation, as well as inhibited from interaction with the global regulator NanR potentially via activation of sialic acid through addition of CMP. Where as other organisms with the ability to synthesize sialic acid also encode a hydrolase, able to free sialic acid from CMP attachment, *E. coli* lack such an enzyme; therefore, addition of CMP to Neu5Ac is irreversible in ExPEC, and may be a potential method of sequestration of synthesized material from signaling. Isolation of sialic acid biosynthesis from imported sialic acid, and lack of synthesized sialic acid for mediating genetic regulation, further supports sialic acid as a regulatory molecule in ExPEC. Interestingly, a Region 1 mutant is stimulated significantly more by exogenous sialic acid than wildtype, suggesting a build-up of activated sialic acid (CMP-Neu5Ac) may have an indirect regulatory role. Experiments in a Δ*neuA* K1 background have yet to be performed.

We observed free sialic acid increases in the urine during infection, and both uninflamed and inflamed urine stimulates increased transcription of genes necessary for sialic acid importation. However, it is important to note that ExPEC will most likely only encounter urine when in the lumen of the bladder. Post epithelial invasion, increased expression of genes requisite for sialic acid importation would presumably be down-regulated due to lack of urine. Additionally, free sialic acid may not be as readily
available post ExPEC invasion, as intracellular accessibility of sialic acid could be limited. Additionally, we know that (1) sialic acid synthesis (Region 2) is most likely constitutively expressed, (2) capsule export machinery (Region 1) is down regulated post T1P binding mannosylated receptors (Schwan et al., 2004), (3) nanAT expression increases in urine both in vitro and in vivo, and (4) an increase in activated sialic acid stimulates NanR mediated-signaling presumably via importation of exogenous sialic acid (Chapter 4). Therefore, we conclude that binding of T1P down-regulates capsule export, and a build-up of activated sialic acid (CMP-Neu5AC) occurs. This build-up of CMP-Neu5AC indirectly stimulates increased NanR-mediated signaling via increasing sialic acid importation, as seen by UTI89ΔregI in the presence or absence of exogenous sialic acid. Importation of sialic acid leads to a change in gene expression, perhaps preparing K1 encapsulated bacteria for life either intracellularly or disseminated disease.
More specifically, we propose the following mechanism. ExPEC invade the urinary tract, expressing both capsule and T1p (Hultgren, etc). Interaction of ExPEC with uninflamed urine stimulates expression of nanAT, ompC, capsule assembly genes, and LPS synthesis genes, amongst others (Snyder et al., 2004). T1P bindmannosylated receptors on bladder epithelial cells, and capsule is down-regulated (Abraham, Mulvey, Hultgren, Schwan). Down-regulation of capsule exposes bacterial LPS, which in turn
stimulates a TLR-4 response, releasing cytokines, and causing neutrophil movement via chemotaxis (Ref). Activated neutrophils cleave sialic acid on host cells via translocated sialidases, activating additional resting neutrophils, and signaling infection to the host immune system; free sialic acid is thus released into the urine. The first round of bacterial invasion occurs (based on in vitro data within the first 30 minutes of attachment); intracellular ExPEC are no longer stimulated by urine, nor in direct attack of the host immune system.

ExPEC that are not among the first invaders remain extracellular in the lumen of the bladder. However, first round invaders have already stimulated the innate immune response via LPS/TLR-4, creating a more hostile environment for subsequent invasion (Hedlund et al., 2001; Mulvey et al., 1998; Samuelsson et al., 2004; Wull et al., 1998). Neutrophils are likely to generate an oxidative burst post TLR-4 stimulation (Remer et al., 2002). Oxygen radicals, along with the harsh urine environment, potentially damage ExPEC LPS; *E. coli* cannot survive without LPS. Due to urine pre-stimulation of *nanAT* and *ompC* expression, ExPEC are able to import increased free sialic acid in the urine due to neutrophil cleavage.

While imported sialic acid has 3 possible fates, in the wake of LPS damage, *E. coli* (most likely) preferentially degrade imported sialic acid to UDP-GlcNAc, the raw material for LPS synthesis, peptidoglycan, and capsule. While increased free sialic acid
in the urine, and increased ability to import sialic acid suggests ExPEC have an
abundance of raw material to make both capsule and LPS simultaneously, both LPS and
capsule synthesis require CMP-KDO; LPS synthesis employs CMP-KDO via KdsB to
create Lipid-A, while sialic acid synthesis engages CMP-KDO to begin capsule
elongation through KpsU (Tzeng et al., 2002). Limiting levels of CMP-KDO and
abundant levels of UDP-GlcNAc, in addition to the need for ExPEC LPS repair,
generates LPS and activated sialic acid monomers (CMP-Neu5Ac); however, without
CMP-KDO, capsule is unable to elongate/polymerize, and extra-cellular capsule
expression is reduced regardless of CMP-Neu5Ac availability (Tzeng et al., 2002;
reviewed by Whitfield, 2006). Because E. coli do not encode a hydrolase, activated sialic
acid cannot interact with NanR, therefore stimulating positive regulation of NanR
controlled genes. Our reg1 and nanA mutants modeled this situation; imported sialic
acid was either shunted toward biosynthesis and LPS (reg1), or biosynthesis, bypassing
UDP-GlcNAc formation (nanA), causing an internal buildup of CMP-Neu5Ac. Though
other sugar sources are employed to make UDP-GlcNAc, CMP-KDO is still a limiting
resource.

Based on the data we have presented here, we conclude that sialic acid is a
regulatory molecule which ExPEC employ to determine niche appropriate gene
expression; genes necessary for invasion in a hostile extra-cellular environment, those
required for increased intracellular survival, and genes necessary for more invasive disseminated disease. We propose that ExPEC use sialic acid as a fine-tuning mechanism to identify location within a host.

6.4 Sialic acid synthesis correlates with symptomatic infections

Our study of current UTI isolates has shown a significant increase in Group 2 encapsulated ExPEC over the past 20 years. In Chapter 3 we concluded that the dominant known capsule type of ExPEC isolated from women who exhibited symptomatic UTI episodes is K1. This suggests a potential correlation between sialic acid synthesis and encapsulation and increased symptomatic episodes.

With an increase of sialic acid transport (nanT), as seen by Snyder et al., in a K2 encapsulated background, we have concluded that increased sialic acid import is conserved among ExPEC strains (Snyder et al., 2004; Chapter 4). However, Group 2 strains expressing different capsule sugars, and lacking the ability to synthesize sialic acid, would presumably have three functions for imported sialic acid: catabolism, signaling, and/or LPS/peptidoglycan synthesis; thus, unlike K1 encapsulated ExPEC, activated sialic acid will not buildup in ExPEC strains of different capsule types. Increased importation of free Neu5Ac from the host luminal space would, therefore, be more likely to interact with NanR, and virulence factors positively regulated by NanR, such as T1P, would be down regulated in ExPEC strains unable to synthesize sialic acid.
In lieu of our data, we suggest this as a possible reason as to why K1 is more likely to cause increased symptomatic disease during UTI.

6.5 A Shift in Capsule Type – Evolution At Work

Our data have shown a significant increase of UTI associated with ExPEC encoding Group 2 capsule in the past 20 years, and that the significant increase in Group 2 ExPEC correlates with a significant increase in strains encoding for Group 2 capsule with an unknown capsule type, Group 2 Undefined (IIUD). IIUD genomes encode conserved Group 2 capsule machinery, are able to produce extracellular capsule, as seen by capsule release experiments and alcian blue staining, and are comprised of phylotypes most associated with pathogenesis, B2 (76%) and D (20%). IIUD isolates are significantly associated with both symptomatic and asymptomatic disease as compared to K1 & K5 isolates combined.

E. coli are continually evolving to fit specific niches within a host. Such an example is ASB strain 83972, isolated from a young girl with 3 years of asymptomatic colonization (Klemm et al., 2006). ASB 83972, which is a Group 2 encoding B2 strain, neither expresses capsule nor pili (Klemm et al., 2006). Survival within a host is presumed to hinge on increased biofilm formation and shorter doubling time implicating that ASB strains are able to out compete symptomatic strains and colonize the urinary tract in a protective manner (Hancock and Klemm, 2007; Hancock et al.,
It has been argued that ASB and commensal strains are separate derivations of pathogenic strains (reviewed by Klemm et al., 2007). It is important to note, however, that ASB strains still have the ability to cause symptomatic infections, further implicating recent divergence from ExPEC strains as well as the host role in UTI susceptibility and disease (Chapter 3). Data show two major scenarios for infection with ASB strains. 1) Initial symptomatic infection results in prolonged ASB with the same organism. Based on our level of resolution, we approximate 90% of those labeled ASB in our study meet these criteria. 2) Initial symptomatic disease caused by K1 encapsulated organisms proceeds to asymptomatic infection with a group IIUD strain. Based on these data, we suggest ASB strains most likely fall into at least two different groups based on host interaction; however, we recognize that these two groups are neither mutually exclusive, nor the only way to define ASB isolates.

Those ASB strains which cause initial cystitis, and then quietly colonize the host, post initial infection are able to cause both symptomatic and asymptomatic episodes within the same host, implicating host susceptibility and predisposition is not the mechanism for a symptomatic to asymptomatic shift; isolates were collected from hosts
with uncomplicated UTI, who were otherwise healthy, suggesting a compromised immune system did not engender a symptomatic infection. We hypothesize that strains able to colonize post initial cystitis are undergoing a genomic content shift; however, based on our level of genomic resolution, it is difficult to conclude a shift in virulence profile is due to genetic loss, or due to a change in ExPEC strain, as we did not include isolates from hosts presenting with initial symptomatic cystitis, shifting to ASB with a different profile. Also, due to the quantity of ASB strains, we did not perform pulse-field gel electrophoresis (PFGE), therefore limiting our ability to track genomic markers across strains.

It is feasible that the shift of IIUD strains from symptomatic to asymptomatic may not be directly from genomic reduction and loss of virulence, but due to a shift in genetic regulation. We have suggested that K1 are able to regulate virulence factors via in sialic acid availability. It is possible that a shift in genetic regulation by sialic acid or another molecule predisposes a bacterial strain to shut down inflammatory virulence factors once in the lumen of the bladder.

With the majority of symptomatic infections caused by known capsular types K1 and K5, UTI induced host inflammation and exfoliation of bladder epithelia potentially creates a unique niche in which IIUD strains are able to colonize. We propose colonization occurs in one of two ways. 1) ASB strains lacking in canonical virulence
factors are introduced to the bladder. IIUD ASB strains, do not stimulate host inflammation due to lack of virulence factors (Chapter 3), and are able to colonize the bladder because of increased growth rates and biofilm formation (Roos et al., 2006; Hancock and Klemm, 2007; Klemm et al., 2007); potential increased fitness in the inflamed bladder enables ASB IIUD strains to outcompete the more symptomatic strains, much like ASB 83972 has a competitive advantage over the pyelonephritic strain, CFT073. This is most likely an advantageous relationship for both host and ASB strain. With the symptomatic strain out-competed, the ASB strain colonizes an environment and flourishes, potentially reducing the frequency of UTI in patients (Hull et al., 2000; Sundén et al., 2006; Wullt et al., 1998; Andersson et al., 1991). 2) Symptomatic strains may be cleared from the host via inflammatory response, etc., and ASB strains, uncontested, are able to colonize the bladder. This is most likely the case for hosts, which had an interim collection between symptomatic infection and asymptomatic infection that lacked in bacterial counts.

Increased prevalence of uniquely encapsulated ExPEC (IIUD), and sustained ASB disease via IIUD is helpful for further studying commonalities among ASB strains, investigating reasons for asymptomatic colonization versus symptomatic disease. These data will help knowledge of UTI move forward, and assist in combating the disease.
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Biography

Tiffany Prest was born June 15, 1984, in Wyandotte, Michigan. She lived in the upper northeast side of Detroit in Clinton Township, for 21 years with her parents and sister before moving to North Carolina in 2006. She attended Michigan State University and graduated Summa Cum Laude in 2006 from the MSU honors college with a Bachelor of Science in Microbiology; concentrations in: medical microbiology, molecular genetics, and virology. She entered the Duke Graduate School, Department of Molecular Genetics and Microbiology, in the fall of 2006; she became a PhD candidate post preliminary exam in spring 2008. Tiffany received the ASM travel grant in 2008, and presented at both the NC-ASM and the national ASM conference in 2008. She is an avid reader, hiker, rock climber and local Durham/Chapel-Hill/Raleigh musician.