

Epigenetic Regulation of the Nitrosative Stress Response and
Intracellular Macrophage Survival by Extraintestinal Pathogenic
Escherichia coli

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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
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ABSTRACT

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Abstract

Escherichia coli is a typical constituent of the enteric tract in many animals, including humans. However, specialized extraintestinal pathogenic *E. coli* strains (ExPEC) may transition from benign occupation of the enteric and vaginal tracts to sterile sites such as the urinary tract, bloodstream, and central nervous system. ExPEC isolates of urinary tract origin express type 1 pili as a critical virulence determinant mediating adherence to and invasion into urinary tract tissues. Type 1 pili expression is under epigenetic regulation by a family of site-specific recombinases, including FimX, which is encoded from a genomic islet called PAI-X for Pathogenicity Islet of FimX. A goal of this study was to determine the prevalence of the type 1 pili epigenetic regulator genes (*fimB*, *fimE*, *fimX*, *ipuA*, *ipuB*) and associated PAI-X genes (*hyxR*, *hyxA*, *hyxB*) present among an extended, diverse collection of pathogenic and commensal *E. coli* isolates. Using a new multiplex PCR, *fimX* and the additional PAI-X genes were found to be highly associated with ExPEC (83.2%) and more prevalent in ExPEC of lower urinary tract origin (87.5%) than upper urinary tract origin (73.6%) or human commensal isolates (20.6%; $p < 0.05$, all comparisons). Fim-like recombinase genes *ipuA* and *ipuB* also had a significant association with ExPEC compared to commensal isolates, but had a low overall prevalence (23.8% vs. 11.1%; $p < 0.05$). PAI-X also showed a strong positive correlation with the presence of virulence genes in the genomes of pathogenic isolates. Combined, our molecular epidemiology studies indicate PAI-X is highly associated with ExPEC isolates, and its high prevalence suggests a potential role in the ExPEC lifestyle.

Further investigation into the regulation of PAI-X factors showed that FimX is also an epigenetic regulator of a LuxR-like response regulator HyxR, encoded on PAI-X. In multiple clinical ExPEC isolates, FimX regulated *hyxR* expression through bidirectional phase inversion of its promoter region at sites different from the inversion sites of the type 1 pili promoter and independent of integration host factor IHF. Additional studies into the role of HyxR during ExPEC pathogenesis uncovered that HyxR is involved in regulation of the nitrosative stress response. *In vitro*, transition from high to low HyxR expression produced enhanced tolerance of reactive nitrogen intermediates (RNI), primarily through de-repression of *hmpA*, encoding a nitric oxide detoxifying flavohemoglobin. However, in the macrophage, HyxR expression produced large effects on intracellular survival in the presence and absence of RNI, and independent of Hmp. Collectively, we have shown that the ability of ExPEC to survive in macrophages is contingent upon the proper transition from high to low HyxR expression through epigenetic regulatory control by FimX.

ExPEC reside in the enteric tract as commensal reservoirs, but can transition to a pathogenic state by invading normally sterile niches, establishing infection, and disseminating to invasive sites like the bloodstream. Macrophages are required for ExPEC dissemination, suggesting the pathogen has developed mechanisms to persist within professional phagocytes. This study demonstrates the functional versatility of the FimX recombinase and identifies novel epigenetic and transcriptional regulatory controls for ExPEC tolerance to RNI challenge and survival during intracellular macrophage

infection. Further investigation of these pathways may shed light on the regulatory cues and programming that provoke the commensal to pathogen transition.

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List of Abbreviations

ExPEC	Extraintestinal pathogenic <i>E. coli</i>
IPEC	Intestinal pathogenic <i>E. coli</i>
UPEC	Uropathogenic <i>E. coli</i>
NMEC	Neonatal meningitis-associated <i>E. coli</i>
EHEC	Enterohemorrhagic <i>E. coli</i>
AIEC	Adherent Invasive <i>E. coli</i>
VF	Virulence factor
T1P	Type 1 pili
PAI	Pathogenicity island
GI	Genomic island
IBC	Intracellular bacterial community
UTI	Urinary tract infection
ASB	Asymptomatic bacteriuria
CY	Cystitis
PY	Pyelonephritis
UTI-BL	Urosepsis-blood
CNS	Central nervous system
HBMEC	Human brain microvascular endothelial cells
NO	Nitric oxide
RNI	Reactive nitrogen intermediates
ROS	Reactive oxygen species
L-NAME	L-NG-Nitroarginine methyl ester
iNOS	Inducible nitric oxide synthase
PMN	Polymorphonuclear neutrophil
DC	Dendritic cell
MOI	Multiplicity of infection
CFU	Colony forming units

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Chapter 1. Introduction

1.1 Extraintestinal Pathogenic *Escherichia coli*

Escherichia coli (*E. coli*) is a Gram-negative, facultative anaerobe present in the gastrointestinal tract of most mammals as a commensal bacterium, and is a non-spore-forming, rod-shaped bacterium measuring approximately 2 μm in length and 0.5 μm in diameter first discovered in 1885. As part of the Enterobacteriaceae Order, *E. coli* is a well-known inhabitant of the gastrointestinal tract of humans; although, it makes up only 0.1% of the gut flora in adults in 16S sequencing studies (Eckburg *et al.*, 2005).

Colonization of the enteric tract by commensal *E. coli* strains provides nutrients to the host like Vitamin K (Bentley & Meganathan, 1982). Moreover, commensal *E. coli* acts as a barrier to disease by preventing incursion by more pathogenic bacteria, such as *Salmonella* (Hudault *et al.*, 2001) or intestinal pathogenic *E. coli* (Leatham *et al.*, 2009).

E. coli is one of the most well-studied organisms with regard to basic biology; however, much remains to be elucidated regarding to how *E. coli* causes disease. While mainly regarded as a commensal in the gastrointestinal tract of mammals, specialized *E. coli* strains can also produce a large spectrum of disease. *E. coli* are broadly classified into three subtypes: 1) commensal, which do not cause disease in healthy individuals; 2) extraintestinal pathogenic *E. coli* (ExPEC), which cause disease at numerous anatomical sites outside of the intestinal tract; and 3) intestinal, or enteric, pathogenic *E. coli* (IPEC) which cause diseases confined to the intestine (Russo & Johnson, 2003). IPEC produce a

wide variety of intestinal diseases, such as diarrheagenic enteritis or colitis (Russo & Johnson, 2003), while ExPEC produces a greater variety of infections including urinary tract infections, septicemia, pneumonia, osteomyelitis, and meningitis (Orskov & Orskov, 1992a). ExPEC are a commensal–pathogen group of *E. coli*, which are of agricultural, veterinary, and medical importance in both avian and mammalian hosts.

1.1.1 Phylogeny and Genomics of Escherichia coli

E. coli belongs to the Enterobacteriaceae Family, and, along with other members of the Family, generally resides within the gastrointestinal tract of mammals. Diverging from the closely-related bacterium *Salmonella* by approximately 100 million years ago (Lawrence & Ochman, 1998), the *Escherichia* Genus split into five species: *E. albertii*, *E. coli*, *E. fergusonii*, *E. hermannii*, and *E. vulneris*. Among all *E. coli* strains there is a high degree of genomic and phenotypic diversity, with only 20% of the genome common to all *E. coli* strains (Lukjancenko *et al.*, 2010). It has been estimated that up to 18% of the current MG1655 (K12 *E. coli* strain) genome has been horizontally acquired since *E. coli* diverged from *Salmonella* 100 mya (Lawrence & Ochman, 1998). Given the high degree of heterogeneity among *E. coli*, intestinal pathogenic *E. coli* strains are often more genetically similar to *Shigella* sp. (Lan & Reeves, 2002) or *Salmonella* than to K-12 *E. coli* laboratory strains. Among pathogenic *E. coli*, two divisions exist, intestinal pathogenic *E. coli* (IPEC) and extraintestinal pathogenic *E. coli* (ExPEC). IPEC is subdivided into six widely- accepted pathotypes that cause intestinal disease such as

colitis, enteritis, and enterocolitis (Nataro & Kaper, 1998). ExPEC, on the other hand, is subdivided into just two main pathotypes: uropathogenic *E. coli* (UPEC) and neonatal meningitis-associated *E. coli* (NMEC) (Table 1). The UPEC and NMEC pathotypes share phenotypic and genomic characteristics that

Table 1. Taxonomic classification of *Escherichia coli*.

Domain	Bacteria
Phylum	Proteobacteria
Class	Gammaproteobacteria
Order	Enterobacteriales
Family	<i>Enterobacteriaceae</i>
Genus	<i>Escherichia</i>
Species	<i>E. albertii</i> , <i>E. coli</i> , <i>E. fergusonii</i> , <i>E. hermannii</i> , <i>E. vulneris</i>
Human <i>E. coli</i> Pathotypes	
Intestinal	EHEC, EPEC, ETEC, EAEC, EIEC, DAEC
Extraintestinal	UPEC, NMEC

Abbreviations: EHEC, enterohemorrhagic *E. coli*; EPEC, enteropathogenic *E. coli*; ETEC, enterotoxigenic *E. coli*; EAEC, enteroaggregative *E. coli*; EIEC, enteroinvasive *E. coli*; DAEC, diffusely adherent *E. coli*; UPEC, uropathogenic *E. coli*; and NMEC, neonatal meningitis *E. coli*.

have caused some to support using the more inclusive term ExPEC to refer to these strains (Johnson & Russo, 2002a; Russo & Johnson, 2000).

While specific pathotypes of *E. coli* are difficult to define given a high degree of genomic heterogeneity, certain genetic features can distinguish pathogenic strains from commensal strains (Groisman & Ochman, 1994). Phylogenetic analyses have shown that *E. coli* strains fall into four main groups: A, B₁, B₂, and D (Herzer *et al.*, 1990; Lecointre *et al.*, 1998). IPEC strains of all pathotypes fall mainly into phylogenetic Groups B₁, A, or D (Girardeau *et al.*, 2003), whereas most commensal strains belong to Group A and B₁. ExPEC, on the other hand, belong mainly to phylotype Group B₂ and, to a lesser extent, to Group D (Boyd & Hartl, 1998; Picard *et al.*, 1999). Recently, however, there has been wide-spread emergence of clonal group A (CgA), part of the Group D phylotype, as the etiologic agent of up to 50% of trimethoprim-sulfamethoxazole (TMP-SMZ)-resistant acute, uncomplicated cystitis and pyelonephritis (Manges *et al.*, 2004; Manges *et al.*, 2001). CgA ExPEC exhibit similar virulence factor profiles compared with other phylotypes, but display a conserved, multi-drug resistance profile (Manges *et al.*, 2001). Coupled with geographic sampling and pulse-field gel-electrophoresis profiles, these data suggest the recent dissemination of a newly-emerged virulent clonal group (Johnson *et al.*, 2002; Stamm, 2001). Generally, phylotype B₂ predominates over Group D as etiologic agents of UTI; however, that trend is reversed among TMP-SMZ-resistant UTI

isolates, with approximately 23% of phylogenetic Group D isolates representing CgA (Johnson *et al.*, 2005).

However, even among the pathogenic B2 and D phylotypes, ExPEC are genetically heterogeneous with an assortment of factors either directly demonstrated or epidemiologically-linked to virulence (discussed in detail in Section 1.3), including adhesive factors like pili (P pili, type 1 pili, S pili), toxins (HlyA and CNF1), and invasins (Hek and IbeA) (Groisman & Ochman, 1994). Much of the genomic heterogeneity stems from massive horizontal exchange of genetic information in the form of genomic islands (GI) or pathogenicity-associated islands (PAIs). Indeed, numerous PAIs and GIs are present in the genomes of ExPEC strains, leading to a mosaic genome structure that contributes to variety extraintestinal disease processes (Diard *et al.*, 2010; Lloyd *et al.*, 2009; Tourret *et al.*, 2010).

1.1.2 Role in Extraintestinal Disease

The urinary tract is the most frequent site of extraintestinal infection caused by ExPEC, producing approximately 1% of ambulatory care visits in the US each year (Hooton & Stamm, 1997; Stamm & Hooton, 1993). Urinary tract infection (UTI) is the second most common infection requiring hospitalization, next to respiratory infections; however, unlike the myriad of respiratory infection etiologic agents, greater than 80% of the approximately 10 million annual cases of community-acquired UTIs are caused by ExPEC (Russo & Johnson, 2003). UTI is a leading infection of children, women, and the

elderly (Downs, 1999; Foxman, 2010; Foxman *et al.*, 2000). Clinically, approximately 20-25% of women who get a UTI will get a recurrent infection within 6 months with a clonal isolate from the primary infection (Ejrnaes *et al.*, 2006; Foxman, 1990). While the majority of UTIs involve bladder infection (cystitis, CY), ExPEC may also produce ascending infections to the kidneys, causing pyelonephritis (PY). ExPEC is the causative agent in 85-95% cases of acute, uncomplicated cystitis and 90% of uncomplicated pyelonephritis cases in pre-menopausal women (Hooton & Stamm, 1997; Stamm & Hooton, 1993; Talan *et al.*, 2000).

ExPEC can also produce more advanced diseases such as bacteremia, sepsis, pneumonia, osteomyelitis, and meningitis among certain patient groups (Laupland *et al.*, 2002; Rushton, 1997b). Neonates, patients with diabetes or urogenital anomalies, and immunocompromised individuals (including those that are immunocompromised due to an underlying disease, chemotherapy, or age) are at particular risk of disseminated disease with resultant bacteremia, sepsis or urosepsis, and meningitis (Rushton, 1997a). Bacteremia can result from a primary infection, such as a UTI, or from direct translocation from the intestine, especially in patients with an increase in intestinal permeability, as is the case for neonates or patients with neutropenia (Johnson *et al.*, 2003). Bacteremia can occur with or without accompanying sepsis syndrome, and is required for the progression to meningitis (Dietzman *et al.*, 1974). Meningitis caused by *E. coli* is a particular problem among neonates and infants, particularly infection with the ExPEC K1 capsule type (Rushton, 1997b; Stoll *et al.*, 2002). Between 80-90% of ExPEC

neonatal meningitis is caused by K1 encapsulated strains (Bonacorsi *et al.*, 2003; Pong & Bradley, 1999).

Gram-negative bacilli are also the most frequent cause of nosocomially-acquired pneumonia, and are responsible for 60-70% of the cases, with *E. coli* producing the largest proportion of infections (Craven *et al.*, 1990); although, ExPEC does not generally cause pneumonia in otherwise healthy individuals. ExPEC has also been associated with a small number of additional infections, including surgical wound infections, osteomyelitis, and myositis; however, the small case number suggests ExPEC is not as proficient at causing these infections (Johnson & Russo, 2002b). Overall, ExPEC is responsible for a wide spectrum of infections outside the intestinal tract and have a diverse repertoire of virulence factors to establish disease in these varied host environments (discussed in more detail in Section 1.3).

1.2 Pathobiology of Extraintestinal *Escherichia coli* Infections

1.2.1 Pathogenesis

Like many other mucosal pathogens, ExPEC follows a similar pattern of steps to initiate and perpetuate disease, including colonization of a mucosal site, adherence, evasion of host defenses, replication and persistence, and, finally, host damage through direct bacterial effectors and induction of inflammation leading to disease symptoms. Even though ExPEC colonization of the gastrointestinal tract is benign, it is the first step toward infection of sterile sites such as the urinary tract, blood stream, and central

nervous system (Orskov & Orskov, 1992b). To effectively colonize the gastrointestinal tract, ExPEC must successfully compete with commensal *E. coli* strains as well as other endogenous microflora for both nutrients and space. As already described, commensal *E. coli* is a typical constituent of the enteric tract in many animals, including humans; however, not all *E. coli* strains colonize the colon in the same manner. Commensal *E. coli* must compete with numerous other endogenous, commensal microflora and occupies a highly specific metabolic niche by utilizing gluconate in the colon more efficiently than other flora (Sweeney *et al.*, 1996). On the other hand, EHEC utilizes a distinct carbon profile for metabolism different than a K-12 commensal strain in the gut, thereby, creating a nutritional and spatial niche partition between various *E. coli* strains (Fabich *et al.*, 2008; Miranda *et al.*, 2004). It remains to be determined whether ExPEC overlaps with these niches or occupies a separate microenvironment conducive to its own colonization.

The next step in ExPEC pathogenesis is to transition from benign colonization of the gastrointestinal tract to previously sterile sites of infection like the urinary tract, bloodstream, or central nervous system (CNS). It is essential that bacteria are able to adhere to host tissues in newly occupied host sites to establish infection. For instance, ExPEC contaminating the peri-urethral area can only establish infection if they can gain access to the urinary tract and adhere to urethral or bladder cells to elude flushing by the normal action of bladder voiding. ExPEC express type 1 pili (T1P) to adhere to and invade into superficial bladder epithelial cells (Martinez *et al.*, 2000; Mulvey *et al.*, 1998)

(Fig. 1, step 2). Maintaining an intracellular infection also avoids innate immune clearance (Mulvey *et al.*, 2001; Mulvey *et al.*, 1998); therefore, bacteria that are unable to express TIP are highly attenuated secondary to reduced adherence to and invasion into superficial bladder epithelial cells. Once the bacteria are intracellular, they are able to form biofilm-like structures called intracellular bacterial communities (IBCs), replicate to high numbers within IBCs, form filaments, flux out of infection cells, and disperse to infect additional bladder cells (Anderson *et al.*, 2003; Justice *et al.*, 2004) (Fig. 1, step 2). Even after appropriate antibiotic treatment, approximately 25% of women will develop a recurrent infection with the identical *E. coli* clone within 6 months of the initial episode (Ejrnaes *et al.*, 2006; Foxman, 1990), suggesting that a sub-set of the intracellular bacterial population may establish a quiescent reservoir capable of producing recurrent infections (Mulvey *et al.*, 2001).

If cystitis (Fig. 1, step 1) is untreated, the bacteria can ascend to the kidneys, causing pyelonephritis (Fig. 1, step 3). Uncomplicated cystitis isolates generally elaborate TIP during the course of infection, causing those strains to tightly adhere to and invade into the superficial bladder epithelium, and, therefore, confining the infection to the bladder. ExPEC that transition to expression of P pili, a different adhesive fiber, are able to establish pyelonephritis by adhering to digalactoside receptors present on kidney the epithelium (Dodson *et al.*, 2001; Korhonen *et al.*, 1986; Lane & Mobley, 2007). ExPEC expression of the toxin hemolysin (HlyA) in the kidney has been shown to extensively damage the kidney epithelium (Trifillis *et al.*, 1994) and is implicated in penetration of

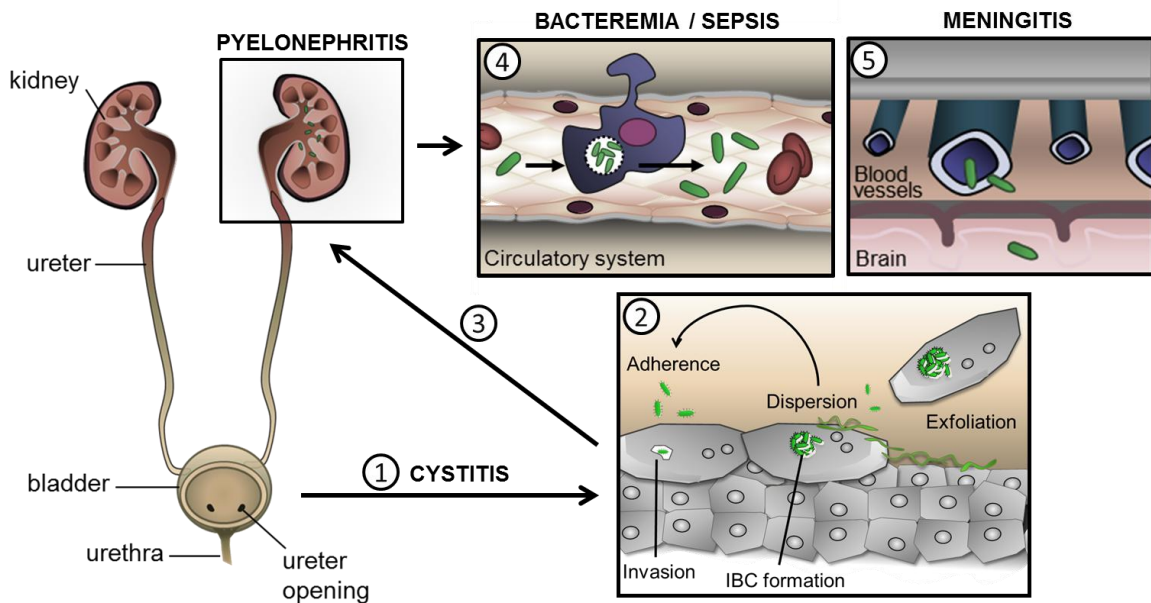


Figure 1: Pathogenesis of extraintestinal pathogenic *Escherichia coli*.

ExPEC contaminating the peri-urethral area can transit to the bladder where bacteria can replicate to high numbers and cause cystitis [1]. To establish cystitis, ExPEC must elaborate type 1 pili to adhere to and invade into the superficial bladder epithelial cells [2]. Once intracellular, ExPEC escape to the cytosol, proliferate, and form tight aggregate communities, called IBCs, which resemble a biofilm-like state [2]. As a host defense mechanism, bladder epithelial cells containing IBCs are shed or exfoliated into the lumen of the bladder and flushed during voiding. Finally, bacteria in late-stage IBCs can disperse from the epithelial cell in a filamentous morphology, potentially leading to re-adherence and continuation of the infection cycle [2]. ExPEC can also ascend the ureters to cause infection of the kidney, or pyelonephritis [3], which can spread hematogenously to the bloodstream, or through direct translocation from the intestine, causing bacteremia and/or sepsis [4]. Following bacteremia, ExPEC can invade the blood-brain barrier to cause meningitis [5], which appears to be dependent on macrophage trafficking.

the bloodstream barrier through destruction of the renal proximal tubule cells (Mansson *et al.*, 2007), leading to bacteremia (Fig. 1, step 4). ExPEC virulence factors that play a role in the various disease processes are discussed in detail in Section 1.3.

E. coli can transit to the bloodstream by translocation from the kidneys or from the gut. In fact, rRNA sequences from bacteria, including *E. coli*, can be detected in the blood of healthy individuals (Nikkari *et al.*, 2001); although, these bacteria rarely cause disease in healthy individuals. However, once established in the bloodstream, ExPEC can result in sepsis and meningitis, with high levels of bacteremia in the blood correlating with the development of meningitis (Dietzman *et al.*, 1974) (Fig. 1, step 5). The high level of bacteremia necessary for establishment of meningitis has been shown to require macrophages (Mittal *et al.*, 2010b), either as a direct vehicle for trafficking or as a protected cellular niche for bacterial replication (Fig. 1, step 4). ExPEC are then able to cross the blood-brain barrier from the blood to the CNS via a transcytosis mechanism that involves numerous adhesive and invasive factors (Huang *et al.*, 2000; Kim, 2001) (discussed in Section 1.3.1 and 1.3.2).

1.2.2 Interaction with Professional Phagocytes

As ExPEC transitions from the enteric, for instance, it may encounter professional phagocytes such as macrophages and polymorphonuclear leukocytes (PMNs). In the urinary tract, dendritic cells (DCs), macrophages, and PMN cell populations vary depending on tissue location, and the relative contribution of each cell type to bacterial clearance is varied.

In the microenvironment of the bladder, there are small numbers of resident macrophages, DCs, and PMNs at baseline (Engel *et al.*, 2006), and many groups have

shown these cells populations are rapidly expanded through recruitment post-infection (Engel *et al.*, 2006; Horvath *et al.*, 2011). Despite a robust influx of multiple DC-subtypes post-infection, the contribution of this immune cell-type to bacterial control in ExPEC-associated UTI is limited. For instance, both CD11b⁻ myeloid and F4/80⁺ DCs are resident in the uninfected bladder, and, along with CD11b^{INT} Tip-DC cells, are rapidly recruited to the ExPEC-infected bladder in higher numbers (Engel *et al.*, 2006). Neither TNF- α and iNOS-producing Tip-DC cells nor DC cells in general were required for granulocyte recruitment or bacterial clearance in the bladder. Specifically, CCR2^{-/-} mice (Tip-DC deficient) and CD11c-DTR mice depleted of all DCs by diphtheria toxin administration still recruited granulocytes to the bladder with wild-type kinetics and abundance, and were not required for ExPEC bacterial clearance (Engel *et al.*, 2006), suggesting that neutrophils have the predominant role in clearance of the bacterial burden in the bladder during UTI.

In the kidney, however, CD11c⁺ DC cells are the most abundant immune cells present (Kruger *et al.*, 2004), forming a connected network throughout the kidney interstitium (Soos *et al.*, 2006). Unlike the bladder, DC counts actually decrease slightly over the course of infection, while the numbers of recruited PMNs and, to a lesser extent, macrophages increase over the course of infection (Tittel *et al.*, 2011). Dendritic cells in the kidney secrete the chemokine CXCL2, or MIP-2, by 3 h post-infection (Tittel *et al.*, 2011), which is required for neutrophilic granulocyte transmigration across the urinary tract epithelium (Hang *et al.*, 1999). PMNs infiltrated the infected kidney prior to

macrophages and were responsible for the majority of bacterial phagocytosis. In mice conditionally depleted of CD11c⁺ DCs, there was a delay in PMN recruitment and a marked reduction in bacterial clearance (Tittel *et al.*, 2011), suggesting that DCs in the kidney respond to ExPEC-associated pyelonephritis by recruiting PMNs, which are responsible for the majority of bacterial clearance.

PMNs are critical effectors of the host immune response in the urinary tract and the primary cellular effectors mediating bacterial clearance during UTI. Inhibition of neutrophil recruitment by depletion with a granulocyte-specific antibody resulted in increased bacterial counts in the bladder and kidneys of infected mice (Haraoka *et al.*, 1999). Although large numbers of PMNs are recruited to both the bladder and kidney during infection, pathogenic ExPEC strains actually suppress many pro-inflammatory cytokines *in vitro* and during infection. For instance, ExPEC can suppress TNF- α -mediated secretion of cytokines IL-8 and IL-6 from cultured urothelial cells compared to K-12 commensal *E. coli* (Billips *et al.*, 2007; Hunstad *et al.*, 2005). Additionally, MIP-2 and IL-6 expression during *in vivo* infection with ExPEC was suppressed compared to a non-pathogenic strain, leading to a decrease in detectable myeloperoxidase (and, by proxy, PMNs), in the bladder and urine of ExPEC-infected animals (Billips *et al.*, 2007).

Work from other groups has shown that ExPEC are able to suppress transepithelial migration of PMNs compared to non-pathogenic *E. coli* (Loughman & Hunstad, 2011), suggesting ExPEC has evolved many mechanisms to modulate the host immune response, thereby, leading to more successful infection. In addition to PMN

migration, ExPEC are also able to alter PMN function to better resist phagocytic killing and dampen production of reactive oxygen species (ROS) compared to their non-pathogenic counterparts (Loughman & Hunstad, 2011). *In vitro*, human PMNs are able to actively kill approximately 10-20% of invading ExPEC; however, bacteria that survive the initial infection are able to replicate and, by 2 h post-infection, out-number the initial inoculum (Loughman & Hunstad, 2011). In comparison, incubation of several non-pathogenic *E. coli* strains with human PMNs resulted in killing of 50% of the initial inoculum of commensal *E. coli*. Several bacterial mechanisms known to influence the interaction between ExPEC and professional phagocytes are discussed in more detail in Section 1.3.

Macrophages are also an important innate immune component rapidly recruited to the bladder (Engel *et al.*, 2006; Horvath *et al.*, 2011) and kidney (Tittel *et al.*, 2011), although, to a lesser extent. In the kidney, macrophages are recruited to a lesser extent than PMNs and are responsible for minimal bacterial phagocytosis and clearance (Tittel *et al.*, 2011). However, the overall contribution of this cell type to bacterial clearance in the urinary tract remains vague. *In vitro*, ExPEC can survive within bone marrow-derived macrophages for greater than 24 h post-infection within a LAMP1⁺ vesicular compartment, and ExPEC strains, in particular, are better adapted to intracellular macrophage survival than commensal strains (Bokil *et al.*, 2011). Other groups have investigated the role of bacterial morphology to intramacrophage survival, with filamentous bacteria surviving better than bacillary forms of ExPEC (Horvath *et al.*,

2011); however, the contribution of macrophage recruitment to UTI resolution remains unclear.

Some host innate immune clearance mechanisms, however, can be co-opted for the benefit of invading bacteria. Recent research suggests that the K1 serotype of ExPEC, a leading cause of neonatal meningitis (Gaschignard *et al.*, 2011; Harvey *et al.*, 1999), requires macrophages for dissemination to the central nervous system, spleen, and lungs (Mittal *et al.*, 2010b) after intranasal challenge. Despite accumulation of bacteria in the bloodstream by 6 h post-infection, bacteria are rapidly cleared in macrophage-depleted animals through as-yet-undefined mechanisms. Similar results were obtained for ExPEC infections of mice that lacked iNOS, the inducible nitric oxide synthase (Mittal *et al.*, 2010a), suggesting that nitric oxide may enhance bacterial survival within macrophages or, conversely, dampen the ability of macrophages to kill intracellular bacteria, which can result in more disseminated disease. These results suggest that macrophages can directly traffic intracellular bacteria to sites of dissemination or, alternatively, provide a protected cellular niche for bacterial persistence. The regulatory cues that provoke the commensal to pathogen transition and the factors required for survival in macrophages as a potential vehicle for disseminated infections are incompletely understood.

1.3 Virulence Determinants

The majority of ExPEC-acquired virulence factors are different from those found in IPEC strains (Picard *et al.*, 1999; Russo & Johnson, 2003), not surprisingly, given that

these two groups of pathogenic *E. coli* cause different diseases in mutually exclusive host sites. Unlike for IPEC pathotypes, there is not a uniform set of virulence factors among ExPEC, or even the UPEC pathotype. Instead of a common virulence profile, however, ExPEC express a large variety of different virulence genes (Brzuszkiewicz *et al.*, 2006), leading to a mosaic genome structure among various ExPEC strains. Numerous reviews have put together an extensive catalogue of ExPEC virulence factors (Johnson & Russo, 2002a, 2005; Smith *et al.*, 2007); however, the review herein will focus on a limited repertoire of ExPEC VFs important for specific pathogenic processes.

1.3.1 Host Cell Adherence and Invasion

As mentioned in Section 1.2, the first step to establishing infection by ExPEC is adherence to host tissues. To accomplish this step, ExPEC encodes for a multitude of adhesive factors that contribute to binding to numerous host cell types. Subsequently, many of these adhesins also mediate internalization. These adhesive factors fall into two main groups: non-fimbrial and fimbrial.

1.3.1.1 Non-fimbrial Factors Contributing to Adherence and Invasion

The major factors that promote adherence of ExPEC to urinary tract tissues are fimbrial adhesins, and will be discussed in the following section. Recently, though, several afimbrial factors belonging to the autotransporter family have been shown to mediate adherence to urinary tract tissues. The afimbrial adhesin antigen 43 (Ag43), for

instance, has been implicated in ExPEC persistence in the urinary tract (Ulett *et al.*, 2007), although, the precise role is not well understood. Ag43 is a self-associating autotransporter (SAAT) protein that mediates bacterial auto-aggregation and biofilm formation in K-12 *E. coli* (Hasman *et al.*, 1999). Although the precise role of Ag43 in urinary tract persistence is not well understood, Ag43 is expressed during IBC formation in bladder epithelial cells during UTI (Anderson *et al.*, 2003) possibly contributing to its biofilm-like state. Other autotransporter proteins have also been shown to play a role in UTI. UpaB, for instance, mediates binding to various extracellular matrix proteins *in vitro* and contributes to urinary tract colonization (Allsopp *et al.*, 2011). ExPEC strains encode for many additional autotransporters, and future work will be necessary to ascertain the individual contributions of these adhesins to urinary tract tissues or other host cell types.

Many afimbrial adhesins have been implicated in adherence to cell types generally encountered during disseminated disease, including both brain endothelial cells and macrophages. To establish meningitis, ExPEC of the K1 capsule type must cross the blood-brain barrier, which separates the circulatory system from the central nervous system. The brain endothelium prevents against entry of bacteria and toxins present in the circulatory system (Huang & Jong, 2001). The presence of certain ExPEC virulence factors, however, can bypass the brain microvascular endothelial cells (BMECs) that comprise the host barrier mechanism, leading to the establishment of meningitis. Numerous bacterial adherence and invasive factors contribute to BMEC penetration both

in vitro (Xie *et al.*, 2004b) and *in vivo*, including the invasin IbeA, the major outer membrane protein OmpA, the toxin Cytotoxic Necrotizing Factor 1 or CNF1, and the adhesive factor SfaS (discussed in the following section) (Badger *et al.*, 2000; Kim, 2001; Prasadarao *et al.*, 1999a; Prasadarao *et al.*, 1999b).

Both OmpA and CNF1 are known to induce rearrangement of the actin cytoskeleton (Khan *et al.*, 2003; Prasadarao *et al.*, 1999b; Xie *et al.*, 2004b), facilitating invasion of BMECs by K1-encapsulated ExPEC. Actin rearrangement is an important component of BMEC invasion as treatment with actin microfilament inhibitors cytochalasin D and latrunculin A blocks K1 ExPEC invasion (Khan *et al.*, 2003; Kim, 2001). CNF1, while not a classical adhesin, is still able to mediate bacterial internalization. CNF1 binds the laminin receptor on BMECs (Kim *et al.*, 2005), leading to RhoA activation, actin cytoskeletal rearrangement, and invasion (Khan *et al.*, 2003). OmpA, however, binds to the N-glucosamine (GlcNAc) epitopes of host surface protein gp96 (Datta *et al.*, 2003; Prasadarao *et al.*, 1996). Interestingly, in macrophages, OmpA is also required for bacterial internalization; however, OmpA appears to interact with Fc γ R1alpha chain as a cellular receptor on macrophages to mediate internalization (Mittal *et al.*, 2010b).

IbeA, on the other hand, facilitates BMEC invasion through receptor-mediated, caveolae-dependent entry, in the absence of actin microfilament rearrangement. In detail, IbeA interaction with its primary host cell receptor vimentin leads to vimentin phosphorylation, subsequent ERK-mediated signaling, and modulation of ExPEC K1

invasion (Chi *et al.*, 2010). Although approximate 80-90% of ExPEC neonatal meningitis isolates are K1-encapsulated (Bonacorsi *et al.*, 2003; Pong & Bradley, 1999), the K1 sialic acid capsule does not appear to facilitate BMEC binding or invasion; however, K1 capsule does appear to enhance intracellular survival post-invasion (Kim *et al.*, 2003) (discussed in Section 1.3.2). There are many other putative virulence factors that contribute to ExPEC K1 invasion of the blood-brain-barrier on the basis of genomic comparisons between ExPEC neonatal meningitis isolates and commensal *E. coli* (Xie *et al.*, 2004a); however, the mechanism of action of many of these factors remains to be elucidated.

1.3.1.2 Fimbrial Structures Contributing to Adhesion and Invasion

Fimbriae, or pili, are multimeric structures assembled into rods anchored to the cell surface of the bacterium and capped by a sugar-binding lectin molecule, targeting the bacteria to glycoproteins and glycolipids present on host tissues. ExPEC encode for and express multiple types of adhesive fibers, including well-studied examples such as type 1 pili (T1P), P pili, and S pili; however, some estimates based on sequence homology in sequenced ExPEC genomes suggest that ExPEC strains can encode for up to 12 fimbrial gene clusters (Johnson *et al.*, 1998; Welch *et al.*, 2002). Although clinical ExPEC strains show a diverse fimbriae profile, encoding for as few as 2 fimbrial clusters or as many as 12 (Spurbeck *et al.*, 2011), many of these fimbriae remain uncharacterized in terms of expression patterns or host cell receptors. Three classical fimbrial systems and one

atypical amyloid fiber known to be important for ExPEC pathogenesis and, arguably, the best characterized, are diagramed in Fig. 2.

Two of the most well-studied examples, type 1 and P pili, dictate ExPEC tropism for the urinary bladder and kidneys, respectively, and vaccination studies with subcomponents of T1P pili (Langermann *et al.*, 2000a; Palaszynski *et al.*, 1998) or P pili (Roberts *et al.*, 2004) reduce the severity and duration of infection. Pili also mediate tissue tropism through specific host cell receptor binding, for instance P pili adhere to digalactoside receptors present on kidney the epithelium (Dodson *et al.*, 2001; Korhonen *et al.*, 1986) while type 1 pili specifically bind manosylated glycoproteins on the bladder epithelium including Uroplakin 1a (Zhou *et al.*, 2001) and $\alpha 3\beta 1$ Integrin (Eto *et al.*, 2007).

Pili function is niche specific, and different host microenvironments can induce the expression of certain fimbrial structures over others. For instance, ExPEC switch off type 1 pili expression upon systemic dissemination because type 1 piliation leads to increased clearance by phagocytes in the bloodstream (Xie *et al.*, 2006b). In contrast, ExPEC introduced directly into the bloodstream in a non-piliated state have been isolated from the CSF predominately piliated by the S type (Saukkonen *et al.*, 1988). S pili bind to sialydated glycoproteins present on the brain microvascular endothelium (Korhonen *et al.*, 1985; Prasadarao *et al.*, 1997), leading to increased intimate association of ExPEC with HBMECs. S pili expressing ExPEC were also more virulent in a neonatal rat model

of bacteremia than either a non-piliated strain or a type 1 piliated strain based on CFU counts and mortality (Saukkonen *et al.*, 1988).

Curli fibers are also expressed by a majority of ExPEC isolates, and mediate cell-

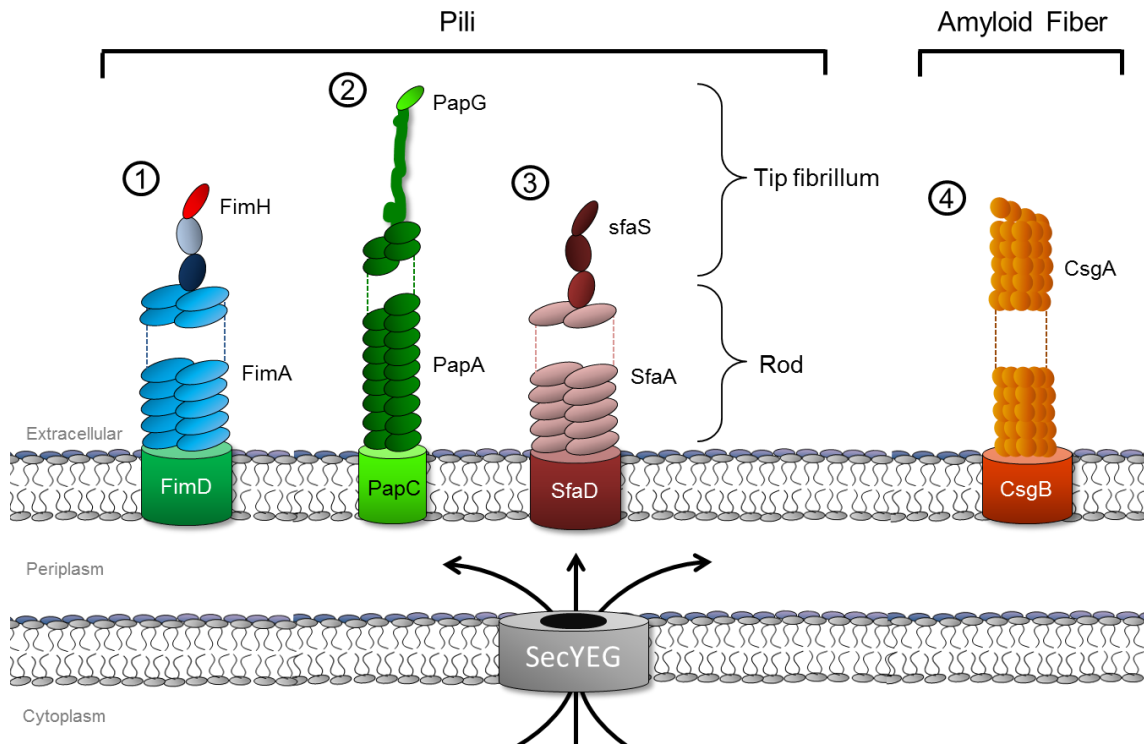


Figure 2: The major surface-associated fimbrial structures of ExPEC that mediate host cell adherence and invasion.

The four most well-studied fimbrial structures of ExPEC are illustrated as follows: [1] type 1 pili, [2] P pili, [3] S pili, and [4] curli. In [1] through [3], the outer membrane translocation pore, major structural subunit, and adhesive factor is shown. Curli [4], however, is composed of repeating subunits of CsgA, the major structural component of the amyloid fiber as well as the adhesive component, and does not have the typical tip fibrillum structure associated with pili. All four fimbrial structures are secreted as subunits through the SecYEG system and assembled on the bacterial cell surface.

cell and abiotic interactions that result in biofilm formation *in vitro* (Cegelski *et al.*, 2009; Kikuchi *et al.*, 2005). Curli are composed of repeating structural subunits that form an amyloid structure (Chapman *et al.*, 2002) (Fig. 2). In commensal K-12 *E. coli*, curli expression results in binding of bacteria to urothelial cells (Kikuchi *et al.*, 2005). Although curli are not abundantly expressed at physiologic temperature (Olsen *et al.*, 1993), expression can result in induction of nitric oxide (NO) (Bian *et al.*, 2001) and proinflammatory cytokine IL-8 (Kai-Larsen *et al.*, 2010). Most studies, however, have looked at commensal K-12 *E. coli* expressing curli. For instance, intraperitoneal infection of mice with a K-12 *E. coli* strain expressing curli mediated significant induction of NO through inducible NO synthase (Bian *et al.*, 2001). Curli can also bind fibronectin (Olsen *et al.*, 1989) and the host antimicrobial peptide LL-37 (Kai-Larsen *et al.*, 2010); however, the overall contribution of curli fibers to *in vivo* disease processes is incompletely understood.

In summary, ExPEC utilize a variety of pili for diverse tissue tropism, and the expression of the different pili classes must be specifically coordinated to allow adaptation and transition between host tissues and environmental niches. Although type 1 and P pili have well demonstrated roles in the establishment and maintenance of urinary tract infection (Lane & Mobley, 2007; Wright *et al.*, 2007), and S pili have a well demonstrated role in meningitis, many other fimbrial systems have only been characterized by sequence homology and do not yet have experimentally demonstrated roles during colonization, transmission, or infection.

1.3.2 Intracellular Survival

One of the major mechanisms used by professional phagocytes such as macrophages to control intracellular bacterial infections is through the generation of reactive nitrogen intermediates, or RNI (Bogdan *et al.*, 2000; De Groote & Fang, 1995). However, ExPEC have evolved multiple virulence pathways to respond to RNI. Clinical ExPEC strains are more resistant to RNI challenge *in vitro* than commensal *E. coli* (Bower *et al.*, 2009; Svensson *et al.*, 2006). One of the key bacterial NO detoxification enzymes is the flavohemoglobin Hmp. Hmp has been shown to play an important role in NO consumption in both pathogenic and non-pathogenic *E. coli* strains, as well as other pathogenic species, including *Salmonella enterica* serovar *typhimurium* (*S. typhimurium*) (Stevanin *et al.*, 2007; Stevanin *et al.*, 2002; Svensson *et al.*, 2010). Hmp expression is responsive to nitrosative stress, and ExPEC isolate J96 challenged with RNI from the generator DETA/NO has been shown to result in increased *hmp* expression (Svensson *et al.*, 2010). Together these results suggest that host RNI is sensed by ExPEC, which in turn upregulates the major Hmp-mediated NO detoxification pathway.

The role of NO in controlling ExPEC infection appears to vary drastically depending on anatomical site. For instance, an *hmp* deletion in ExPEC isolate J96 shows modestly reduced colonization of the urinary tract (Svensson *et al.*, 2010), suggesting that 1) Hmp is a minor contributor to RNI detoxification during UTI or 2) that NO is not a large component of the host response to ExPEC infection in the urinary tract. In fact, work from another group showed that iNOS^{-/-} mice are equally susceptible to ExPEC UTI

as their wild-type counterparts (Poljakovic & Persson, 2003), suggesting that NO induction plays a limited role in bacterial clearance in the urinary tract. However, iNOS^{-/-} mice were actually better at controlling pathogen load in a disseminated model of infection. iNOS-deficient mice were significantly less likely to develop meningitis, and peritoneal macrophages isolated from these animals controlled ExPEC intracellular burden more effectively (Mittal *et al.*, 2010a).

While K1 capsule has classically been defined as an anti-phagocytic virulence factor, new work suggests that it may also play a role in intracellular survival in various cell types (Goller & Seed, 2010a). K1-encapsulated ExPEC show a fitness advantage during ascending urinary tract infection even in the absence of host TLR4-mediated inflammation, suggesting a non-classical role for capsule in pathogenesis. Confocal microscopy confirmed that un-encapsulated bacteria did not form efficient IBCs in bladder epithelial cells; thereby, failing to undergo high level replication within the bladder (Anderson *et al.*, 2010). During the initiation of meningitis, K1 ExPEC invade into BMECs with internalized bacteria found in membrane-bound vacuoles; however, K1 capsule inhibits the maturation of the ExPEC-containing vacuoles by blocking lysosomal fusion (Kim *et al.*, 2003), allowing increased intracellular survival of K1-expressing ExPEC. It has yet to be investigated whether other ExPEC capsule types promote intracellular survival of ExPEC in the same manner as the K1, sialic acid-based capsule.

1.3.3 Evasion of the Innate Immune Response and Survival in the Circulatory System

To mount successful infections after breaching host barriers, ExPEC must circumvent, suppress, or tolerate the host innate immune system, including chemical stresses such as reactive nitrogen and oxygen species as well as cellular immune effectors. PMNs are critical effectors of the host immune response in the urinary tract and the primary cellular effectors mediating bacterial clearance during UTI. Many of the bacterial factors described in the previous section could, in fact, be considered important to evasion of the innate immune response as one of the primary mechanisms of evading detection is maintaining an intracellular lifestyle. Several other bacterial factors have been shown to have anti-phagocytic and/or anti-complement properties, effectively shielding ExPEC from assault by the host innate immune system.

CNF1-expressing ExPEC have decreased phagocytic uptake by PMNs through alteration of complement receptor 3 (CR3) distribution, leading to enhanced survival in neutrophil killing assays (Davis *et al.*, 2005). Additionally, exogenous addition of CNF1-containing outer membrane vesicles attenuated PMN antimicrobial activity and chemotaxis (Davis *et al.*, 2006), suggesting that CNF1 can result in evasion of PMN function in multiple ways including inhibition of migration, phagocytosis, and antimicrobial activity. K capsule is another example of a bacterial virulence factor that blocks phagocytosis of ExPEC by PMNs. For instance, K5 capsule has been shown to increase resistance to phagocytosis by PMNs, with a K5 deletion strain exhibiting

Table 2. ExPEC virulence factors.

Virulence Factor	Function
Adhesins/Invasins	
OmpA	Adherence to GlcNAc epitopes of gp96 on HBMECs and cytoskeleton rearrangement leading to invasion; FC γ -mediated invasion of macrophages
IbeA	Vimentin-mediated HBMEC binding and invasion
type 1 pili	Adherence to manosylated host glycoproteins resulting in bladder epithelial cell invasion; IBC formation; TLR4-mediated inflammatory induction
P pili	Adherence to digalactoside receptor in kidney
S pili	Adherence to sialylglycoproteins on HBMECs
curli	Fibronectin binding; binding to antimicrobial peptide LL-37
Toxins	
CNF1	Tissue necrosis; laminin receptor-mediated invasion through RhoA activation and cytoskeletal rearrangement in HBMECs; impairment of PMN chemotaxis, phagocytosis, and antimicrobial activity
HlyA	RTX toxin; cell lysis leading to breach of renal tubule cell barrier
LPS	Endotoxin; TLR4 stimulation of cytokine production
RNI Tolerance	
Hmp	Flavo-hemoglobin; NO detoxification
Iron Acquisition	
IroN	Siderophore receptor for Salmochelin iron uptake
Other	
Flagella	Motility; stimulates TLR5 cytokine production
K capsule	Anti-phagocytic; host mimetic (K1 type); intracellular survival

TraT	Serum-resistance factor
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Abbreviations: GlcNAc, N-glucosamine; RNI, reactive nitrogen intermediate; NO, nitric oxide.

significant phagocytosis by 15 min post-infection (Burns & Hull, 1999). Inhibition of K1-encapsulated ExPEC with capsule-specific small molecules increases complement factor C3b binding to the surface of the bacterium and sensitizes inhibitor-treated bacteria to serum killing (Goller & Seed, 2010b). LPS O-antigen also contributes to protecting ExPEC during passage through the circulatory system by inhibiting complement-mediated lysis in a K5-encapsulated ExPEC isolate (Burns & Hull, 1998).

As previously described, bacteremia is a key step that is necessary for the initiation of meningitis; therefore, bacterial factors that influence survival in the circulatory system are also important for understanding the underlying causes of meningitis. Many factors have been identified that are important for ExPEC survival in the circulatory system, including OmpA and iron siderophore receptor IroN. The circulatory system is also a site where iron is in notoriously short supply due to sequestration by the host. One particular iron uptake system, the IroN-Salmochelin receptor-siderophore pair, was required for ExPEC to establish high levels of bacteremia in the neonatal rat model (Negre *et al.*, 2004).

One mechanism for survival in the circulatory system is to “hide” from the host immune response in a protected intracellular niche. As discussed in Section 1.3.1.1,

OmpA stimulated actin rearrangement following gp96 binding, leading to internalization into BMECs (Datta *et al.*, 2003; Khan *et al.*, 2003; Prasadarao *et al.*, 1996). However, OmpA also facilitates ExPEC survival in the circulatory system by two distinct mechanisms. First, OmpA mediates macrophage uptake through the host-cell receptor Fc γ Receptor 1 alpha chain (CD64) with ExPEC ending up in LAMP1+ vesicles (Mittal *et al.*, 2010b). The intramacrophage survival appears to support bacterial replication in the blood, as mice that were depleted of macrophages showed enhanced blood counts that ultimately led to meningitis. Secondly, OmpA expression in the bloodstream protects ExPEC from complement-mediated opsonophagocytic killing, thereby, contributing to the ability of ExPEC to survive and replicate in the bloodstream (Weiser & Gotschlich, 1991; Xie *et al.*, 2004a).

1.4 The Major Virulence Factor, Type 1 Pili

1.4.1 Role during Infection

Fundamental to its pathogenic capacity, ExPEC encodes for several cell surface-associated, multimeric adhesive fibers, or pili that, through an adhesive tip lectin, mediate tissue-specific interactions. Of the different fibers, type 1 pili have been shown to be crucial for establishing bladder infections through which ExPEC may proceed to more invasive diseases. Bacteria lacking type 1 pili are attenuated during experimental cystitis secondary to reduced adherence and invasion (Connell *et al.*, 1996; Wright *et al.*, 2007), and corroborating vaccination studies with pili subcomponents have been shown to

reduce the severity and duration of infection (Abraham *et al.*, 1985; Langermann *et al.*, 2000b; Palaszynski *et al.*, 1998). The type 1 pili operon is ubiquitous among both pathogenic and commensal *E. coli*, although, differences in expression among clinical ExPEC isolates do exist (Hultgren *et al.*, 1986), suggesting some strains have undergone adaption of the regulatory program that controls expression of T1P.

In the murine model of cystitis, type 1 pili is a preeminent virulence determinant; bacteria lacking type 1 pili are deficient in adherence to or invasion into superficial bladder epithelial cells, rendering the bacteria essentially avirulent (Connell *et al.*, 1996; Wright *et al.*, 2007). T1P binds β -integrins (Eto *et al.*, 2007), leading to internalization, and can also bind uroplakin 1a (Zhou *et al.*, 2001); however, whether different T1P-dependent entry mechanisms result in altered intracellular compartments or functional niches remains to be elucidated. Once internalized, T1P are necessary for IBC-formation. IBCs have been observed during human infections; however, much of what we know regarding the t1P-dependent formation of IBCs has been demonstrated in mice (Rosen *et al.*, 2008; Wright *et al.*, 2007). Specifically, ExPEC genetically modified for de-repression of type 1 pili expression in the presence of anhydrotetracycline (AHT) treatment colonized the bladder and invaded the superficial bladder epithelium to similar levels as an isogenic control strain during experimental UTI (Wright *et al.*, 2007). However, upon intracellular invasion the strain no longer remained in contact with AHT, became non-piliated, and was unable to form typical IBCs, suggesting the type 1 pili, a

prototypical extracellular adhesive factor, is able to mediate intracellular survival and bacterial community formation.

1.4.2 Type 1 pili Epigenetic Regulation

One major, but perhaps still underexplored, form of virulence regulation is at the epigenetic level. Epigenetic regulation refers to heritable changes in gene expression or phenotype that are not due to underlying changes in the coding sequence, i.e. DNA mutation. The epigenetic change is also heritable, meaning that it can be transmitted to daughter cells, which, in the case of bacteria, can lead to rapid phenotypic changes in the population. Since epigenetic variation does not involve an underlying change to the coding sequence of the DNA, it is a reversible process, either through replicative dilution or a reversal in the precipitating condition. A well-known example of epigenetic regulation in *E. coli* is that of type 1 pili (T1P), a virulence determinant required for adherence to and invasion of the bladder epithelium (Martinez *et al.*, 2000; Mulvey *et al.*, 1998).

Type 1 pili is encoded by the *fim* operon (*fimAICDFGH*) and its expression is controlled at multiple regulatory levels (Corcoran & Dorman, 2009; Kelly *et al.*, 2006; McClain *et al.*, 1991). Type 1 pili has phase variable expression in *E. coli*, which refers to absolute OFF vs. ON expression of the pili on the bacterial cell surface, resulting from inversion of the T1P promoter (*fimS*) (Abraham *et al.*, 1985). The promoter region orientation is controlled by the opposing actions of small tyrosine recombinases that act

as invertases. The recombinases recognize inverted DNA repeat sequences flanking the promoter for the type 1 pili operon and produce a genomic rearrangement, orienting the promoter in favor or disfavor of pilus gene expression. The orientation *fimS* is determined by the action of multiple tyrosine recombinases acting on 9 bp inverted repeats.

Among virtually all *E. coli*, two DNA recombinases called FimB and FimE act as site-specific invertases, controlling the orientation of the type 1 pili promoter such that it is positioned in an OFF or ON orientation (Abraham *et al.*, 1985). FimB and FimE recombination acts in concert with integration host factor (IHF), an essential DNA-bending accessory factor, without which the promoter remains phase-locked (Blomfield *et al.*, 1997; Dorman & Higgins, 1987; Eisenstein *et al.*, 1987). This binary switch produces the dominate control over the expression of T1P such that in the OFF orientation, transcription of the T1P operon is completely eliminated. In the ON orientation, like most promoters, there are subsequent rheostat controls that alter the level of transcription, including CRP-cAMP and H-NS, among others (Muller *et al.*, 2009; O'Gara & Dorman, 2000; Schembri *et al.*, 1998).

1.4.3 The Fim-Like Family of Tyrosine Recombinases

Site-specific recombinases are classified into two main groups based on the mechanism of catalysis, namely the serine and tyrosine recombinases (Grindley, 1997; Stark *et al.*, 1992). Tyrosine recombinases, or lambda integrase superfamily, are traditionally classified by their sequence and functional relationships. The diverse family

of tyrosine recombinases can rearrange DNA duplexes by means of conservative, site-specific recombination reactions, leading to integration, excision, and inversion events (Stark *et al.*, 1992). DNA inversion events are catalyzed by a series of single strand breaks and a Holliday junction intermediate. The recombinases religate the DNA in the opposite orientation. The direction of the repeats, as well as DNA bending, dictate whether the DNA is integrated, excised, or inverted. Lambda integrase family members share a core, conserved catalytic motif contained within the C-terminal half of the protein: Arg, His-X-X-Arg, Tyr, with the tyrosine occurring near the most C-terminal end (Abremski & Hoess, 1992). Mutation of any single putative catalytic residue in FLP recombinase, a lambda recombinase family member, resulted in an inactive recombinase, which is consistent with these residues comprising a catalytic active site (Abremski & Hoess, 1992; Parsons *et al.*, 1988).

Based on sequence similarity, the Fim invertases have been assigned to the tyrosine family of recombinases (Esposito & Scocca, 1997). Tyrosine recombinases utilize a catalytic tyrosine to mediate strand cleavage and require bacterial DNA-bending accessory factor Integration Host Factor (IHF) (Segall *et al.*, 1994). The Fim recombinases are the smallest members of the family (approximately 200 amino acids in length), yet, mediate complex inversion of DNA. ExPEC strains are known to possess up to five Fim-like tyrosine recombinases (FimB, FimE, FimX, IpuA, IpuB) based on sequence homology (Fig. 3A), and all but IpuB have been shown to invert *fimS* (Bryan *et al.*, 2006; Gally *et al.*, 1996; Hannan *et al.*, 2008; Klemm, 1986; McClain *et al.*,

1991; Xie *et al.*, 2006a) with differing biochemical activities: FimE inverts the promoter from ON to OFF, FimB catalyzes bidirectional inversion, but favors OFF to ON, IpuA also has bi-directional activity, while FimX is able to invert *fimS* from OFF to ON (Fig. 3B). However, despite its similarity to the other Fim recombinases, IpuB seems unable to

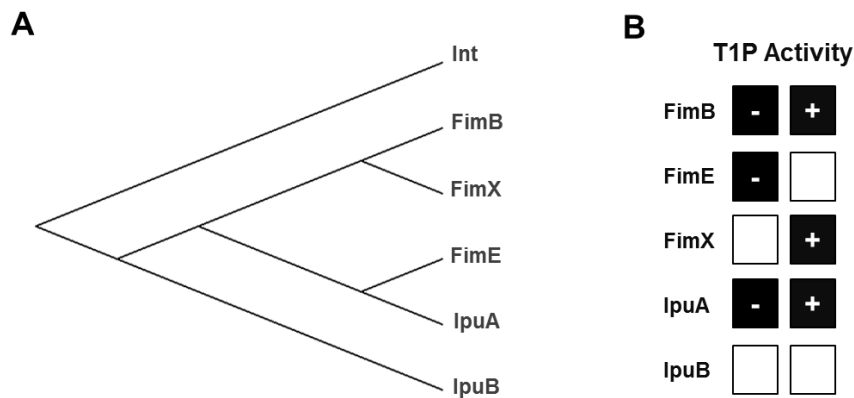


Figure 3: Phylogenetic and functional comparison of the Fim family of tyrosine recombinases.

A) Phylogenetic tree reconstruction (GeneBee web-based program) of tyrosine recombinase family members Int, FimB, FimE, FimX, IpuA, and IpuB using the ClustalW multiple alignment algorithm. Protein sequences for FimB, FimE, and FimX are from UTI89, and protein sequences for IpuA and IpuB are from CFT073. B) Fim recombinase family member function and directionality at inverting T1P. Positive and negative symbols indicate functional activity inverting from OFF to ON and ON to OFF, respectively. A blank box indicates no function.

invert the T1P promoter and has unknown target specificity (Bryan *et al.*, 2006). Not surprisingly, based on protein sequence analysis, IpuB shares the least phylogenetic relationship to the other Fim recombinase family member (Fig. 3A)

The Fim recombinases control the promoter inversion of T1P, thereby causing the absolute OFF vs. ON expression of the pili on the bacterial cell surface. The

recombinases recognize two 9 bp inverted DNA repeat sequences flanking the T1P operon promoter and produce a genomic rearrangement, orienting the promoter in favor or disfavor of pilus gene expression. UTI89, a prototypic cystitis strain used throughout the current study, encodes for three tyrosine recombinases FimB, FimE, and FimX.

Chapter 2. Materials and Methods

2.1 Bacterial Strains and Cultivation

All strains used in this study are listed in Table 3. Clinical isolates used for the molecular epidemiology studies are described below (Section 2.2) and are listed in Table 3 and Appendix A, Table 11. The prototypic cystitis strain, UTI89, was obtained from an adult patient with cystitis and has been previously described (Mulvey *et al.*, 1998) and fully sequenced (Chen *et al.*, 2006). Cystitis isolate NU14 (Hultgren *et al.*, 1986), pyelonephritis isolate J96 (Hall, 1999), asymptomatic bacteriuria isolates ASB1298 (Garofalo *et al.*, 2007), and pyelonephritis/bacteremia isolate CFT073 (Mobley *et al.*, 1990) have all been previously described.

Bacteria were routinely cultured in Luria–Bertani (LB) broth (Genese Scientific) containing, where appropriate, kanamycin 50 $\mu\text{g ml}^{-1}$, ampicillin 100 $\mu\text{g ml}^{-1}$ and chloramphenicol 20 $\mu\text{g ml}^{-1}$. For all *in vitro* studies with UTI89, other ExPEC strains, and their derivatives, cultures were grown overnight (typically 16-18 h) shaking at 37°C then back-diluted 1:100 into fresh Luria–Bertani (LB) broth and grown shaking at 37°C for 6 h, with the addition of 0.2% arabinose as necessary to induce expression of pBAD constructs in trans. IPTG was not included for induction of pTrc constructs, which were found to have sufficient expression in the absence of chemical induction. For *in vitro* nitrosative challenge, cultures were grown as just described, normalized to $\text{OD}_{600} = 0.8$, then sub-cultured 1:100 into MES acid-buffered LB (100 mM, pH 5.0) containing the indicated concentration of freshly added sodium nitrite. For all RAW 264.7 murine

macrophage infections, UTI89 and derivatives were grown overnight shaking at 37°C, back-diluted 1:100 into fresh media, and grown statically at 37°C for 18-24 h to induce expression of type 1 pili.

2.2 Clinical Isolates and Patient Descriptions

Clinical isolates used for the molecular epidemiology studies are listed in Table 3. An extensive list of all individual clinical *E. coli* isolates with descriptive information and multiplex PCR results are listed in Appendix A, Table 11. Bacterial strains were routinely cultured in LB broth without antibiotics. Isolates were derived from three sources: 1) well characterized, published representative pathogenic and commensal strains; 2) isolates collected in previously published clinical studies in Seattle, representing various clinical syndromes of urinary tract infection (UTI) (Czaja *et al.*, 2009a; Garofalo *et al.*, 2007; Hooton *et al.*, 1996; Johnson *et al.*, 1987; Johnson *et al.*, 1988; Johnson *et al.*, 1991) as well as control isolates (Stapleton *et al.*, 1991); and 3) strains from the *E. coli* reference (ECOR) collection (Ochman & Selander, 1984).

The characterized representative strains included K-12 strain MG1655 (Blattner *et al.*, 1997); cystitis isolates UTI89 (Mulvey *et al.*, 1998) and NU14 (Hultgren *et al.*, 1986); pyelonephritis isolates 536, DS17, GR12, and J96 (Berger *et al.*, 1982; Hull *et al.*, 1981; Svanborg Eden *et al.*, 1983; Tullus *et al.*, 1984); blood isolate CFT073 from a patient with concurrent pyelonephritis (Mobley *et al.*, 1990); and enterohemorrhagic isolate EDL933 (Riley *et al.*, 1983).

Table 3. Bacterial strains used in this study.

Strain	Relevant genotype or features	Resistance(s)	Reference
UTI89	<i>Escherichia coli</i> cystitis isolate		(Mulvey <i>et al.</i> , 1998)
UTI89 $\Delta fimBEX_{T1P-OFF/hyxR-OFF}$	UTI89 $\Delta fimBEX$ segregated phase OFF and T1P and <i>hyxR</i>		This Study
UTI89 $\Delta fimBEX_{T1P-OFF/hyxR-ON}$	UTI89 $\Delta fimBEX$ segregated phase OFF and T1P and phase ON at <i>hyxR</i>		This Study
UTI89 $\Delta fimBEX_{T1P-ON/hyxR-OFF}$	UTI89 $\Delta fimBEX$ segregated phase ON and T1P and phase OFF at <i>hyxR</i>		This Study
UTI89 $\Delta fimBEX_{T1P-ON/hyxR-ON}$	UTI89 $\Delta fimBEX$ segregated phase ON and T1P and <i>hyxR</i>		This Study
UTI89 $\Delta fimB$	UTI89 $\Delta fimB$; T1P phase OFF		(Hannan <i>et al.</i> , 2008)
UTI89 $\Delta hyxR$	UTI89 $\Delta hyxR$; T1P phase variable		This Study
UTI89 $\Delta fimX$	UTI89 $\Delta fimX$; T1P phase OFF		(Hannan <i>et al.</i> , 2008)
UTI89 Δhns	UTI89 Δhns		This Study
UTI89 $\Delta himA$	UTI89 $\Delta himA$; IHF null		This Study
BW25113 Δhmp	$\Delta hmp::kan$ strain used as the basis for creating P1 lysogen for transfer into UTI89	Kan ^r	(Baba <i>et al.</i> , 2006)
UTI89 Δhmp	P1 phage transduction from BW $\Delta hmp::kan$ into UTI89	Kan ^r	This Study
UTI89 $\Delta hyxR \Delta hmp$	P1 phage transduction from BW $\Delta hmp::kan$ into UTI89 $\Delta hyxR$ (Kan ^s)	Kan ^r	This Study
NU14	<i>Escherichia coli</i> cystitis isolate		(Hultgren <i>et al.</i> , 1986)
J96	<i>Escherichia coli</i> pyelonephritis isolate		(Hull <i>et al.</i> , 1981)
CFT073	<i>Escherichia coli</i> pyelonephritis/bacteremia isolate		(Mobley <i>et al.</i> , 1990)
ASB1298	<i>Escherichia coli</i> asymptomatic bacteriuria isolate		(Garofalo <i>et al.</i> , 2007)
MG1655	K-12 <i>E. coli</i> isolate		(Blattner <i>et al.</i> , 1997)
536	<i>E. coli</i> pyelonephritis isolate		(Berger <i>et al.</i> , 1982)

DS17	<i>E. coli</i> pyelonephritis isolate	(Tullus <i>et al.</i> , 1984)
GR12	<i>E. coli</i> pyelonephritis isolate	(Svanborg Eden <i>et al.</i> , 1983)
EDL933	Enterohemorrhagic <i>E. coli</i> isolate	(Riley <i>et al.</i> , 1983)
ECOR strain collection	<i>E. coli</i> reference set (n = 70)	(Ochman & Selander, 1984)
Clinical CY isolates	Single and recurrent clinical <i>E. coli</i> cystitis isolates (n = 68)	(Czaja <i>et al.</i> , 2009a; Garofalo <i>et al.</i> , 2007; Hooton <i>et al.</i> , 1996)
Clinical ASB isolates	Single and recurrent clinical <i>E. coli</i> asymptomatic bacteriuria isolates (n = 45)	(Garofalo <i>et al.</i> , 2007; Hooton <i>et al.</i> , 1996)
Clinical fecal isolates	Clinical <i>E. coli</i> fecal-commensal isolates (n = 20)	(Stapleton <i>et al.</i> , 1991)
Clinical PY isolates	Clinical <i>E. coli</i> pyelonephritis isolates (n = 22)	(Johnson <i>et al.</i> , 1991)
Clinical UTI-BL	Clinical <i>E. coli</i> urosepsis isolates (n = 20)	(Johnson <i>et al.</i> , 1987; Johnson <i>et al.</i> , 1988)
Clinical EHEC isolates	Human enterohemorrhagic <i>E. coli</i> isolates (n = 7)	This Study

Table 4. Plasmids used in this study.

Plasmid	Relevant features	Resistance(s)	Reference
pKD4	Kan ^r antibiotic template for red recombinase-mediated gene knockout	Amp ^r Kan ^r	(Datsenko & Wanner, 2000)
pKM208	Red recombinase expression plasmid; IPTG inducible	T ^s Amp ^r	(Datsenko & Wanner, 2000)
pCP20	<i>P_{ara-flp}</i> ; Ara-inducible Flp recombinase expression plasmid	T ^s Amp ^r	(Datsenko & Wanner, 2000)
pLR92	pBAD33 derivative; <i>araC</i> , <i>P_{ara}</i>	Cm ^r	(Robinson <i>et al.</i> , 2006)
pBAD33	pLR92 digested with SacI/BamHI and religated to create pBAD33 control vector; <i>araC</i> , <i>P_{ara}</i>	Cm ^r	(Guzman <i>et al.</i> , 1995)
pTrc99a	pBR332 derivative; <i>lacIq</i> ; <i>P_{lac}</i> cloning and expression plasmid; IPTG inducible	Amp ^r	(Amann & Brosius, 1985)
pTrc: <i>hyxR</i>	SacI/HindIII-cut PCR product from UTI89 ligated into identical pTrc99a sites to create <i>P_{lac}-6xHIS-hyxR</i> ; IPTG-inducible <i>hyxR</i> expression	Amp ^r	This Study
pTrc: <i>fimB</i>	<i>P_{lac}-HAT-fimB</i> in pTrc99a; source of <i>fimB</i> for cloning of pBAD33: <i>fimB</i>	Amp ^r	(Hannan <i>et al.</i> , 2008)
pBAD: <i>fimB</i>	SacI/BamHI-cut HAT- <i>fimB</i> cloned into pLR92 SacI/BglII sites to create pBAD33 with <i>P_{ara}-HAT-fimB</i> ; Ara-inducible <i>fimB</i> expression	Cm ^r	This Study
pBAD: <i>fimX</i>	pBAD33 with <i>P_{ara}-6xHN-fimX</i> ; Ara-inducible <i>FimX</i> expression	Cm ^r	(Hannan <i>et al.</i> , 2008)
pANT4	<i>P_{lac}-GFP.mut3</i> ; Constitutive, high-level GFP expression	Amp ^r , Kan ^r	(Lee & Falkow, 1998)
FimB:FimX Chimeras	pBAD33 derivatives containing various FimB:FimX protein chimeras; arabinose-inducible	Cm ^r	This Study
CAT Mutants	pBAD33 derivatives containing an alanine substitution in each of four FimX catalytic residues; arabinose-inducible	Cm ^r	This Study

For clinical isolates collected through studies carried out in Seattle, strains were archived in a specimen repository at the University of Washington UTI Research Laboratory and provided de-identified and anonymously through an IRB-approved protocol. Clinical UTI syndromes were defined using the same criteria in all studies. The study population for all studies was non-pregnant, outpatient women ages 18 to 49 seen in the Hall Health Primary Care Center (student health clinic) at the University of Washington (Czaja *et al.*, 2009a; Garofalo *et al.*, 2007; Hooton *et al.*, 1996) or Group Health Cooperative of Puget Sound (Garofalo *et al.*, 2007; Hooton *et al.*, 1996), except for isolates collected from inpatients hospitalized with pyelonephritis and/or urosepsis, which were collected from women ages 18 to 45 with bacteremia arising from a urinary tract source, hospitalized in one of four hospitals in the Seattle, WA (Johnson *et al.*, 1987; Johnson *et al.*, 1988; Johnson *et al.*, 1991). Exclusion criteria for all studies of outpatients included known anatomical or functional abnormalities of the urinary tract, chronic illness requiring medical supervision, pregnancy or planned pregnancy during the next three months, and, except for studies of pyelonephritis, symptoms or signs of acute pyelonephritis. Acute, uncomplicated cystitis (CY; n = 68 isolates) was defined as the presence of typical symptoms (dysuria, frequency, and/or urgency), and a midstream urine culture containing $\geq 10^2$ CFU mL⁻¹ of bacteria (Czaja *et al.*, 2009a; Garofalo *et al.*, 2007; Hooton *et al.*, 1996). Asymptomatic bacteriuria (ASB; n = 45) was defined as a midstream urine culture containing $> 10^5$ CFU per milliliter of bacteria documented twice at least 24 hours apart in a subject with no typical symptoms of UTI (Garofalo *et al.*,

2007; Hooton *et al.*, 1996). Acute uncomplicated pyelonephritis (PY; n = 22) was defined as costovertebral angle pain and/or tenderness, pyuria, and bacteriuria with $\geq 10^4$ CFU mL⁻¹ (Garofalo *et al.*, 2007; Johnson *et al.*, 1991). Among subjects with pyelonephritis, 20 were women ages 18 to 45 hospitalized in one of four hospitals in Seattle (Johnson *et al.*, 1991) and 2 were outpatients (Garofalo *et al.*, 2007). Urosepsis (UTI-BL; n = 20) was defined as bacteremia arising from a urinary tract source (Johnson *et al.*, 1987; Johnson *et al.*, 1988). Commensal isolates (C; n = 20) were defined as fecal isolates (prospectively collected between 1981 and 1987) from healthy, non-pregnant women with no cystitis symptoms, no history of UTI in the prior year, and no antibiotic use in the previous month (Stapleton *et al.*, 1991).

Seventy isolates from the ECOR collection (Ochman & Selander, 1984) representing human and non-human fecal-commensal isolates (C, n = 61), human urinary tract isolates (ASB, n = 1; PY, n = 6; CY, n = 4), and human enterohemorrhagic isolates (n = 2) were used as a well-characterized strain reference. ASB, CY, and PY isolates from the ECOR collection were included as ExPEC isolates for analysis. Phylogenetic Group E isolates from the ECOR collection were included as EHEC isolates for analysis. An additional seven previously unpublished clinical isolates of EHEC were also included for reference.

2.3 Percent G+C Determination

Percent G+C was determined using Microsoft Excel by importing the entire UTI89 genome sequence from *betT* through *ykgE* and dividing the sequence into 100 bp windows. Percent G+C was calculated for each 100 bp window and averaged across the PAI-X region versus flanking genomic segments. G+C values were compared to the average for the entire UTI89 genome, as previously determined (Chen *et al.*, 2006).

2.4 Multiplex PCR Assay Design

Primers for the Fim recombinase genes (*fimB*, *fimE*, *fimX*, *ipuA*, and *ipuB*) and PAI-X genes (*hyxR*, *hyxA*, *hyxB*) were designed to conserved regions from gene alignments based on sequence data from available *E. coli* genomes (NCBI Genomes). All multiplex primers used in this study are listed in Table 5. Primers for *ipuA* and *ipuB* were designed based on the genomic sequence of CFT073 while *fimB*, *fimE*, *fimX*, and PAI-X sequences were designed based on the UTI89 genome. The primer specificity was tested using the program BLAST against the UTI89 genome as a reference to minimize non-specific annealing (Altschul *et al.*, 1997).

2.5 Multiplex PCR Analysis

One microliter of bacterial culture suspension was used as template for multiplex PCR. All strains were analyzed by multiplex PCR to determine the presence of the Fim-

like recombinases and PAI-X genes. Up to four primer pairs were used per reaction (for primer sequences see Table 1). Primer groups were as follows: Group 1 (*ipuB*, *fimE*, *hyxB*, *fimB*), 94°C (5 min), then 30 cycles of 94°C (30 s), 51°C (30 s), 72°C (30 s), followed by 72°C (7 min) and 4°C hold; Group 2 (*ipuA*, *fimX*, *hyxA*, *hyxR*), 94°C (5 min), then 30 cycles of 94°C (30 s), 52°C (30 s), 72°C (30 s), followed by 72°C (7 min) and 4°C hold.

Since ExPEC also colonize the enteric tract, we assayed the ECOR reference strains (n = 70) and fecal isolates (n = 20) for the presence of additional ExPEC-associated virulence factors (VFs) to determine if any of the fecal isolates represent potential extra-intestinal pathogens using the multiplex primers listed in Table Blank. The ECOR strain set has been previously analyzed by multiplex PCR for various VFs (Johnson *et al.*, 2001a). Primers and programs were grouped as follows: Group 3 (*kpsMT* II, *kpsMT* III, *ibeA*), 94°C (5 min), then 30 cycles of 94°C (30 s), 55°C (30 s), 72°C (35 s), followed by 72°C (7 min) and 4°C hold; Group 4 (*sfa/foc*, *traT*, *papA*), 94°C (5 min), then 30 cycles of 94°C (30 s), 55°C (30 s), 72°C (50 s), followed by 72°C (7 min) and 4°C hold; Group 5 (*cnf1*, *hlyA*, *fyuA*), 94°C (5 min), then 30 cycles of 94°C (30 s), 55°C (30 s), 72°C (70 s), followed by 72°C (7 min) and 4°C hold; and phylogenetic groups (*chuA*, *yjaA*, TSPE4.C2), 94°C (5 min), then 30 cycles of 94°C (30 s), 55°C (30 s), 72°C (30 s), followed by 72°C (7 min) and 4°C hold. For analysis, we defined commensal strains as those strains with < 4 out of 9 VFs assayed. Those strains carrying ≥ 4 VFs were

Table 5. Multiplex PCR primers used in this study.

Primers (forward/reverse)	Target	Relevant features / Sequence (5' → 3')	Size (bp)	Reference
fimE MPX 1 / fimE MPX2	<i>fimE</i>	CTAACTGGAAAGGCGCTGAC/GAATATTTTCGATGCCCCGAGA	225	This study
fimB MPX 1 / fimB MPX2	<i>fimB</i>	GCCTCATGCTGCACGTAAT/CAATCGACAAATTTCACTCG	79	This study
<i>fimX</i> MPX 1 / <i>fimX</i> MPX 2	<i>fimX</i>	CCAGAGCATGTCCTTTCCCTG/TTCCTCGCTTAAGCCACAAC	216	This study
ipuA MPX 1 / ipuA MPX 2	<i>ipuA</i>	GCGATGTTTGCATGATTTTA/TTTTACCCGCAGCAGAAACT	303	This study
ipuB MPX 1 / ipuB MPX 2	<i>ipuB</i>	TGCGCAAATTTATTACTCATAGTG/TGTCTCGAGATTTTATTTCCTTGA	334	This study
hyxR MPX 1 / hyxR MPX 2	<i>hyxR</i>	TCGATGAGCGGAATGTTGTC/GGCTGCTCTATACGGGATGC	89	This study
hyxA MPX 1 / hyxA MPX 2	<i>hyxA</i>	GCATTTCCATCACCGTGAAA/GTGCAGTTCCTCAAAAACG	142	This study
hyxB MPX 1 / hyxB MPX 2	<i>hyxB</i>	GGGTATCACCCAGCATT/CAGGATGCTGTCCGTCTGAG	139	This study
cnf1 / cnf2	<i>cnf1</i>	AAGATGGAGTTTCTATGCAGGAG/CATTCAGAGTCCTGCCCTCATTATT	498	(Yamamoto <i>et al.</i> , 1995)
hly f / hlyR	<i>hlyA</i>	AACAAGGATAAGCACTGTTCTGGCT/ACCATATAAGCGGTCATTCCCGTCA	1177	(Yamamoto <i>et al.</i> , 1995)
ibe10 f / ibe10 R	<i>ibeA</i>	AGGCAGGTGTGCGCCGCGTAC/TGGTGCTCCGGCAAACCATGC	170	(Huang <i>et al.</i> , 1995)
sfa1 / sfa2	<i>sfa/focDE</i>	CTCCGGAGAAGTGGGTGCATCTTAC/CGGAGGAGTAATTACAAACCTGGCA	410	(Le Bouguenec <i>et al.</i> , 1992)
papA f / papA r	<i>papAH</i>	ATGGCAGTGGTGTCTTTTGGTG/CGTCCCACCATACGTGCTCTTC	720	(Johnson & Stell, 2000)
kpsII f / kpsII r	<i>kpsMT II</i>	GCGCATTTGCTGATACTGTTG/CATCCAGACGATAAGCATGAGCA	272	(Johnson & Stell, 2000)
kpsIII f / kpsIII r	<i>kpsMT III</i>	TCCTCTTGCTACTATTCCTCCCT/AGGCGTATCCATCCCTCCTAAC	392	(Johnson & Stell, 2000)
traT f / traT r	<i>traT</i>	GGTGTGGTGCGATGAGCACAG/CACGGTTCAGCCATCCCTGAG	290	(Johnson & Stell, 2000)
fyuA f / fyuA r	<i>fyuA</i>	TGATTAACCCCGCAGCGGAA/CGCAGTAGGCACGATGTTGTA	880	(Johnson & Stell, 2000)
ChuA.1 / ChuA.2	<i>chuA</i>	GACGAACCAACGGTCAGGAT/TGCCGCCAGTACCAAAGACA	279	(Clermont <i>et al.</i> , 2000)
YjaA.1 / YjaA.2	<i>yjaA</i>	TGAAGTGTCAGGAGACGCTG/ATGGAGAATGCGTTCCTCAAC	211	(Clermont <i>et al.</i> , 2000)
TspE4C2.1 / TspE4C2.2	TSPE4.C2	GAGTAATGTCGGGGCATCA/CGCGCCAACAAAGTATTACG	152	(Clermont <i>et al.</i> , 2000)

excluded from analysis as commensal strains (n=18) as they likely represent commensal-pathogens.

All PCR reactions were performed using APEX Taq polymerase and accompanying buffers (Genesee Scientific). Amplicons were visualized on a 2% TBE gel stained with ethidium bromide and photographed with a UV transilluminator (Bio-Rad). Each reaction was performed a minimum of three times, as biological replicates.

2.6 Alignment of PAI-X Coding Sequences from O157:H7 EHEC Strains

The coding sequences for *fimX*, *hyxR*, *hyxA*, and *hyxB* were gathered for UTI89 (EMBL coding sequence: ABE05839) and O157:H7 strain ELD933 (EMBL coding sequence: AAG54651) from the EMBL-EBI Integr8 web portal (Kersey *et al.*, 2005). The resulting sequences were aligned using the ClustalW multiple alignment algorithm (Thompson *et al.*, 1994) in the BioEdit Sequence Alignment Editor (Hall, 1999), using the ‘Toggle’ feature to translate the coding sequence. Mutations in EHEC, relative to UTI89, that resulted in a premature stop codon were noted.

2.7 Determination of Phi Coefficients

Phi coefficients, or mean square contingency coefficients, measure the association of two binary variables, similar to a Pearson correlation, between -1 and 1. Values close to 1 or -1 indicate a strong positive or negative association between variables,

respectively. The correlation of PAI-X with other virulence factors was determined using 2x2 contingency tables as previously described (Johnson *et al.*, 2001b).

2.8 Specificity and Sensitivity Calculations

Specificity and sensitivity metrics were calculated as previously described (Altman & Bland, 1994) and were used in this study as a common metric to compare the performance of detecting different *E. coli* genes to classify strains. Calculations were based on data derived from Table 7. The sensitivity metric measured the accuracy of a target gene PCR test to categorize an isolate as ExPEC. The specificity metric indicated the degree to which a negative gene PCR test excluded an isolate as ExPEC. The formulas used for calculating sensitivity and specificity are listed below:

$$\text{Sensitivity} = \text{Number True Positive} / (\text{Number True Positive} + \text{Number False Negative})$$

$$\text{Specificity} = \text{Number True Negative} / (\text{Number True Negative} + \text{Number False Positive})$$

For the purposes of this study, a true positive was regarded as a positive PCR test for a specific *E. coli* gene when the test isolate was recovered from a study subject with extraintestinal disease. A true negative was scored when the PCR test was negative and the test isolate was recovered from a commensal (fecal) reservoir in a non-symptomatic subject. A false positive was scored when a human commensal strain produced a positive

test for a given genomic marker, while a false negative was scored when the PCR test for a genomic marker was negative and the test isolate was from a symptomatic subject with extraintestinal disease.

2.9 Construction of Deletion Mutants

Complete deletions for the coding regions of *hyxR*, *hns*, and *himA* were made using the Red recombinase method, as previously described (Datsenko & Wanner, 2000; Murphy & Campellone, 2003). Briefly, Red recombinase is expressed from a helper plasmid (pKM208) to facilitate homologous recombination of a linear construct into the genome of the recipient strain. We used the pKD4 plasmid as a template for PCR amplification of a linear template containing a Kanamycin resistance cassette, flanking FRT recombination sites, and approximately 50 bp homologous ends to the gene to be knocked-out. All primers (Integrated DNA Technologies) used to create deletions are listed in Table 6. The deletion was confirmed by PCR with flanking primers (Table 6). The kanamycin antibiotic insertion was removed through FLP-mediated excision by transforming the mutant strain with the temperature-sensitive plasmid pCP20 expressing the FLP recombinase (Cherepanov & Wackernagel, 1995). The resultant strains had no antibiotic resistance compared with the parental strain.

The *hmp* deletion derivatives were created through P1 phage transduction of a disruption of *hmp* carrying the kanamycin cassette from the Keio collection as previously described (Baba *et al.*, 2006). The *hmp::kan^R* lesion was transduced into both UTI89 and

Table 6. Primers used in this study for deletion strain generation, plasmid construction, qRT-PCR analysis, and phase orientation.

Primers (forward/reverse)	Target	Relevant features / Sequence (5' → 3')	Reference
Generation of Deletion Strains			
ORF0336 5' pKD4	<i>hyxR</i>	ATTTTGAATCAAAATGTCAGGTTTTGTAGCAATGGCGTGGACGAGTCTGAGTGTAGGCTGGAGCTGCTTC	This Study
ORF0336 3' pKD4	<i>hyxR</i>	GTAGCGAGTCCGTATGCGCCGAGTGGTGGTAGTAACAACGCTGCTGTGATGGGAATTAGCCATGGTCC	This Study
<i>hns</i> KD4 #1	<i>hns</i>	GCATGTGCAATCTACAAAAGATTATTGCTTGATCAGGAAATCGTCGAGGGAATGGGAATTAGCCATGGTCC	This Study
<i>hns</i> KD4 #2	<i>hns</i>	CCCAATATAAGTTTGAGATTACTACAATGAGCGAAGCACTTAAAAATCTGAAGTGTAGGCTGGAGCTGCTTC	This Study
<i>himA</i> KD4 #1	<i>himA</i>	TAGTTAGATCAGATTACTCGTCTTTGGGCGAAGCGTTTTTCGACCCGGCTTATGGGAATTAGCCATGGTCC	This Study
<i>himA</i> KD4 #1	<i>himA</i>	TGTGTAGAGGCATTAAGAAGCGATCCAGGCATCATTGAGGGATTGAACCT	This Study
Confirmation of Deletions			
X HypoII large promoter #1	<i>hyxR</i>	CAGGTCGCCCTGCCGATATC	This Study
X HypoI Promoter #2	<i>hyxR</i>	GGTGTGGAAAAAAGCCTCGTTAA	This Study
<i>hns</i> 5' flank/3' <i>hns</i> flank	<i>hns</i>	GGCGGCCCAAAATAAAGAACAATT/TTAGGTTACATCCAGGCCTTCGTTG	This Study
<i>himA</i> 5' flank/3' <i>himA</i> flank	<i>himA</i>	TCTCGGTTCTGTGCCAGATACTCAGG/ACGTAAGAGTATTAAGTGATGATAA	This Study
<i>hmp</i> flank #1/ <i>hmp</i> flank #2	<i>hmp</i>	GATTACCTTCAGGCTACGCAAG/CGACATTGTCGATACCTGTGTCG	This Study
Plasmid Construction			
HAT-B 5' SacI/ <i>fimB</i> HAT-5	<i>fimB</i>	ATCATTGAGCTCAAGAAGGAGATATA/TAATATGGATCCCTATAAAACAGCGT	This Study
X HypoI 5'	<i>hyxR</i>	CATCATCATCATCATGTTCTAAGAAGCCTCTGGTTATTATCAGTGCC	This Study
X HypoI 3'	<i>hyxR</i>	TGATATAAGCTTTCATAACAATCCTGGTCTTTTTTTAGATATAIT	This Study
Generation of FimB::X Chimeras			
B:X.1 (B 3')	<i>fimB</i>	CGGCGTTCCGGACAGAAATACC	This Study
B:X.1 (X 5')	<i>fimX</i>	GCATGCTGAGAGCGAGTGGGTATTT	This Study
B:X.2 (B 3')	<i>fimB</i>	CGCGAATATTAAGTTGTTTTCCAGA	This Study

B:X.2 (X 5')	<i>fimX</i>	CCGATTGAGGATTTCCGGATATTGATC	This Study
B:X.3 (B 3')	<i>fimB</i>	GCCACAACCTTGATCAAATTATATTCA	This Study
B:X.3 (X 5')	<i>fimX</i>	CTTTTCAACAACGCACCCGCTAATGC	This Study
B:X.4 (B 3')	<i>fimB</i>	GGGCTGCATCCATCAGCCTGTAGATT	This Study
B:X.4 (X 5')	<i>fimX</i>	GAACTTCCTGACCATAGTGAAATC	This Study
B:X.5 (B 3')	<i>fimB</i>	GAGGATGTGCTTTTAAGCTAATCCT	This Study
B:X.5 (X 5')	<i>fimX</i>	CCATATTATCTCGACTTCCGGTGGTA	This Study
FimB::X (5' SacI)	<i>fimB</i>	ATCATTGAGCTCAAGAAGGAGATATA	This Study
FimB::X (3' BglII)	<i>fimX</i>	TGATATAGATCTTTAACGAGGCTTTT	This Study

qRT-PCR

<i>hmp</i> qPCR R/ <i>hmp</i> qPCR L	<i>hmp</i>	AGCGCACTTATCACCAGCTT/TACGATAGCCTTTGCCATCC	This Study
HypoI pCR L/HypoI qPCR R	<i>hyxR</i>	TCGATGAGCGGAATGTTGTC/GGCTGCTCTATACGGGATGC	This Study
16S qPCR #1/16S qPCR #2	<i>16S rRNA</i>	GTTAGCCGGTGCTTCTTCTG/CAGCCACACTGGAAGTGAAGA	This Study
<i>fimB</i> qPCR R/ <i>fimB</i> qPCR L	<i>fimB</i>	CTGTTGCCGAGAAAGTGGAT/CCGATTGAGGATTTCCGGATA	This Study
HypoII qPCR L/HypoII qPCR R	<i>hyxA</i>	GCATTTCCATCACCGTGAAA/GTGCGCAGTTTCTCAAAAACG	This Study
HypoIII qPCR L/HypoIII qPCR R	<i>hyxB</i>	GGGTATCACCAACCAGCATT/CAGGATGCTGTCTGTCTGAG	This Study

hyx Promoter Orientations

X hypoI promoter FAR LEFT	P_{hyxR}	GCCCGGCATCACTCCGCAAAC	This Study
X HypoI promoter #2	P_{hyxR}	GGTGTGGAATAAAGCCTCGTTAA	This Study
X HypoII large promoter #1	P_{hyxA}	CAGGTCGCCCTGCCGATATC	This Study
X HypoII large Promoter #2	P_{hyxA}	CATGCTGACAGCGAGTCGTTATC	This Study
X HypoIII promoter #1	P_{hyxB}	CATCGCCAGTTGAATCAATGACC	This Study
X HypoIII Promoter #2	P_{hyxB}	CGACCTGCCGAATGCGCTG	This Study

T1P Phase (BstU1 Digest)

PHASE 1	<i>fimS</i>	CCGTAACGCAGACTCATCCTC	(Hannan <i>et al.</i> , 2008)
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PHASE 2	<i>fimS</i>	GACAGAACAACAATTGCCAG	(Hannan <i>et al.</i> , 2008)
Fim 14	<i>fimS</i>	TGCTATCGATTCCAGGAAATACACAGTCTG	(Hannan <i>et al.</i> , 2008)
TIP Phase (Specific)			
PHASE 2 (anchor)	<i>fimS</i>	GACAGAACAACAATTGCCAG	(Hannan <i>et al.</i> , 2008)
P _{OFF} fimS.1	<i>fimS</i> _{OFF}	TTGGGGCCAAACTGTCTATATCATAA	This Study
P _{ON} fimS.1	<i>fimS</i> _{ON}	GAGGAAGATGCGTCGAGCCACAG	This Study
<i>hyxR</i> Phase			
X HypoI promoter #1 (anchor)	P _{<i>hyxR</i>}	ACTGATAATAACCAGAGGCTTCTT	This Study
P _{HypoI} (WT) orientation #2	P _{<i>hyxR</i>} -OFF	CAGTGATTAACITTCGAACATATTG	This Study
P _{HypoI} (WT+X) orientation #3	P _{<i>hyxR</i>} -ON	GCGAAAGTTAATCACTGGTATGACC	This Study

UTI89 Δ *hyxR* (*kan^S*) backgrounds and screened with flanking primers. The single *fimB* and *fimX* deletions in UTI89 were created as described previously (Hannan *et al.*, 2008).

2.10 Creation of T1P and *hyxR* Phase-Locked Strains

Deletions in the coding sequences for *fimB*, *fimE*, and *fimX* to create UTI89 Δ *fimBEX* were constructed using the Red recombinase method (Datsenko & Wanner, 2000; Murphy & Campellone, 2003) as previously published (Hannan *et al.*, 2008). Briefly, a linear knock-out product was created to adjacent genes *fimB* and *fimE* to create UTI89 Δ *fimBE::kan^R* as previously published. The kanamycin antibiotic insertion was removed through FLP-mediated excision by transforming the mutant strain with the temperature-sensitive plasmid pCP20 expressing the FLP recombinase (Cherepanov & Wackernagel, 1995). This strain served as the basis for subsequent knock-out of the *fimX* gene, again using the Red recombinase method and a linear construct containing homologous ends and a kanamycin resistance cassette. UTI89 Δ *fimBE* Δ *fimX::kan^R* was transformed with pCP20, and the resultant strains had no antibiotic resistance compared with the parental strain.

Subsequently, this strain was assayed for the promoter orientation of T1P and *hyxR*. After passage and screening, individual colonies were isolated that were in the OFF or ON orientation at each loci, leading to the generation of four UTI89 Δ *fimBE* Δ *fimX* phase-locked variants: UTI89 Δ *fimBE* $X_{\text{T1P-OFF/hyxR-OFF}}$, UTI89 Δ *fimBE* $X_{\text{T1P-OFF/hyxR-ON}}$, UTI89 Δ *fimBE* $X_{\text{T1P-ON/hyxR-OFF}}$, and UTI89 Δ *fimBE* $X_{\text{T1P-ON/hyxR-ON}}$ (Table 6).

2.11 Construction of *fimB* and *hyxR* Expression Plasmids

The *fimB* expression plasmid was created by digesting pLR92 (Robinson *et al.*, 2006), a pBAD33 derivative, with SacI/BglII to create a linearized vector. The pLR92 vector backbone was gel-purified from a 1% TBE agarose gel (APEX) using the Qiagen gel purification kit. The *fimB* insert was amplified from pTrc:HAT-*fimB* using Accuprime pfx high fidelity polymerase (APEX) and primers HAT-B 5' SacI and *fimB*-HAT-5 (Table 6). The *fimB* amplicon was digested with SacI/BamHI, gel-purified as described above, and ligated into the similarly cut linearized pLR92 vector. Putative clones were confirmed by PCR and sequencing. For comparison, pBAD33 (vector control) was created by religating SacI/HindIII digested pLR92 as previously described (Hannan *et al.*, 2008).

To create the *hyxR* expression plasmid, pTrc99a was digested with SacI/HindIII to create a linearized vector as described above for pBAD:*fimB*. The *hyxR* insert was amplified from UTI89 with primers X HypoI 5' and X HypoI 3' (Table 6), digested with SacI/HindIII, and ligated with the pTrc99a linearized vector. Putative clones were confirmed by PCR and sequencing.

2.12 Type 1 Pili Phase Orientation and Expression

Type 1 pili expression was routinely checked by phase PCR to determine the orientation of *fimS* (the type 1 pili invertible promoter) as well as by immunoblot to detect protein expression. Phase PCR was assayed using one of two methods. The first

method takes advantage of an asymmetric BstUI restriction site in the promoter of *fimA* and was performed using a modified method (Horcajada *et al.*, 2005) as previously described (Hannan *et al.*, 2008). Briefly, the *fimA* promoter was amplified with primers PHASE 1, PHASE 2, and Fim 14 (Table 6), which allowed amplification in both wild-type and Δ *fimBE* strain backgrounds, digested with BstUI, and visualized by gel electrophoresis.

Phase PCR assays were also performed by using a 1:1:1 mixture of three phase primers that included 1) an anchor primer (PHASE 2), 2) a phase-specific primer for the OFF orientation ($P_{\text{OFF}}\text{fimS.1}$), and 3) a phase-specific primer for the ON orientation ($P_{\text{ON}}\text{fimS.1}$) to increase sensitivity in detecting small phase changes and to more closely resemble the phase PCR assay for *hyxR* (Table 6). Mixed phase populations showed 2 bands by PCR, corresponding to the OFF and ON promoter orientations. Products were run on a 2% TBE agarose (APEX) gel. Western blot analysis was performed as previously described (Wright *et al.*, 2005) for expression of type 1 pili using an antibody raised against the major subunit, FimA (Hannan *et al.*, 2008). PCR and Western blot results represent at least three biological replicate experiments.

2.13 Determination of the Location of DNA Inversion Upstream of *hyxR*

The orientation of the putative promoter regions for *hyxR*, *hyxA*, or *hyxB* was determined using a PCR-based assay that exploited a restriction fragment length dimorphism due to possible orientation-dependent restriction sites. The 5' UTR plus

flanking sequence for each gene was amplified and digested with NspI, SspI, or TspRI for *hyxR*; SspI, SphI, or PstI for *hyxA*; and BsgI, SphI, or BstUI for *hyxB*. Size determination of bands resulting from inversion of the promoter allowed approximate localization of the sites of inversion.

The precise sites of FimX-mediated inversion at the type 1 pili promoter as well as upstream of *hyxR* were determined by sequencing (Duke University DNA Analysis Facility) both strands of PCR products in both the phase ON and phase OFF orientations. Individual phase orientations were amplified using phase-specific primer pairs across the proximal inversion site from UTI89 expressing pBAD:*fimX in trans* to increase the proportion of the phase ON population (Table 6). The resulting sequences in both orientations were aligned using the ClustalW multiple alignment algorithm (Thompson *et al.*, 1994) in the BioEdit Sequence Alignment Editor (Hall, 1999). Sequence alignments were compared between each orientation to determine the maximal region of inversion, indicated by the region of DNA that did not show sequence alignment. Sequence alignment conservation was observed starting at the *hyxR* proximal 16 bp inverted repeat and extending outward into the flanking region, indicating that the 16 bp repeat was the site of DNA inversion. Similarly, FimX-mediated inversion upstream of the type 1 pili operon was conserved starting at the proximal 9 bp inverted repeat that had been previously defined for FimB and FimE inversion.

2.14 Phase-Specific *hyxR* Phase PCR

The promoter orientation of *hyxR* was determined using a phase-specific PCR approach similar to *fimS* phase PCR. Briefly, a 1:1:1 mixture of three phase primers (Table 6) was used to assay the promoter orientation. Mixed phase populations show two bands by PCR, corresponding to the OFF and ON promoter orientations, which are discriminated by size (Fig. 4). PCR reactions were visualized on a 2% TBE agarose gel.

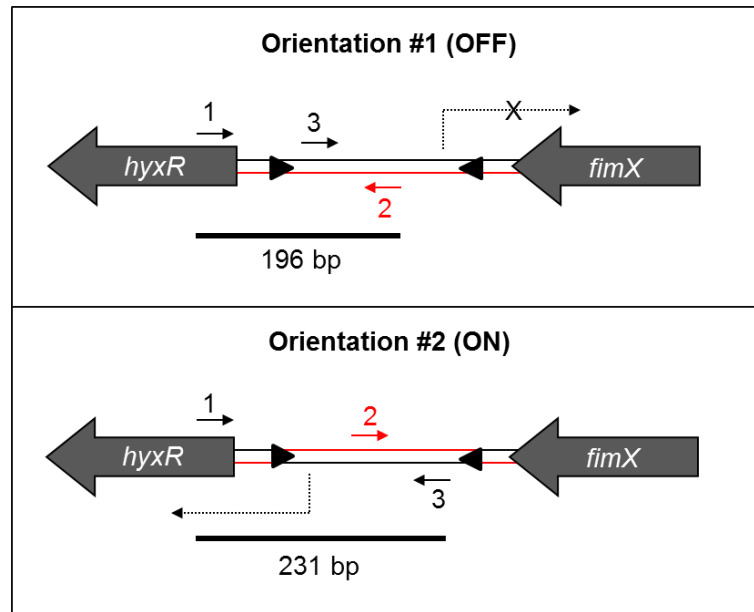


Figure 4: PCR-based assay to discriminate *hyxR* promoter phase orientation.

Incorporation of three primers to the PCR reaction can distinguish *hyxR* promoter orientation based on size.

2.15 Quantitative Reverse Transcription PCR (qRT-PCR)

RNA was collected from 5 ml of cultures grown in LB shaking for 3 h using the Masterpure system (Epicentre). For nitrosative stress conditions, duplicate cultures were challenged with 3 mM ASN for the last hour of growth, compared with pH-matched MES-LB as described above. One microgram of RNA was reverse transcribed using 1X iScript buffer (Bio-Rad) containing random hexamers and 1 μ l iScript (Bio-Rad). cDNA was diluted four-fold in DEPC-treated water, and 2 μ l of each dilution was used per 25 μ l of qPCR reaction. Amplicons were detected using the inclusion of 20x EvaGreen (Biotium) diluted to 1X (final) in a mixture of 1 \times APEX Taq polymerase buffer (Genesee Scientific), 1 U of APEX Taq polymerase, 2.5 mM MgCl₂ (final) and 0.2 μ M primers (IDT). Relative transcript abundance was assayed for *hyxR* and *hmpA*, which were normalized to 16S rRNA transcript levels. cDNA for 16S rRNA analysis (calibrator) was performed by 1 cycle of 95°C (10 min) then 40 cycles of 95°C (10 s), 55°C (30 s), 72°C (15 s), 80°C (5 s) and plate reading. *hyxR* and *hmpA* cDNA analysis was performed by 1 cycle of 95°C (10 min) then 40 cycles of 95°C (10 s), 57°C (30 s), 72°C (15 s), 80°C (5 s) and plate reading, with the exception that the annealing temperature for *hyxR* cDNA analysis was 55°C. Melting curve analysis and gel electrophoresis was used to ensure amplicon homogeneity. Relative fold change was derived by the $\Delta\Delta C(t)$ method in comparison with UTI89 *ΔfimBEX*/pBAD33, UTI89, UTI89 *ΔfimBEX*_{TIP-OFF/hyxR-OFF}, or UTI89/pTrc99a, depending on the experiment (ABI User Bulletin #2). Reactions lacking reverse transcriptase (–RT) were performed to ensure the adequacy of DNase treatment.

All qRT-PCR reactions were performed on the MJ Mini MiniOpticon Personal Thermal Cycler (Bio-Rad).

2.16 Construction of FimB:X Chimeras

pTrc-HAT-*fimB* was used as a template for *fimB* amplification with the primers listed in Table 6, listed under the FimB::X Chimera section. The N-terminal region of *fimB* was amplified using primer FimB::X (5' SacI) and one of the five primers listed (primers with a B 3' designation). pBAD33:*fimX* was used as a template for the similar amplification of *fimX* using primer FimB::X (3' BglII) and one of the five primers listed (primers with a X 5' designation). Primers internal to FimB and FimX have end-regions of homology such that when those individual reaction products are combined and amplified with the external primer pair (the SacI- and BglII-containing primers) in a weaving PCR, a full length construct is generated that contains various portions of the N-terminal region of FimB translationally fused, in-frame, to the C-terminal region of FimX. The woven PCR product was then digested with SacI and BglII, and cloned into similarly digested pLR92. The break-points of the five FimB::X chimeras are shown in Fig. 19.

2.17 Swimming Motility Assay

Bacterial strains UTI89/pTrc99a, UTI89Δ*hyxR*/pTrc99a, and UTI89Δ*hyxR*/pTrc:*hyxR* were assayed for swimming motility using a modification of the

swimming-in-agar technique. The medium consisted of 1% tryptone, 0.5% NaCl, and 0.3% agar. The strains were grown overnight (16 h) in LB at 37°C with aeration, at which point a sterile open inoculating loop was used to inoculate the soft agar medium. The plates were then incubated at 37°C for 16 h. The diameter of the motility ring was measured (mm) in at least two locations. Mean diameter of the motility rings were calculated from at least two experiments representing at least five technical replicates each. Statistical significance between strains was determined using a two-tailed Student t-test (unpaired).

2.18 Macrophage infections

RAW 264.7 murine macrophages were routinely grown in DMEM (HG) + 10% FBS (Sigma) and sub-cultured 1:4 approximately every 3 days using a cell scraper to detach adherent cells for maintenance. For infections with *E. coli*, RAW 264.7 cells were seeded into 24 well trays at a density of 1×10^5 cells/well and allowed to adhere and grow to confluence for 36-42 h prior to infection. Where indicated, cells were pretreated with 1 mM L-Arginine or the iNOS-specific inhibitor L-NAME (Chemicon) for 1h prior to infection. L-Arginine and L-NAME were subsequently included in the media for the duration of the experiments. In some cases, cells were pretreated with 1 ng ml^{-1} IFN γ for 16-18h prior to infection to stimulate NO expression and the production of cytokines. IFN γ was not included in the media after the initiation of infections. Infections were done

by adding 10 μ l of bacterial suspension (1×10^7 CFU or multiplicity of infection (MOI) of 10) in PBS to confluent cell monolayers ($\sim 1 \times 10^6$ cells/well). Plates were centrifuged at low speed to bring bacteria in contact with the cell monolayer (5 min @ 1000 rpm) and then incubated for 1 h at 37°C with 5% CO₂. Initial adherence/invasion (1 h) of UTI89 derivatives was used to normalize CFU/well at 24h post-infection; however, there were no significant differences in the 1 h adherence/invasion between the strains tested. Infected monolayers were washed 3 times with PBS and incubated for the remainder of the 24 h experiment using a step-down gentamicin protocol (100 μ g ml⁻¹ for 2 h then 50 μ g ml⁻¹ for 21 h). Finally, cells were washed 3 times with PBS, lysed in 1 mL PBS + 0.1% Triton-X with vigorous pipetting, and bacterial CFU were enumerated by dilution plating on LB with ampicillin.

2.19 Epifluorescent Microscopy

GFP-expressing strains (UTI89/pANT4 and UTI89 Δ *hyxR*/pANT4) were allowed to infect L-Arginine-treated RAW 264.7 cells for 24 h as described in the previous section. Cells were fixed in 3% paraformaldehyde for 20 min. Cell nuclei were counterstained with DAPI by using a mounting media containing 1x DAPI. Coverslips were mounted on glass slides and visualized by epifluorescence microscopy on a Zeiss AxioImager. Images were captured and processed using Metamorph software at the Duke University Light Microscopy Core Facility.

2.20 Determination of RNI Concentration

To measure RNI, we used Griess Reagent (Biotium) and followed the manufacturer's protocol. Briefly, supernatants from infected macrophage monolayers were centrifuged at 13,000 rpm for 10 minutes to pellet any contaminating eukaryotic or prokaryotic cells then filter sterilized using a 0.2 μm low-protein binding filter. 50-150 μl of supernatant was mixed with 20 μl Griess reagent and 130-230 μl dH₂O. Samples were measured at 548 nm, and the values were converted to μM nitrite by using a standard curve generated with sodium nitrite.

2.21 Statistical Analyses

All statistical determinations were made using GraphPad Prism (GraphPad Software). Student's t-test (unpaired) or one-sample t tests were used to determine statistical differences in mean values as indicated in the text. qRT-PCR results were analyzed using a one-sample t test to compare whether the means were different from either 1 or -1. Two-tails were used in the determination of statistical significance, which was defined by attaining p-values ≤ 0.05 . Statistical significance is indicated in figures with * p-value < 0.05 , ** p-value ≤ 0.01 , and *** p-value ≤ 0.001 .

The molecular epidemiology associations were examined using the Fisher's exact test were performed using GraphPad Prism (GraphPad Software) where indicated in figure legends. Two-tails were used in the determination of statistical significance, which

was defined by attaining p-values ≤ 0.05 . Statistical determinations were not weighted for epistatic relationships.

Chapter 3. The Type 1 Pili Epigenetic Regulator Gene *fimX* and Associated Pathogenicity Island are Highly Associated with Extraintestinal Pathogenic *Escherichia coli*

3.1 Introduction

Escherichia coli is a typical constituent of the enteric tract in many animals, including humans. Specialized *E. coli* strains produce a wide variety of intestinal and extra-intestinal diseases, such as diarrhea, urinary tract infections, septicemia, and meningitis (Orskov & Orskov, 1992a). Urinary tract infection (UTI) is a leading infection of children, women, and the elderly (Foxman, 2010; Foxman *et al.*, 2000), and ExPEC is responsible for over 80 percent of the approximately 10 million annual cases of community-acquired UTIs in the US (Russo & Johnson, 2003). While the majority of UTIs involve bladder infection, ExPEC may also produce ascending infections to the kidneys, causing pyelonephritis or more advanced diseases such as urosepsis (Laupland *et al.*, 2002; Rushton, 1997b).

Animal and human studies suggest that type 1 pili is a critical virulence determinant in the pathogenesis of ExPEC cystitis, and bacteria lacking type 1 pili are attenuated secondary to reduced adherence and invasion into superficial bladder epithelial cells (Connell *et al.*, 1996; Mulvey *et al.*, 1998; Wright *et al.*, 2007). Corroborating the molecular pathogenesis studies of type 1 pili in UTI, vaccination with pili subcomponents in animal studies has been shown to reduce the severity and duration of infection

(Palaszynski *et al.*, 1998). Type 1 pili are encoded by the *fim* operon (*fimAICDFGH*) and their expression is controlled at multiple regulatory levels (Corcoran & Dorman, 2009; Kelly *et al.*, 2006; McClain *et al.*, 1991). The orientation of the invertible type 1 pili promoter region (*fimS*) (Abraham *et al.*, 1985) is determined by the action of multiple tyrosine recombinases. ExPEC strains possess up to five Fim-like tyrosine recombinases (*fimB*, *fimE*, *fimX*, *ipuA*, *ipuB*) based on sequence homology, and all but IpuB have been shown to invert *fimS* (Bryan *et al.*, 2006; Gally *et al.*, 1996; Hannan *et al.*, 2008; Klemm, 1986; McClain *et al.*, 1991; Xie *et al.*, 2006a) with differing biochemical activities: FimE inverts the promoter from ON to OFF, FimB catalyzes bidirectional inversion, but favors OFF to ON, IpuA also has bi-directional activity, while FimX is able to invert *fimS* from OFF to ON.

While *fimB* and *fimE* are closely linked to the type 1 pili operon, *fimX* is located at an unlinked genetic locus we have termed PAI-X (Fig. 5). Among a small collection of *E.*

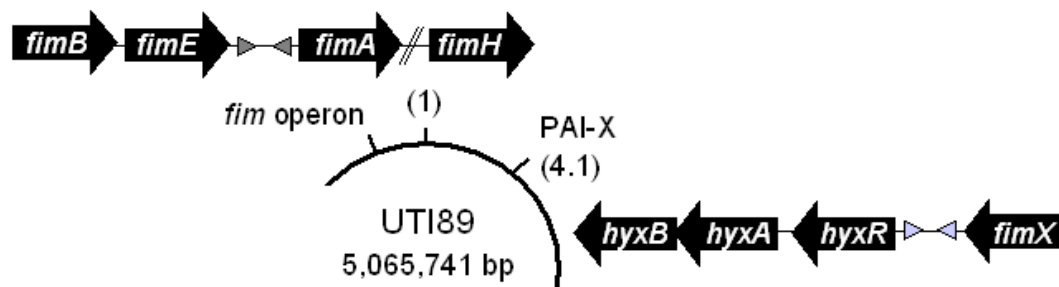


Figure 5: Chromosomal location of the type 1 pili operon, recombinase genes *fimB* and *fimE*, and PAI-X.

coli isolates, we previously demonstrated that *fimX* was found in high association with pathogenic isolates (Hannan *et al.*, 2008). Therefore, a goal of this study was to determine the prevalence of the type 1 pili epigenetic regulator genes present among a more extended, diverse collection of pathogenic and commensal *E. coli* isolates. We hypothesized that *fimX* would have the highest prevalence among ExPEC isolates. Furthermore, by establishing the prevalence of the additional PAI-X genes among *E. coli*, we aimed to determine if PAI-X is associated with ExPEC isolates from a particular UTI syndrome, suggesting a possible role for the factors encoded by these genes in the ExPEC pathogenic lifestyle.

3.2 Results

3.2.1 Identification, Genomic Organization, and Conservation of the *fimX*-Associated Island, PAI-X

Three recombinases FimB, FimE, and FimX regulate the phase variation of type 1 pili in the prototypic cystitis strain UTI89 (Hannan *et al.*, 2008). *fimB* and *fimE* are located immediately upstream of the type 1 pili operon, while *fimX* is located at an unlinked genetic locus we have termed PAI-X (located at approximately 4.2 minutes on the UTI89 chromosome) (Fig. 5), adjacent to the *betABIT* choline-glycine betaine locus (Fig. 6A). The ~6.2 kb region contains four open reading frames (>100 aa): *fimX*, *hyxR*, *hyxA*, and *hyxB* for Hypotheticals of PAI-X (Fig. 6A). Translation and BLAST analysis of *hyxR* suggest it is a putative helix-turn-helix, LuxR-like response regulator. The

additional two conserved open reading frames found in this locus appear to code for a putative outer membrane autotransporter (*hyxB*) and a hypothetical protein (*hyxA*) of which the predicted translation product has homologs only in the genomes of *E. coli* and *S. enterica* (protein BLAST E-score < 5e-05). Although prior work demonstrated that FimX plays a role in the regulation of type 1 pili during acute cystitis (Hannan *et al.*, 2008), the role of the *hyx* gene products during infection remains unknown.

PAIs are common among ExPEC (Blum *et al.*, 1995; Chen *et al.*, 2006; Lloyd *et al.*, 2007) and have some characteristic features, including 1) disparate G+C content from the core genome, 2) association with pathogenic organisms, but not commensal counterparts, 3) contain virulence-associated genes, 4) insertion sites or flanking direct repeats, 5) a high frequency of insertion at tRNA sites, and 6) often mobility (pseudo)genes (Dobrindt *et al.*, 2004; Gal-Mor & Finlay, 2006; Hacker & Kaper, 2000). The *fimX* genomic locus (PAI-X) meets most, if not all, criteria for a pathogenicity island as defined by Hacker and Kaper (Hacker & Kaper, 2000). For instance, PAI-X has disparate G+C content compared to the rest of the UTI89 genome: PAI-X has 42.9% G+C content relative to 50.6% G+C content for UTI89 generally (Fig. 6B) (Chen *et al.*, 2006; EnCor Biotechnology). As shown later in the results, PAI-X is more prevalent among pathogenic *Escherichia coli* strains than commensal strains (Table 7), indicating PAI-X is associated with virulence. Prior studies have also clearly demonstrated that FimX-mediated control of type 1 pili expression is activated *in vivo* and that

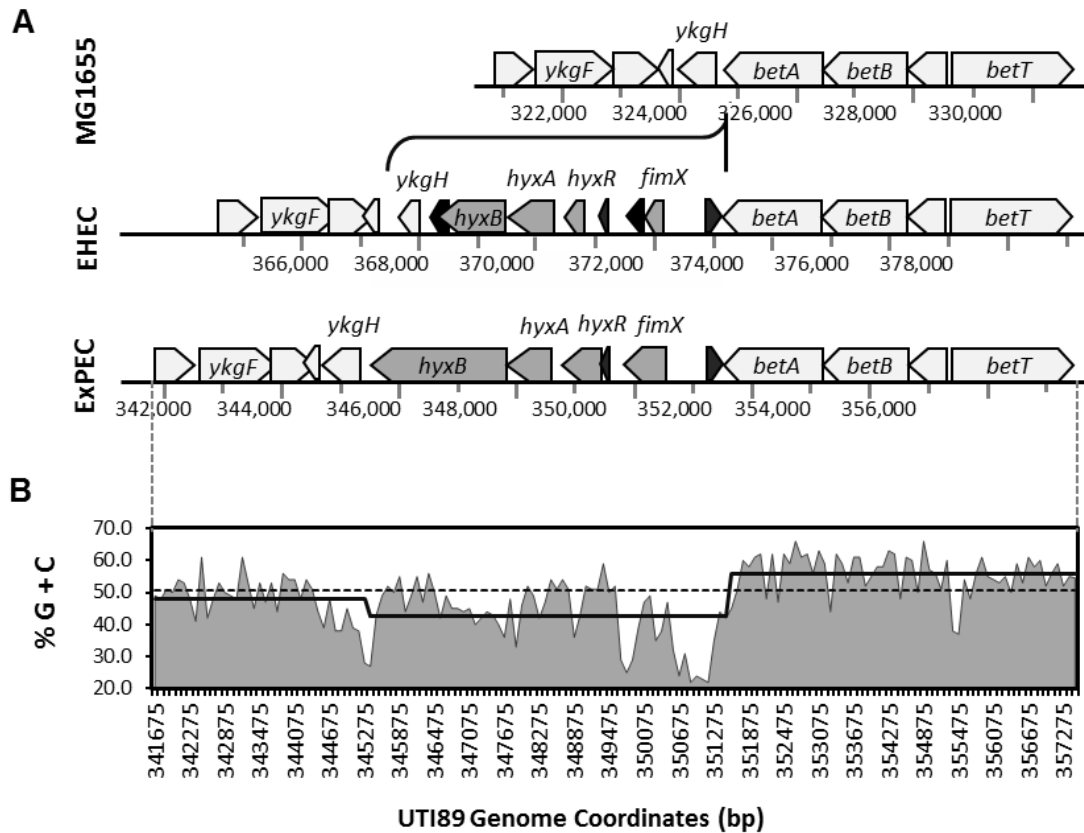


Figure 6: Overview of the genomic location, features, and organization of PAI-X among sequenced *E. coli* isolates.

A) Genomic organization and conservation of PAI-X among sequenced isolates from EHEC (EDL933) and ExPEC (UTI89) pathotypes. MG1655 is used as a K-12, commensal reference. B) % G+C trace of PAI-X/UTI89 and surrounding flanking regions. The average UTI89 genome % G+C (dotted line), the average PAI-X region % G+C (black, stepped line), and average G+C content in 100 bp windows (gray fill) are indicated.

independent expression of type 1 pili by FimX *in vivo* is sufficient to produce acute cystitis (Hannan *et al.*, 2008; Xie *et al.*, 2006a). PAI-X is not associated with a tRNA and direct repeats or insertion (IS) elements were not immediately apparent. Many islands are

also associated with phage genes and transposases and, while FimX is part of the Lambda phage integrase superfamily, it has never been observed to function as an integrase or excisionase, rather mediating site-specific recombination or inversion.

FimX, HyxR, HyxA, and HyxB are highly conserved at the amino acid level with greater than 98% percent identity, respectively, among the prototypic extraintestinal pathogenic *E. coli* of urinary tract isolates UTI89, 536, and CFT073. Sequence analysis of PAI-X in EHEC O157:H7 strains EDL933 and EC4115 indicated the presence of a premature nonsense mutation in *fimX* (Tyr128→Stop), *hyxB* (Ser370→Stop), and *hyxR* (Ser56→Stop) (Fig. 6A). In EHEC strains, there is also a potential in-frame secondary translational start site at residue 107 of *hyxR*, which would create a significant N-terminal truncation. Thus, it is likely that *fimX*, *hyxR*, and *hyxB* do not yield functional products in these EHEC strains. In contrast, HyxA is 97% identical at the amino acid level between sequenced EHEC isolate EDL933 and prototypic ExPEC isolate UTI89, suggesting that PAI-X may have a common provenance.

3.2.2 The Type 1 Pili Epigenetic Regulator, FimX, is the Only Fim-Like Recombinase that is Specifically Associated with ExPEC

To determine the prevalence and complement of Fim-like recombinase genes present in the genomes of a range of *E. coli* isolates as well as the association of *fimX* with the other PAI-X genes, a multiplex PCR approach was developed to detect the Fim-like recombinase genes (*fimB*, *fimE*, *fimX*, *ipuA*, *ipuB*) and *hyx* genes (*hyxR*, *hyxA*, *hyxB*)

(Fig. 7A and B). The pathogenic strains were isolated from women with a variety of clinical syndromes, including single acute cystitis (sUTI), recurrent cystitis (rUTI), asymptomatic bacteriuria (ASB), pyelonephritis (PY), and urosepsis (UTI-BL). For comparison, the assay was performed on non-human and the human commensal

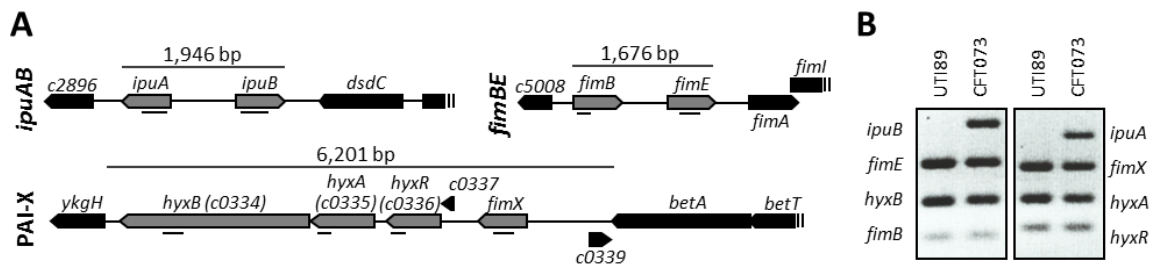


Figure 7: Multiplex PCR design and assay development.

A) Gray arrows represent open reading frames detected by multiplex PCR. Directionality of the open reading frames represents forward or reverse strand orientation. Amplicons are indicated (—). B) Representative results of the multiplex PCR for the Fim-like recombinases and associated genomic islands using multiplex PCR.

isolates from the ECOR reference set (Ochman & Selander, 1984), an independent set of fecal isolates from healthy women, and a small group of EHEC isolates.

As expected, *fimB* and *fimE* are ubiquitous among both pathogenic and non-pathogenic *E. coli* as both *fimB* and *fimE* were present in >98% of ExPEC and commensal strains (Table 7). We found that *ipuA* and *ipuB* were always present together, consistent with previous findings (Bryan *et al.*, 2006), and that *ipuA* and *ipuB* were associated with ExPEC, but were found in only about one fourth of ExPEC isolates

(23.7% in ExPEC vs. 10.0% in commensals; p-value = 0.0248). In contrast, *fimX* was highly associated with ExPEC (83.2% in ExPEC vs. 25.0% in commensal; p-value <0.0001) and that *fimX* is more prevalent in lower UTI isolates (ASB, sUTI, and rUTI isolates) than upper UTI (PY and urosepsis isolates) (87.5% in lower UTI vs. 73.6% in upper UTI isolates; p-value = 0.0288) (Table 7); however, there were no significant differences in the prevalence of *fimX* among the various UTI syndromes (data not shown). *ipuA* and *ipuB*, on the other hand, were equally distributed among upper vs. lower UTI isolates (23.3% in lower UTI vs. 24.5% in upper UTI; p-value = 1.0). Fim-like recombinase genes *ipuA*, *ipuB*, and *fimX* showed higher association with ExPEC isolates; however, *fimX* was the only recombinase gene highly prevalent among extraintestinal pathogenic isolates.

3.2.3 Distribution of the Fim-Like Recombinase and PAI-X Genes by Phylogenetic Group

Phylogenetic analyses have shown that *E. coli* strains fall into four main phylogenetic groups: A, B1, B2, and D (Herzer *et al.*, 1990; Lecointre *et al.*, 1998). Virulent, ExPEC isolates belong mainly to Group B2 and, to a lesser extent, to Group D (Boyd & Hartl, 1998; Picard *et al.*, 1999), whereas most commensal strains belong to Group A. To determine if the molecular prevalence of the Fim recombinase genes and PAI-X was associated with particular *E. coli* phlotypes, phylogenetic group analysis was performed as previously described (Clermont *et al.*, 2000; Gordon *et al.*, 2008).

Table 7. Molecular epidemiology of the Fim-like recombinases and PAI-X factors in commensal and pathogenic *Escherichia coli* isolates.

Target	Commensal						ExPEC						EHEC	
	Human (n = 34)		Non-Human (n = 26)		Total (n = 60)		Lower UTI (n = 120)		Upper UTI (n = 53)		Total (n = 173)		EHEC (n = 10)	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
<i>fimB</i>	33	97.1	26	100.0	59	98.3	119	99.2	53	100.0	172	99.4	10	100.0
<i>fimE</i>	34	100.0	26	100.0	60	100.0	120	100.0	53	100.0	173	100.0	10	100.0
<i>ipuA</i>	5	14.7	1	3.8	6	10.0	28	23.3	13	24.5	41	23.7 [†]	1	10.0
<i>ipuB</i>	5	14.7	1	3.8	6	10.0	28	23.3	13	24.5	41	23.7 [†]	1	10.0
<i>fimX</i>	7	20.6	8	30.8	15	25.0	105	87.5	39	73.6 [*]	144	83.2 ^{HH}	7	70.0
<i>hyxR</i>	7	20.6	8	30.8	15	25.0	105	87.5	39	73.6 [*]	144	83.2 ^{HH}	6	60.0
<i>hyxA</i>	7	20.6	8	30.8	15	25.0	105	87.5	39	73.6 [*]	144	83.2 ^{HH}	7	70.0
<i>hyxB</i>	7	20.6	8	30.8	15	25.0	105	87.5	39	73.6 [*]	144	83.2 ^{HH}	7	70.0
<i>ibeA</i>	2	5.9	0	0.0	2	3.3	56	46.7	11	20.8	67	38.7 ^{HH}	2	20.0
<i>traT</i>	10	29.4	8	30.8	18	30.0	94	78.3	43	81.1	137	79.2 ^{HH}	6	60.0

<i>fyuA</i>	15	44.1	5	19.2	20	33.3	109	90.8	48	90.6	157	90.8 ^{HHH}	5	50.0
<i>hlyA</i>	1	2.9	0	0.0	1	1.7	45	37.5	20	37.7	61	37.6 ^{HHH}	3	30.0
<i>cnfI</i>	0	0.0	0	0.0	0	0.0	69	57.5	16	30.2 ^{**}	85	49.1 ^{HHH}	4	40.0

^a Abbreviations: UTI, urinary tract infection; ASB, asymptomatic bacteriuria; CY, cystitis; PY, pyelonephritis; EHEC, enterohemorrhagic *E. coli*.

^b Commensal strains represent the ECOR reference set as well as fecal isolates from healthy women that encoded for < 4 out of 8 VFs as determined by multiplex PCR. This eliminated 18 strains from analysis. Only phylogenetic groups A, B1, B2, and D were included. ECOR isolates that were human ASB, CY, or PY isolates were included in the ExPEC group for analysis and phylogenetic group E ECOR isolates (n = 2) were included in the EHEC group for analysis.

^c Lower UTI isolates represent single and recurrent ASB and CY isolates. Upper UTI isolates represent PY and UTI-BL isolates.

^d P-values (Fisher's exact) were calculated using two-tails. Symbols indicating significance for group comparisons are as follows:

Lower UTI vs. Upper UTI: * p-value ≤ 0.05 , ** p-value ≤ 0.01 .

Commensal Total vs. ExPEC Total: † p-value ≤ 0.05 , ‡ p-value ≤ 0.01 , § p-value ≤ 0.001 , ¶ p-value ≤ 0.0001 .

Consistent with previous results (Carlos *et al.*, 2010), commensal *E. coli* of non-human origin contained a larger percentage of Group B1 strains (46.2%) than those of human origin (11.8%; p-value = 0.0038) while, not surprisingly, fecal isolates of human origin contained a larger percentage of Group B2 strains (26.5%; p-value = 0.0329 compared with fecal isolates of non-human origin; Fig. 8). Of the ExPEC isolates, the majority were Group B2 (76.3%; p-value < 0.0001 compared with commensal isolates). Group D strains

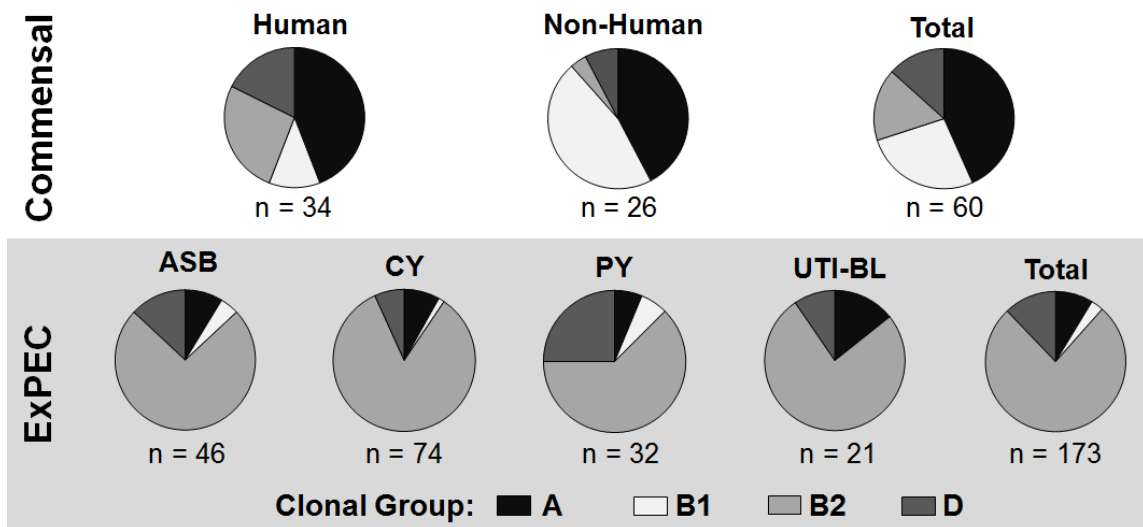


Figure 8: Distribution of the Fim-like recombinase and PAI-X genes by phylogenetic group.

The percentage of isolates in each of the four phylogenetic groups is shown. Out of 50 commensal strains of human-origin, 14 were excluded from this analysis as commensals as they carried ≥ 4 VFs and looked like commensal-pathogens genomically and 2 were excluded as they were previously determined to be phylogenetic group E (Johnson *et al.*, 2001a) (see Chapter 2 for greater detail). Out of 32 total commensal strains of non-human origin, two were excluded because they were phylogenetic group E and 4 were excluded because they carried ≥ 4 VFs.

were at least 2.5-fold more highly associated with pyelonephritis isolates (25.0%; Fig. 8), than isolates from other syndromes (13/141 = 9.2%; p-value = 0.0302).

Analysis of the Fim-like recombinases and PAI-X association with phylogenetic group revealed that *fimB* and *fimE*, not surprisingly, were equally distributed among all phylogenetic groups (Table 8). *ipuA* and *ipuB*, on the other hand, were associated with either Group B2

Table 8. Phylogenetic distribution of the Fim-like recombinases and PAI-X among all *E. coli* isolates.

Target	A n = 42		B1 n = 22		B2 n = 154		D n = 33	
	No.	%	No.	%	No.	%	No.	%
<i>fimB</i>	42	100.0	22	100.0	151	98.1	33	100.0
<i>fimE</i>	42	100.0	22	100.0	154	100.0	33	100.0
<i>ipuA</i>	1	2.4 ^(***)	0	0.0 ^(**)	44	28.6 ^{***}	9	27.3
<i>ipuB</i>	1	2.4 ^(***)	0	0.0 ^(**)	44	28.6 ^{***}	9	27.3
PAI-X	4	9.5 ^(****)	10	45.5 ^(*)	147	95.5 ^{****}	11	33.3 ^(****)

^a All *E. coli* isolates (n = 251), excluding 14 EHEC isolates, were analyzed for phylogenetic group by PCR. PAI-X factors were assayed separately but grouped together for analysis.

^b P-values (Fisher's exact) were calculated using two-tails. Statistical significance is indicated in the table as * p-value ≤ 0.05, ** p-value ≤ 0.01, *** p-value ≤ 0.001, and **** p-value ≤ 0.0001. Significance was calculated for indicated group vs. all other strains. Parentheses () indicate a negative association.

(28.6%) or group D (27.3%), but were rarely found in Group A strains (2.4%) and altogether absent in Group B1 strains (0%; Table 8). PAI-X was also found in almost all Group B2 isolates (95.5%) but few Group A strains (9.5%; p-value < 0.0001), consistent with Group A strains being more highly associated with commensal isolates (Fig. 8). PAI-X showed a much lower association with Group D strains (33.3%), and all 11 of these isolates were from the human ExPEC group (Table 8). PAI-X was also present in 45.5% of B1 isolates (Table 8), but the majority (8/10 = 80.0%) were from non-human commensals.

3.2.4 Extraintestinal Pathogenic E. coli Have Acquired Additional Fim-Like Recombinase Genes

There were four main recombinase profiles observed among isolates: strains that encode for *fimB fimE* only, strains that encode for *fimB fimE* in addition to *fimX*, strains that encode for *ipuA ipuB* in addition to *fimB fimE*, and strains that encode for all five recombinase genes, *fimB fimE fimX ipuA ipuB* (Fig. 9). The majority of commensal strains (both human and animal origin) only carried the genes for *fimB* and *fimE* (67.8%; Fig. 9). In both commensal and ExPEC isolates, addition of *ipuA ipuB* in the absence of *fimX* was infrequent (6.8% in commensals vs. 1.7% in ExPEC; p-value = 0.0726). Interestingly, ExPEC strains generally contained a higher number of recombinase genes. For instance, 61.0% of ExPEC contained *fimB fimE fimX* and 22.1% contained all five recombinase genes, compared with 22.0% and 3.4% of commensal isolates, respectively

(p-value < 0.02 for both comparisons; Fig. 9). These data suggested that strains carry *fimB* and *fimE* as part of the “core genome” then can acquire *ipuA* and *ipuB* subsequent to *fimX* acquisition in pathogenic isolates. Our findings are consistent with the notion that ExPEC strains have acquired additional regulatory inputs to control and possibly fine-tune the expression of the major virulence factor, type 1 pili.

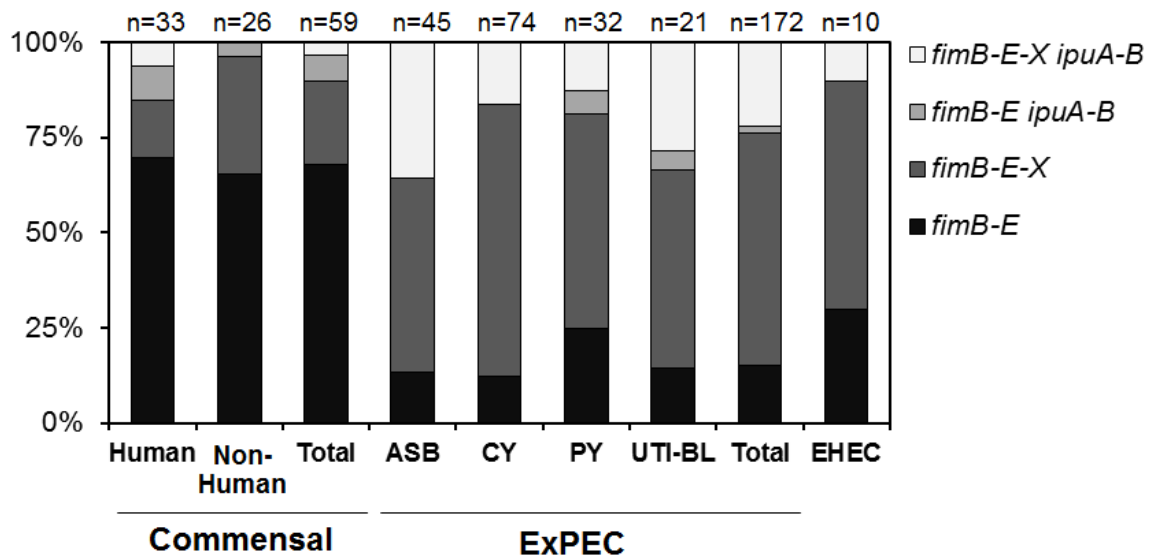


Figure 9: Extraintestinal pathogenic *E. coli* have acquired additional Fim-like recombinase genes.

The percentage of isolates that encode for one of four recombinase profiles observed, grouped as follows: *fimB fimE* only (similar to MG1655); *fimB fimE* with addition of *fimX* (similar to UTI89); *fimB fimE* with addition of *ipuA* and *ipuB* (rarely observed); and all five recombinases present, *fimB fimE fimX ipuA ipuB* (similar to CFT073). One human commensal isolate and one ASB isolate were excluded from analysis as they did not carry the *fimB* gene by PCR analysis.

3.2.5 PAI-X is Prevalent among Extraintestinal Pathogenic E. coli and is Correlated with Virulence Potential

Like *fimX*, *hyxRAB* was also more significantly associated with pathogenic versus commensal isolates and the proportion of *E. coli* isolates carrying PAI-X increased with the total number of virulence factor genes (Fig. 10A and B). In ExPEC isolates, the *hyx* genes were always present with *fimX*, and thus, were also more significantly associated with ExPEC isolates (83.2% in ExPEC vs. 25.0% in commensals; p-value < 0.0001; Table 7), demonstrating that, when present, the major genetic features of PAI-X_{UT189} are highly conserved among other extraintestinal pathogenic isolates.

Multiple EHEC isolates (n=10) were also assayed for the presence of PAI-X. Seven out of ten isolates (70%; Table 7 and Figure 10A) were positive by multiplex PCR for PAI-X; however, genome analysis of several sequenced O157:H7 isolates revealed premature stop mutations in *fimX*, *hyxR*, and *hyxB* (see Section 3.2.1 for greater detail). The PCR assay detected the PAI-X genes, but as noted earlier for the sequenced EHEC genomes, it is likely these EHEC genes have nonsense mutations not detect by this PCR analysis. In one EHEC isolate (ECOR 43), *hyxR* was not detected by PCR while the other three PAI-X genes were detected (Appendix A, Table 11). BLAST analysis (Altschul *et al.*, 1997) also revealed PAI-X is present in additional *E. coli* pathotypes, including neonatal meningitis ExPEC isolate RS218, avian pathogenic isolate APEC01, and adherent/invasive *E. coli* (AIEC) isolates LF82 and NRG 857c. These data indicate that PAI-X is likely widely distributed among ExPEC and may include the AIEC group,

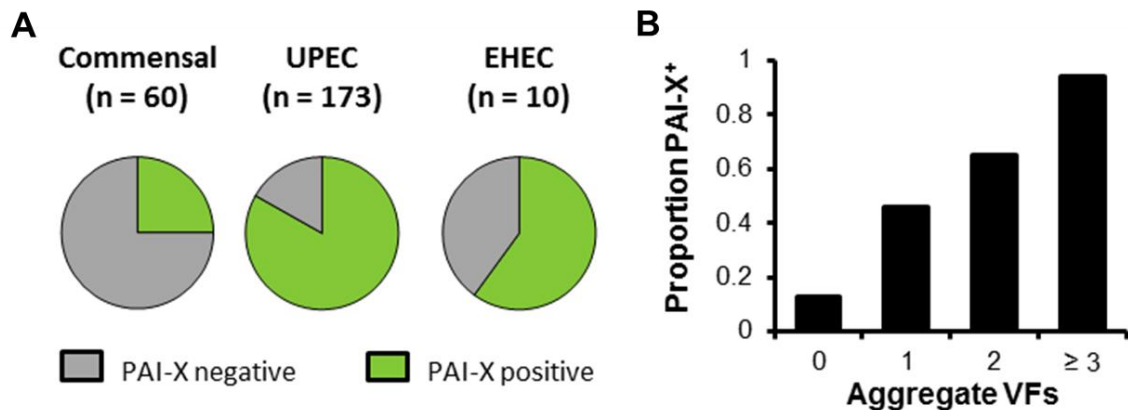


Figure 10: PAI-X is highly associated with ExPEC and acquisition strongly correlates with virulence.

A) PAI-X is highly associated with uropathogenic *E. coli* representing multiple syndromes by multiplex PCR analysis. B) All *E. coli* isolates (n = 265), regardless of clinical syndrome, were scored based on the number of selective virulence factors (VFs) present by PCR analysis (*hlyA*, *cnf1*, *ibeA*, *traT*, and *fyuA*). Sample sizes varied between groups: 0 VFs (n = 31), 1 VF (n = 46), 2 VFs (n = 69), ≥ 3 VFs (n = 119).

although sequencing efforts will reveal if these strains carry polymorphisms producing coding sequence disruptions as found in many EHEC isolates.

Phi coefficients, also called mean square contingency coefficients, were determined to ascertain if PAI-X was co-associated with any of the virulence factors present in the genomes of PCR-tested ExPEC isolates. Phi coefficients measure the association of two binary variables, similar to a Pearson correlation. For instance, whether virulence factor A is associated, either positively or negatively, with the presence of virulence factor B. In this case, Phi coefficients were calculated between PAI-X and the presence of 5 additional virulence factors tested by multiplex PCR. Coefficients can

Table 9. Phi coefficient and PAI-X virulence factor association.

Factor	Phi Coefficient
<i>ibeA</i>	0.329
<i>cnf1</i>	0.438
<i>hlyA</i>	0.864
<i>traT</i>	0.265
<i>fyuA</i>	0.379

range in value between -1 and 1, with values close to 1 or -1 indicating a strong positive or negative association between variables, respectively, and were determined using 2x2 contingency tables. The virulence genes *ibeA*, *cnf1*, *traT*, and *fyuA* all showed weak positive association with PAI-X; however, virulence gene *hlyA* showed a very strong positive association with PAI-X with a Phi coefficient of 0.864 (Table 9). PAI-X and *hlyA* are located in distinct regions of the UTI89 genome, suggesting they are not genetically linked loci. Therefore, it is possible that PAI-X and *hlyA* co-associate with a particular as-yet-undefined subset of ExPEC isolates or that HlyA and PAI-X may participate in similar disease processes.

3.2.6 PAI-X is a Genetic Marker for Human ExPEC

To elucidate whether specific genetic features of *E. coli* were predictive of isolates being urinary tract-associated (ExPEC), we evaluated the sensitivity and specificity of virulence factor genes for predicting or excluding an *E. coli* isolate as ExPEC (see Chapter 2 for a detailed explanation). These served as common metrics to compare the likelihood that specific *E. coli* genes were predictive of an isolate being part of the ExPEC group. Applying this analysis to the data from Table 7, several genes have high sensitivity (equivalent to prevalence) as a positive indicator for an isolate being ExPEC. For, instance *fyuA* and PAI-X have the two highest sensitivities at 90.8% and 83.2%, respectively, indicating that these genomic markers are highly associated with

Table 10. Sensitivity and specificity of VFs predictive of ExPEC.

	Sensitivity	Specificity
<i>ibeA</i>	67/173 = 38.7%	32/34 = 94.1%
<i>traT</i>	137/173 = 79.2%	24/34 = 70.6%
<i>fyuA</i>	157/173 = 90.8%	19/34 = 55.9%
<i>hlyA</i>	61/173 = 37.6%	33/34 = 97.0%
<i>cnfI</i>	83/173 = 49.1%	34/34 = 100.0%
PAI-X	144/173 = 83.2%	27/34 = 79.4%

^a Sensitivity and specificity are described in the Materials and Methods section. Calculations are based on data derived from Table 1.

ExPEC (Table 10). Conversely, a high specificity would indicate those genes that, when absent, predict an isolate is not ExPEC. Genes like *cnf1* and *hlyA*, which are almost never found in commensal isolates, have specificities $\geq 97\%$, indicating that negative tests for these genomic markers are useful in excluding an isolate from the ExPEC group.

Although *hlyA* and *cnf1* have high specificities, their low prevalence in the ExPEC population yields a low sensitivity value, making their presence less useful in predicting if an isolate is ExPEC. Of the VFs tested, PAI-X had the best combination of sensitivity and specificity, at 83.2% and 79.4%, respectively (Table 10).

3.3 Discussion

PAI-X was associated with *E. coli* isolated from patients experiencing a variety of clinical syndromes, including asymptomatic bacteriuria, cystitis, pyelonephritis, and urosepsis. Our molecular epidemiology data provide strong evidence that this locus is widespread among a diverse group of ExPEC clinical isolates of urinary tract origin. Among extraintestinal pathogenic *E. coli* in general, *fimX* was always found associated with *hyxR*, *hyxA*, and *hyxB* in isolates so far tested, suggesting that when this locus is present, it is broadly structurally conserved. Although sequence data from pathogenic *E. coli* suggests these genes reside together at the same locus, hybridization and/or sequencing studies will be necessary to ascertain if *fimX* and *hyxRAB* are always linked.

Our molecular epidemiology data also suggest that this locus is less prevalent among pyelonephritis and urosepsis isolates than lower UTI isolates (ASB, CY);

although, the majority of upper UTI strains still carry PAI-X. Future studies will be needed to determine if there is a biological significance to this statistical difference. Many isolates that cause urosepsis are genetically related to strains known to cause cystitis and pyelonephritis, suggesting that the simple presence or absence of any genetic factors is insufficient to define urosepsis isolates as a group. As illustrated by several of the sequenced EHEC isolates, the simple presence or absence of PAI-X by PCR analysis is unlikely to predict the functional state of the factors. PAI-X, including *fimX*, was highly conserved in sequenced ExPEC isolates but disrupted in sequences EHEC isolates, suggesting a potential evolutionary benefit of this locus specific to the colonization, transmission, or pathogenesis of ExPEC isolates.

A role for PAI-X in virulence is supported by our molecular epidemiology data and the strong, positive correlation between the number of VFs present in the genome of an isolate and the presence of PAI-X. Previous studies have shown that FimX is capable of regulating virulence during experimental cystitis (Hannan *et al.*, 2008). FimX is sufficient but not necessary to mediate type 1 pili expression during cystitis, thereby promoting ExPEC bladder epithelial invasion. As presented in Chapter 4, we also show that FimX exclusively regulates the PAI-X gene *hyxR*, and that HyxR regulates the intracellular survival of ExPEC during macrophage infection. Further work will be needed to discern the individual contributions of the other PAI-X genes in colonization and extraintestinal disease. PAI-X is also highly associated with *hlyA*, the expression of which has recently been shown to exacerbate inflammation and cause hemorrhage in the

bladder during murine experimental cystitis with extraintestinal pathogenic *E. coli* (Smith *et al.*, 2008). The high prevalence of PAI-X genes distributed among ExPEC may suggest that PAI-X encoded factors like FimX and HyxR have conserved roles in host interactions, as further investigated in Chapters 4 and 5.

Chapter 4. The Type 1 Pili Epigenetic Regulator FimX Regulates a Second Target, *hyxR*, Through Phase Variation

4.1 Introduction

Through molecular epidemiology studies presented in Chapter 3, we found that *fimX* is the only Fim-like recombinase family member highly associated with ExPEC strains, being present in >80% of such isolates, while only present in <25% of commensal strains. Prior work has shown that FimX is sufficient to mediate the phase OFF to ON transition and thus drive T1P expression in the urinary tract, ultimately resulting in the initiation and perpetuation of cystitis (Hannan *et al.*, 2008). However, the apparent functional redundancy among the Fim-like recombinases, particularly type 1 pili positive regulators FimB and FimX, combined with the molecular epidemiology data suggested that FimX may have activities beyond regulating type 1 pili that are important for maintaining the ExPEC lifestyle.

Prior studies showed that FimX promotes OFF to ON phase switching of type 1 pili in ExPEC and was sufficient to drive expression of T1P during an ascending model of UTI *in vivo*, leading to the establishment of cystitis (Hannan *et al.*, 2008). Notably, FimX is encoded at an unlinked locus from T1P, while FimB and FimE, the remaining two homologs present in UTI89 that regulate T1P expression, are encoded directly upstream of the T1P operon. FimX is encoded on a small pathogenicity islet PAI-X (Fig. 5) with three additional, conserved open reading frames greater than 100 amino acids in

length (Fig. 6). Many recombinases regulate genes adjacent to their coding loci, similar to FimB- and FimE-mediated recombination of T1P. Therefore, we hypothesized that FimX, whose gene is immediately adjacent to *hyxR*, *hyxA*, and *hyxB* on the pathogenicity islet PAI-X, has a role in phase variation of their respective promoters, thus providing epigenetic control over their expression.

4.2 Results

4.2.1 *FimX Epigenetically Regulates hyxR Through Bidirectional Phase Inversion of the Promoter*

To monitor inversion of the *hyx* gene promoters on the PAI-X islet, we amplified the 5' intergenic region plus flanking sequences of each gene (*hyxR*, *hyxA*, *hyxB*) and then digested the amplicon with multiple restriction enzymes, predicting that phase inversion would result in re-orientation of the asymmetric restriction site evident by gel electrophoresis. We amplified the putative promoter regions in human ExPEC strain UTI89 derivatives expressing arabinose-inducible constructs under control of the P_{BAD} promoter (Guzman *et al.*, 1995) to ensure that both FimB and FimX were present at high levels, given previous data suggesting that FimX is poorly expressed under laboratory conditions (Hannan *et al.*, 2008). We predicted that phase variation in any of the intergenic regions due to FimX or FimB activity would yield a unique four band pattern, compared to a double band pattern in the UTI89/pBAD33 control background (Fig. 11A).

We determined that FimX was able to phase vary the promoter of *hyxR* (Fig. 11A), but not *hyxA* or *hyxB* (Fig. 12B and C), based on the restriction sites tested. Digestion of the *hyxR* 5' UTR amplicon with the restriction enzyme NspI showed the expected bands at 466 bp and 117 bp when only vector control was present (Fig. 11A). Expression of FimX produced changes in the *hyxR* promoter region consistent with phase variation, creating two additional fragments of approximately 350 and 225 bp (Fig. 11A). In contrast, we found no evidence of FimX phase variation upstream of *hyxA* or *hyxB* (Fig. 12B and C). Expression of FimB did not produce any change in restriction patterns

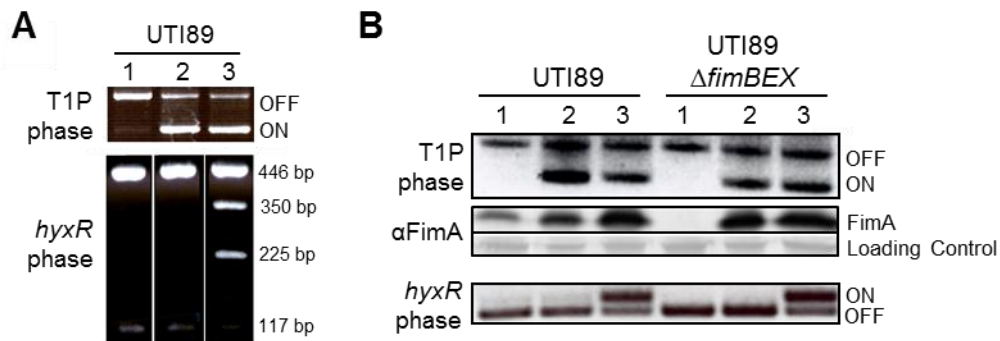


Figure 11: FimX phase variation of the *hyxR* promoter region.

A) Comparison of FimX and FimB phase variation of T1P (*fimS*) and *hyxR* promoter regions. Lanes 1, 2, and 3 indicate UTI89/pBAD33, UTI89/pBAD:*fimB*, and UTI89/pBAD:*fimX*, respectively. B) FimB and FimX inversion of the type 1 and *hyxR* promoters in wild-type and recombinase-null backgrounds using phase-specific primers and PCR analysis. Promoter orientation is labeled relative to transcriptional state. Type 1 pili expression was measured by immunoblot against the major subunit, FimA. Lanes represent UTI89 or $\Delta fimBEX$ with the following plasmids: 1=pBAD33, 2=pBAD:*fimB*, and 3=pBAD:*fimX*.

for any of the regions tested (Fig. 11A and Fig. 12). To confirm that the expression of FimX produced phase inversion of the *hyxR* 5' UTR, we designed phase-specific primers that discriminate between both orientations of the promoter based on size. We compared the inversion patterns of the type 1 promoter and *hyxR* promoter produced by FimB and FimX expression in wild-type and recombinase-null (Δ *fimBEX*) strains. Δ *fimBEX* is unable to undergo T1P or *hyxR* phase variation in the absence of expression of FimB, FimE, or FimX, creating a clean background to assay individual recombinase

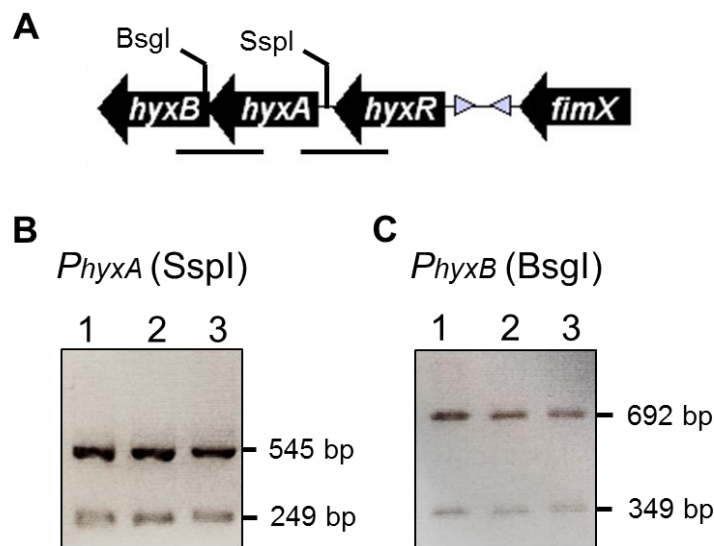


Figure 12: FimB and FimX do not induce phase variation of the promoters of *hyxA* or *hyxB*.

A) Overview of the *hyxA* and *hyxB* promoter regions amplified by PCR and digested using the indicated restriction enzymes. B) Digest of the *hyxA* promoter with SspI shows the predicted fragments at 545 bp and 249 bp regardless of *fimB* or *fimX* expression. No additional bands are present upon *fimB* or *fimX* induction (compare with Fig. 8A, lane 3). Lanes represent UTI89 with the following plasmids: 1= pBAD33, 2= pBAD:*fimB*, and 3= pBAD:*fimX*. C) Digest of the *hyxB* promoter with BspI shows the predicted fragments at 692 bp and 349 bp regardless of *fimB* or *fimX* expression. Lanes are labeled the same as panel B.

activities. The promoter orientation is labeled relative to the transcriptional state as “OFF” or “ON”, as previously published for type 1 pili (Gally *et al.*, 1996; Klemm, 1986; Olsen & Klemm, 1994) and as determined later in this study for *hyxR* through transcript analysis. We and others have shown, as previously described, that both FimB and FimX can mediate phase inversion of the *fimS* promoter to the ON orientation in a wild-type background and also in a recombinase-null background (Fig. 11B) (Bryan *et al.*, 2006; Gally *et al.*, 1996; Hannan *et al.*, 2008; Klemm, 1986; McClain *et al.*, 1991; Xie *et al.*, 2006a). Concordant with the T1P phase PCR results, both FimB and FimX expression in wild-type and recombinase-null backgrounds showed increased levels of the major T1P pilus protein, FimA, as demonstrated by Western blot (Fig. 11B). However, only FimX expression showed phase inversion of the *hyxR* promoter (Fig. 11B).

In UTI89 Δ *fimBEX* starting with the *hyxR* promoter in the OFF orientation, we observed that the expression of FimX *in trans* produced only an ~ 50% ON population. This suggested that a large proportion of the population was either unresponsive to FimX-associated recombination or that FimX had bidirectional activity. This led us to determine if FimX and FimB expression results in bidirectional phase variation of the *hyxR* promoter region. Accordingly, we derived strains with all four possible combinations of phase-locked states at T1P and *hyxR* (Fig. 13A, compare lane 1 for each strain). Using the recombinase null, phase-locked derivatives, we monitored phase inversion at both loci following FimX or FimB expression. FimX and FimB were

expressed *in trans* under induction of the P_{BAD} promoter to ensure tightly-controlled expression of each. As previously observed, expression of FimX, but not FimB, produced inversion at the *hyxR* promoter from OFF to ON (Fig. 13A). Interestingly, FimX was also able to invert the *hyxR* promoter from ON to OFF in contrast to its unidirectional activity turning T1P from OFF to ON (Fig. 13A). FimB, which produced bidirectional inversion at the T1P promoter, was unable to mediate inversion in either direction at *hyxR* (Fig. 12A). FimX-associated inversion of T1P was independent of HyxR, as FimX expressed *in trans* was capable of producing T1P inversion in an *hyxR* deletion background (Fig. 13C). Our results suggest that FimX is a unique site-specific, tyrosine recombinase family member that recognizes two divergent targets and has differing biochemical functions at each locus.

We have shown that FimX is specifically able mediate regulation of *hyxR* through phase variation of its promoter region (Fig. 11A and B and Fig. 13A); therefore, our next goal was to ascertain whether FimX-associated inversion of the *hyxR* promoter affected the expression of *hyxR*. To determine *hyxR* transcript levels, qRT-PCR was performed with FimX, FimB, or vector control expressed *in trans* in the triple recombinase-null strain UTI89 $\Delta fimBEX$. Expression of FimX *in trans* resulted in an approximately 50:50 *hyxR* phase population, corresponding to a two-fold increase in *hyxR* transcript levels compared to vector control (p-value = 0.0345, one-sample t test) (Fig. 13B), which suggests there may be non-inversion transcriptional controls governing *hyxR* expression. Transcript levels of *hyxR* were also compared between wild-type and isogenic *fimX* or

fimB deletion strains. Compared to the wild-type strain, UTI89 Δ *fimX* had a 3.5-fold decrease in *hyxR*

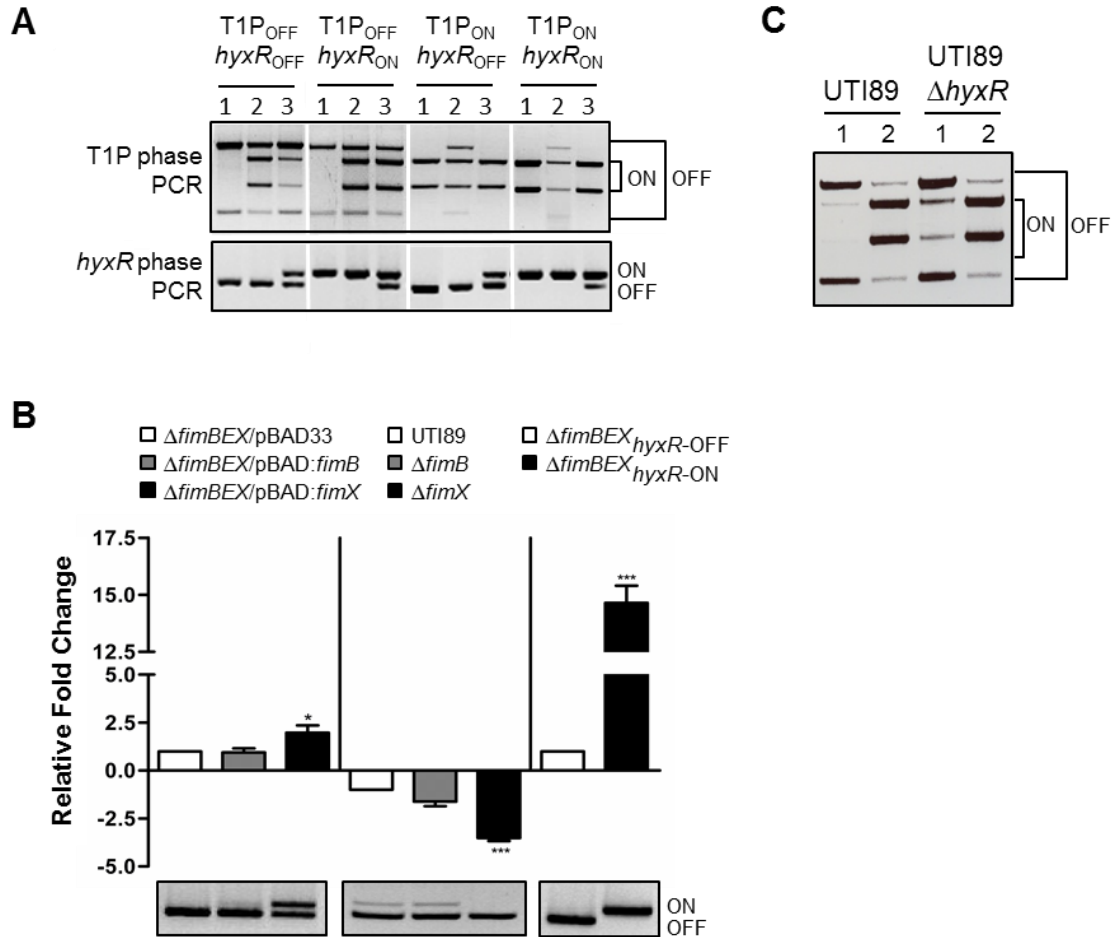


Figure 13: *fimX* expression results in bidirectional inversion of the *hyxR* promoter region, correlating with *hyxR* expression.

A) *fimX* expression results in bidirectional inversion of *hyxR* as assayed by phase PCR. Lanes represent Δ *fimBEX* phase-locked derivatives with the following plasmids: 1= pBAD33, 2= pBAD:*fimB*, and 3= pBAD:*fimX*. B) *hyxR* transcript measured by qRT-PCR in recombinase-null strains expressing *fimX* or *fimB* *in trans*, in single *fimX* or *fimB* deletion strains, and phase-locked derivatives of UTI89 Δ *fimBEX*. P-values that reached significance, defined as reaching a p-value < 0.05, are indicated as follows: * p-value < 0.05; *** p-value \leq 0.001. C) *fimS* phase PCR in UTI89 or Δ *hyxR* backgrounds expressing [1] pBAD33 vector control or [2] pBAD:*fimX* *in trans*.

transcript (p-value = 0.0006, one-sample t test). A single *fimB* deletion showed a non-significant 1.6 fold reduction in *hyxR* transcript levels compared to wild-type (p-value = 0.0806, one-sample t test) (Fig. 13B). To determine *hyxR* transcript levels in the *hyxR* phase-locked OFF or ON strains, we again performed qRT-PCR. The *hyxR* phase-lock ON strain showed a 14.6-fold increase in *hyxR* transcript levels compared with the phase-lock OFF baseline (p-value < 0.0001, One-sample t test) (Fig. 13B). Taken together, FimX, but not FimB, is able to mediate the phase inversion of the *hyxR* promoter region, thereby promoting *hyxR* transcription. Accordingly, the respective *hyxR* promoter orientations were designated as OFF or ON.

4.2.2 Coordinate Transcriptional Regulation of PAI-X Factors and Type 1 Pili Mediated by FimX and FimB

In addition to investigating the effect of phase variation on *hyxR* transcript levels, we also investigated phase-independent transcriptional effects of FimX and FimB expression on T1P and PAI-X genes. Interestingly, expression of FimX *in trans* induced *fimB* expression by approximately 5-fold (Fig. 14A). FimB expression, however, had no apparent effect on *fimX* transcript levels (not shown). The mechanism of this regulation is presently unknown although we speculate that it is likely through an indirect FimX effect through a secondary regulator such as HyxR. This prediction is based on bioinformatic scanning of the *fimB* promoter that did not reveal any sequences that bear resemblance to

either the 9 bp *fimS* repeats or the 16 bp *hyxR* repeats (described in Chapter 4 section 2.4). Alternatively, FimX may be able to bind DNA sequences in the *fimB* promoter in

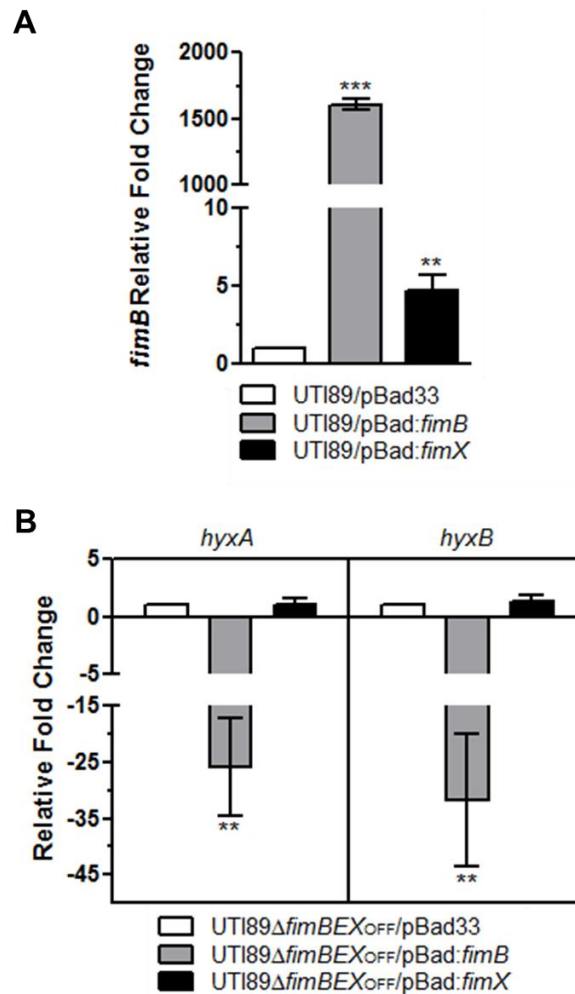


Figure 14: FimX can upregulate *fimB* transcription, which can in turn repress *hyxA* and *hyxB*.

A) *fimB* transcript measured by qRT-PCR with pBAD constructs in trans after 5 h static growth and 0.2% arabinose induction. B) *hyxA* and *hyxB* transcript levels in a triple-recombinase null strain at 24 h static growth and 0.2% arabinose induction.

the absence of inversion, acting as a more canonical transcriptional activator.

In the absence of the other recombinases, FimB expression repressed the transcript levels of *hyxA* and *hyxB*, the other two hypothetical proteins encoded on PAI-X (Fig. 14B). This regulation appears independent of any phase inversion upstream of these genes as several PCR-based analyses failed to uncover any genetic rearrangement in the putative promoter region(s) upon FimB expression (Fig. 12A and B). These results lead us to speculate that the regulation of type 1 pili and PAI-X, may be coordinately regulated under specific conditions.

4.2.3 Inversion of the *hyxR* Promoter is Conserved in Multiple Clinical Isolates and is Independent of the Accessory DNA-Bending Factor IHF

To determine if FimX induced phase inversion of the *hyxR* 5' UTR was conserved in unrelated ExPEC carrying the PAI-X locus, we expressed the UTI89 FimX *in trans* under arabinose induction in four epidemiologically-unrelated clinical isolates and assayed *hyxR* phase variation by PCR. The pathogenic isolates selected were isolated from patients with different clinical presentations, including asymptomatic bacteriuria, cystitis, pyelonephritis, and pyelonephritis/urosepsis and were previously identified as carrying the PAI-X locus in these strains by multiplex PCR analysis (Appendix A, Table 11). FimX, expressed *in trans*, was able to mediate phase inversion of the *hyxR* promoter in all four clinical isolates tested (Fig. 15). FimB was unable to mediate phase inversion of the *hyxR* promoter in three of the four isolates. However, in pyelonephritis isolate J96,

the expression of FimB produced a minor ON population at the *hyxR* intergenic region, but not to nearly the same extent as FimX expression (Fig. 15). Together, these data suggest that FimX is the principle epigenetic regulator of *hyxR* among a diverse group of UTI strains.

The Fim-like recombinases are part of the larger tyrosine recombinase family in prokaryotes for which integration host factor (IHF) has been regarded as an essential co-factor for the inversion reaction. Previous work has established the essential role of integration host factor (IHF), a DNA-bending accessory factor that binds to the invertible T1P promoter, in providing scaffolding for the correct conformation of the FimB and FimE recombinase-mediated inversion reaction (Blomfield *et al.*, 1997). FimB- and FimE-mediated recombination of the T1P promoter does not occur in the absence of IHF and the conformational change induced by IHF binding to the *fimS* promoter region

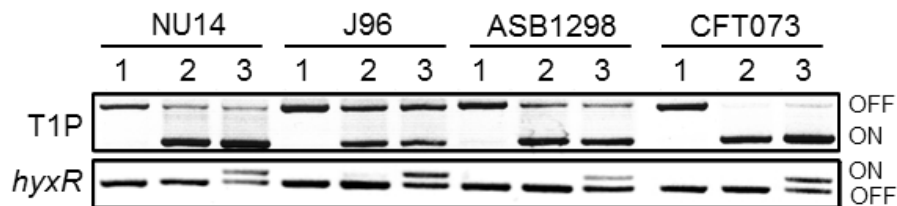


Figure 15: FimX-associated inversion of the *hyxR* promoter region is conserved among pathogenic clinical isolates.

Investigation of FimX and FimB phase inversion of T1P and *hyxR* promoter region was undertaken in four additional pathogenic strains representing multiple UTI clinical presentations: J96 (pyelonephritis), CFT073 (pyelonephritis/urosepsis), ASB1298 (asymptomatic bacteriuria), and NU14 (cystitis). Results denote representative experiment.

(Dorman & Higgins, 1987; Eisenstein *et al.*, 1987). We sought to investigate whether the action of IHF was required for FimX-mediated inversion of the T1P and *hyxR* promoter regions. In accordance with previous work, we observed that FimB-mediated inversion of T1P required IHF. In both cases, inversion of the T1P promoter was locked in strains carrying an isogenic deletion in *himA*, encoding the α subunit of IHF (Fig. 16). As anticipated, IHF was required for both FimB- and FimX-mediated inversion of the T1P promoter (Fig. 16). Overexpression of FimB or FimX *in trans* was unable to overcome the absence of IHF, and no inversion at T1P was observed in these strains (Fig. 16). In contrast, FimX inversion of the *hyxR* promoter region was independent of IHF. Baseline *hyxR* inversion was maintained in the *himA* deletion background, and overexpression of FimX was still able to robustly invert the *hyxR* promoter from OFF to ON, even in the absence of IHF; however, expression of FimB still did not result in *hyxR* phase inversion even in the absence of IHF (Fig. 16).

Previous research has shown that the nucleoid-associated protein H-NS can influence the inversion of the T1P invertible promoter (Kawula & Orndorff, 1991; O'Gara & Dorman, 2000), primarily through repression of *fimB* and *fimE* (Olsen & Klemm, 1994; Olsen *et al.*, 1998). However, follow-up studies revealed that although IHF plays the dominant role in biasing the T1P invertible switch towards the ON state, H-NS may also play a role in the orientation bias of the T1P promoter in an IHF-null background (Corcoran & Dorman, 2009). Recent work has demonstrated the role of H-

NS in binding AT-rich regions of the chromosome, particularly within pathogenicity islands, and silencing their expression via transcriptional repression (Fang & Rimsky, 2008; Lucchini *et al.*, 2006; Navarre *et al.*, 2006). Given that both FimX and HyxR are encoded on a small pathogenicity islet (PAI-X) with low GC content and that IHF appears dispensable for FimX inversion at *hyxR*, we hypothesized that H-NS may play a role in regulating the promoter orientation of *hyxR*. However, FimX, expressed *in trans*, was capable of inverting both the T1P and *hyxR* promoters in the absence of H-NS (Fig. 16). Interestingly, although H-NS and IHF were dispensable for FimX-associated recombination of the *hyxR* promoter, both IHF and H-NS do appear to promote an orientation bias towards OFF, as noted by the increased basal level of *hyxR* phase ON in either an *hns* or *himA* deletion background (Fig. 16). This same effect was not noticed at

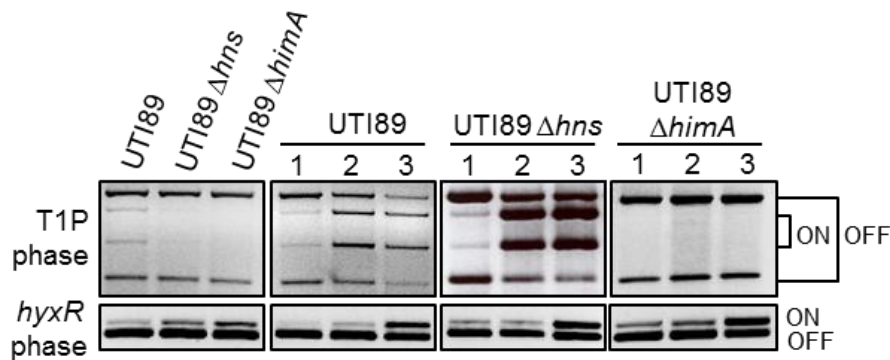


Figure 16: Inversion of the *hyxR* promoter independent of the accessory DNA-bending factor IHF and the global regulator H-NS.

FimX and FimB phase inversion of the type 1 promoter and *hyxR* in either UTI89, Δhns , or $\Delta himA$ (IHF-) deletion strains. Results denote representative experiment.

the T1P promoter region. Even though both H-NS and IHF are not required for FimX-mediated inversion of *hyxR*, either one or both factors may play some role in the directional orientation of the promoter.

4.2.4 The *hyxR* Promoter is Inverted at 16 bp Inverted Repeats that are Dissimilar in Length and Sequence Composition from the T1P Repeats

We next sought to localize the sites of FimX-associated inversion at P_{hyxR} . Canonical sites of inversion resembling those flanking the T1P promoter were not apparent in a sequence analysis of the *hyxR* 5' UTR. Based on the NspI and SspI restriction pattern with and without FimX expression (Fig. 11A and data not shown), we determined that the *hyxR* promoter invertible region was between 270 and 290 bp in length. The location of the inversion sites was mapped to within 40 bp: the proximal inversion site (P_{hyxR} -IR_{proximal}) was located approximately 30 to 70 bp upstream of the putative translational start site of *hyxR*, while the distal inversion site (P_{hyxR} IR_{distal}) was located approximately 290 to 330 bp upstream (Fig. 17A). To determine the exact sites of inversion upstream of *hyxR*, we amplified the proximal inversion region plus flanking sequence using phase-specific primer pairs from UTI89 expressing pBAD:*fimX in trans* and then sequenced through the region of inversion in both orientations (for detailed methods see Chapter 2). We determined that FimX expression resulted in inversion of the *hyxR* promoter starting at 16 bp inverted repeats (Fig. 17A). These sequencing results concur with the mapping of the inversion sites by restriction digest size analysis. The 16

bp inverted repeats are located 278 bp apart with the proximal repeat located 39 bp upstream of the putative translational start site for *hyxR* (Fig. 17A).

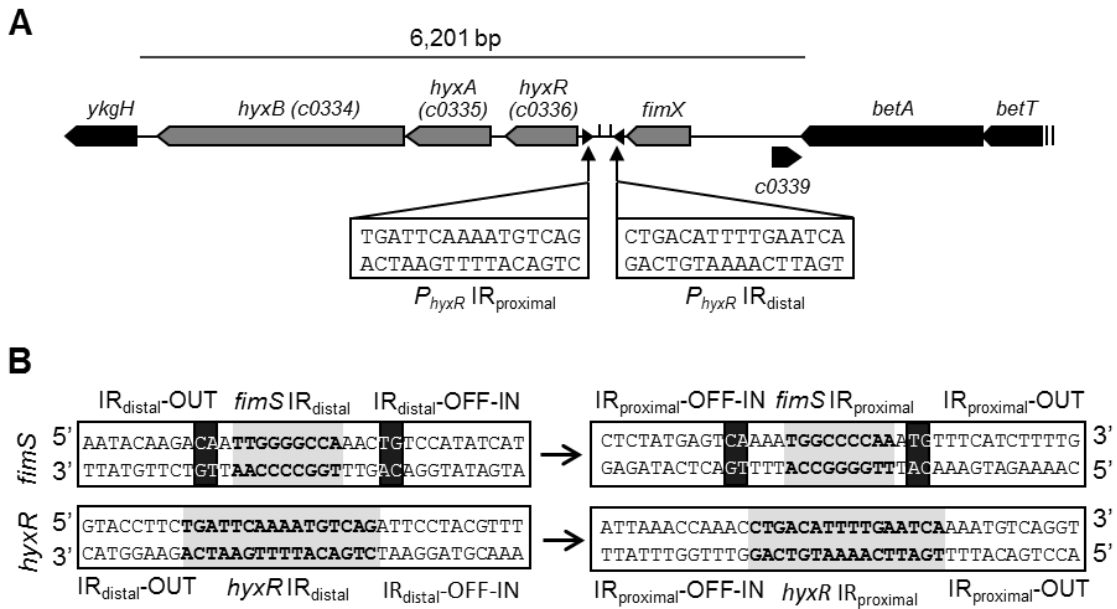


Figure 17: The *hyxR* 5' UTR is inverted at 16 bp inverted repeats.

A) Overview of the genomic organization of PAI-X_{UT189} and location of the *hyxR* inverted repeats. Directionality of the open reading frames, representing forward or reverse strand orientation, is shown. The 16 bp inverted repeats (►) for the *hyxR* 5' UTR (P_{hyxR} -IR_{proximal} and P_{hyxR} -IR_{distal}) are shown with the repeat sequence listed below. The NspI (closest to *hyxR*) and SspI (closest to *fimX*) restriction sites used to map the approximate region of inversion are indicated by vertical lines. B) The region shown is known to be important for FimB and FimE binding to *fimS* (Dove *et al.*, 1996; Gally *et al.*, 1996; Kulasekara *et al.*, 1999; Holden *et al.*, 2007). The conserved 5' CA dinucleotides of the *fimS* repeats are shown in white text on a black background. The 9 bp repeats upstream of *fimS* and the 16 bp repeats upstream of *hyxR* are shown in bold text on a gray background. Both sequences are shown in the 5' → 3' direction, although the *hyxR* sequence has been flipped to help align the proximal and distal repeats in the same orientation as the loci are on opposite strands.

The dissimilarity between the *hyxR* sites of phase inversion and the sites in the type 1 pili promoter region (Fig. 17B) raised questions about the actual sites of inversion for FimX-mediated phase variation of type 1 pili. We hypothesized that, like FimX homologs FimB and FimE, FimX-mediated inversion upstream of type 1 pili would occur at the same 9 bp inverted repeats, although this had not yet been experimentally demonstrated (Gally *et al.*, 1996; Holden *et al.*, 2007; Kulasekara & Blomfield, 1999). To determine inversion sites of FimX upstream of the type 1 pili operon, we used a similar approach as used to determine the sites of *hyxR* promoter inversion, performing PCR and sequencing the regions of inversion flanking the T1P promoter in strains segregated phase OFF or ON. We were able to confirm that FimX-mediated inversion of the *fimS* promoter occurs at the previously defined 9 bp inverted repeats (data not shown).

The 16 bp inverted repeats in the 5' UTR of *hyxR* have minimal similarity to the 9 bp inverted repeats centered 314 bp apart that are the sites of phase inversion for type 1 pili (Fig. 17B) (Abraham *et al.*, 1985; Abraham *et al.*, 1986). Previous *in vivo* footprinting analysis has defined 13 bp half-sites adjacent to the type 1 pili inverted repeats that serve as FimB and FimE binding sites (Gally *et al.*, 1996; Holden *et al.*, 2007; McCusker *et al.*, 2008). Comparison of the inverted repeats and half-site flanking sequence at the two loci shows that the sites of inversion, and analogous putative DNA binding sites upstream of *hyxR*, are dissimilar in length and sequence composition to the known binding sites upstream of T1P (Fig. 17B). Therefore, to ascertain the binding substrate of FimX using an unbiased approach, we used a bacterial-one-hybrid system to

assay the binding of a DNA-binding protein (FimX) with a DNA substrate (random library).

The system works on the principal that a DNA-binding protein fused to the alpha-subunit of RNA polymerase will activate expression of downstream reporter genes if the protein can bind to the DNA sequence inserted upstream of the reporter genes (Fig. 18B). We generated a random library of clones where 40 randomized nucleotides were cloned in the bait vector upstream of the reporter genes *his3* and *ura3*. The binding site library contained approximately 7.2×10^8 individual clones and was used to screen for FimX

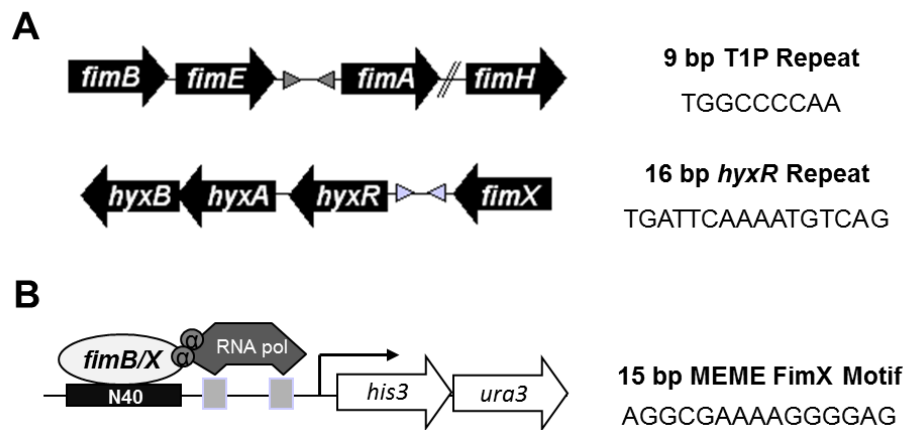


Figure 18: Investigation of the FimX binding site using a bacterial-one-hybrid system.

A) Comparison of the 9 bp inverted repeats present upstream of T1P and the 16 bp repeats present in the *hyxR* promoter. B) Schematic diagram of the bacterial-one-hybrid principle to elucidate the FimX binding site. Sequence analysis of activating clones were examined for over-represented motifs using a MEME algorithm, yielding a 15 bp motif present in FimX activating clones.

DNA binding sequences. As a control, the sequence for the T1P proximal inverted repeat in the OFF orientation plus flanking sequence (35 bp insert) was cloned into the bait vector and assayed for expression of the reporter genes after FimX expression. As expected, FimX expression resulted in activation of the reporter genes and growth of the strains on selective media (data not shown), suggesting that FimX was able to bind to the T1P proximal inverted repeat in the OFF orientation.

Clones that allowed FimX-mediated expression of the reporter genes were sequenced to determine which DNA sequences from the randomized prey library supported FimX binding. After sequencing clones that were capable of binding the target sequence, as evidenced by expression of the reporter genes *his3* and *ura3*, we ran MEME algorithms to identify over-represented motifs. Analysis of FimX binding yielded a 15 bp motif that bears similarity to the 16 bp inverted repeat upstream of *hyxR* (Fig. 18A and B). In addition to the similar length of the *hyxR* repeats and the bacterial-one-hybrid motif, both sequences also contain a core 4 bp homopolymeric stretch flanked to either side with several identical nucleotides. One caveat of this system, however, is that it identifies binding targets, but these sequences may be different than optimal inversion substrates.

4.2.5 Identification and Characterization of the FimX Protein Domains Responsible for DNA Specificity and Directionality

The disparate sites of T1P and *hyxR* inversion suggest that FimX may have dual sequence specificity, which significantly diverges from the functionality of the close homolog FimB. Therefore, we investigated the protein domains of FimX that are responsible for the differential binding of FimX, but not FimB, to the *hyxR* promoter. We hypothesize that there is a domain of FimX that is at least partly responsible for recognition and/or recombination at the *hyxR* promoter, distinct from the domains responsible for recognition/recombination at *fimS*. Therefore, we have constructed a

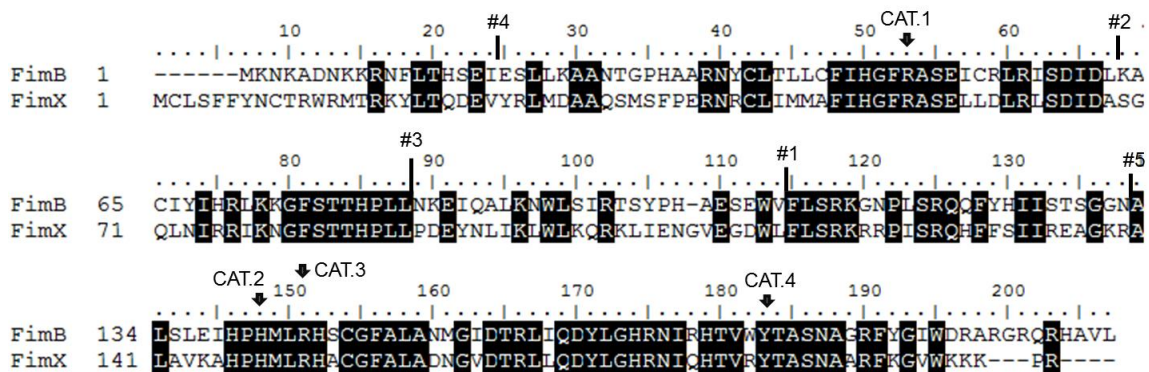


Figure 19: Comparison of FimB and FimX amino acid identity, noting catalytic point mutants and FimB:X chimera demarcations.

Amino acid sequences for FimB and FimX are from UTI89. The catalytic residues that, based on sequence conservation, are part of the catalytic motif (Arf, His-X-X-Arg, Tyr) are indicated with black downward-pointing arrows, and are labeled CAT.1-CAT.4. FimB::X chimeras were created between the N-terminal region of FimB and the C-terminal region of FimX. The boundary demarcation for chimeras #1 through #5 are indicated.

series of FimB::FimX chimeras to ascertain which regions of FimX are responsible for the recognition of the repeats upstream of both type 1 pili and *hyxR* (Fig. 19).

Five FimB:X chimeras were constructed that translationally fused various sized fragments of the N-terminal region of FimB in-frame to the more C-terminal region of FimX (Fig. 19). All construct residues refer to the FimX protein sequence, which has an annotated start site 6 amino acids prior to FimB.

FimB::X chimera #4 was the construct with the smallest N-terminal swap, replacing the most N-terminal 24 amino acids of FimX with FimB residues 1 through 18. Despite the small region altered, chimera #4 showed an approximately 50% reduction in functionality in switching *hyxR* from OFF to ON and almost no activity at switching *hyxR* from ON to OFF (Fig. 20A and B). Chimera #4 was completely non-functional at switching T1P from OFF to ON, suggesting that the N-terminal region of FimX may be important for this activity. FimB residues 1 through 18 did not confer FimB-like activity to chimera #4 as the chimera remained non-functional at switching T1P from ON to OFF (Fig. 20A and B). FimB::X chimera #2 and #3 are the next most conservative swaps, replacing up to FimX amino acid residue 67 or residue 88, respectively, with the N-terminal region of FimB (Fig. 19). Neither chimera #2 or #3 showed activity at switching *hyxR* or T1P in either direction (Fig. 20), suggesting that these chimeras may alter DNA binding domains or overall protein structure. Future work will be needed to discern if these constructs are expressed to the same extent as FimX, as a lack of protein stability would also account for the non-functional phenotype.

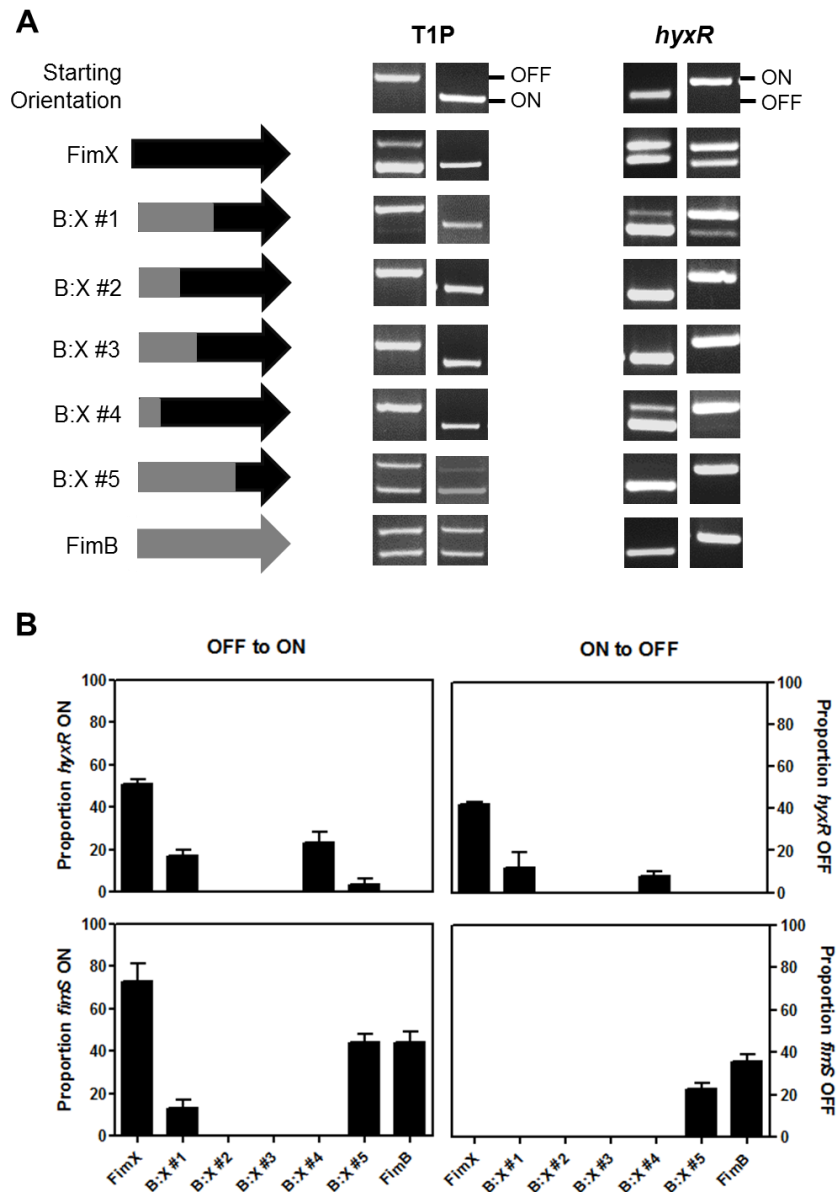


Figure 20: FimB::X protein chimera functionality.

A) FimB, FimX, and FimB::FimX chimeras were investigated for function by assessing inversion of T1P and *hyxR* in both directions. B) To quantify directional activity, densitometry was performed on PCR bands corresponding to phase OFF or phase ON. Subsequently, the proportion of the promoter in either orientation after expression of the various constructs was calculated. Results were quantitated based on three biological replicates in technical duplicate.

The small amino acid change from FimB:X chimera #1 to chimera #5 conferred gain-of-function activity of FimX in turning T1P from ON to OFF. The same small amino acid swap, however, completely abrogated function at *hyxR* in both directions (Fig. 20B). FimB:X chimera #1 (C-terminal FimX residues 115 through 120 fused to the N-terminal region of FimB) still retains activity at *hyxR*, suggesting that this small amino acid stretch (24 amino acid difference between chimera #1 and #5) may confer FimX specificity at *hyxR* and FimB ON to OFF specificity at T1P. Future work will be necessary to determine whether functional differences between constructs are at the level of DNA recognition, recombination, or protein stability.

Lambda integrase family members share a core, conserved catalytic motif contained within the C-terminal half of the protein: Arg, His-X-X-Arg, Tyr, with the tyrosine occurring near the most C-terminal end (Abremski & Hoess, 1992). Based on sequence homology to the catalytic tetrad, we created single alanine substitution mutants at each position (R53A, H148A, R151A, and Y183A) (Fig. 19). In tyrosine recombinase family members, the DNA cleavage event results in a DNA-protein intermediate with a transient covalent bond between the catalytic tyrosine and the cleaved DNA strand, hence, the naming convention. Therefore, we would predict that if FimX Y183 is indeed the catalytic tyrosine, FimX CAT.4 should be catalytically inactive, which would be equivalent to a non-functional recombinase in our phase PCR assay. All four individual catalytic mutants were analyzed for function at T1P and *hyxR*. Any single alanine

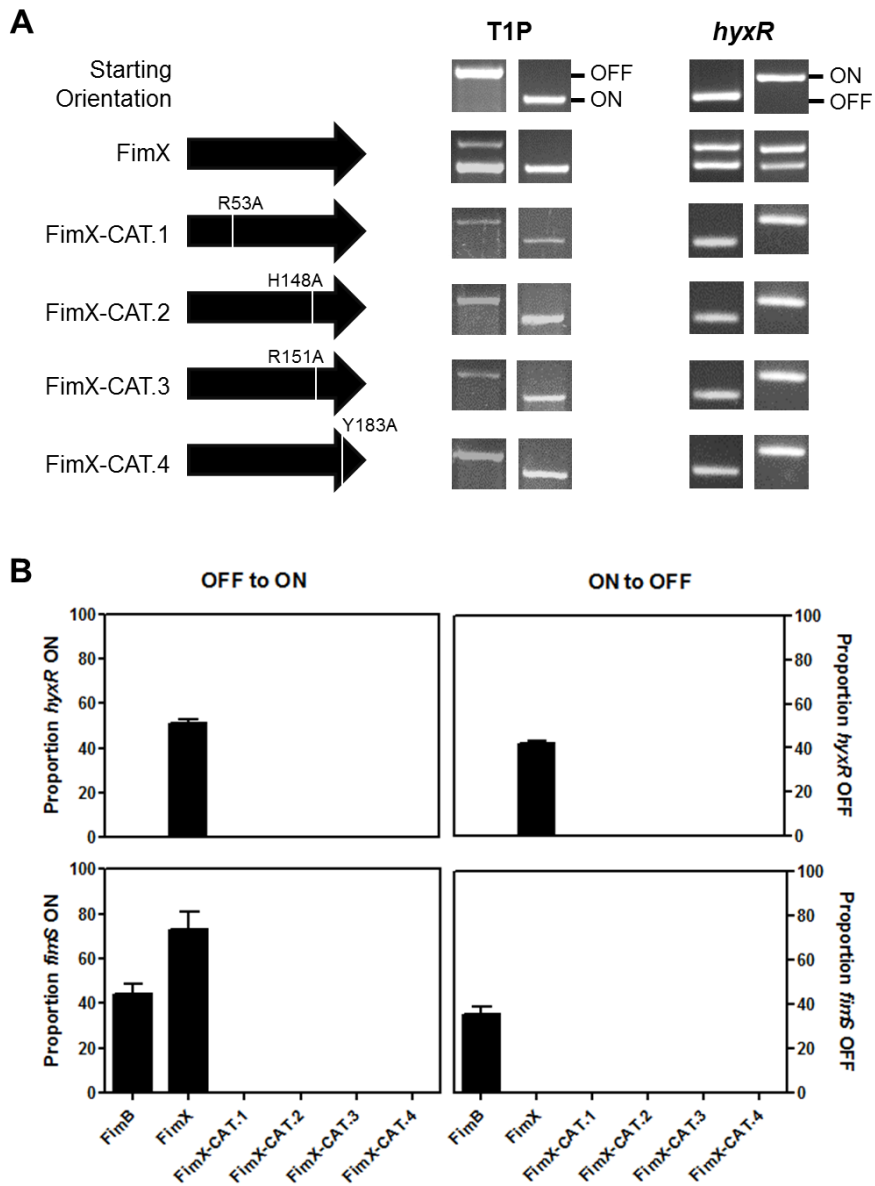


Figure 21: FimX catalytic point mutants are non-functional.

A) FimX catalytic point-mutants (R53A, H148A, R151A, and Y183A) were investigated for function by assessing inversion of T1P and *hyxR* in both directions. B) Quantification of directional activity was calculated based on densitometry as described in the previous figure legend.

substitution resulted in non-functional phase inversion at T1P, meaning that FimX was not longer able to mediate phase inversion of T1P from OFF to ON (Fig. 21A and B). Any single catalytic mutation also resulted in complete abrogation of FimX activity in both directions at *hyxR*, indicating that FimX expression no longer results in OFF to ON or ON to OFF inversion of the *hyxR* promoter. These results are consistent with the predicted, conserved catalytic motif residues mediating catalytic DNA strand exchange and recombination of T1P and *hyxR* by FimX. Future investigations will determine if, as expected, the functional defects are at the level of recombination and not DNA recognition or protein stability.

4.3 Discussion

Among prokaryotic epigenetic mechanisms, phase variation acts to couple environmental signals to rapid expression or repression of a specific factor, with one of the well-studied examples being T1P. The recombinases FimB and FimE, members of the lambda integrase superfamily, are preeminent examples of prokaryotic epigenetic regulation through phase variation. The Fim recombinase family members are the smallest members of the superfamily; yet, despite their small size, they still retain complex functions as site-specific invertases (Esposito & Scocca, 1997; Nunes-Duby *et al.*, 1998). Like other lambda integrase family members, FimB and FimE require the DNA bending factor IHF for phase inversion of the T1P promoter (Blomfield *et al.*, 1997; Dorman & Higgins, 1987; Eisenstein *et al.*, 1987). However, phase variation of the

hyxR promoter by FimX regulation does not require IHF, which deviates significantly from the canonical phase inversion precedence of other integrase superfamily members. Future studies will investigate whether other accessory factors, like LRP, have compensated for the roles of IHF and H-NS in phase inversion or whether there is something intrinsic about the *hyxR* promoter that alleviates the necessity for DNA-bending accessory factors. Unlike the T1P promoter region, P_{hyxR} phase variation is also independent from H-NS.

FimX expression produces phase inversion at the T1P and *hyxR* promoters, suggesting that FimX is responsible for mediating the observed inversion at both loci and that FimX may coordinately regulate cross-talk between the loci through divergent DNA binding specificities. However, the sites of inversion between the two invertible regions are significantly different in length and sequence composition. Our data to this point are sufficient to draw a strong association that FimX mediates direct interaction with each promoter region. Alternatively, FimX may regulate, either transcriptionally or through phase inversion, another factor that may be responsible for inversion at the *hyxR* promoter. This scenario seems unlikely given that FimX expression is capable of producing inversion at T1P and *hyxR* in a $\Delta fimBEX$ background in which no other FimB-like homologs are found (based on BLAST analysis for homologs in the UTI89 genome); however, at this time, we cannot rule out the possibility that FimX regulates another recombinase that is responsible for *hyxR* inversion.

Additional ongoing studies should provide direct biochemical evidence of FimX binding and define direct interactions between FimX and the DNA to model specificity. These studies will complement our continuing effort to define the FimX protein domain(s) responsible for recognition and directional recombination at both target loci. FimX interaction with these very different invertible elements would constitute a significant functional divergence between FimX and other lambda int homologs and run contrary to the site-specific precedence of this family of proteins.

Chapter 5. HyxR Suppresses the Nitrosative Stress Response and Intracellular Macrophage Survival in Extraintestinal Pathogenic *Escherichia coli*

5.1 Introduction

In humans, ExPEC may transition from benign occupation of the enteric and vaginal tracts to sterile sites such as the urinary tract, blood stream, and central nervous system (Orskov & Orskov, 1992b). To mount successful infections after breaching host barriers, ExPEC must circumvent, suppress, or tolerate the host innate immune system, including chemical stresses such as reactive nitrogen and oxygen species as well as cellular immune effectors. As ExPEC transitions from the enteric and urinary tracts, for instance, it may encounter professional phagocytes such as macrophages. Recent research suggests that the K1 serotype of ExPEC, a leading cause of neonatal meningitis (Gaschignard *et al.*, 2011; Harvey *et al.*, 1999), requires macrophages for dissemination to the central nervous system, spleen, and lungs (Mittal *et al.*, 2010b). The regulatory cues that provoke the commensal to pathogen transition and the factors required for survival in macrophages as a potential vehicle for disseminated infections are incompletely understood.

In the studies described in Chapter 4, we demonstrate that FimX expression produces unidirectional phase inversion of the T1P promoter and bidirectional inversion of the promoter for *hyxR*, a gene immediately adjacent to *fimX* and encoding a LuxR-like

response regulator. In this chapter, we demonstrate the consequence of HyxR expression is suppression of ExPEC tolerance to reactive nitrogen intermediate (RNI) stress. We further show that HyxR acts to suppress RNI-dependent and -independent intracellular survival during experimental infection of macrophage-like cells. Together, this study demonstrates the functional versatility of the FimX recombinase and identifies novel epigenetic and transcriptional regulatory controls for ExPEC survival under RNI and macrophage challenges.

5.2 Results

5.2.1 HyxR Suppresses RNI Tolerance In Vitro

After demonstrating that FimX provides epigenetic control over type 1 pili and *hyxR*, our next goal was to determine the functional consequences of HyxR expression on how ExPEC responds to environmental stresses encountered during infection. For instance, UTI infection results in recruitment of nitric oxide (NO)-producing professional phagocytes to the bladder, which constitutes a key host defense mechanism (Hang *et al.*, 1999; Haraoka *et al.*, 1999) and leads to a 3- to 50-fold increase in NO levels (Lundberg *et al.*, 1996; Poljakovic *et al.*, 2001). ExPEC may also encounter RNIs outside the urinary tract, possibly during passage through the gastrointestinal tract, where intestinal epithelial cells can secrete high levels of nitrate (NO₃⁻) and nitrite (NO₂⁻) (Kolios *et al.*, 1995), or during invasive infections such as meningitis, where ExPEC interacts with macrophages (Mittal *et al.*, 2010b), key cellular RNI producers. NO is a precursor to a variety of RNIs,

such as peroxyxynitrite, nitrous oxide, and nitrosothiols, which can lead to extensive damage of nucleic acids, lipids, and proteins. Compared to K-12 reference strains, most ExPEC isolates have increased resistance to acidified sodium nitrite (Bower *et al.*, 2009) and other NO generators (Svensson *et al.*, 2006).

To model RNI generation, we used acidified sodium nitrite or ASN, which has been used widely to generate RNI and test resistance to its effects *in vitro* (Bower *et al.*, 2009; Firmani & Riley, 2002). Sodium nitrite, when added to acidic media, is converted to nitrous acid, which spontaneously forms NO and other RNI. We investigated the tolerance of UTI89, an isogenic *hyxR* deletion, and a HyxR constitutive expression strain to nitrosative stress *in vitro*. Each strain was sub-cultured into MES acid-buffered LB (pH 5.0) with or without the addition of 3 mM ASN. All strains tested grew equally well in pH-matched control media lacking RNI (Fig. 22A); however, there were differences in growth rate and tolerance when the strains were exposed to 3 mM ASN (Fig. 22B). The *hyxR* deletion strain had a growth advantage under high RNI conditions that was reversible if HyxR was overexpressed (Fig. 22B). The consequence of constitutive HyxR expression was suppressed tolerance to RNI, whereas, conversely, mutation of *hyxR* resulted in a significant growth advantage to ExPEC under RNI stress modeled *in vitro*.

We hypothesized that if HyxR was acting, either directly or indirectly, as a repressor of RNI resistance, then HyxR expression might be regulated by nitrosative stress. To address this hypothesis, UTI89 was grown in the presence or absence (MES-LB control) of 3 mM ASN. Samples were taken at initial sub-culture, mid-log ($OD_{600} =$

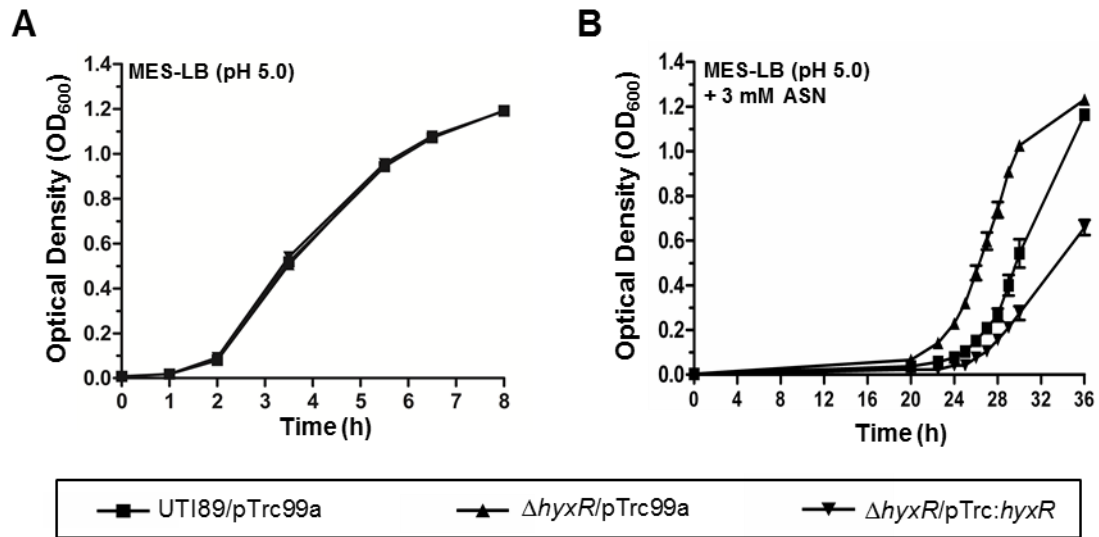


Figure 22: HyxR negatively regulates RNI tolerance *in vitro*.

A) Growth kinetics of UTI89, $\Delta hyxR$, and a constitutive $hyxR$ expressing strain in MES-LB (100 mM, pH 5.0). All strains grew equally well in pH-matched MES-LB. B) Growth kinetics of UTI89/pTrc99a, $\Delta hyxR$ /pTrc99a, and a constitutive $hyxR$ expressing strain in MES-LB (100 mM, pH 5.0) + 3 mM ASN.

0.5), and stationary ($OD_{600} = 1.2$) phase to assess the $hyxR$ promoter orientation by phase PCR. If HyxR represses RNI resistance, then we predicted that, upon challenge with RNI, $hyxR$ would be repressed; however, we did not know whether this would occur at the level of phase inversion or transcription. After growth in ASN, the invertible $hyxR$ promoter region was predominantly in the OFF orientation at the population level (Fig 23A, middle panel). The invertible T1P promoter did not undergo a significant shift in orientation following RNI exposure (Fig. 23A, top panel). To increase the basal $hyxR$ phase ON state from the start of the experiment, we grew UTI89/pBAD: $fimX$ with arabinose (Fig. 23A, bottom panel). As we expected, the $hyxR$ promoter showed a

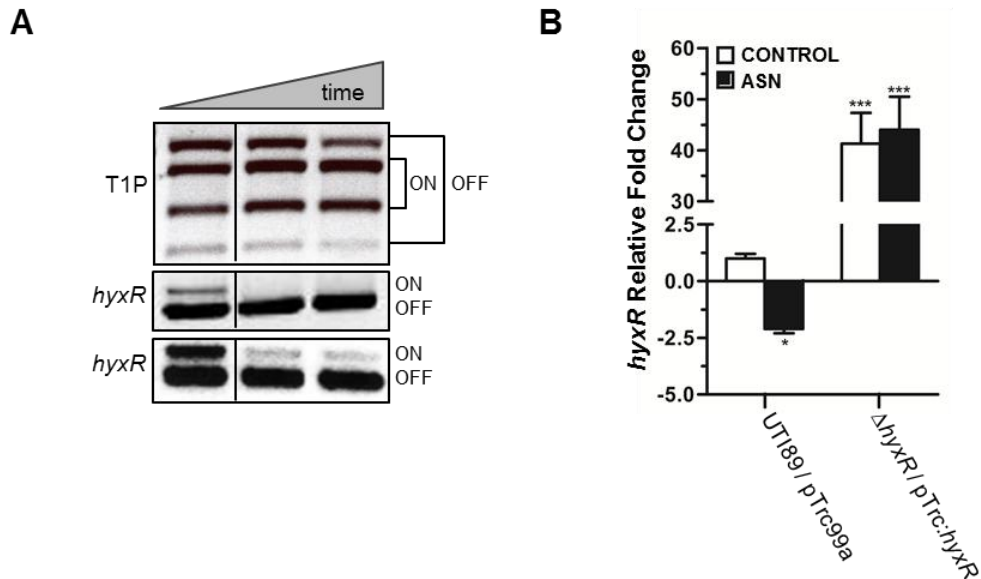


Figure 23: *hyxR* expression is responsive to RNI challenge.

A) T1P and *hyxR* promoter orientation in UTI89 (top two panels) post ASN challenge. Bottom panel shows UTI89/pBAD:*fimX* after arabinose induction of *fimX* expression, increasing the starting *hyxR* phase ON population prior to challenge with ASN. B) Transcript analysis of *hyxR* by qRT-PCR in each of the strains +/- ASN challenge. Transcript levels are shown as relative fold change using the $\Delta\Delta c(t)$ method and were normalized the expression of 16S rRNA with fold changes are shown relative to UTI89/pTrc99a (untreated control). * p-value < 0.05; *** p-value \leq 0.001.

more dramatic shift in the population from phase ON to phase OFF after exposure to ASN (Fig. 23A, bottom panel). Sampling at earlier time-points (1, 4, and 6 h post ASN challenge) indicated that the shift OFF of the *hyxR* promoter was not rapid (data not shown), suggesting that the phase OFF state may have a fitness benefit, but was not likely being actively inverted. Therefore, to investigate whether HyxR repression was occurring at the level of transcription, we quantified *hyxR* transcript levels by qRT-PCR with or without a 1 h exposure to ASN. At 1 h we did not observe phase inversion of the *hyxR*

promoter following ASN exposure, suggesting that any change in *hyxR* transcript levels by qRT-PCR were due to transcriptional or post-transcriptional regulation rather than phase inversion. Challenge of UTI89 with ASN resulted in a 2.1-fold reduction in *hyxR* transcript relative to the no ASN control (p-value = 0.0081, Student's t test) (Fig. 23B), which was statistically indistinguishable from RNA collected from the negative Δ *hyxR* genetic control (p-value = 0.4636). As expected, expression of HyxR from *P_{trc}* *in trans* resulted in a ~40-fold up-regulation of *hyxR* transcript levels compared to UTI89 wild-type under control conditions (p-value < 0.0004, Student's t test). However, *hyxR* transcript levels did not vary with ASN challenge when driven by *P_{trc}*, suggesting that regulation of *hyxR* by RNI occurs at the transcriptional level (Fig. 23B). Together, these data indicate that HyxR represses RNI tolerance *in vitro* and that nitrosative challenge results in suppression of *hyxR* transcript levels.

5.2.2 HyxR Represses Hmp, a Key Bacterial Nitric Oxide (NO) Detoxification Enzyme

We sought to determine the mechanism through which HyxR controls tolerance to RNI. We hypothesized that HyxR may regulate the expression of the NO detoxification flavohemoglobin called Hmp (see Section 1.3.2 for detailed description). As shown in Figure 24, *hmp* transcription under non-RNI conditions was similar between the wild-type and *hyxR* deletion strains. However, *hmp* transcript levels were repressed when measured

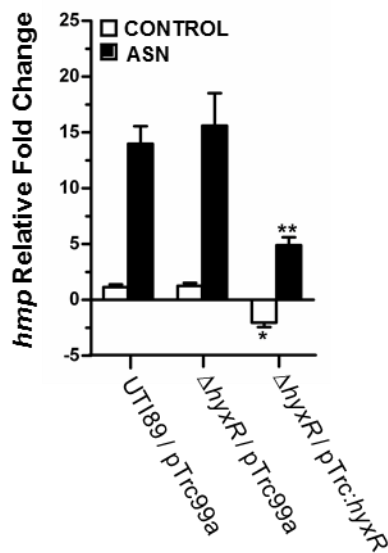


Figure 24: HyxR represses transcript of a key bacterial NO detoxification enzyme, HmpA.

hmpA transcript levels were measured by qRT-PCR in the indicated strains +/- exposure to 3mM ASN for 1 h. Transcript levels are shown as relative fold change using the $\Delta\Delta$ c(t) method and were normalized the expression of 16S rRNA with fold changes are shown relative to UTI89/pTrc99a (untreated control). * p-value < 0.05; ** p-value \leq 0.01.

under the same conditions with UTI89 constitutively expressing HyxR (2.0-fold decrease; p-value = 0.0154, one-sample t test). Transcript levels of *hmp* were subsequently measured in each strain after growth in 3 mM ASN to induce RNI stress. After 1 hr growth in ASN, the UTI89 wild-type and *hyxR* deletion strains had significantly increased levels of *hmp* transcript (14.0 and 15.6 fold, respectively; p-value < 0.0001 for both strains compared to UTI89/pTrc99a without ASN, Student's t test) (Fig. 24). Constitutive expression of HyxR in ASN growth conditions resulted in significantly lower *hmp* transcript compared to either the wild-type or *hyxR* deletion strains (5-fold up-regulation under ASN; p-value < 0.02 for both comparisons, Student's t

test) (Fig. 24). These data indicate that HyxR is a repressor of *hmp* *in vitro* with and without RNI stress.

We predicted that HyxR control over *hmp* transcription would have a major impact on the tolerance of ExPEC to RNI stress. The growth dynamics of UTI89 wild-type and isogenic derivatives with combinations of *hmp* and *hyxR* deletions and HyxR expression *in trans* were measured in media with and without RNI stress. All strains grew equally well in pH-matched MES-LB without ASN (Fig. 25A). In media containing

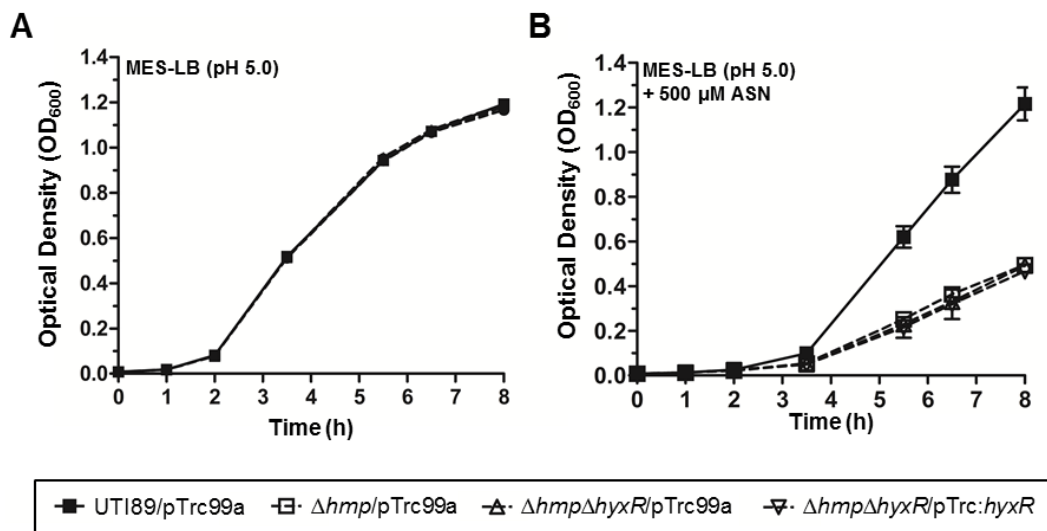


Figure 25: HyxR suppression of *hmp* expression correlates with a defect in growth under RNI challenge.

A) Growth kinetics UTI89/pTrc99a, Δhmp /pTrc99a, $\Delta hmp\Delta hyxR$ /pTrc99a, and $\Delta hmp\Delta hyxR$ /pTrc:*hyxR* in MES-LB (100 mM, pH 5.0). All strains grew equally well in pH-matched MES-LB. B) Growth kinetics of *hmp* and *hyxR* deletion derivatives in MES-LB (100 mM, pH 5.0) + 3 mM ASN.

ASN, strains carrying a deletion in *hmp* had a significant lag in growth (Fig. 25B).

Constitutive expression of HyxR did not produce an Hmp-independent effect on RNI tolerance by UTI89, suggesting that it is through regulation of *hmp* that HyxR exerts its major effect on RNI tolerance (Fig. 25B).

We next assessed if HyxR regulation of *hmp* altered the overall detoxification of RNI by ExPEC strain UTI89. Strains in early logarithmic growth were challenged with 500 μ M ASN (Fig. 26A). At varying times following ASN challenge, nitrite

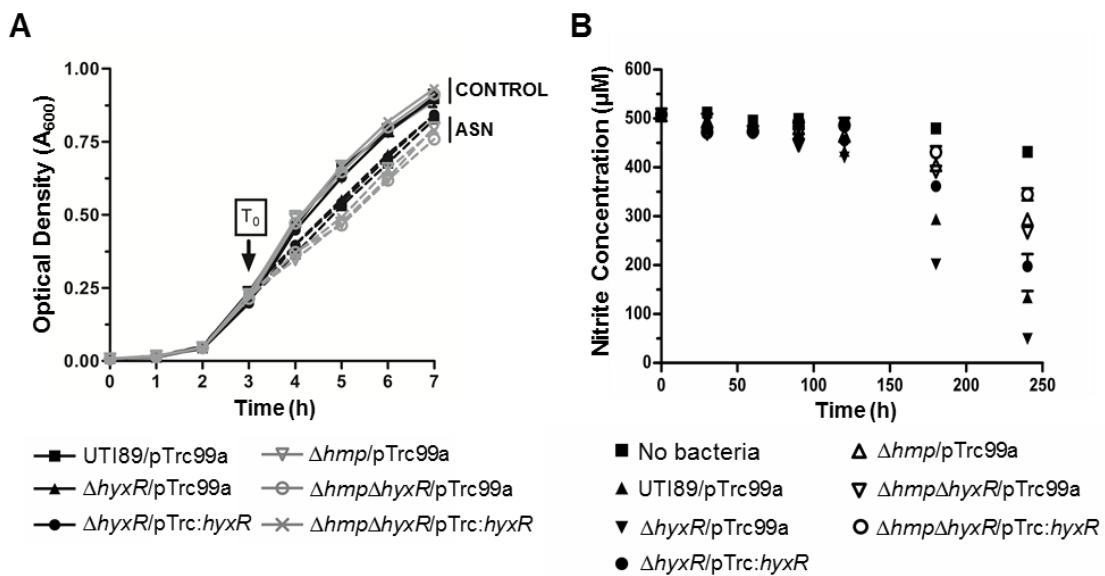


Figure 26: Constitutive *hyxR* expression results in a defect in detoxification of RNI, which is at least partially dependent on Hmp.

A) Strains were grown to mid-log (3 h). At an approximate $OD_{600} = 0.25$, 500 μ M ASN was added to half of the cultures. Growth was monitored for the duration of the experiment at indicated time-points. B) At T_0 (addition of ASN) and subsequent indicated times, aliquots of the cultures were taken, centrifuged to remove bacteria, and assayed for nitrite levels using Greiss Reagent.

concentrations were determined. In the absence of bacteria to consume NO, nitrite levels remained essentially constant at ~500 μ M (Fig. 26B). All of the strains carrying deletions in *hmp*, including those with or without HyxR expression, had significant defects in detoxification of RNI as measured as higher nitrite level relative to UTI89/pTrc99a (Fig. 26B). In the wild-type and the single *hyxR* deletion backgrounds, detoxification was reciprocal to the levels of HyxR expression: The strain with constitutive HyxR expression had the highest nitrite levels while the non-complemented *hyxR* deletion strain had the lowest levels of nitrites (Fig. 26B).

5.2.3 HyxR Suppresses Intracellular Survival in Macrophages through RNI-Dependent and -Independent Mechanisms

The apparent role of HyxR in regulating RNI resistance *in vitro* led us to investigate whether this system has a role during intracellular survival in macrophage-like cells. This question is particularly relevant given evidence that *E. coli* potentially traffics within macrophages during disseminated infection (Mittal *et al.*, 2010b). UTI89 and a single *hyxR* deletion strain, both carrying a plasmid-encoded GFP marker, were assayed for intracellular survival at 24 h post-infection in untreated RAW 264.7 cells. Under control conditions, UTI89 and Δ *hyxR* were competent for intracellular survival in macrophage-like cells to a similar extent (Fig. 27A). At 24 h post-infection, the intracellular bacterial burden was visualized by epifluorescent microscopy. Both strains produced large intracellular collections of bacteria in the RAW 264.7 cells (Fig. 27B),

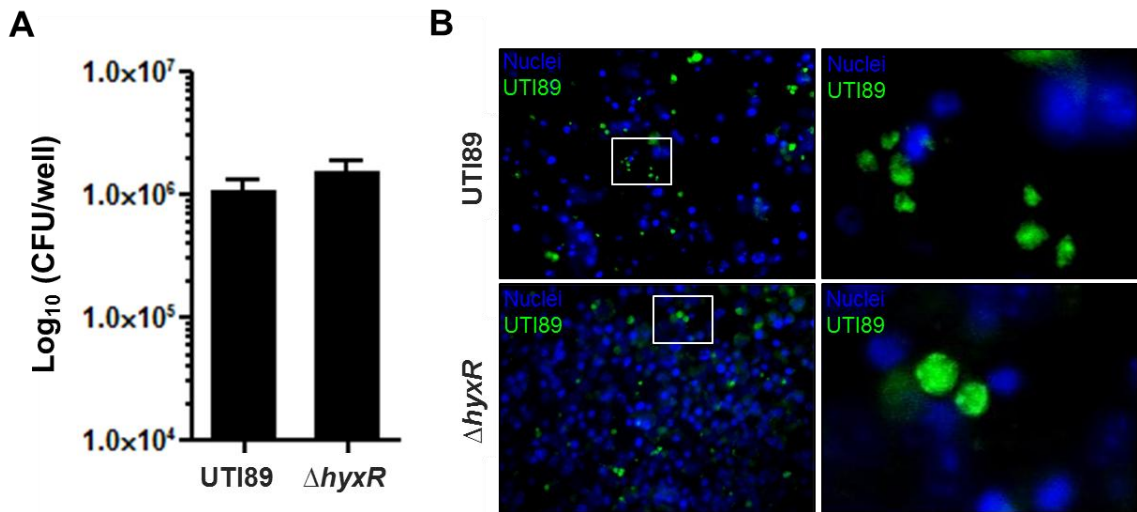


Figure 27: RAW 264.7 murine macrophage-like cells support intracellular ExPEC bacterial communities.

A) Bacterial burden, measured as CFU/well, of UTI89 and $\Delta hyxR$ at 24 h post-infection. B) Epifluorescent microscopy of GFP-expressing UTI89 and derivatives after 24 h Gentamicin-protection infection. Blue indicates nuclei by DAPI staining, green indicate bacteria.

suggesting that macrophage-like cells support large intracellular communities of ExPEC bacteria. UTI89 and $\Delta hyxR$ produce robust intracellular bacterial burdens that are quantitatively and qualitatively similar in un-stimulated macrophage-like cells.

To investigate the role of HyxR in regulating RNI resistance, RAW 264.7 murine macrophage-like cells were pre-incubated with either L-arginine, an NO precursor, or L-NAME, an inducible NO synthase (iNOS)-specific inhibitor, to yield high and low NO physiological states, respectively. Macrophage cells were then infected with UTI89 and isogenic derivatives to determine the contribution of HyxR in responding to RNI and intracellular stress *in vivo* as shown schematically in Fig. 28A.

Paralleling our *in vitro* results, we found that HyxR played a significant role in repressing intracellular survival. A *hyxR* deletion strain showed an increase in

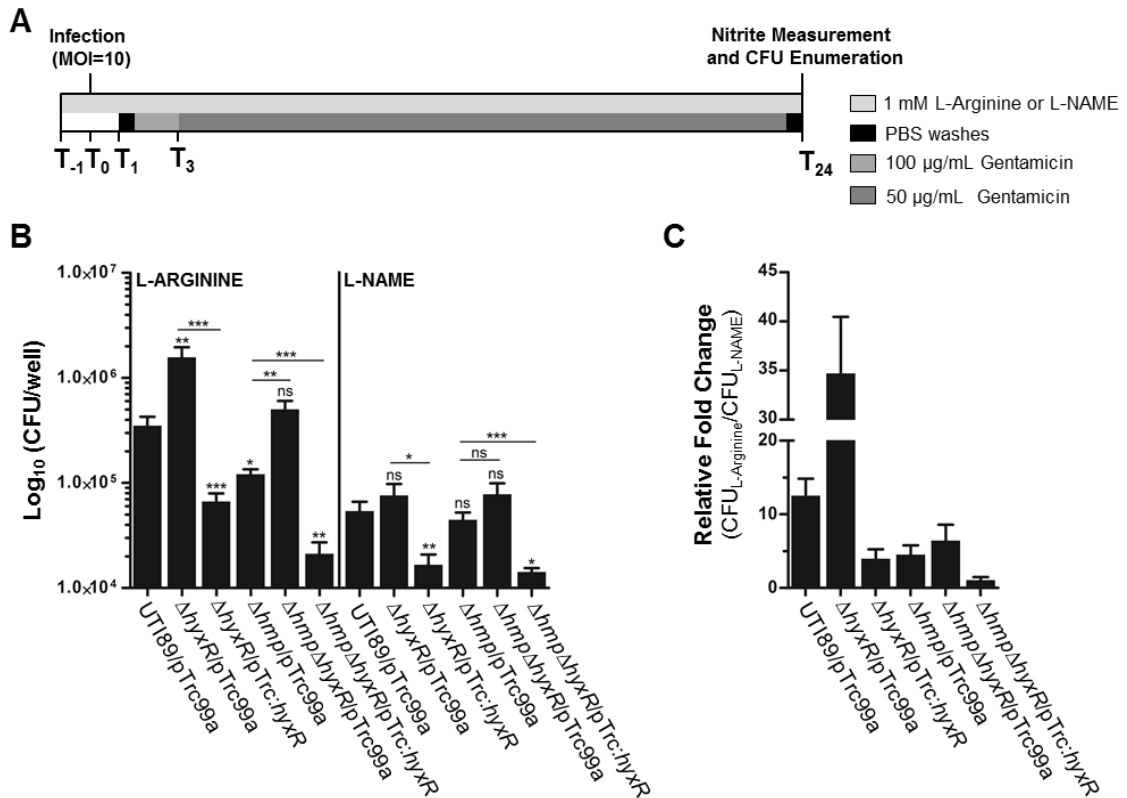


Figure 28: HyxR negatively regulates intracellular survival in macrophages through RNI-dependent and -independent mechanisms.

A) Schematic diagram of the experimental design and treatment regimen. B) RAW 264.7 cells were pre-treated with 1 mM L-arginine, an NO precursor, or L-NAME, an iNOS-specific inhibitor 1 h prior to infection and for the duration of the experiment. Infections were allowed to proceed with gentamicin treatment for 24 hours, at which time, bacterial counts were determined. Statistical significance indicated as follows: ns = not significant; * p-value < 0.05; ** p-value ≤ 0.01; *** p-value ≤ 0.001. C) Relative fitness of different strains under high NO conditions by assessing the ratio of the bacterial burden at 24 h for each strain between a high NO condition (L-arginine) and a low NO condition (L-NAME).

intracellular bacterial burden compared to the wild-type strain (p-value = 0.0043, Student's t test), while the HyxR overexpression strain showed a reduced capacity for intracellular survival (p-value \leq 0.001 compared with UTI89/pTrc99a or $\Delta hyxR$ /pTrc99a; Student's t test) (Fig. 28B). The intracellular survival defect was partially dependent on RNI generation as constitutive expression of HyxR still produced a significant survival defect even under low RNI conditions (p-value = 0.0079 for UTI89/pTrc99a vs. $\Delta hyxR$ /pTrc:*hyxR* under L-NAME treatment, Student's t test) (Fig. 28B). Unexpectedly, all three UTI89 derivatives had higher intracellular survival rates in macrophages in the presence of elevated RNI (L-Arginine treated) compared to the low RNI state (L-NAME treated) (Fig. 28B, compare left and right panels). Under conditions to stimulate NO production, the survival advantage of the non-complemented *hyxR* deletion was most evident (Fig. 28C).

Given our previous work showing the cross-regulation between type 1 pili and PAI-X, we tested whether *hyxR* deletion or overexpression had an impact on type 1 pili expression and swimming motility, which could have a large impact on the outcome of intracellular infections. Neither the single *hyxR* deletion nor *hyxR* overexpression impacted the expression of type 1 pili or motility (Fig. 29A and B), increasing our confidence that the HyxR-dependent intracellular survival is due to non-type 1 pili effects.

Our previous results indicating that HyxR suppression of RNI tolerance *in vitro* is dependent on Hmp led us to investigate the contribution of Hmp to growth during

macrophage infections in the context of HyxR deregulation. A single *hmp* deletion showed a small but significant reduction in intracellular survival under high RNI conditions (p-value = 0.0187 compared to UTI89/pTrc99a, Student's t test) that was completely restored under low RNI conditions (p-value = 0.6498 compared to

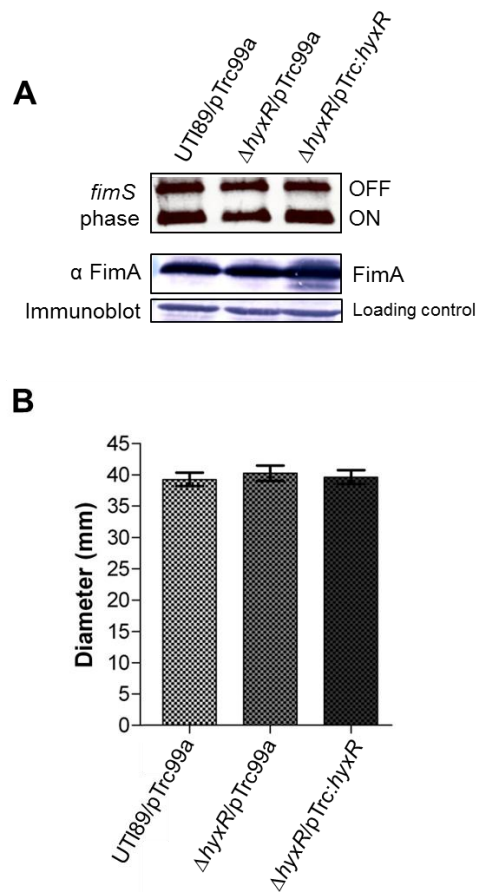


Figure 29: Type 1 pili expression and swimming motility by *hyxR* derivatives.

A) T1P expression by *hyxR* derivatives by phase PCR and western blot against the major T1P subunit FimA. B) Swimming motility as measured by zone diameter on motility agar. None of the comparisons were significantly different.

UTI89/pTrc99a, Student's t test) (Fig. 28B). Under high RNI conditions, the $\Delta hyxR\Delta hmp$ double deletion strain showed a significant increase in intracellular bacterial burden compared to the single *hmp* deletion strain (p-value = 0.0012, Student's t test), while the HyxR overexpression strain still demonstrated a reduced capacity for intracellular survival in the *hmp* deletion background (p-value \leq 0.0001 compared to $\Delta hmp/pTrc99a$, Student's t test) (Fig. 28B). The intracellular survival defect due to constitutive HyxR expression was still evident even in the *hmp* deletion background even under low RNI conditions (p-value = 0.0002 compared to $\Delta hmp/pTrc99a$, Student's t test) (Fig. 28B, right panel). Despite lacking the major NO detoxifying enzyme Hmp, $\Delta hmp/pTrc99a$ still showed a relative fitness advantage under high RNI conditions compared with L-NAME treatment conditions (4.53-fold) (Fig. 28C). Overexpression of HyxR in the context of the *hmp* deletion ablated the relative fitness benefit of high RNI (1.08-fold) (Fig. 28C), again, suggesting that HyxR controls RNI-dependent, but Hmp-independent pathways.

We next quantified NO in the supernatant of infected macrophages to begin to elucidate whether HyxR-dependent, Hmp-mediated detoxification of NO was occurring during intracellular growth in macrophages. Supernatants of cells infected with $\Delta hyxR/pTrc99a$ contained a reduced amount of NO (measured as nitrite) compared with infection with UTI89/pTrc99a (Fig. 30A). Infection with $\Delta hyxR/pTrc:hyxR$ produced NO significantly greater than the non-complemented deletion or UTI89 wild-type and a level comparable to all of the strains carrying *hmp* deletions (Fig. 30A). These data suggest that the levels of NO in the macrophage supernatant reflect detoxification of NO by Hmp

and the degree of repression of *hmp* by HyxR. Alternatively, the HyxR expressing strain could have altered induction of NO through iNOS. Therefore, to test that hypothesis we assessed the ability of UTI89/pTrc99a, $\Delta hyxR$ /pTrc99a, and $\Delta hyxR$ /pTrc:*hyxR* to stimulate NO generation after heat-inactivation or inhibition of protein synthesis. Suppression of NO production by the low *hyxR* expression strains required metabolically active bacteria, as heat-inactivation and inhibition of protein synthesis abrogated the

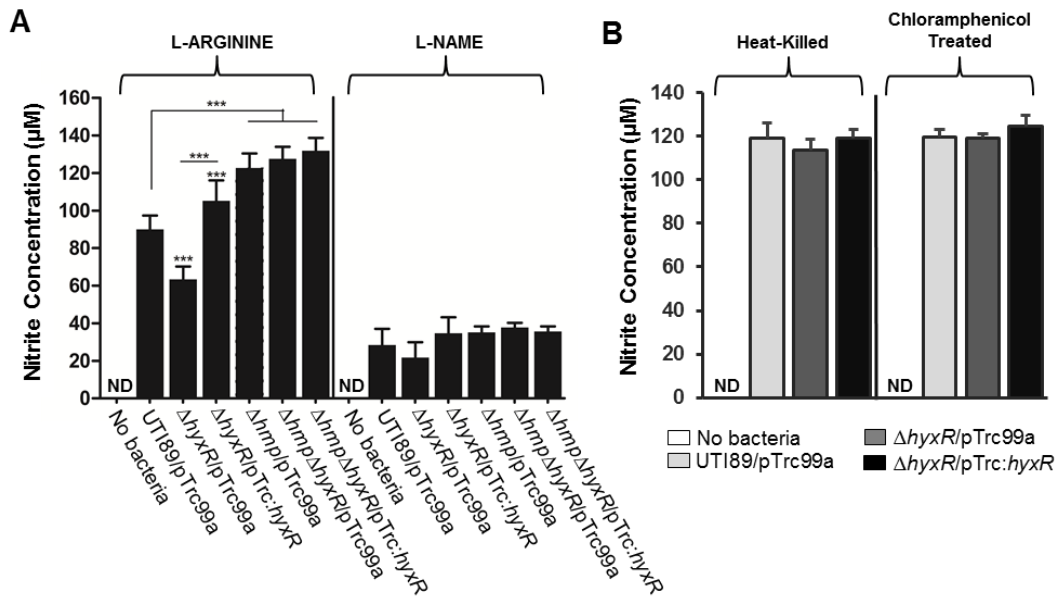


Figure 30: HyxR-dependent NO generation by macrophages is dependent on Hmp.

A) Cell-free supernatant nitrite levels were measured from the supernatant of infected RAW 264.7 cells using Greiss Reagent (see Chapter 2 for detailed methods). Asterisk (***) represents a p-value ≤ 0.001 . B) Nitrite levels were measured from the supernatant of RAW 264.7 cells infected with heat-killed (80°C for 15 min) or arrested (inclusion of 20 $\mu\text{g ml}^{-1}$ chloramphenicol to inhibit protein synthesis) bacteria using Greiss Reagent. No comparisons reached statistical significance. Abbreviations: ND, not detected.

differences between the HyxR high and low expression strains (Fig. 30B). Together, we have shown that HyxR is able to repress *hmp* *in vitro* both at baseline and upon exposure to nitrosative stress; however, HyxR regulation of *hmp* is unable to fully explain the observed HyxR-dependent intracellular survival defect under high RNI conditions.

5.2.4 HyxR-Dependent and –Independent Intracellular Survival and Nitric Oxide Consumption in IFN γ -Activated Macrophages

As another physiologically-relevant condition with high NO induction, we looked at the ability of *hyxR* derivatives for intracellular survival in IFN γ -treated macrophages. Figure 31A shows a schematic of the treatment and sampling protocol. IFN γ produced an ~1 log decrease in the bacterial counts for each of the strains (Fig. 31B and C), to a level similar to that observed previously under L-NAME-treated conditions (Fig. 28B). Of all the strains, constitutive expression of HyxR produced the lowest survival in IFN γ -treated and untreated cells (Fig. 31B and C). The *hyxR* deletion strain showed the largest difference between untreated and treated conditions (Fig. 31D).

We next quantified NO in the supernatant of infected macrophages that had been pre-activated with IFN γ treatment or no treatment as a control. Uninfected, IFN γ -treated macrophages showed a high level of basal NO induction (~78 μ M; Fig. 32). Upon infection, NO levels were suppressed to some extent by all of the strains relative to the IFN γ -treated, uninfected control (Fig. 32). However, constitutive expression of HyxR continued to result in higher relative NO levels as seen in previous experiments (Fig. 32).

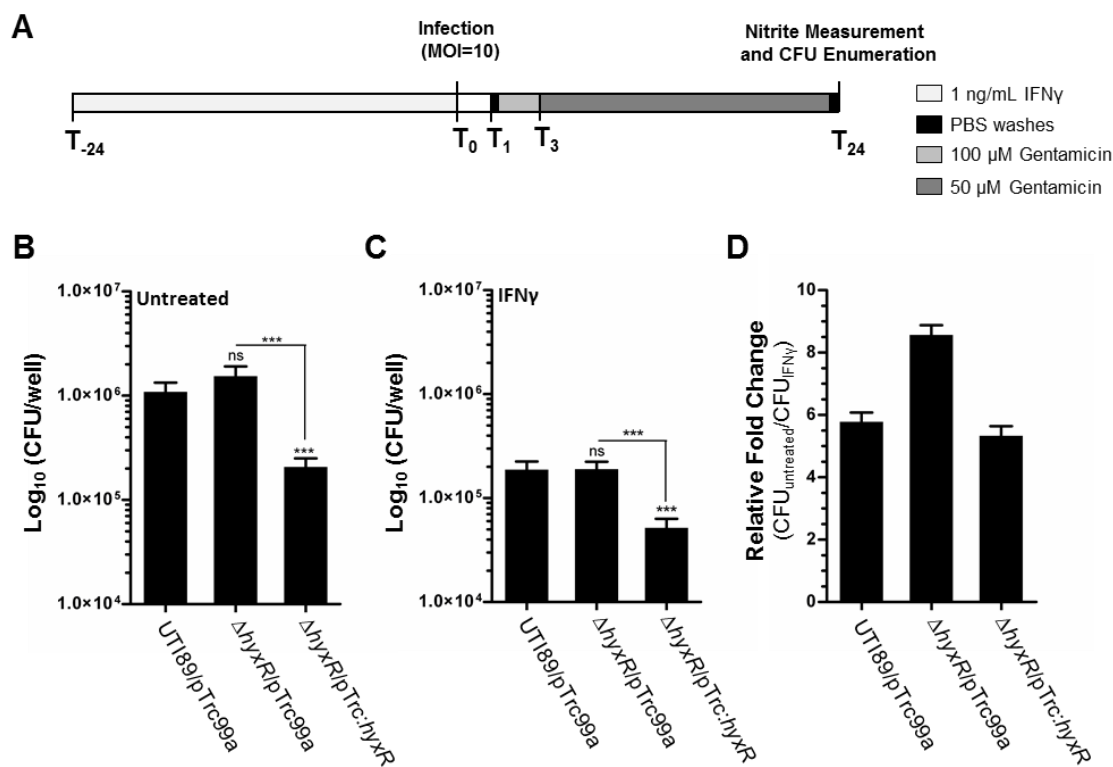


Figure 31: HyxR negatively regulates intracellular survival in IFN γ -activated macrophages through RNI-dependent and -independent mechanisms.

A) Schematic diagram of the experimental design and treatment regimen. B and C) RAW 264.7 cells were not treated (B) or treated with 1 ng ml⁻¹ IFN γ (C) 18 h prior to infection. Infections were allowed to proceed under gentamicin-protection for 24 h, at which time, bacterial burden was determined. Statistical significance indicated as follows: ns = not significant; * p-value < 0.05; ** p-value \leq 0.01; *** p-value \leq 0.001. D) Relative fitness of different strains in activated macrophages by assessing the ratio of the bacterial burden at 24 h for each strain between IFN γ -activated (C) and unactivated (B) macrophages.

IFN γ was able to mediate some modest control of the bacterial burden, as relative fitness was higher in untreated cells (Fig. 31D). We still observed a HyxR-dependent survival defect in IFN γ -treated cells, again suggesting HyxR may control other pathways contributing to intracellular survival (Fig. 31C).

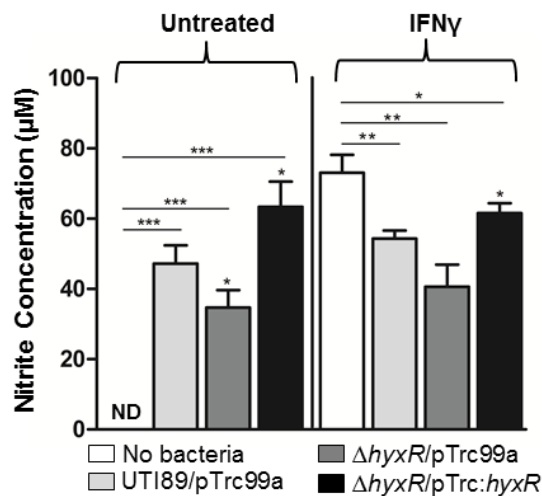


Figure 32: ExPEC actively suppresses NO production by IFN-activated macrophages through HyxR-dependent and -independent mechanisms.

Nitrite levels were measured in the supernatant of infected RAW 264.7 cells using Greiss Reagent. Statistical significance indicated as follows: * p-value < 0.05; ** p-value \leq 0.01; *** p-value \leq 0.001. Error bars indicate standard deviation. Abbreviations: ND, not detected.

5.3 Discussion

Through regulation of *hyxR*, FimX controls tolerance to RNI in ExPEC. A major mechanism through which the FimX-HyxR axis regulates RNI tolerance is by HyxR-mediated repression *hmp* transcription. Although Hmp is a key NO consumption enzyme under negative regulation by HyxR *in vitro* and *in vivo*, Hmp does not appear to play a significant role in intracellular survival in macrophages alone or during HyxR suppression of intracellular persistence. As evident in Figure 28B, Hmp has only a small role in intracellular survival in macrophages under high RNI conditions without strict correlation between detoxification of RNI and intracellular survival. Independent of

Hmp, HyxR has substantial control over ExPEC intracellular survival. Future studies will be aimed at investigating the level of HyxR control of *hmp* expression, either direct or indirect, and investigating the effectors of HyxR-mediated suppression of RNI-independent intracellular survival.

HyxR is not a virulence determinant in the classical sense, as deletion does not cause attenuation. From the perspective of intracellular survival in macrophages, HyxR appears to suppress virulence. Other anti-virulence or virulence-modulating factors are known in other pathogenic organisms, such as the transcription factor Lrp in *S. Typhimurium* (Baek *et al.*, 2009), the ACE2 transcription factor in *Candida glabrata* (Kamran *et al.*, 2004), and the RcsC/YojN/RcsB phosphorelay system in *S. Typhimurium* (Cano *et al.*, 2001; Mouslim *et al.*, 2004). Deletion of the genes for these virulence moderators leads to increased proliferation, invasion, and, sometimes, immune response compared to the wild-type isogenic parent. Lrp, for instance, is known to transcriptionally repress key virulence genes, indicating that proper regulation of Lrp expression in the host environment is central to *Salmonella* pathogenesis (Baek *et al.*, 2009). Similarly, our data suggest that proper regulation of the presumptive HyxR regulon is important for intracellular survival in macrophages, as a constitutive HyxR-expressing strain shows RNI-dependent and -independent effects on intracellular survival.

The macrophage is increasingly becoming known as an important cellular niche for K1 *E. coli* establishment of distal invasive sites of infection, including the brain, spleen, liver, and kidney in animal models infected intranasally (Mittal *et al.*, 2010b). It

may then be beneficial to ExPEC to evolve multiple regulatory systems to tightly control the expression of RNI tolerance systems in response to certain environmental stimuli or host niches. It is also interesting that FimX appears to play a pivotal role in regulating both T1P and *hyxR*, suggesting coordinated regulation between a major adherence/invasion factor and the nitrosative stress response. Proper phase variable expression of ExPEC T1P in the bloodstream is important for binding and invasion of human brain microvascular endothelial cells (Teng *et al.*, 2005) and establishment of bacteremia (Smith *et al.*, 2010; Xie *et al.*, 2006a). Epigenetic control by a factor like FimX may coordinate pilus expression with the physiologic alterations necessary to prepare a bacterium for host cellular entry and persistence. In host sites where ExPEC may encounter professional macrophages or high RNI concentrations, constitutive expression of HyxR has a survival cost to ExPEC.

Chapter 6. Concluding Remarks and Future Directions

6.1 Summary

In summary, we have shown that FimX, a regulator of the major virulence factor type 1 pili, is able to coordinately regulate an additional target through epigenetic phase variation. This previously unrecognized ExPEC-associated target, HyxR, can suppress the nitrosative stress response and contributes to intracellular survival in macrophage-like cells. Combined, these data indicate that 1) FimX is an important ExPEC-associated regulator that epigenetically regulates *hyxR* and that 2) the ability of ExPEC to survive RNI-mediated stresses within macrophages is contingent upon the proper regulation of HyxR, a negative regulator of RNI response pathways. We have shown that HyxR represses transcription of *hmp*, which encodes a major RNI detoxification enzyme and that without appropriate relief of HyxR repression, ExPEC is severely attenuated under RNI stress *in vitro* and *in vivo* during intracellular persistence in macrophage-like cells. In summary, we have used a combination of genetic, biochemical, and *in vivo* techniques to begin to evaluate the regulation of *hyxR* by FimX and its role in the regulation and coordination of ExPEC virulence.

6.2 Current Model

Pathogens like ExPEC have complex regulatory networks to coordinate the transition between commensal and pathogenic states and to maximize survival during

their engagement with the host. The transition from commensal to pathogen must be tightly controlled since premature induction of virulence may have significant costs; therefore, to keep tight regulatory control over this transition, a hierarchy of regulatory controls may be employed, including epigenetic, transcriptional, and post-transcriptional regulation. Many pathogenic organisms have acquired unique genomic segments associated with novel regulatory inputs, such as acquiring additional Fim recombinases to control the expression of type 1 pili. Alternatively, many global regulators are part of the “core genome,” meaning they can be found in the genomes of related commensal organisms, but may have altered functionality in pathogenic isolates.

In the case of T1P, phase inversion provides a mechanism to rapidly transition between piliated and non-piliated states during the progression of ExPEC through different host niches. While T1P are critical for adherence to and invasion into the bladder epithelium during UTI (Anderson *et al.*, 2003; Mulvey *et al.*, 1998), the expression of T1P in the bloodstream can result in opsonization by phagocytes (Silverblatt & Ofek, 1983; Weinstein & Silverblatt, 1983) and rapid clearance (Xie *et al.*, 2006a). Phase variation through the Fim recombinases provides a rapid and complete switch needed for the transition. Phase orientation is also a population dynamic, meaning that a minority population can be in alternative state that may be favored once the transition is made to a new host niche. For instance, non-type 1 piliated bacteria fare poorly in the bladder; however, that minority population of cells would be primed for survival once transitioned to the bloodstream.

The results presented in Sections 2.1 and 2.2 in Chapter 4 demonstrate that T1P and PAI-X can be coordinately regulated through epigenetic and transcriptional mechanisms by the Fim-like recombinases FimB and FimX (Fig. 33). It is interesting that FimX appears to play a pivotal role in regulating both T1P and *hyxR*, suggesting coordinated regulation between a major adherence/invasion factor and the nitrosative stress response. FimX may enhance adherence and invasion further by up-regulating *fimB* through an as yet unknown mechanism. FimB, in turn, appears to cross-regulate PAI-X

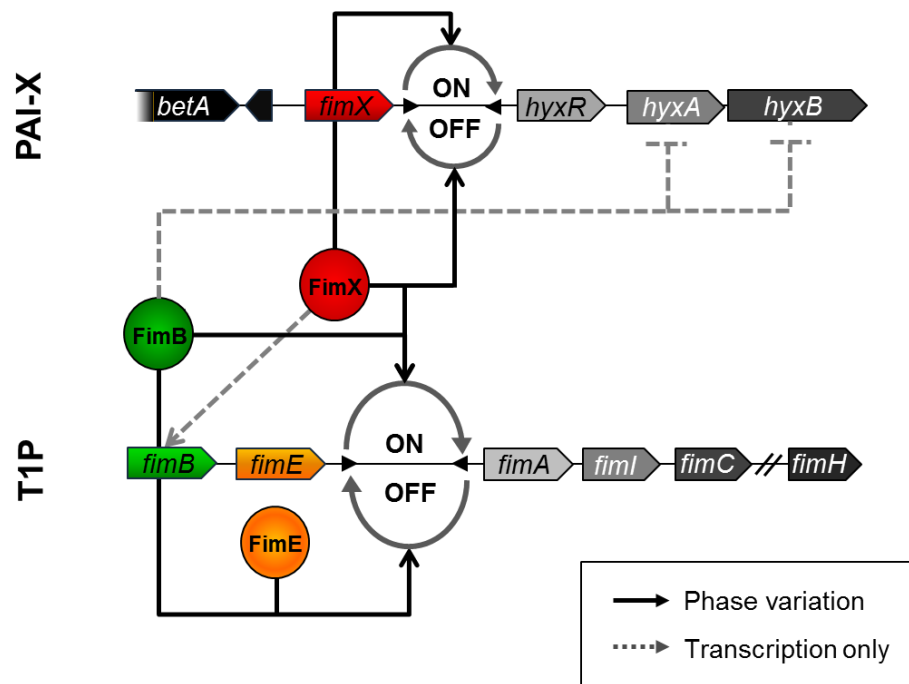


Figure 33: Epigenetic and transcriptional coordinate regulation of type 1 pili and PAI-X.

by repressing the transcript levels of *hyxA* and *hyxB*, although the phenotypic consequences of this regulation are not yet understood. Despite being highly conserved among ExPEC (Chapter 3), HyxA and HyxB have unknown roles in ExPEC colonization, transmission, and/or pathogenesis.

The evolution of bacterial pathogen genomes often includes the acquisition of virulence genes or genomic segments, predominantly through horizontal exchange. We present evidence in Chapter 3 that ExPEC has specifically acquired additional regulatory inputs, namely FimX and, to a lesser extent, IpuA and IpuB, to control the expression of type 1 pili. Interestingly, PAI-X-encoded factor FimX appears to cross-regulate the major adherence factor type 1 pili with expression of HyxR, a negative regulator of the nitrosative stress response and intracellular macrophage survival (Chapter 5). As demonstrated in Chapter 5, HyxR is not a virulence factor in the classical sense as deletion causes a gain-of-virulence phenotype. PAI-X, including *hyxR*, may be maintained in the genomes of ExPEC isolates as a pathoadaptive acquisition to moderate virulence or enhance survival in a new niche. Certainly, the data presented in Chapter 5 indicates that there is a fitness loss to constitutive expression of HyxR under nitrosative stress or during intracellular macrophage survival; however, the contribution of HyxR expression to other niche adaptations has yet to be elucidated. Epigenetic control by FimX may coordinate pilus expression with the physiologic alterations necessary to prepare a

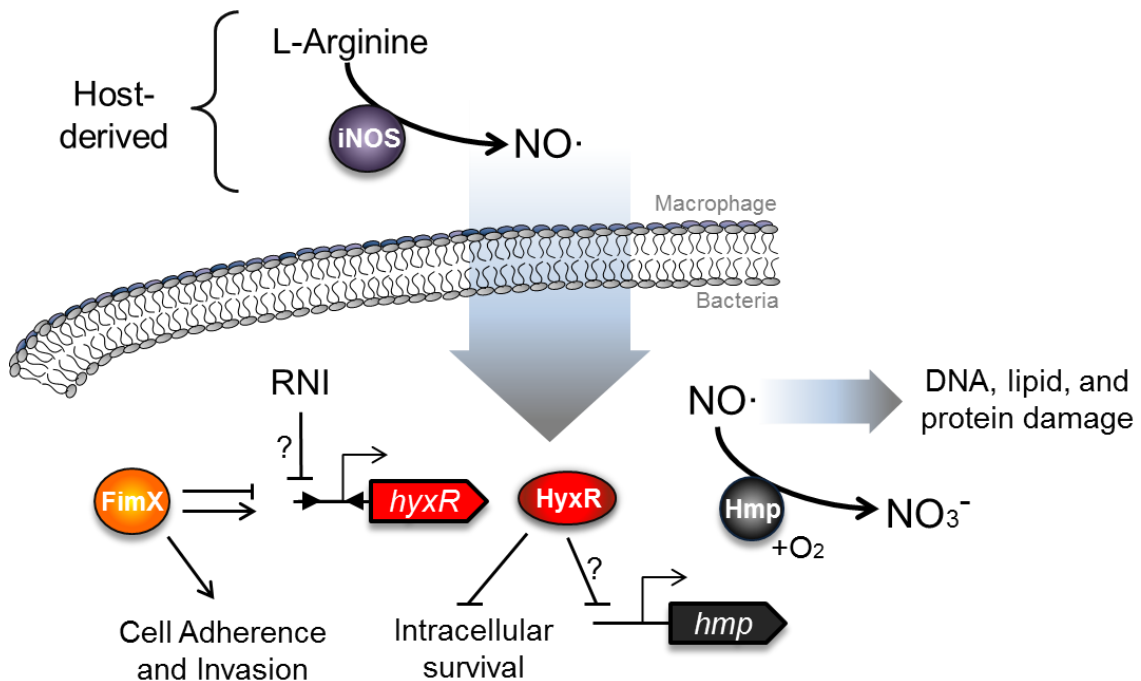


Figure 34: Role of HyxR in regulating *hmp* and intracellular survival during macrophage infection.

FimX coordinates the expression of the major adherence/invasion factor type 1 pili with *hyxR* expression, priming bacteria for cell entry and survival. During nitrosative stress generation within the intracellular macrophage compartment, HyxR is presumably turned OFF, resulting in de-repression of *hmp*. Hmp then converts NO to the less toxic nitrate in the presence of oxygen, leading to detoxification of RNIs and bacterial survival. HyxR also independently regulates intracellular survival through an unknown mechanism independent of Hmp and RNI generation.

bacterium for host cellular entry and persistence into a cell type with high RNI concentrations, such as macrophages (Fig. 34). These results lead us to hypothesize that the regulation of type 1 pili and PAI-X factors are coordinated during ExPEC pathogenesis.

6.3 Future Directions

6.3.1 Elucidating *FimX* DNA Specificity and Directionality

Preliminary studies have shown that *FimX* expression produces recombination of the *hyxR* promoter via 16 bp inverted repeats that have limited sequence homology to the known inversion sites upstream of T1P (Fig. 17), suggesting that *FimX* has dual sequence specificity. Therefore, we hypothesize that *FimX* is a unique tyrosine recombinase with multiple targets of regulation which are mediated by at least two different DNA binding specificities. Based on preliminary data presented in Chapter 4, we propose a two-fold approach to investigate *FimX* DNA specificity. First, we would like to confirm direct *FimX* binding to the promoter of T1P and *hyxR* through electromobility shift assays (EMSA) with DNA probes to the invertible repeats plus flanking sequence of both loci in each orientation. Second, we will continue to investigate the *FimX* protein domains that are responsible for recognition/recombination at each loci through the use of selective deletions, point-mutants, and *FimE:X* chimeras. This work will confirm that *FimX* is directly responsible for *hyxR* promoter recombination as well as determine the *FimX* protein domains responsible for recognition and directionality. Ultimately, comparative crystal structures of the *Fim* recombinases would provide insights into the function of this unique sub group of proteins. These studies will inform our understanding of protein domains that control DNA binding and directional switching.

6.3.2 Elucidate the Regulation of *FimX*

FimX appears to act as a node coordinating the expression of the major adherence factor type 1 pili and a regulator controlling response to nitrosative stress during intracellular macrophage survival. Very little is known about the factors or stimuli that are responsible for FimX expression. Therefore, we plan to map the promoter architecture of *fimX* and identify its major transcriptional regulators. To start, we will characterize the promoter architecture of *fimX*, including mapping the transcriptional start site and any promoter elements that enhance or repress *fimX* expression through promoter deletions coupled to a β -galactosidase reporter. We can combine this to bioinformatic analysis of promoter elements controlling *fimX* expression by utilizing known *E. coli* transcription factor binding sites with software like ProDoric (Grote *et al.*, 2009; Munch *et al.*, 2005). We will also use a $P_{tet}/tetR$ Tn5-based transposon system to elucidate activators and repressors of *fimX* expression using FimX-mediated inversion of *hyxR* as a surrogate marker for *fimX* expression. Deletions in potential regulators identified through promoter mapping or transposon analysis will be examined for their effects on *hyxR* phase inversion, type 1 pili phase switching PCR assays, type 1 and FimX immunoblots, and tissue culture adherence/invasion assays.

6.3.3 The *HyxR* Regulon

Given our results that HyxR regulation of *hmp* does not entirely explain RNI-dependent intracellular survival effects in macrophages, we suspect that HyxR may

regulate additional factors. Therefore, we would like to ascertain whether HyxR regulates any additional factors by investigating differential gene expression and HyxR-binding sites on a genome-wide approach. To accomplish this aim, we will utilize RNA-seq to identify differentially-expressed genes between UTI89/pTrc99a, UTI89 Δ hyxR/pTrc99a, and UTI89 Δ hyxR/pTrc:hyxR. We will couple this analysis with ChIP-Chip utilizing an anti-HyxR antibody in UTI89 Δ hyxR/pTrc99a (background control) and UTI89 Δ hyxR/pTrc:hyxR to determine whether predicted HyxR-regulated genes are direct or indirect targets. The combination of these experiments will allow us to predict the role of HyxR on the balance between ExPEC commensalism and pathogenesis.

6.3.4 Role of HyxR In Vivo in Disseminated Disease

Our results indicate that the ability of ExPEC to survive RNI-mediated stresses both *in vitro* and within macrophages is contingent upon the proper regulation of HyxR, a negative regulator of RNI response pathways. We have also shown that HyxR represses transcription of *hmp*, which encodes a major RNI detoxification enzyme, and that constitutive expression of HyxR leads to attenuation of ExPEC during intracellular persistence in macrophage-like cells. This previously unrecognized ExPEC-associated regulator contributes to intracellular survival in macrophage-like cells in both an RNI-dependent and -independent manner. To further investigate the role of HyxR to disease progression by ExPEC, we propose to utilize a neonatal mouse model with an intranasal route of infection that has been previously established to require macrophages for

disseminated disease (Mittal *et al.*, 2010b). To address this question, I will infect mice intranasally with *hyxR* deletion or overexpression derivatives at baseline (control) or post carrageenan-treatment to reduce the macrophage population by >95%, as previously described (Goldmann *et al.*, 2004; Mittal *et al.*, 2010b).

We would also like to assess the role of nitric oxide production by iNOS to HyxR-dependent *in vivo* infections by administering iNOS-specific inhibitors (L-NAME or aminoguanidine (AG)) at various time-points pre- and post-infection using a previously established protocol (Mittal *et al.*, 2010a). Unexpectedly, RAW 264.7 cells treated with L-NAME to inhibit NO production actually supported a lower intracellular bacteria burden, suggesting that NO produced by iNOS may be beneficial for *E. coli* to survive within macrophages (Chapter 5). Induction of NO by *E. coli* through iNOS may provide a signal to alter the host environment towards a more conducive atmosphere for bacterial survival. Alternatively, NO could serve as a regulatory cue to promote altered bacterial virulence programs, leading to increased bacterial replication or resistance.

Although induction of NO through iNOS during infection leads to increased bacterial burden and CNS-disseminated disease (Mittal *et al.*, 2010a), inhibition of host iNOS is not a viable therapeutic strategy to treat neonatal *E. coli* meningitis, due to the role NO plays in bone plate development (Wang *et al.*, 2011). Therefore, further investigation of the bacterial factors that coordinate the bacterial response to increased NO induction during intracellular macrophage infections, like HyxR, may elucidate new pathways to target therapeutically.

6.3.5 Contribution of Other PAI-X Factors to ExPEC Lifestyle

Finally, we wish to further investigate the contribution of the constituents of PAI-X to the pathogenicity of ExPEC using a combination of *in vivo* animal models and tissue culture. We will investigate the contribution of the PAI-X genes to ExPEC pathogenicity using an *in vivo* model of ascending UTI in the adolescent animal and intranasal infection in the neonatal animal. We will assay several defined deletions in *fimX*, *hyxA*, and *hyxB* compared to UTI89 wild-type for bacterial titers in various organ tissues (bladder, kidneys, spleen, blood, and brain). gDNA from the various infected tissues can be collected to assess *hyxR* promoter orientation as a proxy for FimX expression. Given the high prevalence and conservation of PAI-X factors among ExPEC, we hypothesize that HyxA and HyxB may have roles during ExPEC pathogenesis. However, we cannot rule out the possibility that these factors are involved in other aspects of the ExPEC lifestyle such as colonization of the intestine. Therefore, we will also investigate defined deletions in a streptomycin-treated mouse model of intestinal colonization.

6.4 Concluding Remarks

Extraintestinal pathogenic *E. coli* is a primary cause of UTIs in all patient groups and is a leading cause of sepsis and meningitis in neonates, immunocompromised patients, and hospitalized patients. In the studies described herein, we demonstrate that ExPEC has acquired the PAI-X locus that encodes FimX, a novel, multi-target tyrosine recombinase, and HyxR, a major repressor of RNI tolerance that is regulated through

FimX-associated epigenetic control and by RNI stress itself. These studies have elucidated key regulatory mediators in virulence, thus providing important new insights into pathogenesis.

Appendix A

Table 11. Multiplex PCR analysis for the presence of extraintestinal virulence factors, Fim-like recombinases, and PAI-X genes in all clinical strains used in this study.

Strain	Reference	Source	Clinical Syndrome	Clonal Group	Virulence Factors										Fim-like recombinases				PAI-X		
					<i>sfa/focDE</i>	<i>papA</i>	<i>ibeA</i>	<i>traT</i>	<i>fyuA</i>	<i>hlyA</i>	<i>cnf1</i>	<i>Kps MT II</i>	<i>Kps MT III</i>	<i>fimB</i>	<i>fimE</i>	<i>ipuA</i>	<i>ipuB</i>	<i>fimX</i>	<i>hyxR</i>	<i>hyxA</i>	<i>hyxB</i>
MG1655	(Blattner <i>et al.</i> , 1997)	N/A	C	A	0	0	1	0	0	0	0	0	0	1	1	0	0	0	0	0	0
UTI89	(Mulvey <i>et al.</i> , 1998)	HU	CY	B2	1	1	1	1	1	1	1	1	0	1	1	0	0	1	1	1	1
NU14	(Hultgren <i>et al.</i> , 1986)	HU	CY	B2	1	1	1	1	1	1	1	1	0	1	1	0	0	1	1	1	1
536	(Berger <i>et al.</i> , 1982)	HU	PY	B2	N/D	N/D	0	1	1	0	0	N/D	N/D	1	1	0	0	1	1	1	1
DS17	(Tullus <i>et al.</i> , 1984)	HU	PY	B2	N/D	N/D	0	1	1	1	1	N/D	N/D	1	1	0	0	1	1	1	1
GR12	(Svanborg Eden <i>et al.</i> , 1983)	HU	PY	B2	N/D	N/D	1	1	1	0	1	N/D	N/D	1	1	1	1	1	1	1	1
J96	(Hull <i>et al.</i> , 1981)	HU	PY	B2	1	1	0	1	0	1	1	0	1	1	1	0	0	1	1	1	1
CFT073	(Mobley <i>et al.</i> , 1990)	HU	UTI-BL	B2	1	1	0	1	1	1	1	0	0	1	1	1	1	1	1	1	1
EDL933	(Riley <i>et al.</i> , 1983)	HU	E	N/D	0	0	0	1	1	1	0	0	0	1	1	0	0	1	1	1	1
ECOR-1	(Ochman & Selander, 1984)	HU	C	A	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0
ECOR-2	(Ochman & Selander, 1984)	HU	C	A	0	1	0	0	1	0	0	0	0	1	1	0	0	0	0	0	0
ECOR-3	(Ochman & Selander, 1984)	dog	C	A	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
ECOR-4	(Ochman & Selander, 1984)	HU	C	A	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0
ECOR-5	(Ochman & Selander, 1984)	HU	C	A	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0

ECOR-6	(Ochman & Selander, 1984)	HU	C	A	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	
ECOR-7	(Ochman & Selander, 1984)	orangutan	C	A	0	0	0	1	1	0	0	0	0	0	1	1	0	0	1	1	1	1
ECOR-8	(Ochman & Selander, 1984)	HU	C	A	0	0	0	0	1	0	0	1	0	1	1	0	0	0	0	0	0	
ECOR-9	(Ochman & Selander, 1984)	HU	C	A	0	0	0	1	1	0	0	0	0	1	1	0	0	0	0	0	0	
ECOR-10	(Ochman & Selander, 1984)	HU	C	A	0	0	0	0	1	0	0	0	0	1	1	0	0	0	0	0	0	
ECOR-11	(Ochman & Selander, 1984)	HU	CY	A	0	1	0	1	1	0	0	1	0	1	1	0	0	0	0	0	0	
ECOR-12	(Ochman & Selander, 1984)	HU	C	A	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	
ECOR-13	(Ochman & Selander, 1984)	HU	C	A	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	
ECOR-14	(Ochman & Selander, 1984)	HU	PY	A	0	0	0	0	0	0	0	1	0	1	1	0	0	0	0	0	0	
ECOR-15	(Ochman & Selander, 1984)	HU	C	A	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	
ECOR-16	(Ochman & Selander, 1984)	leopard	C	A	0	0	0	0	1	0	0	0	0	1	1	0	0	0	0	0	0	
ECOR-17	(Ochman & Selander, 1984)	pig	C	A	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	
ECOR-18	(Ochman & Selander, 1984)	celebese ape	C	A	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	
ECOR-19	(Ochman & Selander, 1984)	celebese ape	C	A	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	
ECOR-20	(Ochman & Selander, 1984)	steer	C	A	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	
ECOR-21	(Ochman & Selander, 1984)	steer	C	A	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	
ECOR-22	(Ochman & Selander, 1984)	steer	C	A	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	
ECOR-23	(Ochman & Selander, 1984)	elephant	C	A	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	
ECOR-24 [†]	(Ochman & Selander, 1984)	HU	C	A	0	1	0	1	1	0	1	0	1	1	0	0	0	0	0	0	0	
ECOR-25	(Ochman & Selander, 1984)	dog	C	A	0	0	0	1	0	0	0	0	0	1	1	0	0	0	0	0	0	
ECOR-26	(Ochman & Selander, 1984)	infant	C	B1	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	
ECOR-27	(Ochman & Selander, 1984)	girafe	C	B1	0	0	0	1	0	0	0	0	0	1	1	0	0	0	0	0	0	

ECOR-28	(Ochman & Selander, 1984)	HU	C	B1	0	0	0	1	0	0	0	0	0	1	1	0	0	0	0	0
ECOR-29	(Ochman & Selander, 1984)	kangaroo rat	C	B1	0	0	0	1	0	0	0	0	0	1	1	0	0	1	1	1
ECOR-30	(Ochman & Selander, 1984)	bison	C	B1	0	0	0	1	0	0	0	0	0	1	1	0	0	1	1	1
ECOR-32	(Ochman & Selander, 1984)	giraffe	C	B1	0	0	0	0	0	0	0	0	0	1	1	0	0	1	1	1
ECOR-33	(Ochman & Selander, 1984)	sheep	C	B1	0	0	0	0	0	0	0	0	0	1	1	0	0	1	1	1
ECOR-34	(Ochman & Selander, 1984)	dog	C	B1	0	0	0	0	0	0	0	0	0	1	1	0	0	1	1	1
ECOR-35 [†]	(Ochman & Selander, 1984)	HU	C	D	0	1	0	1	1	0	0	1	0	1	1	0	0	0	0	0
ECOR-36 [†]	(Ochman & Selander, 1984)	HU	C	D	0	1	0	1	1	0	0	1	0	1	1	0	0	0	0	0
ECOR-38	(Ochman & Selander, 1984)	HU	C	D	0	0	0	0	1	0	0	1	0	1	1	1	1	0	0	0
ECOR-39	(Ochman & Selander, 1984)	HU	C	D	0	1	0	0	1	0	0	1	0	1	1	1	1	0	0	0
ECOR-40	(Ochman & Selander, 1984)	HU	PY	D	0	1	0	0	1	0	0	1	0	1	1	1	1	0	0	0
ECOR-41	(Ochman & Selander, 1984)	HU	C	D	0	1	0	0	1	0	0	1	0	1	1	1	1	0	0	0
ECOR-42	(Ochman & Selander, 1984)	HU	C	E	0	0	0	1	0	0	0	0	0	1	1	0	0	1	1	1
ECOR-43	(Ochman & Selander, 1984)	HU	C	E	0	0	0	0	0	1	1	0	0	1	1	0	0	1	0	1
ECOR-44	(Ochman & Selander, 1984)	cougar	C	D	0	0	0	0	0	0	0	1	0	1	1	1	1	0	0	0
ECOR-45	(Ochman & Selander, 1984)	pig	C	B1	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0
ECOR-46 [†]	(Ochman & Selander, 1984)	ape	C	D	0	1	0	1	1	0	0	1	0	1	1	1	1	0	0	0
ECOR-47	(Ochman & Selander, 1984)	sheep	C	D	0	0	0	1	0	0	0	0	0	1	1	0	0	0	0	0
ECOR-48	(Ochman & Selander, 1984)	HU	CY	D	0	1	0	1	1	1	1	1	0	1	1	0	0	0	0	0
ECOR-49 [†]	(Ochman & Selander, 1984)	HU	C	D	0	1	0	1	1	0	0	1	0	1	1	0	0	0	0	0
ECOR-50	(Ochman & Selander, 1984)	HU	PY	D	0	1	0	1	1	0	0	1	0	1	1	0	0	0	0	0
ECOR-51 [†]	(Ochman & Selander, 1984)	infant	C	B2	1	1	0	0	1	1	1	1	0	0	1	1	1	1	1	1

ECOR-52 [†]	(Ochman & Selander, 1984)	orangutan	C	B2	1	1	0	0	1	1	1	1	0	1	1	1	1	1	1	1
ECOR-53 [†]	(Ochman & Selander, 1984)	HU	C	B2	1	1	0	0	1	1	1	1	0	1	1	0	0	1	1	1
ECOR-54 [†]	(Ochman & Selander, 1984)	HU	C	B2	1	0	0	0	1	1	1	1	0	1	1	1	1	1	1	1
ECOR-55	(Ochman & Selander, 1984)	HU	PY	B2	0	0	0	0	1	0	0	1	0	1	1	1	1	1	1	1
ECOR-56	(Ochman & Selander, 1984)	HU	C	B2	0	1	0	0	1	1	0	0	0	1	1	1	1	1	1	1
ECOR-57 [†]	(Ochman & Selander, 1984)	gorilla	C	B2	1	1	0	0	1	0	0	1	0	1	1	1	1	1	1	1
ECOR-58	(Ochman & Selander, 1984)	lion	C	B1	1	0	0	1	0	0	0	0	0	1	1	0	0	1	1	1
ECOR-59	(Ochman & Selander, 1984)	HU	C	B2	0	0	0	0	1	0	0	0	1	1	1	0	0	1	1	1
ECOR-60	(Ochman & Selander, 1984)	HU	CY	B2	1	1	0	0	1	1	1	0	1	1	1	0	0	1	1	1
ECOR-61	(Ochman & Selander, 1984)	HU	C	B2	0	0	0	0	1	0	0	1	0	1	1	0	0	1	1	1
ECOR-62	(Ochman & Selander, 1984)	HU	PY	B2	0	1	0	1	1	0	0	1	0	1	1	0	0	1	1	1
ECOR-63 [†]	(Ochman & Selander, 1984)	HU	C	B2	1	1	1	0	1	1	1	1	0	1	1	0	0	1	1	1
ECOR-64	(Ochman & Selander, 1984)	HU	CY	B2	1	0	0	0	1	0	0	1	0	1	1	0	0	1	1	1
ECOR-65	(Ochman & Selander, 1984)	celebese ape	C	B2	1	0	0	0	1	0	0	0	0	1	1	0	0	0	0	0
ECOR-66 [†]	(Ochman & Selander, 1984)	celebese ape	C	B1	1	1	1	0	1	0	0	1	0	1	1	0	0	1	1	1
ECOR-67	(Ochman & Selander, 1984)	goat	C	B1	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0
ECOR-68	(Ochman & Selander, 1984)	girafe	C	B1	0	0	0	0	1	0	0	0	0	1	1	0	0	1	1	1
ECOR-69	(Ochman & Selander, 1984)	celebese ape	C	B1	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0
ECOR-70	(Ochman & Selander, 1984)	gorilla	C	B1	0	0	0	1	1	0	0	0	0	1	1	0	0	0	0	0
ECOR-71	(Ochman & Selander, 1984)	HU	ASB	B1	0	0	0	1	1	0	0	0	0	1	1	0	0	0	0	0
ECOR-72	(Ochman & Selander, 1984)	HU	PY	B1	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0
C 62 [†]	(Stapleton <i>et al.</i> , 1991)	HU	C	B2	0	0	1	1	0	1	0	1	0	1	1	0	0	1	1	1

C 65	(Stapleton <i>et al.</i> , 1991)	HU	C	B2	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0
C 68 [†]	(Stapleton <i>et al.</i> , 1991)	HU	C	B2	1	1	0	0	1	1	1	1	0	1	1	1	1	1	1	1
C 73	(Stapleton <i>et al.</i> , 1991)	HU	C	B2	0	0	0	0	1	0	0	1	0	1	1	1	1	1	1	1
C 77	(Stapleton <i>et al.</i> , 1991)	HU	C	A	0	0	0	1	1	0	0	0	0	1	1	0	0	0	0	0
C 79	(Stapleton <i>et al.</i> , 1991)	HU	C	D	0	1	0	0	1	0	0	0	0	1	1	0	0	0	0	0
C 83 [†]	(Stapleton <i>et al.</i> , 1991)	HU	C	B2	1	1	1	1	1	1	1	1	0	1	1	0	0	1	1	1
C 86	(Stapleton <i>et al.</i> , 1991)	HU	C	A	0	0	0	1	0	0	0	0	0	1	1	0	0	0	0	0
C 89 [†]	(Stapleton <i>et al.</i> , 1991)	HU	C	B2	1	0	0	1	1	0	0	1	0	1	1	1	1	1	1	1
C 92	(Stapleton <i>et al.</i> , 1991)	HU	C	B2	0	0	0	1	1	0	0	1	0	1	1	0	0	1	1	1
C 95	(Stapleton <i>et al.</i> , 1991)	HU	C	B1	0	0	0	1	0	0	0	0	0	1	1	0	0	0	0	0
C 98	(Stapleton <i>et al.</i> , 1991)	HU	C	A	0	0	0	1	0	0	0	0	0	1	1	0	0	0	0	0
C 101	(Stapleton <i>et al.</i> , 1991)	HU	C	B2	0	0	1	1	0	0	0	1	0	1	1	0	0	1	1	1
C 104	(Stapleton <i>et al.</i> , 1991)	HU	C	B1	0	0	0	1	0	0	0	0	0	1	1	0	0	1	1	1
C 107	(Stapleton <i>et al.</i> , 1991)	HU	C	B2	0	0	1	0	0	0	0	1	0	0	1	0	0	0	0	0
C 110 [†]	(Stapleton <i>et al.</i> , 1991)	HU	C	B2	1	1	0	0	0	1	1	1	0	1	1	0	0	1	1	1
C 113	(Stapleton <i>et al.</i> , 1991)	HU	C	D	0	0	0	0	0	0	0	1	0	1	1	0	0	0	0	0
C 116	(Stapleton <i>et al.</i> , 1991)	HU	C	B2	0	0	0	0	1	0	0	1	0	1	1	0	0	0	0	0
C 119	(Stapleton <i>et al.</i> , 1991)	HU	C	D	0	0	0	1	0	0	0	0	0	1	1	0	0	0	0	0
C 122 [†]	(Stapleton <i>et al.</i> , 1991)	HU	C	B2	0	1	0	1	1	0	0	1	0	1	1	0	0	1	1	1
7-279	(Hooton <i>et al.</i> , 1996)	HU	sUTI	B2	N/D	N/D	0	1	1	0	0	N/D	N/D	1	1	0	0	1	1	1
9-365	(Hooton <i>et al.</i> , 1996)	HU	sUTI	B2	N/D	N/D	0	1	1	0	0	N/D	N/D	1	1	0	0	1	1	1
10-439	(Hooton <i>et al.</i> , 1996)	HU	sUTI	B2	N/D	N/D	1	0	1	1	1	N/D	N/D	1	1	0	0	1	1	1
11-487	(Hooton <i>et al.</i> , 1996)	HU	sUTI	D	N/D	N/D	1	1	1	0	0	N/D	N/D	1	1	0	0	1	1	1
11-508	(Hooton <i>et al.</i> , 1996)	HU	sUTI	B2	N/D	N/D	1	0	1	1	1	N/D	N/D	1	1	0	0	1	1	1
15-666	(Hooton <i>et al.</i> , 1996)	HU	sUTI	B2	N/D	N/D	0	0	1	0	0	N/D	N/D	1	1	1	1	1	1	1
25-1332	(Hooton <i>et al.</i> , 1996)	HU	sUTI	B2	N/D	N/D	0	1	1	0	1	N/D	N/D	1	1	0	0	1	1	1
16-745	(Hooton <i>et al.</i> , 1996)	HU	sUTI	B2	N/D	N/D	0	1	1	1	0	N/D	N/D	1	1	1	1	1	1	1
17-806	(Hooton <i>et al.</i> , 1996)	HU	sUTI	D	N/D	N/D	1	1	1	0	0	N/D	N/D	1	1	0	0	1	1	1
18-845	(Hooton <i>et al.</i> , 1996)	HU	sUTI	B2	N/D	N/D	1	1	1	0	0	N/D	N/D	1	1	0	0	1	1	1
21-991	(Hooton <i>et al.</i> , 1996)	HU	sUTI	A	N/D	N/D	0	1	1	0	0	N/D	N/D	1	1	0	0	0	0	0
22-1025	(Hooton <i>et al.</i> , 1996)	HU	sUTI	D	N/D	N/D	0	1	1	0	0	N/D	N/D	1	1	0	0	1	1	1
22-1075	(Hooton <i>et al.</i> , 1996)	HU	sUTI	B2	N/D	N/D	0	0	1	1	1	N/D	N/D	1	1	0	0	1	1	1
23-1127	(Hooton <i>et al.</i> , 1996)	HU	sUTI	B2	N/D	N/D	0	0	1	1	1	N/D	N/D	1	1	0	0	1	1	1

23-1135	(Hooton <i>et al.</i> , 1996)	HU	sUTI	B2	N/D	N/D	0	1	1	0	1	N/D	N/D	1	1	0	0	1	1	1	1
24-1204	(Hooton <i>et al.</i> , 1996)	HU	sUTI	A	N/D	N/D	0	1	0	0	0	N/D	N/D	1	1	0	0	1	1	1	1
24-1218	(Hooton <i>et al.</i> , 1996)	HU	sUTI	B2	N/D	N/D	0	0	1	1	1	N/D	N/D	1	1	1	1	1	1	1	1
24-1236	(Hooton <i>et al.</i> , 1996)	HU	sUTI	B2	N/D	N/D	1	0	1	1	1	N/D	N/D	1	1	1	1	1	1	1	1
24-1272	(Hooton <i>et al.</i> , 1996)	HU	sUTI	B2	N/D	N/D	0	1	1	0	0	N/D	N/D	1	1	0	0	1	1	1	1
25-1326	(Hooton <i>et al.</i> , 1996)	HU	sUTI	D	N/D	N/D	0	1	1	0	0	N/D	N/D	1	1	0	0	1	1	1	1
498	(Czaja <i>et al.</i> , 2009b)	HU	rUTI	B2	N/D	N/D	0	1	1	0	1	N/D	N/D	1	1	0	0	1	1	1	1
578	(Czaja <i>et al.</i> , 2009b)	HU	rUTI	A	N/D	N/D	0	1	0	0	0	N/D	N/D	1	1	0	0	0	0	0	0
587	(Czaja <i>et al.</i> , 2009b)	HU	rUTI	B2	N/D	N/D	0	0	1	0	1	N/D	N/D	1	1	0	0	1	1	1	1
737	(Czaja <i>et al.</i> , 2009b)	HU	rUTI	B2	N/D	N/D	0	0	1	0	1	N/D	N/D	1	1	0	0	1	1	1	1
746	(Czaja <i>et al.</i> , 2009b)	HU	rUTI	B2	N/D	N/D	0	1	1	1	1	N/D	N/D	1	1	1	1	1	1	1	1
797	(Czaja <i>et al.</i> , 2009b)	HU	rUTI	B2	N/D	N/D	0	1	1	0	0	N/D	N/D	1	1	0	0	1	1	1	1
1074	(Czaja <i>et al.</i> , 2009b)	HU	rUTI	B2	N/D	N/D	0	1	1	0	0	N/D	N/D	1	1	0	0	1	1	1	1
1090	(Czaja <i>et al.</i> , 2009b)	HU	rUTI	B2	N/D	N/D	1	0	1	0	1	N/D	N/D	1	1	0	0	1	1	1	1
1235	(Czaja <i>et al.</i> , 2009b)	HU	rUTI	B2	N/D	N/D	0	1	1	0	1	N/D	N/D	1	1	0	0	1	1	1	1
1274	(Czaja <i>et al.</i> , 2009b)	HU	rUTI	B2	N/D	N/D	1	1	1	0	1	N/D	N/D	1	1	0	0	1	1	1	1
1490	(Czaja <i>et al.</i> , 2009b)	HU	rUTI	B2	N/D	N/D	1	1	1	1	1	N/D	N/D	1	1	0	0	1	1	1	1
1648	(Czaja <i>et al.</i> , 2009b)	HU	rUTI	B2	N/D	N/D	0	0	1	1	1	N/D	N/D	1	1	1	1	1	1	1	1
1987	(Czaja <i>et al.</i> , 2009b)	HU	rUTI	A	N/D	N/D	0	0	1	0	0	N/D	N/D	1	1	0	0	0	0	0	0
2199	(Czaja <i>et al.</i> , 2009b)	HU	rUTI	B2	N/D	N/D	0	1	1	0	1	N/D	N/D	1	1	0	0	1	1	1	1
2216	(Czaja <i>et al.</i> , 2009b)	HU	rUTI	B2	N/D	N/D	0	0	1	1	1	N/D	N/D	1	1	1	1	1	1	1	1
2745	(Czaja <i>et al.</i> , 2009b)	HU	rUTI	B2	N/D	N/D	1	1	1	0	0	N/D	N/D	1	1	1	1	1	1	1	1
3481	(Czaja <i>et al.</i> , 2009b)	HU	rUTI	B2	N/D	N/D	0	1	1	1	1	N/D	N/D	1	1	1	1	1	1	1	1
4028	(Czaja <i>et al.</i> , 2009b)	HU	rUTI	B2	N/D	N/D	1	1	1	0	1	N/D	N/D	1	1	0	0	1	1	1	1
1283	(Johnson <i>et al.</i> , 1991)	HU	PY	B2	N/D	N/D	1	1	1	0	1	N/D	N/D	1	1	0	0	1	1	1	1
1303	(Johnson <i>et al.</i> , 1991)	HU	PY	D	N/D	N/D	0	1	1	0	0	N/D	N/D	1	1	1	1	1	1	1	1
1358	(Johnson <i>et al.</i> , 1991)	HU	PY	B2	N/D	N/D	0	1	1	0	0	N/D	N/D	1	1	0	0	1	1	1	1
1394	(Johnson <i>et al.</i> , 1991)	HU	PY	B2	N/D	N/D	0	1	1	0	0	N/D	N/D	1	1	0	0	1	1	1	1
1404	(Johnson <i>et al.</i> , 1991)	HU	PY	D	N/D	N/D	0	1	1	0	0	N/D	N/D	1	1	1	1	0	0	0	0
1420	(Johnson <i>et al.</i> , 1991)	HU	PY	A	N/D	N/D	0	1	1	0	0	N/D	N/D	1	1	0	0	0	0	0	0
1429	(Johnson <i>et al.</i> , 1991)	HU	PY	B2	N/D	N/D	0	1	1	1	1	N/D	N/D	1	1	0	0	1	1	1	1
1430	(Johnson <i>et al.</i> , 1991)	HU	PY	B2	N/D	N/D	1	1	1	1	1	N/D	N/D	1	1	0	0	1	1	1	1
1431	(Johnson <i>et al.</i> , 1991)	HU	PY	D	N/D	N/D	1	1	1	1	1	N/D	N/D	1	1	0	0	1	1	1	1

1438	(Johnson <i>et al.</i> , 1991)	HU	PY	B2	N/D	N/D	1	1	1	0	1	N/D	N/D	1	1	0	0	1	1	1	1
1460	(Johnson <i>et al.</i> , 1991)	HU	PY	B2	N/D	N/D	1	1	1	0	0	N/D	N/D	1	1	0	0	1	1	1	1
1479	(Johnson <i>et al.</i> , 1991)	HU	PY	D	N/D	N/D	0	1	1	1	0	N/D	N/D	1	1	0	0	0	0	0	0
1487	(Johnson <i>et al.</i> , 1991)	HU	PY	B2	N/D	N/D	0	1	1	1	1	N/D	N/D	1	1	1	1	1	1	1	1
1491	(Johnson <i>et al.</i> , 1991)	HU	PY	B2	N/D	N/D	0	1	1	0	0	N/D	N/D	1	1	0	0	1	1	1	1
1499	(Johnson <i>et al.</i> , 1991)	HU	PY	B1	N/D	N/D	0	0	0	0	0	N/D	N/D	1	1	0	0	0	0	0	0
1502	(Johnson <i>et al.</i> , 1991)	HU	PY	B2	N/D	N/D	0	1	1	0	0	N/D	N/D	1	1	0	0	1	1	1	1
1505	(Johnson <i>et al.</i> , 1991)	HU	PY	B2	N/D	N/D	0	0	1	0	0	N/D	N/D	1	1	0	0	1	1	1	1
1506	(Johnson <i>et al.</i> , 1991)	HU	PY	B2	N/D	N/D	0	1	1	0	0	N/D	N/D	1	1	0	0	1	1	1	1
1556	(Johnson <i>et al.</i> , 1991)	HU	PY	D	N/D	N/D	0	1	1	0	0	N/D	N/D	1	1	0	0	0	0	0	0
1568	(Johnson <i>et al.</i> , 1991)	HU	PY	D	N/D	N/D	0	1	1	0	0	N/D	N/D	1	1	0	0	0	0	0	0
2-1-2	(Johnson <i>et al.</i> , 1987; Johnson <i>et al.</i> , 1988)	HU	UTI-BL	D	N/D	N/D	0	1	1	0	0	N/D	N/D	1	1	0	0	0	0	0	0
2-1-5	(Johnson <i>et al.</i> , 1987; Johnson <i>et al.</i> , 1988)	HU	UTI-BL	B2	N/D	N/D	1	1	1	0	1	N/D	N/D	1	1	0	0	1	1	1	1
2-1-7	(Johnson <i>et al.</i> , 1987; Johnson <i>et al.</i> , 1988)	HU	UTI-BL	B2	N/D	N/D	0	1	1	0	0	N/D	N/D	1	1	0	0	1	1	1	1
2-1-9	(Johnson <i>et al.</i> , 1987; Johnson <i>et al.</i> , 1988)	HU	UTI-BL	B2	N/D	N/D	0	0	1	1	1	N/D	N/D	1	1	1	1	1	1	1	1
2-2-1	(Johnson <i>et al.</i> , 1987; Johnson <i>et al.</i> , 1988)	HU	UTI-BL	B2	N/D	N/D	0	1	1	1	0	N/D	N/D	1	1	0	0	1	1	1	1
2-2-3	(Johnson <i>et al.</i> , 1987; Johnson <i>et al.</i> , 1988)	HU	UTI-BL	B2	N/D	N/D	0	1	1	1	0	N/D	N/D	1	1	1	1	1	1	1	1
2-2-5	(Johnson <i>et al.</i> , 1987; Johnson <i>et al.</i> , 1988)	HU	UTI-BL	B2	N/D	N/D	0	0	1	0	1	N/D	N/D	1	1	1	1	1	1	1	1
2-2-7	(Johnson <i>et al.</i> , 1987; Johnson <i>et al.</i> , 1988)	HU	UTI-BL	B2	N/D	N/D	0	0	1	1	0	N/D	N/D	1	1	1	1	1	1	1	1
2-2-9	(Johnson <i>et al.</i> , 1987; Johnson <i>et al.</i> , 1988)	HU	UTI-BL	B2	N/D	N/D	0	1	1	1	0	N/D	N/D	1	1	1	1	1	1	1	1
2-3-1	(Johnson <i>et al.</i> , 1987; Johnson <i>et al.</i> , 1988)	HU	UTI-BL	B2	N/D	N/D	1	1	1	0	0	N/D	N/D	1	1	0	0	1	1	1	1
2-3-3	(Johnson <i>et al.</i> , 1987; Johnson <i>et al.</i> , 1988)	HU	UTI-BL	B2	N/D	N/D	0	1	1	0	1	N/D	N/D	1	1	0	0	1	1	1	1
2-3-5	(Johnson <i>et al.</i> , 1987; Johnson <i>et al.</i> , 1988)	HU	UTI-BL	B2	N/D	N/D	0	1	1	0	0	N/D	N/D	1	1	0	0	1	1	1	1
2-3-7	(Johnson <i>et al.</i> , 1987; Johnson <i>et al.</i> , 1988)	HU	UTI-BL	B2	N/D	N/D	0	1	1	0	0	N/D	N/D	1	1	0	0	1	1	1	1
2-4-3	(Johnson <i>et al.</i> , 1987; Johnson <i>et al.</i> , 1988)	HU	UTI-BL	B2	N/D	N/D	0	1	1	1	0	N/D	N/D	1	1	0	0	1	1	1	1

2-4-7	(Johnson <i>et al.</i> , 1987; Johnson <i>et al.</i> , 1988)	HU	UTI-BL	B2	N/D	N/D	1	1	1	1	1	N/D	N/D	1	1	0	0	1	1	1	1
2-4-9	(Johnson <i>et al.</i> , 1987; Johnson <i>et al.</i> , 1988)	HU	UTI-BL	B2	N/D	N/D	0	1	1	1	0	N/D	N/D	1	1	0	0	1	1	1	1
2-5-1	(Johnson <i>et al.</i> , 1987; Johnson <i>et al.</i> , 1988)	HU	UTI-BL	A	N/D	N/D	0	1	1	1	0	N/D	N/D	1	1	0	0	0	0	0	0
2-5-3	(Johnson <i>et al.</i> , 1987; Johnson <i>et al.</i> , 1988)	HU	UTI-BL	D	N/D	N/D	1	1	1	1	0	N/D	N/D	1	1	0	0	1	1	1	1
2-6-5	(Johnson <i>et al.</i> , 1987; Johnson <i>et al.</i> , 1988)	HU	UTI-BL	A	N/D	N/D	0	1	0	0	0	N/D	N/D	1	1	0	0	0	0	0	0
2-7-1	(Johnson <i>et al.</i> , 1987; Johnson <i>et al.</i> , 1988)	HU	UTI-BL	A	N/D	N/D	0	0	1	1	0	N/D	N/D	1	1	1	1	0	0	0	0
ASB 131	(Hooton <i>et al.</i> , 1996)	HU	ASB	B2	N/D	N/D	1	1	1	1	1	N/D	N/D	1	1	0	0	1	1	1	1
ASB 167	(Hooton <i>et al.</i> , 1996)	HU	ASB	B2	N/D	N/D	0	0	1	1	1	N/D	N/D	1	1	1	1	1	1	1	1
ASB 229	(Hooton <i>et al.</i> , 1996)	HU	ASB	B2	N/D	N/D	1	1	1	0	1	N/D	N/D	1	1	0	0	1	1	1	1
ASB 271	(Hooton <i>et al.</i> , 1996)	HU	ASB	A	N/D	N/D	0	1	1	0	0	N/D	N/D	1	1	0	0	0	0	0	0
ASB 271	(Hooton <i>et al.</i> , 1996)	HU	ASB	B2	N/D	N/D	1	1	1	0	1	N/D	N/D	1	1	1	1	1	1	1	1
ASB 278	(Hooton <i>et al.</i> , 1996)	HU	ASB	D	N/D	N/D	0	1	1	0	0	N/D	N/D	1	1	0	0	0	0	0	0
ASB 389	(Hooton <i>et al.</i> , 1996)	HU	ASB	B2	N/D	N/D	0	0	1	1	0	N/D	N/D	1	1	0	0	1	1	1	1
ASB 408	(Hooton <i>et al.</i> , 1996)	HU	ASB	B2	N/D	N/D	1	1	1	1	1	N/D	N/D	1	1	0	0	1	1	1	1
ASB 451	(Hooton <i>et al.</i> , 1996)	HU	ASB	B2	N/D	N/D	1	1	1	0	1	N/D	N/D	1	1	0	0	1	1	1	1
ASB 452	(Hooton <i>et al.</i> , 1996)	HU	ASB	B2	N/D	N/D	1	1	1	0	0	N/D	N/D	1	1	0	0	1	1	1	1
ASB 506	(Hooton <i>et al.</i> , 1996)	HU	ASB	B2	N/D	N/D	1	0	1	1	1	N/D	N/D	1	1	0	0	1	1	1	1
ASB 556	(Hooton <i>et al.</i> , 1996)	HU	ASB	B2	N/D	N/D	1	1	1	0	1	N/D	N/D	1	1	1	1	1	1	1	1
ASB 586	(Hooton <i>et al.</i> , 1996)	HU	ASB	B2	N/D	N/D	1	1	1	1	1	N/D	N/D	1	1	1	1	1	1	1	1
ASB 640	(Hooton <i>et al.</i> , 1996)	HU	ASB	B2	N/D	N/D	1	1	1	0	1	N/D	N/D	1	1	1	1	1	1	1	1
ASB 781	(Hooton <i>et al.</i> , 1996)	HU	ASB	B2	N/D	N/D	1	1	1	0	1	N/D	N/D	1	1	0	0	1	1	1	1
ASB 795	(Hooton <i>et al.</i> , 1996)	HU	ASB	B1	N/D	N/D	0	1	0	1	0	N/D	N/D	1	1	0	0	1	1	1	1
ASB 933	(Hooton <i>et al.</i> , 1996)	HU	ASB	B2	N/D	N/D	1	1	1	0	0	N/D	N/D	1	1	0	0	1	1	1	1
ASB 934	(Hooton <i>et al.</i> , 1996)	HU	ASB	D	N/D	N/D	0	1	0	0	0	N/D	N/D	1	1	1	1	1	1	1	1
ASB 941	(Hooton <i>et al.</i> , 1996)	HU	ASB	B2	N/D	N/D	1	1	1	0	1	N/D	N/D	1	1	0	0	0	0	0	0
ASB 942	(Hooton <i>et al.</i> , 1996)	HU	ASB	B2	N/D	N/D	0	1	1	0	0	N/D	N/D	1	1	0	0	1	1	1	1
ASB 950	(Hooton <i>et al.</i> , 1996)	HU	ASB	A	N/D	N/D	0	1	1	0	0	N/D	N/D	1	1	0	0	1	1	1	1
ASB 951	(Hooton <i>et al.</i> , 1996)	HU	ASB	A	N/D	N/D	0	1	1	0	0	N/D	N/D	1	1	0	0	1	1	1	1
ASB 957	(Hooton <i>et al.</i> , 1996)	HU	ASB	B2	N/D	N/D	0	1	1	1	1	N/D	N/D	1	1	0	0	1	1	1	1
ASB 967	(Hooton <i>et al.</i> , 1996)	HU	ASB	B2	N/D	N/D	1	0	1	1	1	N/D	N/D	1	1	0	0	1	1	1	1

ASB 1061	(Hooton <i>et al.</i> , 1996)	HU	ASB	B2	N/D	N/D	0	1	1	1	1	N/D	N/D	1	1	0	0	1	1	1	1
ASB 1111	(Hooton <i>et al.</i> , 1996)	HU	ASB	D	N/D	N/D	1	1	1	0	0	N/D	N/D	1	1	0	0	1	1	1	1
ASB 1134	(Hooton <i>et al.</i> , 1996)	HU	ASB	B2	N/D	N/D	1	1	1	0	1	N/D	N/D	1	1	1	1	1	1	1	1
ASB 1135	(Hooton <i>et al.</i> , 1996)	HU	ASB	B2	N/D	N/D	1	1	1	1	1	N/D	N/D	0	1	0	0	1	1	1	1
ASB 1158	(Hooton <i>et al.</i> , 1996)	HU	ASB	B2	N/D	N/D	0	0	1	0	1	N/D	N/D	1	1	1	1	1	1	1	1
ASB 1230	(Hooton <i>et al.</i> , 1996)	HU	ASB	A	N/D	N/D	0	1	1	0	0	N/D	N/D	1	1	0	0	0	0	0	0
ASB 1231	(Hooton <i>et al.</i> , 1996)	HU	ASB	B2	N/D	N/D	0	1	1	1	1	N/D	N/D	1	1	1	1	1	1	1	1
ASB 1273	(Hooton <i>et al.</i> , 1996)	HU	ASB	B2	N/D	N/D	0	1	1	1	1	N/D	N/D	1	1	1	1	1	1	1	1
ASB 1282	(Hooton <i>et al.</i> , 1996)	HU	ASB	B2	N/D	N/D	1	1	1	1	1	N/D	N/D	1	1	1	1	1	1	1	1
ASB 1286	(Hooton <i>et al.</i> , 1996)	HU	ASB	D	N/D	N/D	0	0	1	0	1	N/D	N/D	1	1	0	0	0	0	0	0
ASB 1297	(Hooton <i>et al.</i> , 1996)	HU	ASB	B2	N/D	N/D	1	1	1	1	1	N/D	N/D	1	1	1	1	1	1	1	1
ASB 1298	(Garofalo <i>et al.</i> , 2007; Hooton <i>et al.</i> , 1996)	HU	rASB	B2	N/D	N/D	0	1	1	1	1	N/D	N/D	1	1	1	1	1	1	1	1
rASB 1021-2	(Hooton <i>et al.</i> , 1996)	HU	rASB	B2	N/D	N/D	1	1	1	0	0	N/D	N/D	1	1	0	0	1	1	1	1
rASB 1021-2	(Hooton <i>et al.</i> , 1996)	HU	rASB	D	N/D	N/D	1	1	1	0	0	N/D	N/D	1	1	0	0	1	1	1	1
rASB 1021-3	(Hooton <i>et al.</i> , 1996)	HU	rASB	D	N/D	N/D	1	1	0	0	0	N/D	N/D	1	1	0	0	1	1	1	1
rASB 1154-1	(Hooton <i>et al.</i> , 1996)	HU	rASB	B2	N/D	N/D	1	1	1	0	0	N/D	N/D	1	1	0	0	1	1	1	1
rASB 1154-2	(Hooton <i>et al.</i> , 1996)	HU	rASB	B2	N/D	N/D	1	1	1	1	1	N/D	N/D	1	1	0	0	1	1	1	1
rASB 1154-3	(Hooton <i>et al.</i> , 1996)	HU	rASB	B2	N/D	N/D	1	1	1	0	0	N/D	N/D	1	1	0	0	1	1	1	1
rASB 2289-2	(Hooton <i>et al.</i> , 1996)	HU	rASB	B2	N/D	N/D	1	1	1	0	0	N/D	N/D	1	1	1	1	1	1	1	1
rASB 2289-3	(Hooton <i>et al.</i> , 1996)	HU	rASB	B2	N/D	N/D	1	1	1	1	0	N/D	N/D	1	1	1	1	1	1	1	1
rASB 2289-1	(Hooton <i>et al.</i> , 1996)	HU	rASB	B2	N/D	N/D	1	1	1	0	0	N/D	N/D	1	1	1	1	1	1	1	1
PY2	(Garofalo <i>et al.</i> , 2007; Johnson <i>et al.</i> , 1991)	HU	PY	B2	N/D	N/D	1	1	1	1	1	N/D	N/D	1	1	0	0	1	1	1	1
PY3	(Garofalo <i>et al.</i> , 2007; Johnson <i>et al.</i> , 1991)	HU	PY	B2	N/D	N/D	0	1	1	0	0	N/D	N/D	1	1	0	0	1	1	1	1
rUTI 2-1	(Czaja <i>et al.</i> , 2009b; Garofalo <i>et al.</i> , 2007)	HU	rUTI	B2	N/D	N/D	1	1	1	1	1	N/D	N/D	1	1	0	0	1	1	1	1
rUTI 2-2	(Czaja <i>et al.</i> , 2009b)	HU	rUTI	B2	N/D	N/D	0	1	0	0	0	N/D	N/D	1	1	0	0	1	1	1	1
rUTI 5-1	(Czaja <i>et al.</i> , 2009b; Garofalo <i>et al.</i> , 2007)	HU	rUTI	B2	N/D	N/D	0	1	1	0	1	N/D	N/D	1	1	0	0	1	1	1	1

TB352C	This study	HU	E	N/D	N/D	N/D	1	1	1	0	1	N/D	N/D	1	1	0	0	0	0	0
TBUTI01	This study	HU	E	N/D	N/D	N/D	0	1	0	0	1	N/D	N/D	1	1	0	0	1	1	1

* Abbreviations: N/A, not applicable; N/D, not determined; C, fecal-commensal; ASB, single asymptomatic bacteriuria; rASB, recurrent ASB; CY, cystitis, where no information, regarding patient recurrence is known; sUTI, single cystitis; rUTI, recurrent cystitis; PY, pyelonephritis; UTI-BL, isolated from blood with concurrent culture positive UTI; E, enterohemorrhagic.

† Strains were not included as commensals in determination of prevalence if they encoded for ≥ 4 VFs as assayed by multiplex PCR (n = 18). ECOR isolates designated as human CY (n = 4), PY (n = 6), or ASB (n = 1) were included as part of the ExPEC group for analysis. ECOR isolates designated as human phylogenetic group E (n = 2) were included as part of the EHEC group for analysis.

‡ Discrepancy between VF detection for 11 strains between this study and a previous study (Johnson *et al.*, 2001a). Strains were assayed from the original ECOR stocks received from the Michigan State archive at least 3 times in biological duplicate with previously published primers (Johnson & Stell, 2000). We are unable to account for the discrepancies in the detection of *fyuA* (n = 10, less prevalent in our study compared with (Johnson *et al.*, 2001a)), *traT* (n = 1, detected in this study, but not in the previous study), and *hlyA* (n = 1, detected in this study, but not in the previous study).

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Biography

Stacey Lynn Bateman was born in Somerville, NJ, and lived in central NJ through high school. Stacey graduated from Swarthmore College with a B.A. in Biology in May, 2006. Post-college, Stacey enrolled in the Molecular Genetics and Microbiology program at Duke University. After rotating through several labs, Stacey joined the lab of Dr. Patrick Seed, where she completed the dissertation research contained herein.