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IN VIVO REGULATION OF *FIM* GENE TRANSCRIPTION IN UROPATHOGENIC *ESCHERICHIA COLI*

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IN VIVO REGULATION OF *FIM* GENE TRANSCRIPTION IN

UROPATHOGENIC *ESCHERICHIA COLI*

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We recommend acceptance of this thesis in partial fulfillment of the candidate's requirements for the degree of Master of Science in Biology, Clinical Microbiology Concentration

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ABSTRACT

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Uropathogenic *Escherichia coli* (UPEC) is the primary cause of human urinary tract infections. Type 1 pili of UPEC allow adherence to uroepithelial cells. Expression of type 1 pili is affected by phase variation, where a 314-bp DNA element (called *fimS*) switches between Phase-ON and Phase-OFF orientations as a consequence of binding by two site-specific recombinases FimB and FimE. In this study, we created three *fim-lux* operon transcriptional fusions and moved them into the clinical UPEC isolate NU149. Growth in acidic medium reduced transcription of three *fim* genes (*fimA*, *fimB* and *fimE*) compared to growth in neutral pH medium. In a low pH/high osmolarity environment, expression from all three *fim* genes was markedly decreased compared to the expression in growth media without added NaCl. When NU149 containing each *fim-lux* fusion was used to infect murine urinary tracts, *fimA* and *fimB* expression were high in murine bladders, but transcription of both genes was markedly lower at the fifth day post-inoculation in murine kidneys. In contrast, *fimE* expression was lower than either *fimA* or *fimB* over a five-day post-inoculation period in either UPEC bladders or kidneys. Our results suggest *in vivo* environmental factors in different niches affect *fim* gene expression by UPEC that infect the murine urinary tract.

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TABLE OF CONTENTS

PAGE

LIST OF FIGURES……...vii

LIST OF APPENDICES……..viii

INTRODUCTION……...……....1

Epidemiology of Urinary Tract Infections……..…...….1

Type 1 Pili as a Main UPEC Virulence Factor Leading to UTIs……2

Structure of The *fim* Operon.……..……..……...3

Phase Variation Controls Expression of Type 1 Pili……...6

Site-specific Recombinases That Affect Phase Switching……...……...8

Global Regulatory Factors That Affect Phase Switching……..……....10

Other Proteins That Affect Phase Switching……14

Two-component Regulatory Systems That Affect

fim Gene Transcription……...…..16

Environmental Cues That Affect *fim* Gene Expression……..…….…..18

MATERIALS AND METHODS……...21

Bacterial Strains, Plasmids, and Growth Conditions…….……21

Table 1. Strains and Plasmids Used in this Study…….……...22

Construction of the *fim-lux* Transcriptional Fusions…….…….……...21

Construction of the *ftsZ-lux* Transcriptional Fusion……..…..……...30

Testing *in vitro* Environmental Conditions……...……31

In vitro Spectrophotometric Analysis and Bioluminescence Assays……31

Murine Infection Model……...…….32

PAGE

Statistical Analysis……...…….…….33

RESULTS……..34

Evaluation of the *fim-lux* fusions in UPEC Growing

in Different pH Media……..…….34

Evaluation of the *fim-lux* Fusions in UPEC Growing

in Different Osmotic Conditions……..……...……..37

Transcription of Three *fim* Genes Varies in a UPEC Strain

Infecting Murine Bladders versus Kidneys...…….40

DISCUSSION……45

REFERENCES……..53

APPENDICES……...66

LIST OF FIGURES

FIGURE PAGE

1. Schematic of the *fim* Operon, Including the Characterized Promoter Sites……..…...4

2. Schematic of Type 1 Pilus Structure…….…….…….……....…..…5

3. A Schematic Showing How the FimB and FimE Proteins Orient the *fimS* Element…….....……..7

4. Schematic Model of the Actions of 20 Auxiliary Proteins on the Regulation of Type 1 Pili……..…….….12

5. The Flow Diagram for the Construction of the *E. coli* Strain NU149 Containing Recombinant Plasmids with *fim-lux* or *ftsZ-lux* Fusions……...……...……....…….....23

6. Plasmid Map of pHSS22……..…...24

7. Plasmid Map of pXen-5……..……26

8. Plasmid Map of pPP2-6……..……...…….27

9. Effects of pH and Osmolarity on *ftsZ*, *fimA*, *fimB*, and *fimE* Expression as Determined with *lux* Transcriptional Fusions in Strain NU149……35-36

10. Expression of *ftsZ*, *fimA*, *fimB* and *fimE* in Murine Bladders and Kidneys for Infection with NU149 Containing Recombinant Plasmids with *fim*- or *ftsZ-lux* Transcriptional Fusions over Five-day (0.33, 1, 3 and 5 Days) Post-inoculation Time..……41-42

LIST OF APPENDICES

APPENDIX PAGE

A. Reagents for DNA Agarose Gel Electrophoresis……..……..66

B. Reagents for PCR……68

C. Reagents for Media……...……..70

D. Reagents for Organ Homogenate Buffer…….……...73

INTRODUCTION

Epidemiology of Urinary Tract Infections

The human urinary tract is normally protected from the nearby colonic microflora by non-specific defenses, such as the epithelial barrier, the antibacterial properties of the bladder mucosa, and the flow of urine. Nevertheless, urinary tract infections (UTIs) are one of the most common infections of humans in the United States. The infection can occur at different points in the urinary tract, leading to urethritis, cystitis, pyelonephritis, bacteremia, and sepsis. UTIs affect a wide range of individuals, such as preschool girls, women of childbearing age, men and the elderly. Women tend to get more UTIs than men for anatomical reason. Approximately 40% of women and 12% men will experience at least one symptomatic urinary tract infection during their lifetime. Approximately 7.3 million cases of acute cystitis and 250,000 cases of acute pyelonephritis occur annually, resulting in over 100,000 hospitalizations and an estimated cost of $1.6 billion. More than 80% of all UTIs are due to uropathogenic *Escherichia coli* (UPEC), causing substantial morbidity and mortality, particularly from the risk of sepsis during pyelonephritis (Foxman *et al.*, 2000; Foxman, 2003; Foxman *et al*., 2003). UTIs also have been shown to be an independent risk factor for bladder cancer (Kantor *et al*., 1984) and may play a role in the development of renal cell carcinoma (Parker *et al*., 2004).

Type 1 Pili as a Main UPEC Virulence Factor Leading to UTIs

The ability to bind to epithelial cells lining the human urinary tract is generally considered one of the first steps in UPEC initiated UTIs. This adherence by UPEC strains occurs due to various fimbrial adhesins that allow attachment to uroepithelial cells. The most common fimbriae found on UPEC strains are P fimbriae and type 1 fimbriae.

A number of epidemiological studies have shown that P fimbriae are important in causing upper UTIs. P fimbriae, also called pyelonephritis-associated pili (Pap), recognize receptor epitopes defined by a αGal(1-4)βGal moiety, present in the globoseries of glycolipids predominantly found in human renal glycolipids (Källenius *et al*., 1980a; Leffler & Svanborg-Edén, 1981; Svenson *et al*., 1983). This receptor is also present on human erythrocytes as a part of the P blood group antigen (Källenius *et al*., 1980b). Binding to this receptor enhances bacterial persistence in the urinary tract and elicits a cytokine response in epithelial cells (Hedlund *et al*., 1999).

Another important adherence structure of UPEC strains is the type 1 pilus. Type 1 pili, also called type 1 fimbriae, are produced by many members of the *Enterobacteriaceae* family, and they are common to nearly all UPEC strains (Buchanan *et al*., 1985). The expression of type 1 pili is likely to be important in permitting UPEC to colonize in the early stage of infection, adhering to human bladder epithelial cells (Abraham *et al*., 1985b; Schaeffer *et al*., 1979; van den Bosch *et al*., 1980). These threadlike appendages are composed of repeating subunits polymerized in a helix, with 3 and 1/8 subunits per turn. Type 1 pili are morphologically and functionally distinct both from flagella and sex pili. Type 1 pili were originally characterized based on their ability to agglutinate guinea pig erythrocytes in the absence of mannose (Duguid, 1957). Further

work has shown that type 1 pili can bind to mannose-containing glycoproteins on a variety of host cells, such as epithelial cells in the bladder; human buccal cells; proximal tubular cells of the kidney, lung, and intestine; and various inflammatory cells (Baorto *et al*., 1997; Salit & Gotschich, 1977; Tewari *et al*., 1993). In addition, type 1 pili have been found to be responsible for bacterial invasion and persistence in target host cells (Baorto, *et al*., 1997; Schilling *et al*., 2002).

Structure of the *fim* Operon

Expression of type 1 pili is due to the *fim* operon, a cluster of nine genes and labeled *fimA-I* (Fig. 1). FimA, the product of the *fimA* gene, is the main structural subunit of the type 1 pili, and is arranged in a tight right-handed helical rod (Brinton, 1965) (Fig. 2). The minor subunit, FimH adhesin, encoded by *fimH* gene, is located at the tip of a type 1 pilus, and is interspersed along the fimbrial shaft. The other two minor components are FimF and FimG. These two proteins are located distally to FimA, and are integrated in the fimbrial structure. FimH is an adhesin, followed by FimF and FimG, involved in linking FimA to the shaft of fimbrillin subunits. FimH binds to mannose-containing glycoproteins present in the bladder epithelium and to a number of other tissues in a mannose-sensitive manner (Jones *et al*., 1995; Klemm & Christiansen, 1987; Krogfelt *et al*., 1990; Maurer & Orndorff, 1987). In addition to mediating pilus attachment to the bladder epithelium, the FimH adhesion also enables bacteria to internalize in bladder cells, where they are protected from the flow of urine, the host immune system, and most antibacterial drugs. In this way, bacteria can survive and in some instances replicate inside host cells, which may be the cause of the recurrent infection pattern observed in patients with urinary tract infection (Jones *et al*., 1995).

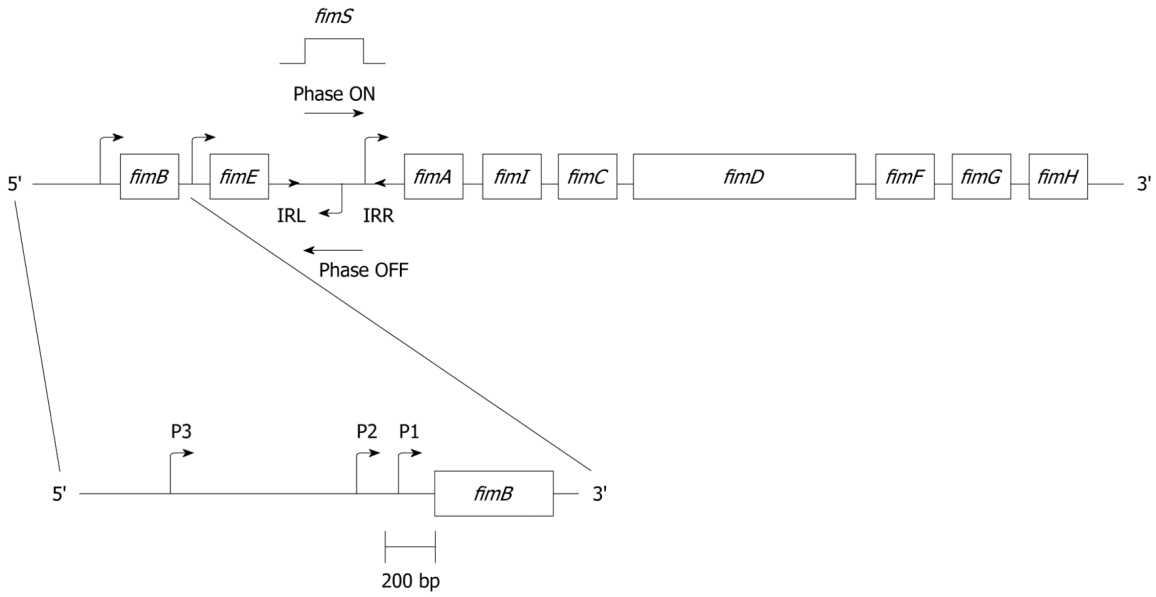


Fig. 1. Schematic of the *fim* operon, including the characterized promoter sites [permission granted by W. R. Schwan from Schwan *et al*. (2011)].

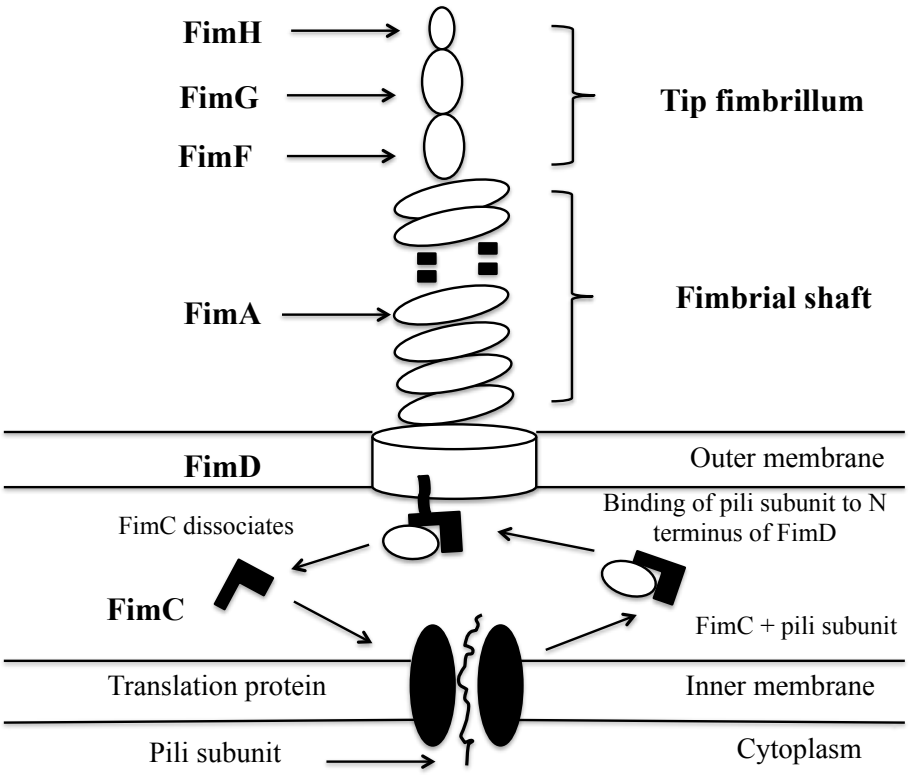


Fig. 2. Schematic of the type 1 pilus structure. The tip fimbrillum comprises the adhesin FimH, followed by one copy of each of the adaptor subunits, FimG and FimF. Pili subunit refers to *fimA*, *fimG*, *fimF* or *fimH* gene products.

Assembly of type 1 pili depends on two proteins that are not a part of the final pilus structure: FimC and FimD (Jones *et al*., 1993; Klemm & Christiansen, 1987) (Fig. 2). FimC is a member of the immunoglobulin-like periplasmic chaperone family. The chaperone binds to pilus subunits as they enter the periplasm, capping their interactive surface (Jones *et al*., 1993). FimD belongs to the outer membrane usher family of proteins. FimD not only anchors the pilus to the cell surface, but also recognizes FimC-subunit complexes in the periplasm, and mediates translocation of folded subunits through the outer membrane (Saulino *et al*., 2000).

Originally, the *fimI* gene was identified as an open reading frame located adjacent and 3' to the *fimA* gene. However, the FimI protein has not been well characterized and is predicted to encode a FimA-like protein necessary for type 1 pili biosynthesis (Valenski *et al*., 2003).

Phase Variation Controls Expression of Type 1 Pili

The expression of type 1 pili is affected by phase variation, a random ON-OFF switching process that allows individual cells to alternate between piliated (Phase-ON) and nonpiliated states (Phase-OFF) (Eisenstein, 1981) (Fig. 3). Phase variation can help the bacteria evade the host immune system and enhance the survival of UPEC cells in a variety of environments, including the human urinary tract. Phase variation is due to the inversion of a 314-bp DNA element called the *fimS* region containing the promoter (*fimAp*) for the *fimA* structural gene (Abraham *et al*., 1985a; Hedlund *et al*., 1999) (Fig. 1). When the *fimAp* is in the Phase-ON orientation, transcription of *fimA* occurs. However, when the *fimAp* is in the Phase-OFF orientation, there is no transcription of *fimA*, resulting in a nonpiliated phenotype (Burns *et al*., 2000; Klemm, 1986) (Fig. 3).

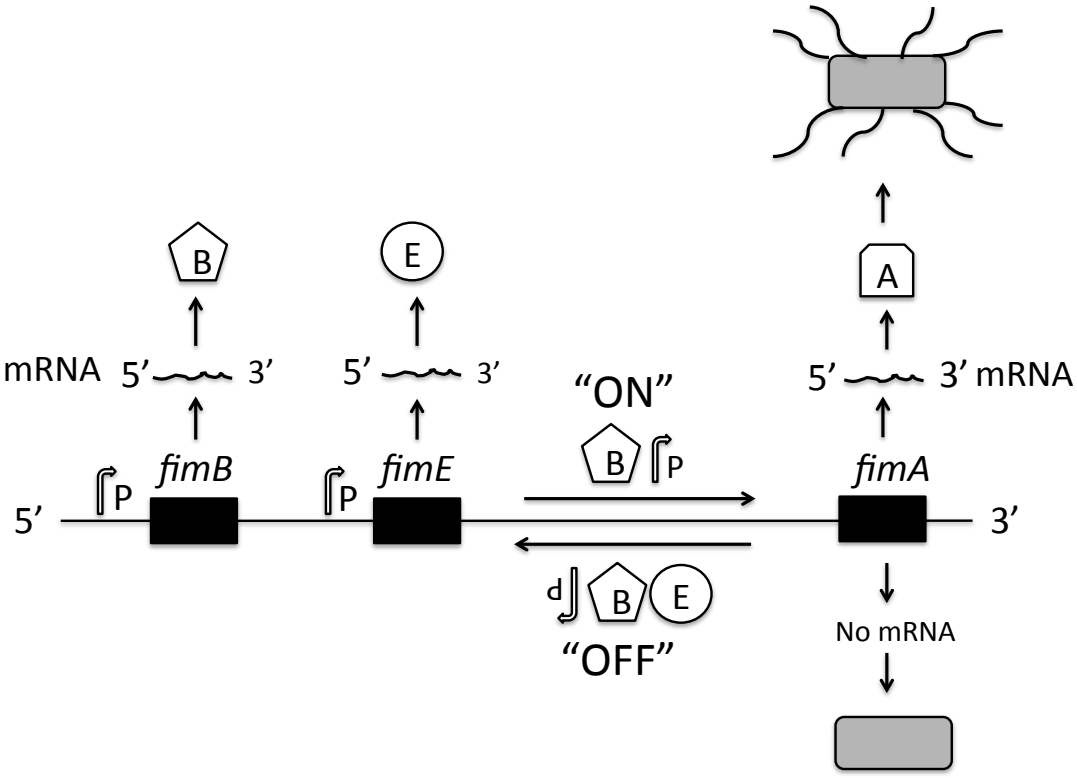


Fig. 3. A schematic showing how the FimB and FimE proteins orient the *fimS* element.

Site-specific Recombinases That Affect Phase Switching

The phase switching of the 314-bp *fimAp* sequence is controlled by the products of two regulatory genes, *fimB* and *fimE*, located upstream of *fimA* (Klemm, 1986). The *fimB* and *fimE* gene products are site-specific recombinases that influence the positioning of the *fimS* region (Klemm, 1986; McClain *et al*., 1991; McClain *et al*., 1993). FimB and FimE are related in their amino acid sequence (48% similar) and are encoded by tandem arranged genes on the chromosome located adjacent to the *fimS* region (Klemm, 1986). Both proteins belong to a tyrosine recombinase family, which rearranges DNA duplexes by means of conservative site-specific recombination reactions (Burns *et al*., 2000; Dorman & Higgins, 1987; Eisenstein *et al*., 1987; Esposito & Scocca, 1997). FimE appears to promote inversion of the promoter-containing DNA fragment from the Phase-ON to Phase-OFF orientation (Johnson & Orndorff, 1991; Kulasekara & Blomfield, 1999; McClain *et al*., 1993), whereas FimB promotes switching in both directions with a slight switching bias toward the Phase-ON orientation (Gally *et al*., 1993; Klemm, 1986; McClain *et al*., 1993).

Both of the *fim* recombinase genes are transcribed independently. The consensus is that there are two *fimB* promoters (Åberg *et al*., 2006, 2008; Donato, 1997; Schwan *et al*., 1994), although one study with an *E. coli* K12 strain has indicated a single promoter for *fimB* (Olsen & Klemm, 1994). A third potential *fimB* promoter was also identified in UPEC strains, but has not been confirmed by others (Schwan *et al*., 1994) (Fig. 1). A single promoter has been identified for *fimE* (Olsen & Klemm, 1994). Regulation of the *fimB* and *fimE* genes in UPEC cells growing in the human urinary tract and other mammals is still largely uncharacterized.

Inversion of the *fimS* region involves site-specific recombination between two 9-bp inverted repeats that border the switching sites. Each repeat is flanked by two Fim recombinase binding sites (also known as half-sites), each of which contains the important core dinucleotide 5'-CA (Gally *et al*., 1996). There are four binding sites for both FimB and FimE in the *fimS* region (Gally *et al*., 1996). These half-sites flank the 9-bp inverted repeats and are designated IRL (left-hand inverted repeat)-outside, IRL-inside, IRR (right-hand inverted repeat)-outside, and IRR-inside. The IRL-outside and IRR-outside sites are constant in Phase-ON and Phase-OFF cells, while the inside sites vary based on whether the position of the *fimS* inversion is oriented in Phase-ON or Phase-OFF (Burns *et al*., 2000; Gally *et al*., 1996). The variation is important for the directionality of the FimB- and FimE-promoted DNA inversion event (Kulasekara & Blomfield, 1999). Both FimB and FimE have been shown to bind to half-sites that flank and overlap the left and right inverted repeats of the *fimS* switching (Burns *et al*., 2000; Gally *et al*., 1996).

Cross-talk between other fimbriae regulatory proteins and the *fim* operon has been investigated. One such example is the *pap* operon, which encodes P fimbriae associated with acute pyelonephritis in UPEC. The PapB protein is a transcriptional regulator of the *pap* operon (Båga *et al*., 1985), and it inhibits FimB-promoted recombination as well as increases *fimE* expression by binding to the promoter regions of *fimB* and *fimE* (Holden *et al*., 2001; Xia *et al*., 2000). When PapB was expressed at wild-type levels, the protein inhibited type 1 pili expression in UPEC (Holden *et al*., 2006).

Cross-talk between the type 1 and S fimbrial operons has also been observed. A regulator encoded by the *sfaB* gene and situated proximal to the main structural *sfaA* gene

is able to activate expression of S fimbriae (Schmoll *et al*., 1990). The SfaB protein is also capable of partially repressing type 1 pili expression (Holden *et al*., 2001). Overexpression of another protein associated with S fimbriae expression, $SfaX_{II}$, caused downregulation of the *fimB* gene, resulting in enhanced switching from the Phase-ON to Phase-OFF orientation (Sjöström *et al*., 2009).

In addition to FimB and FimE, three additional *fimB/E*-like recombinases have been reported to affect the *fimS* switch: IpuA, IpuB, and IpbA encoded by the *ipuA*, *ipuB*, and *ipbA* genes, respectively. Genome analysis of the urosepsis isolate CFT073 demonstrated the three potential recombinases shared a significant degree of amino acid sequence similarity among themselves as well as with FimB and FimE. IpuA and IpbA were able to invert the *fimS* switch from Phase-OFF to Phase-ON. Moreover, the FimB-like protein IpuA, could invert the *fimS* switch in either Phase-ON or Phase-OFF orientations, whereas IpbA only promoted a switch to the Phase-ON position. Although the *ipuB* gene was always found associated with *ipuA* and located in the same gene locus, IpuB didn't affect the *fimS* switch region, suggesting that the IpuB was not a functional Fim recombinase (Bryan *et al*., 2006). Another novel site-specific recombinase, HbiF, was identified and found to regulate the *fimS* switch in *E. coli*. The HbiF protein has an amino acid similarity of 55% and 52% to FimB and FimE, respectively. HbiF functions by mediating Phase-OFF to Phase-ON orientation in the *fimS* switch region (Xie *et al*., 2006).

Global Regulatory Factors That Affect Phase Switching

In addition to the Fim recombinases, expression of type 1 pili is also controlled by at least three global regulatory factors: integration host factor (IHF) (Dorman &

Higgins, 1987; Eisenstein *et al*., 1987), the histone-like protein H-NS (Donato *et al*., 1997; Kawula & Orndorff, 1991), and leucine-responsive regulatory protein (Lrp) (Blomfield *et al*., 1993) (Fig. 4).

The IHF is a heterodimeric site-specific DNA-binding protein, consisting of two subunits: IHFα encoded by the *himA* gene and IHFβ encoded by the *hip/himD* gene (Flamm & Weisberg, 1985; Miller & Nash, 1981; Miller *et al*., 1981; Nash & Robertson, 1981). The IHF protein has been shown to participate in a variety of processes in *E. coli*, including site-specific recombination and transcriptional control (Freundlich *et al*., 1992). The IHF protein recognizes and binds to a specific DNA sequence to introduce sharp bends facilitating recombination events in *E. coli*, which includes playing a significant role in type 1 pilus switching within UPEC strains. Mutations in either *himA* or *hip/himD* locked the *fimS* region in either the Phase-ON or Phase-OFF orientation (Dorman & Higgins, 1987; Eisenstein *et al*., 1987). Two IHF-binding sites were identified that were associated with the *fimS* region: IHF I (site I) was located between the end of *fimE* and IRL-outside of the *fimS* region, and IHF II (site II) proximal to IRR within the *fimS* region (Blomfield *et al*., 1993; Blomfield *et al*., 1997) (Fig. 4). Mutations in site I or site II that lowered the affinity of IHF binding *in vitro* were found to lower the frequency of FimE and/or FimB recombination *in vivo*. IHF I had a relatively pronounced effect on FimB recombination but a marginal effect on FimE recombination, suggesting a direct bias for FimB-promoted recombination (Blomfield *et al*., 1997; Dove & Dorman, 1996; Leathart & Gally, 1998).

In addition to IHF, the DNA-binding protein H-NS is another global regulator in *E. coli*. The 15.6 kDa H-NS protein affects DNA supercoiling (Dürrenberger *et al*.,

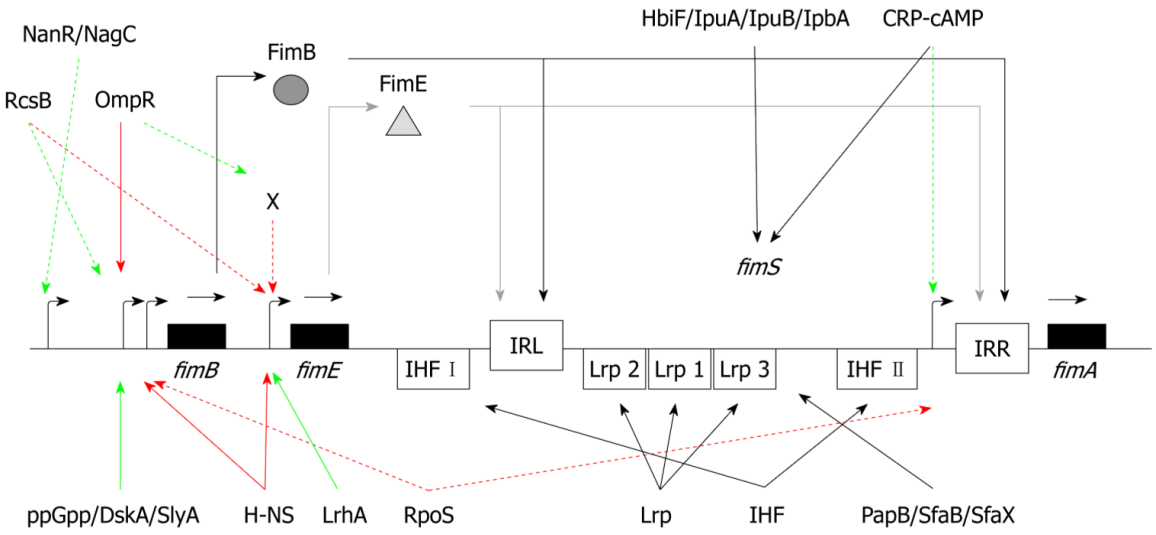


Fig. 4. Schematic model of the actions of 20 auxiliary proteins on the regulation of type 1 pili. The inverted repeat left and right (IRL and IRR) are shown as open boxes. Binding sites for integration host factor (IHF I and II) and leucine-responsive protein (Lrp1, 2, and 3) are also represented as open boxes. Genes are displayed as black boxes and the promoters are shown as bent black arrows. The dark gray arrows correspond to FimB and the light gray arrows are for FimE. Black arrows signify an effect on the *fimS* element. Solid green arrows indicate confirmed binding associated with stimulatory effects, whereas dashed green arrows indicate presumed stimulatory effects. Solid red arrows indicate confirmed binding associated with repressing effects, whereas dashed red arrows indicate presumed repressing effects [permission granted by W.R. Schwan from Schwan *et al*. (2011)].

1991;Tupper *et al*., 1994) and condenses DNA (Dame *et al*., 2000) by preferentially binding to curved DNA *in vitro* (Jordi *et al*., 1997; Yamada *et al*., 1990). H-NS controls expression of over 200 genes, including the *fimB* and *fimI* genes (Hommais *et al*., 2001).

The H-NS protein has an important, but poorly understood, role in modulating the *fimS* switching. H-NS directly effect post-transcription of the *fimS* element by binding to DNA segments adjacent to and within the *fimS* region (Donato & Kawula, 1999; Schembri *et al*., 1998). In addition, H-NS has an indirect effect on the *fimS* region by binding with high affinity to the promoter regions of *fimB* to repress the transcription of *fimB* gene (Donato *et al*., 1997; Donato & Kawula, 1999; Kawula & Orndorff, 1991; O' Gara & Dorman, 2000; Schembri *et al*., 1998) and binding to DNA segments harboring the promoter region of *fimE* acting as a repressor (Olsen *et al*., 1998). Moreover, H-NS represses the *lrp* gene, which promotes DNA recombination of the *fimS* element (Oshima *et al*., 1995).

In addition to H-NS, SlyA has been shown to have overlapping binding sites with H-NS on the *fimB* promoter region and be capable of displacing H-NS from its binding sites *in vitro*. Expression of *fimB* is derepressed in an *hns* mutant and diminished by a *slyA* mutation in the presence of H-NS only. SlyA appeared to activate *fimB* gene expression by preventing H-NS binding to the *fimB* promoter, limiting type 1 pili expression. Unfortunately, this study didn't demonstrate whether growth conditions affected *slyA* gene expression related to *fim* gene transcription (McVicker *et al*., 2011).

Besides H-NS, inversion of *fimS* requires Lrp. Lrp is a site-specific DNA-binding global regulatory protein that controls at least 40 genes in *E. coli*, including fimbrial expression, amino acid transport, degradation, and biosynthesis (Blomfield *et al*.,

1993; Ernsting *et al*., 1992; Nou *et al*., 1993; Wang & Calvo, 1993; Willins *et al*., 1991). Mutations in *lrp* markedly reduced both FimB- and FimE-promoted switching of the *fimS* element, indicating Lrp stimulates switching of the *fimS* element in both directions (Blomfield *et al*., 1993). There are three Lrp binding sites within the *fimS* region (Fig. 4). Lrp binds to two of the sites (Lrp sites 1 and 2) where it acts positively and cooperatively to stimulate DNA recombination (Gally *et al*., 1994; Roesch & Blomfield, 1998). In the presence of leucine, Lrp attaches to Lrp site 3, which inhibits recombination and has little effect on Lrp binding to Lrp sites 1 and 2 (Roesch & Blomfield, 1998). Lrp binding to Lrp sites 1 and 2, together with IHF binding to its IRL-proximal IHF-1 site, maintains the *fimS* region in its Phase-ON orientation (Corcoran & Dorman, 2009; Kelly *et al*., 2006). In the absence of both Lrp and IHF binding, the *fimS* region adopts the Phase-OFF orientation and the H-NS protein is required to sustain this alternative orientational bias (Corcoran & Dorman, 2009). Lrp has no direct effect on the transcription of *fimB* and *fimE* genes.

Other Proteins That Affect Phase Switching

Some other proteins also exert their effects on type 1 pili expression. The LysR-type regulatory protein (LrhA) activates *fimE* expression, promotes Phase-OFF orientation switching, and reduces type 1 pili expression (Blumer *et al*., 2005). The outer membrane protein OmpX is also involved in type 1 pili expression. An *ompX* mutant produced more FimA protein compared to wild-type cells, reflecting a defect in an adaptive response to surface contact that requires type 1 pili (Otto & Hermansson, 2004). Both IbeA and IbeT located on the same GimA genomic island are predicted to be potentially involved in carbohydrate metabolism (Huang *et al*., 2001). Deletion of *ibeA* or

ibeT increased the *fimS* switching to the Phase-OFF orientation and decreased expression of type 1 pili compared with the wild- type parent (Cortes *et al*., 2008). The mechanism by which IbeA and IbeT alter the expression of type 1 pili still remains unclear.

A regulatory alarmone ppGpp has been also implicated in the expression of type 1 pili and biofilm formation in UPEC. Mutations that eliminated ppGpp production reduced type 1 pili production compared with the wild-type strain by modulating transcription of *fimB* (Åberg *et al*., 2006) (Fig. 4). The DksA protein, a cofactor for ppGpp-mediated gene regulation, also alters type 1 pili expression by altering *fimB* transcription (Åberg *et al*., 2008).

Another alarmone regulator that has been associated with type 1 pili expression is 3', 5'-cyclic adenosine monophosphate (cAMP) and its receptor protein (CRP). In the presence of glucose, both the intracellular cAMP and CRP levels drop, causing catabolite repression of more than 200 genes including those involved in the expression of type 1 pili (Eisenstein *et al*., 1981; Eisenstein & Dodd, 1982; Ishizuka *et al*., 1993; Müller *et al*., 2009; Shimada *et al*., 2011).

Other factors that affect type 1 pili expression are RpoS protein and a Rho-dependent RNA terminator. The stationary phase sigma factor, RpoS, is thought to have a negative regulatory effect on *fimA* and *fimB* transcription (Dove *et al*., 1997; Loewen, 1994). A Rho-dependent transcriptional terminator identified in the *fimS* region represses *fimE* transcription and indirectly alters post-regulation of the *fimS* element (Hinde *et al*., 2005; Joyce & Dorman, 2002).

Two proteins involved in the catabolism of sialic acid also affect type 1 pili expression. NanR and NagC, induced by sialic acid, can bind to a 1.4 kbp intergenic

DNA region upstream of the P1 and P2 *fimB* promoters and downstream of the *yihATS* genes and activate the *fimB* gene *in cis* (El-Labany *et al*., 2003; Plumbidge, 2001; Sohanpal *et al*., 2004, 2007) (Fig. 4). The location of the NanR and NagC binding sites within the 1.4 kbp DNA region maps to the location of the P3 *fimB* promoter previously identified (Schwan *et al*., 1994). One tRNA may act as a regulatory molecule to control *fim* gene expression. LeuX, encoded by the *leuX* gene, encodes a tRNA specific for the rare leucine codon UUG. LeuX is thought to be required for translation of *fimB* and subsequent type 1 pili expression (Hannan *et al*., 2008; Klemm, 1986; Newman *et al*., 1994; Ritter *et al*., 1997).

Two-component Regulatory Systems That Affect *fim* Gene Transcription

In addition to the many proteins described above, proteins that comprise two-component regulatory systems can exert a profound effect on type 1 pili expression. Two-component regulatory proteins are expressed under specific growth conditions, some of which are important for growth in the urinary tract. These systems are generally composed of a sensor kinase and a response regulator. The sensor kinase is a histidine protein kinase (HK), usually a membrane protein, which detects the environmental stimulus, uses ATP to autophosphorylate its His residues, and transmits a signal to a response regulator (RR), usually a cytoplasmic protein, which is phosphorylated at its Asp residues. Phosphotransfer from the HK to the RR results in activation of the RR that elicits a response to environmental changes, such as nitrogen, phosphate limitation, pH, osmolarity, anaerobiosis, and chemotaxis, usually at the level of transcription (Hoch, 2000; Ronson *et al*., 1987).

One two-component regulatory system implicated in the regulation of type 1

pili expression is the QseB/QseC system (Hadjifrangiskou *et al*., 2011; Kostakioti *et al*., 2009). QseC is a membrane sensor kinase, which responds to host and bacterial signals by phosphorylating the QseB response regulator to activate or repress its target gene expression. QseC is important for both carbon and nitrogen metabolism (Hadjifrangiskou *et al*., 2011). Deletion of the *qseC* gene impaired intracellular bacterial community formation and attenuated UPEC, while a single *qseB* or double *qseBC* deletion didn't directly affect UPEC virulence. In a murine urinary tract infection model, QseB upregulated its own expression in a *qseC* mutant, resulting in downregulation of type 1 pili, curli, and flagella (Kostakioti *et al*., 2009).

Another *E. coli* two-component response regulatory system that affects type 1 pili expression is RcsC/RcsB. RcsC/RcsB was initially identified as a regulator of the synthesis of the capsular polysaccharide in *E. coli* (Gottesman *et al*., 1985). RcsC is an integral membrane protein that senses the environmental stimulus and transmits a signal by phosphorelay to an effector RcsB, which then acts as a transcription factor. Under a neutral-pH, low-salt growth condition, an *rcsB* mutant displayed significantly reduced type 1 pili expression. RcsB appears to be an activator of *fimB* transcription in a neutral-pH growth environment (Schwan *et al*., 2007).

A phosphate-specific transport (Pst) system specific for inorganic phosphate is controlled by a two-component regulatory system, PhoB/PhoR, in response to phosphate-limiting conditions (Wanner, 1996). The PhoB/PhoR system is also associated with type 1 pili expression. Inactivation of *pst* repressed transcription and production of type 1 pili both *in vitro* and *in vivo* due to reduced expression of the *fimB*, *ipuA*, and *ipbA* recombinase-encoding genes and increased expression of the *fimE* gene (Crépin *et al*.,

2012).

Another two-component regulatory system linked to the regulation of type 1 pili is BarA/UvrY, which functions in several metabolic processes including biofilm formation and the efficient adaption to carbon utilization in UPEC (Mitra *et al*., 2013; Pernestig *et al*., 2003). Deletion of *uvrY* reduced transcription of *fimA* and turned off expression of type 1 pili. Both *fimB* and *fimE* transcription also diminished in the *uvrY* mutants (Mitra *et al*., 2013).

Lastly, the global regulatory protein OmpR is involved in *fim* gene expression. OmpR is part of the EnvZ/OmpR two-component signal transduction regulatory system that responds primarily to an osmotic stress environmental stimulus in bacteria (Mizuno & Mizushima, 1990). In a low pH/high osmolarity growth condition, an *ompR* mutant derepressed *fimB* expression compared to growth in a neutral-pH low-osmolarity condition (Schwan *et al*., 2002). Furthermore, unphosphorylated OmpR bound to the promoter region 2 of *fimB* gene, which confirmed that unphosphorylated OmpR was sufficient to repress *fimB* expression (Fig. 4). Neither unphosphorylated OmpR nor phosphorylated OmpR bound to the *fimE* promoter, suggesting an intermediate is involved (Rentschler *et al*., 2013). These results indicate that osmotic stress has a substantial impact on UPEC type 1 pili expression. Since the human urinary tract has a number of niches with high osmolarity, the EnvZ/OmpR system would be important.

Environmental Cues That Affect *fim* Gene Expression

Two important environmental cues affecting UPEC growth in the murine or human urinary tract are pH and osmolarity. Human urine can allow the growth of UPEC strains in pH values ranging from 5.0 to 8.0 (Ross & Neely, 1983). Besides the variation

in pH, the osmolarities of human urine can vary between 0.038 and 1.4 mol kg^{-1} (0.7 M NaCl). Human urine has been shown to repress expression of type 1 pili (Schwan *et al*., 2002). Both urea and inorganic ions are present in significant levels in urine. Urea induces the transcription of *fim* gene expression *in vitro* (Withman *et al*., 2013).

Previous work in our laboratory has demonstrated that pH and osmotic changes in growth media have an effect on *fim* gene expression (Schwan *et al*., 2002). Transcription of *fimA*, *fimB*, and *fimE* were reduced in the bacteria growing in acidic Luria broth (LB) medium. The combination of an acid pH with high osmolarity in the growth media resulted in a significant decline in *fimB* and *fimA* transcription, but a slight increase was observed in *fimE* transcription. Enzyme immunoassays with type 1 pili antibody showed that type 1 pili expression level was significantly lower in bacteria strains grown in low pH-high osmolarity compared to neutral pH/low osmolarity conditions (Schwan *et al*., 2002).

The effects of the low pH/high osmolarity environmental cues on *fim* gene transcription *in vitro* described above may offer us some insights into the mechanism by which the regulation of *fim* gene takes place. However, they don't tell us whether *fimA*, *fimB*, or *fimE* will be regulated in UPEC growing in the urinary tract nor to what extent these genes may be transcriptionally regulated. Very few studies have examined the regulation of *fim* gene expression in UPEC colonizing a murine urinary tract. A limited number of studies had examined the expression of type 1 pili in UPEC growin *in vivo* (Hultgren *et al*., 1985; Schaeffer *et al*., 1987; Snyder *et al*., 2005). Several studies have examined positioning of the *fimS* element in UPEC strains infected murine bladders and kidneys (Crépin *et al*., 2012; Gunther *et al*., 2001, 2002; Lim *et al*., 1998; Snyder *et al*.,

2006; Struve & Krogfelt, 1999). Very few studies have examined the regulation of specific *fim* gene in UPEC infecting murine bladders. One study examined *fimB* and *fimE* expression over a two-day period (Crépin *et al*., 2012).

In order to address whether *fim* gene expression will be regulated in UPEC cells found in murine tissues in the same manner as conditions that were tested *in vitro*, we constructed *fim-lux* transcriptional fusions to examine *fimA*, *fimB*, and *fimE* transcription in a UPEC strain colonizing murine urinary tracts over a five-day period. This *lux* reporter system had been successfully used to measure regulatory differences in *Staphylococcus aureus* infecting murine urinary tissues (Schwan *et al*., 2006). In this study, we have demonstrated that the *fimA*, *fimB*, and *fimE* genes were differentially regulated in *E. coli* colonizing the bladder versus the kidney. Within UPEC infected murine bladders, *fimA* and *fimB* transcription were high, whereas all of the *fim* genes had lower levels of transcription over time in UPEC colonizing the murine kidneys. Our results may help us understand the temporal regulation of these adhesin genes in *E. coli* colonizing the urinary tract.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Growth Conditions

All strains and plasmids used in this study are listed in the Table 1. *E. coli* DH5α MCR (Gibco/BBL) was used for all of the cloning and vector construction. *E. coli* strain NU149 (Schaeffer *et al.*, 1987) is a UPEC clinical isolate. All strains were grown statically in Luria-Bertani (LB) broth at 37°C or were passaged on Luria agar (LA) plates incubated at 37°C. For recombinant *E. coli* strains, the following antibiotic concentrations were used unless otherwise noted: ampicillin, 100 μg ml^{-1}; chloramphenicol, 12.5 μg ml^{-1}; kanamycin, 40 μg ml^{-1}; and erythromycin, 150 μg ml^{-1}.

Construction of the *fim-lux* Transcriptional Fusions

To create the *fimA-lux* transcriptional fusion on the single copy plasmid pPP2-6, pAON-1 plasmid DNA was extracted using a commercial kit (Qiagen). The pAON-1 DNA containing the promoter for *fimA* was digested with the restriction endonuclease enzymes *Eco*RI and *Bam*HI to separate the *fimA* promoter sequence from the pUJ8 backbone (de Lorenzo *et al.*, 1990) (Fig. 5). Next, pHSS22 plasmid DNA (Fig. 6) was extracted and cut with the same restriction endonuclease enzymes described above. The *fimA* promoter DNA sequence following restriction endonuclease digestion was ligated to cut pHSS22 DNA using T_4 DNA ligase (Schwan *et al.*, 1992), and the ligation products were transformed into *E. coli* DH5α MCR cells. Transformants were selected on LA plates containing 40 μg kanamycin ml^{-1}. Several transformants were screened for the proper sized plasmid DNA. An aliquot of plasmid DNA was cut with *Eco*RI and *Bam*HI

Table 1. Strains and plasmids used in this study

Strains / plasmids	Description	Reference or source
E. coli strains		
NU149	Clinical isolate	Schaeffer *et al*. (1987)
DH5α MCR	General cloning strain	Gibco/BBL
Plasmids		
pAON-1	*fimA-lacZYA* locked on on pUJ8	Schwan *et al*. (2002)
p5-48	*fimB-lacZYA* on pUT-Tc	Schwan *et al*. (2002)
pMP5-2.17	*fimE-lacZYA*	Schwan *et al*. (2002)
pUJ8	*trp´-´lacZ phoA* Ap^r	de Lorenzo *et al*. (1990)
pHSS22	*ori*T Km^r	Schwan *et al*. (2002)
pXen-5	T_s origin, Tn*4001*, promoterless *lux*	Francis *et al*. (2001)
pPP2-6	pPR274 with MCS[§]	Schwan *et al*. (2002)
pWS145-38	*fimB-lux* fusion on pPP2-6	William Schwan
pHD-01	*fimA* promoter on pHSS22	This study
pHD-02	*fimA-lux* fusion on pHSS22	This study
pHD-03	*fimA-lux* fusion on pPP2-6	This study
pHD-04	*fimE* promoter on pHSS22	This study
pHD-05	*fimE-lux* fusion on pHSS22	This study
pHD-06	*fimE-lux* fusion on pPP2-6	This study
pHD-07	*ftsZ* promoter on pHSS22	This study
pHD-08	*ftsZ-lux* fusion on pHSS22	This study
pHD-09	*ftsZ-lux* fusion on pPP2-6	This study

[§] MCS = multiple cloning sites.

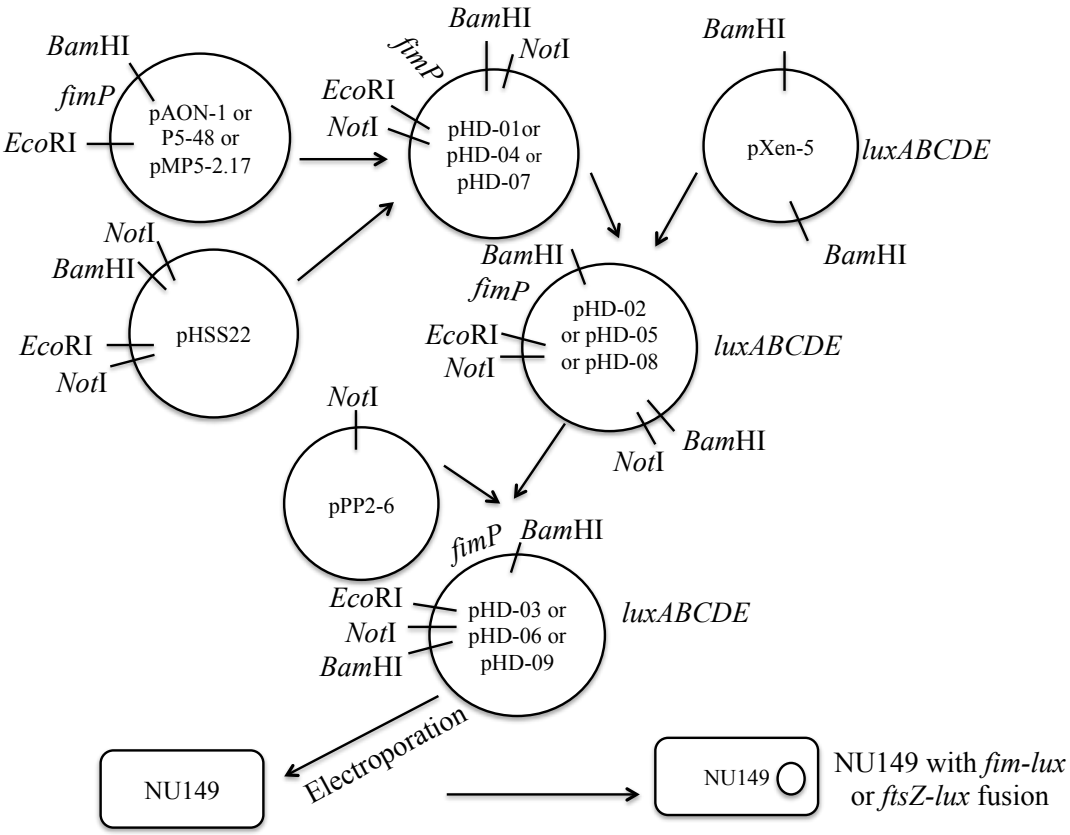


Fig. 5. The flow diagram for the construction of the *E. coli* strain NU149 containing recombinant plasmids with *fim-lux* or *ftsZ-lux* fusions. The *fimP* represents the promoter sequence of *fim* gene. The circles represent the plasmids. The short lines across the circles represent the restriction endonuclease cut site on the plamid. Different restriction endonucleases are indicated.

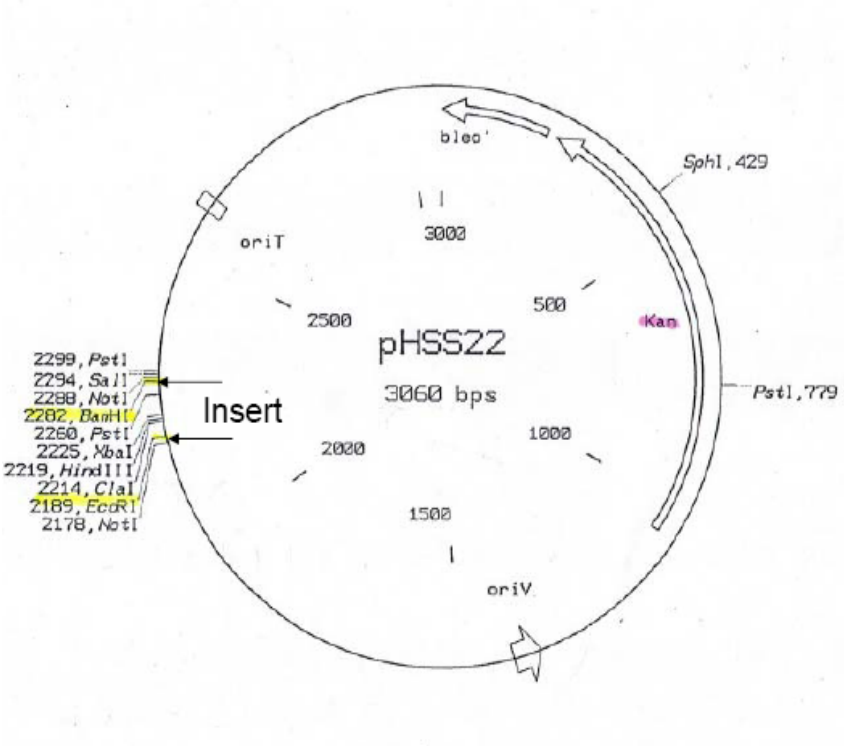


Fig. 6. Plasmid map of pHSS22. The arrows show *Eco*RI and *Bam*HI restriction endonuclease enzyme cut sites.

restriction endonucleases digestion to confirm insertion of the *fimA* promoter DNA. One clone, labeled pHD-01, was chosen for additional processing.

Next, pXen5 (Fig. 7) plasmid DNA was extracted as described above, cut with *Bam*HI, and ligated to *Bam*HI-cut pHD-01 plasmid DNA created above. The ligation DNA was transformed into *E. coli* strain DH5α MCR. Plasmid pXen5 contains a kanamycin resistance gene for use in Gram-positive bacteria, an erythromycin resistance gene for use in Gram-positive and negative bacteria and a promoterless *lux* operon. The transformants were then plated onto LA containing 40 μg kanamycin ml^{-1} incubated at 37°C. All transformants were patched onto LA plates containing 150 μg erythromycin ml^{-1} as well as LA containing 40 μg kanamycin ml^{-1}. Any transformants that were kanamycin-resistant (Kan^R) but erythromycin-sensitive (Erm^S) were inoculated into LB containing 40 μg ml^{-1} kanamycin and statically incubated at 37°C. Bioluminescence measurements were performed on these Erm^SKan^R cultures, and any transformants exhibiting luminescence above background levels had plasmid DNA extracted as described above. Plasmid DNAs were then digested with the restriction endonuclease *Pst*I to confirm the insertion of the *lux* operon into pHD-01 plasmid. One clone that showed bioluminescence as well as the proper restriction endonuclease enzymes digestion pattern was named pHD-02, and was chosen for further analysis.

To avoid problems suffered by multicopy-plasmid-based systems, a single copy plasmid pPP2-6 was used as the final vector for the *fimA-lux* transcriptional fusion (Fig. 8). The pPP2-6 plasmid has a chloramphenicol resistance gene and an origin of replication to replicate a single copy. Plasmid DNAs from pPP2-6 and pHD-02 constructs were digested with the *Not*I restriction endonuclease, ligated together using T_4 DNA

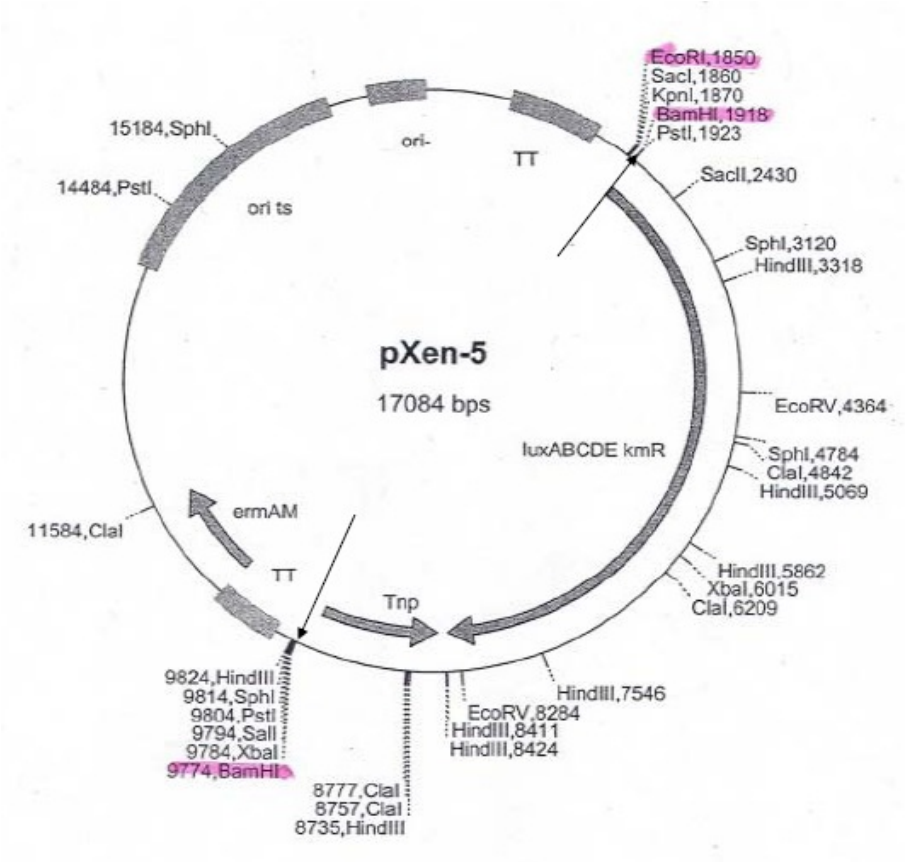


Fig. 7. Plasmid map of pXen-5. Plasmid pXen-5 possesses two *Bam*HI restriction enzyme cut sites shown by the arrows.

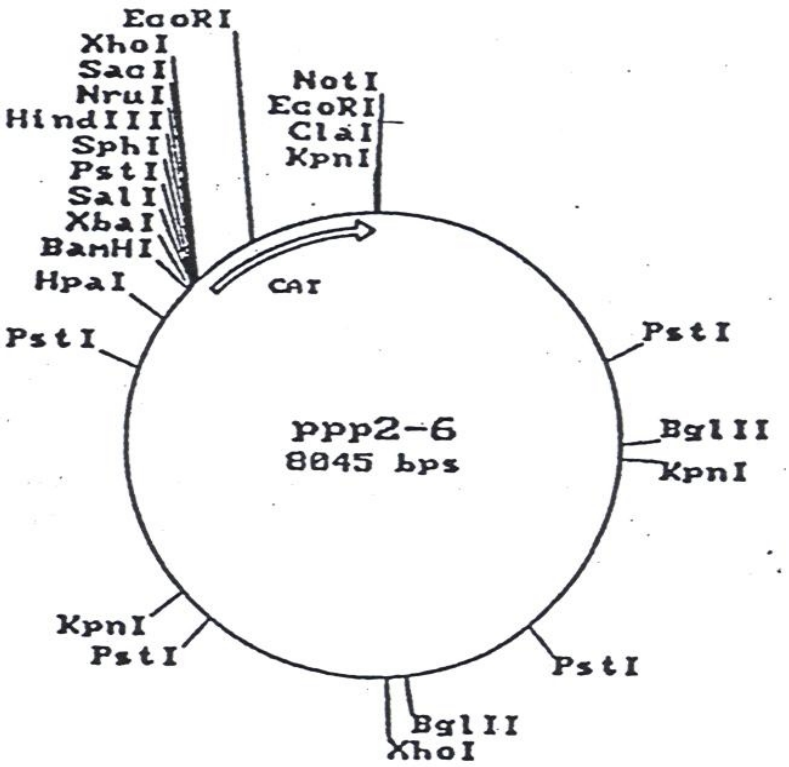


Fig. 8. Plasmid map of pPP2-6.

ligase, and transformed into *E. coli* DH5α MCR cells. Transformants were plated onto LA containing 12.5 μg chloramphenicol ml^{-1} and incubated at 37°C. Any transformants that arose were inoculated into three ml aliquots of LB containing 12.5 μg chloramphenicol ml^{-1} and incubated statically at 37°C. Each broth culture was tested for bioluminescence as previously described. Any transformants that displayed bioluminescence above background levels had their plasmid DNAs extracted and digested with *Not*I to verify the correct recombinant plasmid was constructed. The final *fimA-lux* containing plasmid in the pPP2-6 backbone was named pHD-03.

E. coli strain NU149 is a clinical isolate obtained from a patient with cystitis that expresses type 1 pili, but not P pili (Schaeffer *et al.*, 1987). The NU149 strain has been used for *fim* gene transcriptional analyses and type 1 pili expression (Rentschler *et al*., 2013; Schwan *et al*., 1992; Schwan *et al*., 1994; Schwan *et al*., 2002). Plasmid pHD-03 was electroporated into electrocompetent strain NU149 cells by a procedure described by Casali *et al*. (Casali & Preston, 2003).

Electroporated NU149 cells were plated on LA containing 12.5 μg chloramphenicol ml^{-1} and incubated at 37°C. All the transformants were inoculated into three ml of LB containing 12.5 μg chloramphenicol ml^{-1}, and statically incubated at 37°C. Transformants were screened for bioluminescence. Any transformants that displayed bioluminescence above background levels had their plasmid DNA extracted to confirm the presence of the pHD-03 plasmid. One clone was chosen for further analysis.

For construction of the *fimE-lux* transcriptional fusion on the pPP2-6, plasmid pMP5-2.17 containing the *fimE* promoter was used (Schwan *et al*., 2002). The pMP5-2.17 plasmid DNA was extracted as described above, cut with *Eco*RI and *Bam*HI

restriction endonuclease enzymes, ligated to *Eco*RI and *Bam*HI cut pHSS22 plasmid DNA using T_4 DNA ligase, and transformed into DH5α MCR cell as described above. Transformants were selected on LA containing kanamycin. Plasmid DNAs from several transformants were cut with *Eco*RI and *Bam*HI. One clone, labeled pHD-04, was used for further analysis.

The plasmid DNAs from pHD-04 and pXen-5 were digested with *Bam*HI, ligated with T_4 DNA ligase, and transformed into DH5α MCR cells as previously described. The transformants were selected on LA containing kanamycin and erythromycin, respectively. Several transformants that were $Kan^R Erm^S$ had their bioluminescence measured, plasmid DNAs were extracted and digested with *Pst*I. One of the resulting plasmids was named pHD-05. Plasmid DNA from pHD-05 was cut with *Not*I, ligated to *Not*I digested pPP2-6, and transformed into DH5α MCR cells. Transformants were selected on LA containing 12.5 μg chloramphenicol ml^{-1}. Plasmid DNA from one of the transformants that had the proper *Not*I digested pattern and caused bioluminescence of the *E. coli* was labeled pHD-06. Plasmid DNA from pHD-06 was transformed into *E. coli* strain NU149 cells via electroporation. Following selection on LA with chloramphenicol, several transformants were screened for bioluminescence as described above. One clone was chosen for further analysis.

The *fimB-lux* transcriptional fusion in pWS145-38 was provided by William Schwan. This plasmid has the *fimB-lux* transcriptional fusion in the pPP2-6 backbone. Plasmid DNA from pWS145-38 was electropolated into NU149. Transformants were selected on LA plus 12.5 μg chloramphenicol ml^{-1} and was screening for bioluminescence as described above. One clone was chosen for further analysis.

Construction of the *ftsZ-lux* Transcriptional Fusion

For a housekeeping gene control for this study, the *ftsZ* gene was chosen, which we have used previously (Rentschler *et al*., 2013; Schwan *et al*., 2002, 2007). In order to construct the *ftsZ-lux* transcriptional fusion, the *ftsZ* promoter region was amplified using the primer pair EcFtsZ1 (5'TAGCGGTA TCACCAAAGGACT-3') and EcFtsZ2 (5'-GTGATCAGAGATGCT-3') using DNA sequence obtained from one *E. coli* genome sequencing project (Blattner *et al*., 1997). The DNA fragment was amplified with the total 50 µl volume of PCR nucleotide mix (Appendix B), Taq DNA polymerase, and 50 pmol of each primer using a thermal cycler under the following PCR conditions: initial denaturation at 95°C for 5 min; 35 cycles consisting of denaturation at 95°C for 1 min, annealing at 55°C for 1 min, and elongation at 72°C for 1 min. The final *fts*Z PCR product had an *Eco*RI restriction endonuclease site at the 5' end and a *Bam*HI restriction endonuclease site at the 3' end. This *ftsZ* DNA was cut with *Eco*RI and *Bam*HI, ligated to *Eco*RI and *Bam*HI cut pHSS22 DNA, and transformed into DH5α MCR cells (Fig. 5). Transformants were selected on LA with kanamycin as described above. One of the resulting transformants was named pHD-07. Plasmid pHD-07 DNA was extracted, cut with *Bam*HI, ligated to *Bam*HI digested pXen5 DNA, and the ligation mixture transformed into DH5α MCR cells. Transformants were selected on LA with kanamycin and erythromycin, screened for bioluminescence as previously noted. One plasmid, labeled pHD-08, was created. An aliquot of pHD-05 Plasmid DNA was digested with *Not*I, ligated to *Not*I-cut pPP2-6 DNA, and transformed into DH5α MCR cells. The selection and screening bioluminescence of transformants were performed as described above. One of the plasmids, labeled pHD-09, was transformed into *E. coli* NU149 as

described above and a transformant was used for additional analysis.

Testing *in vitro* Environmental Conditions

A previous study has shown that acidic pH and high osmolarity environmental cues regulate the expression of type 1 pili *in vitro* (Schwan *et al*., 2002). To measure changes following growth in media with different pH and/or osmolarity, LB was buffered using 0.1 M Na_2HPO_4-NaH_2PO_4 buffer and 1% (vol/vol) glycerol as previously described (Schwan *et al*., 2002) (Appendix C). The media was separated into a pH ranging between 5.0 and 8.0 at 0.5-pH unit increments. Osmolarity differences range from 0 mM to 800 mM. Cultures of *E. coli* NU149 containing *fimA-, fimE-, fimB-, or ftsZ-lux* on single copy recombinant plasmids were incubated overnight at 37°C statically in the buffered LB medium at a specific pH. The next day 100 µl of each overnight culture was transferred to another three ml aliquot of buffered LB medium at a specific pH and/or osmolarity, and incubated statically at 37°C until mid-logarithmic phase had been reached. Bioluminescence testing was performed as described below.

In vitro Spectrophotometric Analysis and Bioluminescence Assays

Each culture was incubated at 37°C statically to mid-log phase. A five hundred microliter aliquot of each culture was tested for bioluminescence at optical densities at 600 nm (OD_{600}) using a UV-1601 spectrophotometer (Shimadzu). Next, the same aliquot of each culture was tested using a FB 12 bioluminescence single tube luminometer (Zylux Corporation) and reported as relative luminescence units (RLU). The RLU values were divided by OD_{600} readings to achieve corrected RLU for each culture (Schwan *et al*., 2006).

Murine Infection Model

A murine urinary tract infection model (Schaeffer *et al*., 1987) was used to assess the *in vivo* regulation of the *fim-lux* reporter fusions in *E. coli* NU149. Briefly, each *E. coli* NU149 strain with a specific *fim-lux* transcriptional fusion plasmid was grown in LB medium at 37°C statically overnight. The cultures were pelleted by centrifuging at 6000 x *g* for two minutes at 4°C and poured out the supernatant. The pellets were suspended in 4 ml of phosphate-buffered saline (PBS) with 2 ml of 50% glycerol added, aliquot into four 1.5 ml Sarstedt tubes and stored at -80°C. Next, one aliquot of cultures was thawed. A series of dilution to the culture was perform, plated on LA containing 12.5 μg chloramphenicol ml^{-1}, and incubated at 37°C. The number of colonies per ml was calculated. A 250 μl volume of 10^9 CFU ml^{-1} bacteria was taken from these known quantitive colonies and instilled into the urinary bladders of five to ten female 4- to 6-week-old Swiss Webster mice per time point through a soft polyethylene catheter adapted to a needle. After 0.33, 1, 3, and 5 days post-inoculation, mice were euthanized and the bladder and kidney were removed. Each organ was homogenized in sterile tissue grinders (Kontes, Radnor, PA) with 1 ml of PBS (Sambrook *et al*., 1989) (Appendix D). The homogenized tissues were tested for bioluminescence as described above. Ten-fold serial dilutions of each organ homogenate in PBS for each construct were performed, and aliquots of each dilution were plated in duplicate onto LA containing 12.5 μg chloramphenicol ml^{-1}. The plates were incubated overnight at 37°C and the number of colonies per organ homogenate per construct were calculated. The RLU values were divided by the number of bacteria determined by the viable bacteria counts, generating corrected RLU per time point as reported previously (Schwan *et al*.,

2006).

Statistical Analysis

The student's *t* test was used for statistical analysis of the *in vitro* growth conditions. P values ≤ 0.05 were considered significant. An ANOVA analysis with a Bonferroni correction was used for *in vivo* analysis from the murine urinary tract organ homogenates.

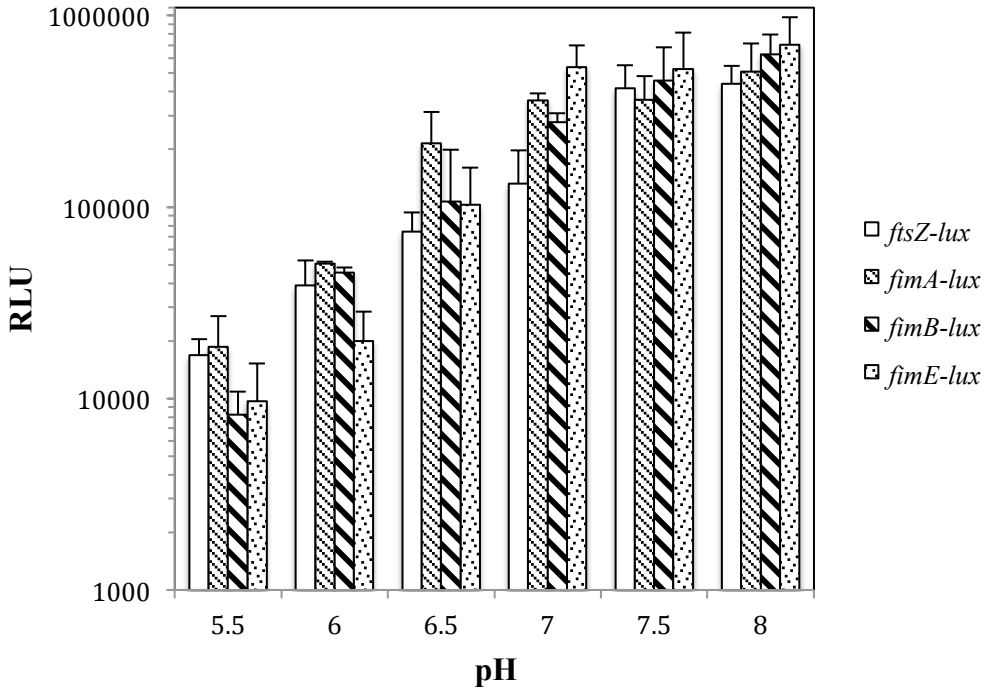
RESULTS

Evaluation of the *fim-lux* Fusions in UPEC Growing in Different pH Media

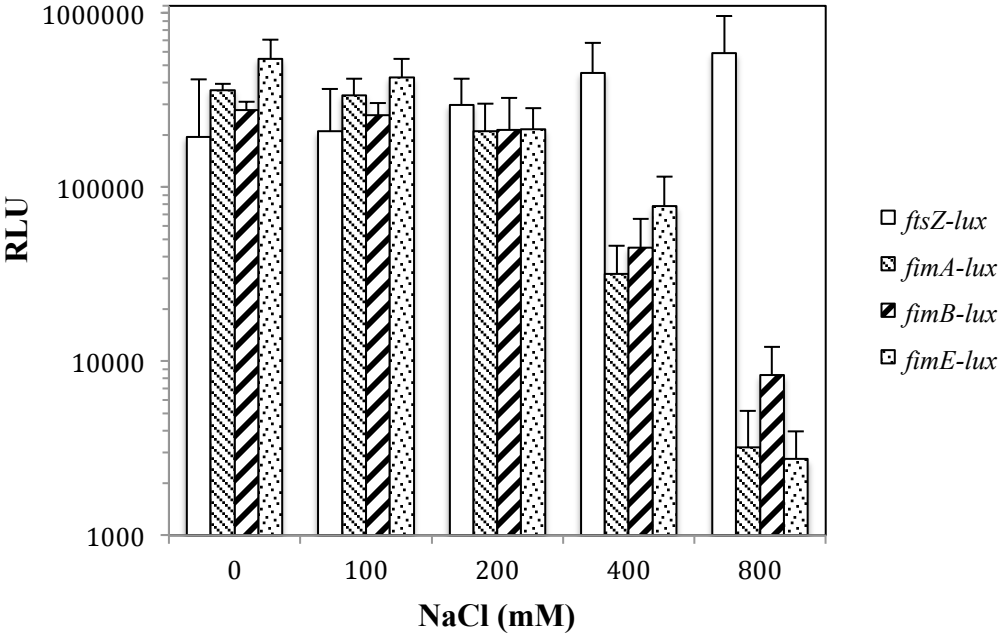
Previously, *fim-lacZYA* fusion were created and tested in *E. coli* strains growing under *in vitro* conditions (Schwan *et al*., 2002). Because of the limitations of using *lacZYA* operon fusions in UPEC growing in animal tissues, several *fim-lux* transcriptional fusions were created. All the *fim-lux* fusions were created on the single-copy number plasmid pPP2-6, and transformed into the clinical *E. coli* strain NU149. Once the *fim-lux* transcriptional fusions on pPP2-6 were electroporated into strain NU149, each strain containing a *fim-lux* transcriptional fusion on a single-copy plasmid was inoculated into LB adjusted to various pHs that ranged from 5.5 to 8.0 to verify the regulatory patterns that were observed using *lux* transcriptional fusions.

When the *E. coli* cells were grown to mid-log phase in various pH media, all three of the *fim-lux* fusions (*fimA-lux, fimB-lux*, and *fimE-lux*) displayed the lowest level of expression at pH 5.5 (*fimA-lux*, 1.9 x 10^4 RLU; *fimB-lux*, 8.2 x 10^3 RLU; and *fimE-lux*, 9.7 x 10^3 RLU) (Fig. 9a). A shift from pH 5.5 to a neutral pH 7.0 in LB media resulted in dramatically increased expression for all three *fim* genes (*fimA-lux*, 19-fold, $P < 0.000005$; *fimB-lux*, 33-fold, $P < 0.006$; and *fimE-lux*, 55-fold, $P < 0.00002$). When the pH was greater than 7.0, expression from all of the *fim-lux* fusions increased and reached the highest level at pH 8.0 (*fimA-lux*, 5.1 x 10^5 RLU; *fimB-lux*, 6.3 x 10^5 RLU; and *fimE-lux*, 7.1 x 10^5 RLU) compared to pH 7.0 LB media. These results confirmed the *fim- lacZYA* fusions results that showed in a low pH environment, transcription of all of the *fim* genes

(a)


1000000
100000
10000
1000
RLU
5.5
6
6.5
7
7.5
8
pH
ftsZ-lux
fimA-lux
fimB-lux
fimE-lux

(b)


1000000
100000
10000
1000
RLU
0
100
200
400
800
NaCl (mM)
ftsZ-lux
fimA-lux
fimB-lux
fimE-lux

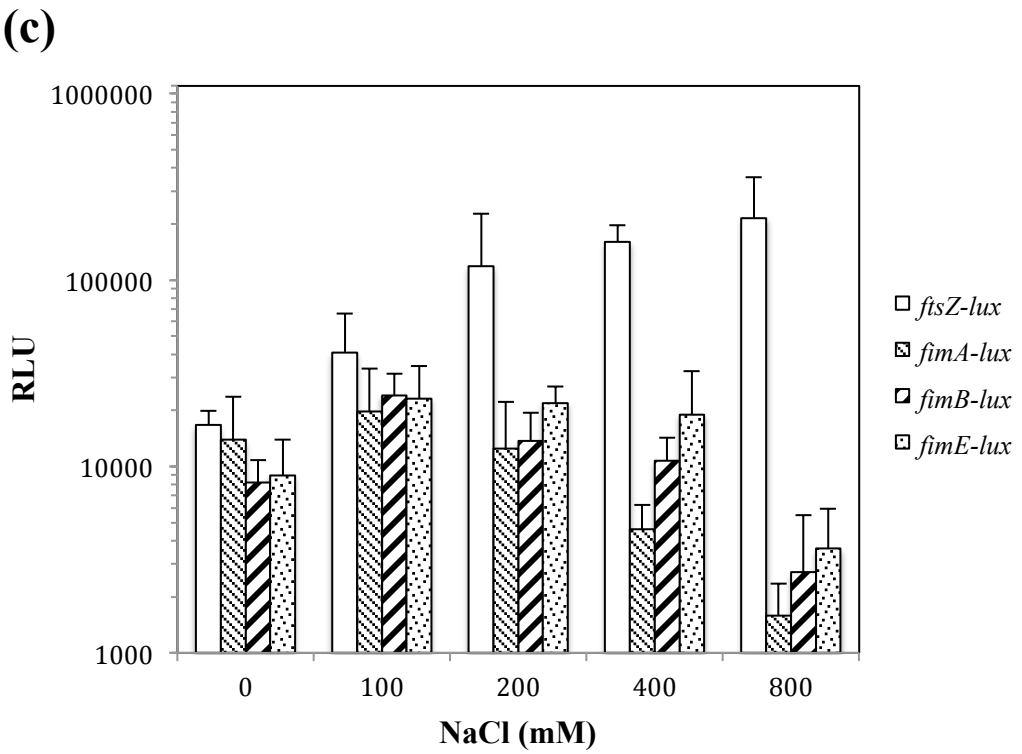


Fig. 9. Effects of pH and osmolarity on *ftsZ*, *fimA*, *fimB*, and *fimE* expression as determined with *lux* transcriptional fusions in strain NU149. The relative luminescence units (RLU) were measured with a luminometer and then divided by the optical densities at 600 nm (OD_{600}). Means ± standard deviations from at least three separate runs are indicated. Osmolarity effects were tested by using NaCl as the osmolyte. (a) Different pHs; (b) different osmolarities in pH 7.0 LB media; and (c) different osmolarities in pH 5.5 LB media.

was repressed.

To assess the possible differential transcription of *fim* gene *in vivo*, the *ftsZ* gene was chosen as an internal control to normalize *fim* genes transcription. Transcription from the *ftsZ-lux* fusion changed in UPEC growing in LB with different pHs. In pH 5.5 LB, the *ftsZ-lux* fusion expression was 1.7 x 10^4 RLU, increasing to 3.9 x 10^4 RLU in pH 6.0 LB, 7.5 x 10^4 RLU in pH 6.5 LB, 1.3 x 10^5 RLU in pH 7.0 LB, and then leveling off to 4.2 x 10^5 RLU in pH 7.5 LB, and 4.4 x 10^5 RLU in pH 8.0 LB, respectively (Fig. 9a). These results suggested that *ftsZ* transcription was affected by the environmental pH *in vitro*.

Evaluation of the *fim-lux* Fusions in UPEC Growing in Different Osmotic Conditions

In addition to pH differences, the human and mouse urinary tracts are affected by variable osmotic stress conditions. The NU149 strains containing an individual *fim-lux* fusion were also tested in LB with variations in the osmolarity and the pH set at either pH 5.5 or 7.0. For all three *fim-lux* transcriptional fusions in strain NU149, the highest level of expression was observed in pH 7.0 medium without added salt (*fimA-lux*, 3.6 x 10^5 RLU; *fimB-lux*, 2.8 x 10^5 RLU; and *fimE-lux*, 5.4 x 10^5 RLU) (Fig. 9b). However, when the osmolarity of the pH 7.0 LB media increased with the addition of 100 mM NaCl, transcription from all three *fim-lux* fusions was lower (*fimA-lux*, 3.4 x 10^5 RLU; *fimB-lux*, 2.6 x 10^5 RLU; and *fimE-lux*, 4.3 x 10^5 RLU). When 200 mM NaCl was added to the pH 7.0 LB, expressions from all three *fim-lux* fusions continued to fall (*fimA-lux*, 2.1 x 10^5 RLU; *fimB-lux*, 2.1 x 10^5 RLU; and *fimE-lux*, 2.15 x 10^5 RLU) compared to LB without added NaCl. In pH 7.0 LB with 400 mM NaCl, transcription of *fimA-lux* fusion dropped

11-fold (3.2 x 10^4 RLU, $P < 0.0001$), *fimB-lux* 6-fold (4.5 x 10^4 RLU, $P < 0.001$), and *fimE-lux* 7-fold (7.8 x 10^4 RLU, $P < 0.0001$) versus pH 7.0 LB without salt addition. An increase in the osmolarity to 800 mM NaCl resulted in a further decline in transcription of all three *fim-lux* fusions (*fimA-lux*, 3.2 x 10^3 RLU; *fimB-lux*, 8.4 x 10^3 RLU; and *fimE-lux*, 2.7 x 10^3 RLU), which were far lower than pH 7.0 LB without salt added. These results indicated in a neutral pH environment, the addition of NaCl to increase the osmolarity caused a substantial decline in transcription of all three *fim* genes.

To further validate the results, the same *fim-lux* fusion containing strains were grown in pH 5.5 LB with variations in the osmolarity. Transcription of *fimB* was at a baseline level of 8.0 x 10^3 RLU in pH 5.5 LB with no salt added (Fig. 9c). When the UPEC strain was grown in LB plus 100 mM NaCl, *fimB-lux* expression increased to 2.4 x 10^4 RLU ($P < 0.053$) as compared to the no salt pH 5.5 LB results. As more NaCl was added to the pH 5.5 LB, transcription of *fimB* fell to 1.4 x 10^4 RLU in 200 mM NaCl LB and 1.1 x 10^4 RLU in 400 mM NaCl LB. When the osmolarity increased to 800 mM added NaCl in the pH 5.5 LB, *fimB* expression fell to its lowest level (2.7 x 10^3 RLU, $P < 0.03$) when compared to pH 5.5 LB without added salt.

For *fimE-lux* expression in the pH 5.5 LB, the baseline RLU was 9.0 x 10^3 RLU without added salt (Fig. 9c). The addition of 100 mM NaCl to the LB media caused a significant rise in *fimE* transcription (2.3 x 10^4 RLU, $P < 0.004$). When the osmolarity of the pH 5.5 LB was increased to 200 mM NaCl, 400 mM NaCl, and 800 mM NaCl, *fimE* transcription fell to 2.2 x 10^4 RLU ($P < 0.0004$), 1.9 x 10^4 RLU ($P < 0.05$), and 3.6 x 10^3 RLU ($P < 0.012$), respectively, as compared to the no salt added pH 5.5 LB.

Transcription of *fimA* followed a pattern similar to the *fimB-lux* fusion results

(Fig. 9c). The baseline *fimA* expression was 1.4 x 10^4 RLU in pH 5.5 LB. When the osmolarity was increased with the addition of 100 mM NaCl, a slight, but not significant increase in *fimA* expression (2.0 x 10^4 RLU, $P < 0.321$) resulted. As the osmolarity was increased, *fimA* expression dropped further (200 mM NaCl, 1.3 x 10^4 RLU; 400 mM NaCl, 5.0 x 10^3 RLU; and 800 mM NaCl, 1.6 x 10^3 RLU). The lowest level of *fimA* transcription was observed in pH 5.5 LB with 800 mM NaCl added (approximately 9 fold) that was significant compared to the baseline no salt pH 5.5 LB results ($P < 0.005$). Therefore, a combination of low pH and high osmolarity caused a significant drop in the transcription of all of the *fim-lux* fusions.

When the *ftsZ-lux* fusion containing UPEC strain was grown in LB with a set pH (5.5 or 7.0), but with variations in the osmolarity, transcription changed as well. In pH 7.0 LB media, the RLU was 1.9 x 10^5. With incremental increases in NaCl concentration, transcription of *ftsZ* increased (100 mM NaCl, 2.1 x 10^5 RLU; 200 mM NaCl, 3.0 x 10^5 RLU; 400 mM NaCl, 4.5 x 10^5 RLU; and 800 mM NaCl, 5.9 x 10^5 RLU) (Fig. 9b). In pH 5.5 LB media, the baseline transcription of *ftsZ* without added NaCl was 1.7 x 10^4 RLU. With the incremental increases in NaCl concentration in pH 5.5 LB media, a substantial increase in *ftsZ* transcription occurred (100 mM NaCl, 4.1 x 10^4 RLU; 200 mM NaCl, 1.2 x 10^5 RLU; 400 mM NaCl, 1.6 x 10^5 RLU; and 800 mM NaCl, 2.2 x 10^5 RLU) (Fig. 9c). These results showed *ftsZ* transcription was affected by a combination of low pH combined with changes in osmolarity.

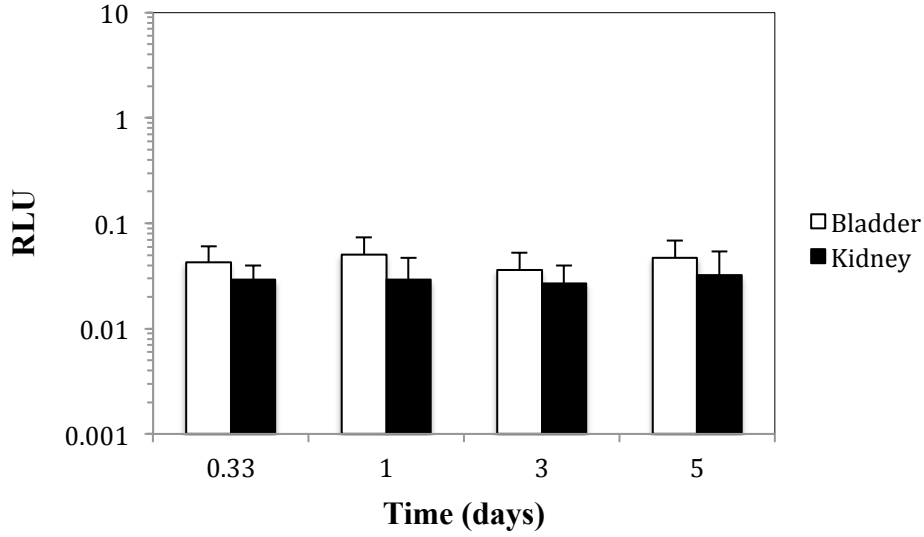
Transcription of Three *fim* Genes Varies in a UPEC Strain Infecting Murine Bladders versus Kidneys

The *in vitro* analysis described above demonstrated that *fim* gene transcription was maximally repressed in the UPEC strains growing in a low pH/high osmolarity environment that would mimic the environment found in regions of murine kidneys. Our next step was to assess expression of the *fim-lux* fusions in the NU149 UPEC strain infecting murine urinary tracts over a five-day period post-inoculation. Strains of NU149 with the pHD-03 (*fimA-lux*), pWS145-38 (*fimB-lux*), pHD-06 (*fimE-lux*), and pHD-09 (*ftsZ-lux*) plasmids were intraurethrally injected into the murine urinary tracts of female Swiss Webster mice. After 0.33, 1, 3, and 5 days post-inoculation, bladders and kidneys were collected, homogenized, tested for their RLU, and plated for viable bacterial counts to obtain the corrected RLU.

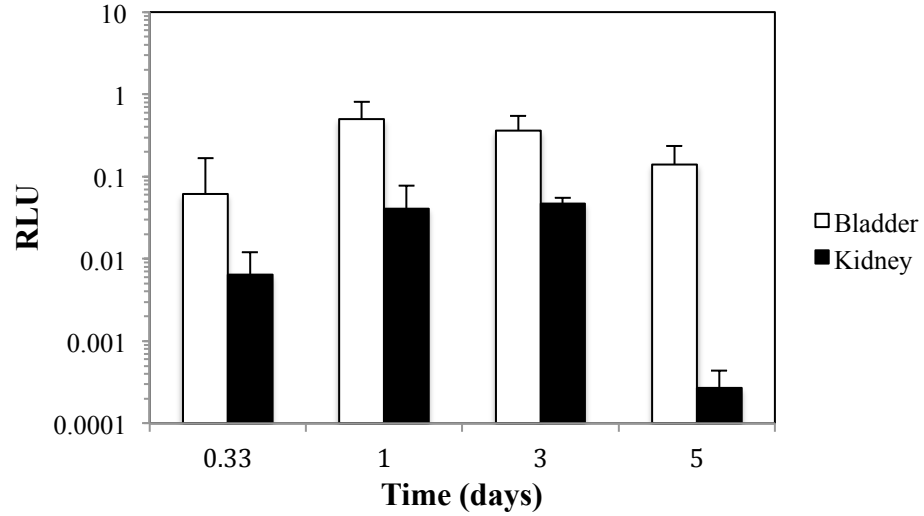
For the *ftsZ-lux* fusion, transcription of *ftsZ* in UPEC infected murine bladders was 0.042 RLU after 0.33 days post-inoculation. After one-day post-inoculation, *ftsZ* expression increased to 0.050 RLU, dropped to 0.036 RLU at day 3, and then rose to 0.047 RLU at day 5 post-inoculation (Fig. 10a). None of these changes were significant. Compared to the regulation in bladders, *ftsZ* transcription in UPEC infected murine kidneys showed less fluctuation among the five-day period post-inoculation (0.33 days, 0.029 RLU; 1 day, 0.029 RLU; 3 days, 0.027 RLU; and 5 days, 0.032 RLU). These results indicated transcription of *ftsZ-lux* fusion was stable with less than 0.3 fold fluctuations in the NU149 infected murine bladders or kidneys.

The *fimA-lux* fusion expression in NU149 infected bladders was at the lowest level at day 0.33 post-inoculation (0.062 RLU), and then increased to the highest level

(a)


10
1
0.1
0.01
0.001
RLU
0.33
1
3
5
Time (days)
Bladder
Kidney

(b)


10
1
0.1
0.01
0.001
0.0001
RLU
0.33
1
3
5
Time (days)
Bladder
Kidney

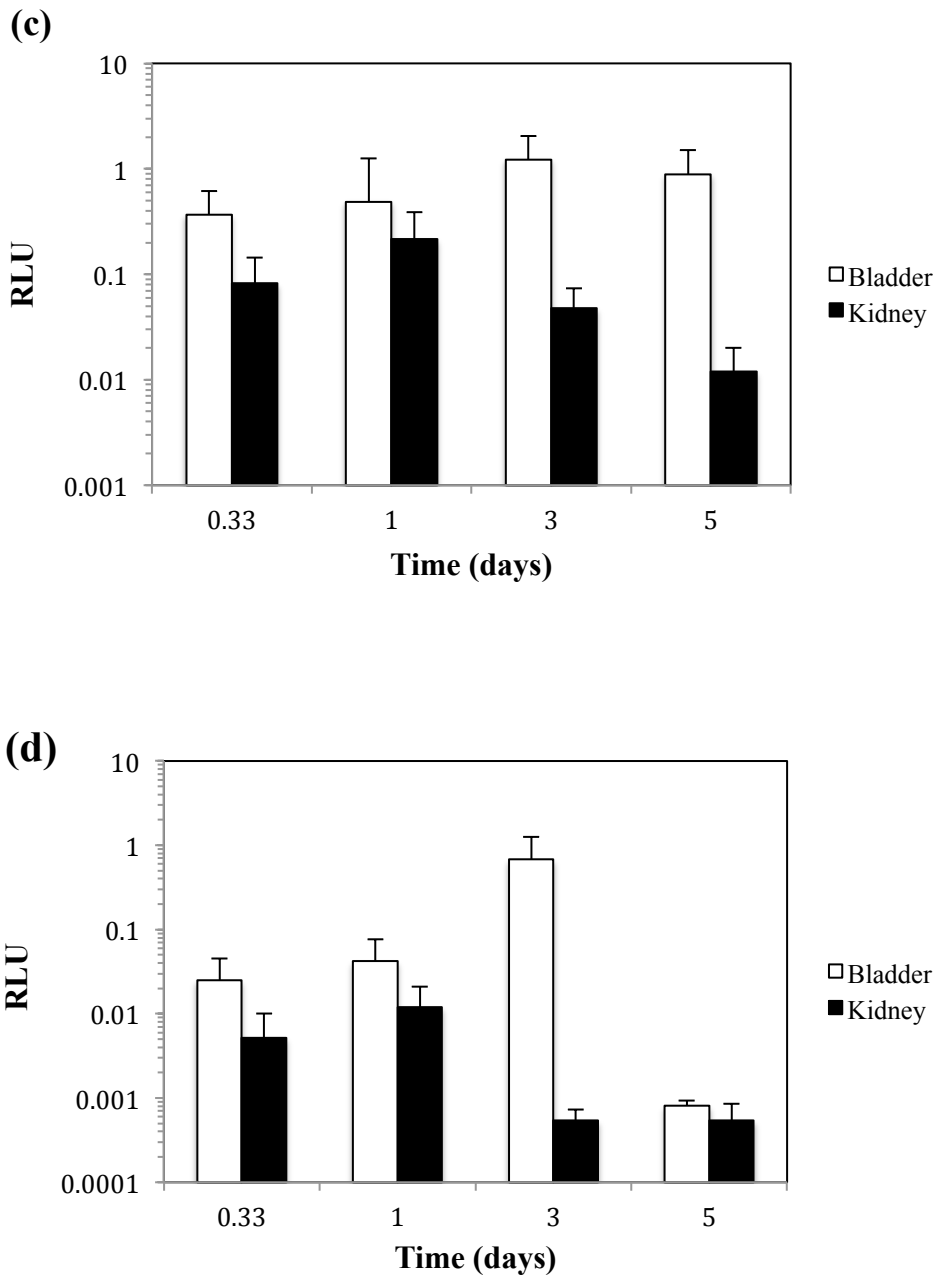


Fig. 10. Expression of *ftsZ*, *fimA*, *fimB* and *fimE* in murine bladders and kidneys for infection with NU149 containing recombinant plasmids with *fim*- or *ftsZ-lux* transcriptional fusions over five-day (0.33, 1, 3 and 5 days) post-inoculation time. The fusions tested included (a) *ftsZ-lux*; (b) *fimA-lux*; (c) *fimB-lux*; (d) *fimE-lux*. The relative luminescence units (RLU) per bacterial cell were measured with a luminometer and then divided by viable bacterial counts. Means ± standard deviations from at least 5-10 mice at each time post-inoculation are indicated.

(0.500 RLU) after one-day post-inoculation (Fig. 10b). Subsequently, *fimA* transcription fell to 0.362 RLU, and 0.141 RLU at day 3 and day 5 post-inoculation, respectively. The difference in *fimA* transcription in NU149 infected bladders was not significant ($P < 0.083$). In contrast, *fimA* transcription in NU149 infected murine kidneys increased initially during three days post-inoculation (day 0.33, 0.006 RLU; day 1, 0.041 RLU; and day 3, 0.047 RLU), but became repressed by the fifth day (0.0003 RLU). At day 5 post-inoculation, *fimA* transcription was barely detectable. However, the variation of *fimA* expression in NU149 infected kidneys was not significant during five-day period post-inoculation ($P < 0.104$). These results showed *fimA* expression was much higher in the UPEC infected bladders than kidneys.

Transcription of *fimB* in NU149 infected bladders showed a trend similar to the *fimA* expression results. At day 0.33 post-inoculation, *fimB* expression was 0.366 RLU (Fig. 10c). After one day post-infection, the RLU increased to 0.486, and reached the highest level at day 3 (1.223 RLU). This increase in *fimB* transcription was significant compared to the day 0.33 post-inoculation (3.3-fold, $P < 0.002$). By the fifth day post-inoculation, *fimB* expression slightly dropped to 0.886 RLU. While *fimB* transcription varied in NU149 infected murine bladders, *fimB* transcription increased slightly from 0.082 RLU at day 0.33 to 0.216 RLU at day 1, and then fell to 0.048 RLU at day 3, and finally to 0.012 RLU at day 5 in UN149-infected murine kidneys. The *fimB* transcription difference between the day 0.33 and day 5 results in UPEC infected murine kidneys was significant (6.8-fold, $P < 0.049$). Moreover, the comparison of UPEC infected murine bladders to kidneys at each time point post-inoculation showed *fimB* expression in NU149 infected bladders was higher than in NU149 infected kidneys (day 0.33, 4.5-fold,

$P < 0.034$; day 1, 2.3-fold, $P < 0.190$; day 3, 25.5-fold, $P < 0.028$; and day 5, 73.8-fold, $P < 0.011$). Thus, *fimB* transcription was favored in UPEC infected murine bladders over kidneys.

Unlike the *fimA* and *fimB* transcription results in UPEC infected murine bladders, *fimE* transcription was much lower than either *fimA* or *fimB* transcription. At 0.33 day post-inoculation, *fimE* expression was 0.025 RLU (Fig. 10d). Transcription of *fimE* rose to 0.042 RLU after one day post-inoculation, and then reached the highest level (0.677 RLU) at day 3 post-inoculation before falling to the lowest level (0.00081 RLU) at day 5 post-infection ($P < 0.0001$) compared to the five-day period post-inoculation. In UPEC infected murine kidneys, *fimE* transcription was markedly different (day 0.33, 0.0052 RLU; day 1, 0.012 RLU; day 3, 0.00054 RLU; and day 5, 0.00054 RLU) during the five days post-infection. Compared the highest level at day 1 to the lowest level at day 5 post-inoculation, the decrease in *fimE* transcription in NU149 infected murine kidneys was significant (222-fold, $P < 0.0001$). These results suggested *fimE* transcription was repressed in both NU149 infected murine bladders and kidneys. This repression in *fimE* expression appeared to more pronounced in NU149 infected murine kidneys than bladders.

DISCUSSION

The binding of type 1 piliated UPEC cells to epithelial cells lining the urinary tract is an important step in pathogenesis within the human or murine urinary tract. Environmental cues, such as pH and osmolarity, within the urinary tract can regulate several key *fim* genes involved in UPEC type 1 pili expression. Even within the urinary tract, there are considerable changes in pH and osmolarity. For example, bladder urine has a higher pH and lower osmolarity than kidney urine (Ross & Neely, 1983). In a previous study, *fim-lacZYA* fusions were used to examine the *in vitro* effects of pH and osmolarity on the expression of *fimA*, *fimB*, and *fimE* gene. Growth of a UPEC strain in a low pH environment led to downregulation of *fimA*, *fimB*, and *fimE* gene involved in type 1 pili expression (Schwan *et al*., 2002). The use of *lacZYA* fusions in bacterial infected animal tissues is limited due to the need for bacterial cell lysis and the requirement for adding substrate when doing β-galactosidase assay. However, a *lux* transcriptional fusion can be used without disruption of the bacterial cell membrane and loss of bacterial cell viability. To assess *fim* gene regulation *in vivo*, we created a series of *fim-lux* transcriptional fusions on a single copy plasmid to assess how environmental cues affect transcription of *fimA*, *fimB*, and *fimE* in a UPEC strain infecting murine urinary tracts.

Initially, NU149 strains containing the *fim-lux* fusions were examined after *in vitro* growth in LB media with differences in pH and osmolarity to determine whether they matched the results using the *fim-lacZYA* fusions (Schwan *et al*., 2002). Most of the *in vitro* results with the *fim-lux* fusions were similar to the observations using the *fim-*

lacZYA fusions. Both present and previous studies showed all of the *fim* genes had the lowest level of transcription in pH 5.5 medium. Furthermore, *fimA* and *fimB* expression reached their lowest level in pH 5.5 LB media with 800 mM NaCl.

However, several differences were also noted between the present and previous studies. All of the *fim* genes displayed the highest transcription in pH 8.0 LB media in the present study, whereas the previous study had optimal expression in pH 7.0 LB media. Transcription from the *fimE-lux* fusion declined as the osmolarity increased, which was contrary to the previous study using a *fim-lacZ*YA fusion under the same growth conditions described above (Schwan *et al*., 2002). One explanation for this discrepancy could be laboratory *E. coli* strains DH5α and AAEC189 used in the previous study are missing many of the virulence factor genes compared to the clinical isolate NU149 that was used for the *fim-lux* analysis. The genetic background differences between NU149 and the laboratory *E. coli* strains could explain some of the *fimE* transcription variations between different strains. Another possibility is the *lux* operon is more sensitive at the post-transcriptional level to pH and osmotic stress changes than the *lacZYA* operon, which may have generated conflicting results for *fimE* grown in low pH/high osmolarity environment. Further experimentation will need to be done to clarify this question.

Besides some differences in *fim* gene transcription using *fim-lux* fusions, there were some variations in the results with the *ftsZ-lux* fusion as well. The *ftsZ-lux* fusion was designed to be a control. However, transcription from the *ftsZ-lux* fusion was not constant when the *in vitro* growth media had pH or osmolarity changes. Our results differed from the quantitative real time polymerase chain reaction (qRT-PCR) *ftsZ* transcription results described previously (Rentschler *et al*., 2013). In this study, the

expression of *ftsZ-lux* fusion fluctuated as the osmolarity increased in pH 5.5 or pH 7.0 LB media, suggesting the luciferase expression in *E. coli* may be transcriptionally or post-transcriptionally affected by pH and osmolarity. The *ftsZ* gene has been widely used as a control for qRT-PCR experiments. However, transcription of the *ftsZ* gene may vary in different species and different growth condition. A recent study emphasized the need for proper validation of reference genes by investigating transcription of several different reference genes during exponential growth, stationary growth and in an iron-depleted environment (Brudal *et al*., 2013). The transcriptional stability of the *ftsZ-lux* fusion should be further investigated.

Because most of the *in vitro fim-lux* fusion results correlated with the previous *fim-lacZYA* fusion study, we next assessed transcription from *fimA-*, *fimB-*, and *fimE-lux* fusions in a UPEC strain colonizing murine urinary tracts over a five-day post-inoculation period. Unlike the *in vitro* experiments, the *ftsZ-lux* control fusion worked well in UPEC infecting murine urinary tracts. Transcription of *ftsZ* did not significantly change in NU149 infecting bladders or kidneys, although *ftsZ* expression was lower on average in kidney homogenates compared to bladder homogenates. Thus, *ftsZ* transcription remained fairly stable over the five-day infection period in mice.

Our *in vivo fimA* expression results were consistent with previous reports that showed the highest level of type 1 pili expression at day 1 post-inoculation in NU149 infected murine bladders. One study observed this temporal regulation of type 1 pili *in vivo* during UPEC infected murine UTIs. In this study, a cystitis isolates maintained the *fimS* Phase-ON orientation throughout the entire four-day period of bladder infection (Gunther *et al*., 2001). Other studies showed type 1 pili expression was most important

for bacterial growth in the early stage (one day post-infection) of a UPEC infection in murine bladders and the *fimS* region remained mostly Phase-ON throughout the entire seven day bladder infection (Gunther *et al*., 2002; Snyder *et al*., 2005, 2006). Previously, *E. coli* strain NU149 was also shown to maintain a consistently high degree of type 1 piliation in UPEC infected murine bladders after five days post-inoculation (Schaeffer *et al*., 1987).

The *fimA* expression results from this study are in agreement with the concept that type 1 pili expression is needed in the initial stages of infection, but their expression is reduced once the UPEC cells attach and/ or penetrate bladder epithelium (Hagan *et al*., 2010; Withman *et al*., 2013). Human and murine bladder epithelial cells present an abundance of mannose moieties on their glycoproteins that may serve as receptors for type 1 pili (Ofek *et al*., 1977), so continued production of type 1 pili would be advantageous for UPEC bladder colonization.

In contrast to *fimA* expression in NU149 infected bladders, NU149 cells in infected murine kidneys expressed the highest level of *fimA* transcription at day 3 and then displayed a significant drop in *fimA* transcription thereafter. No *fimA* transcription was detected in some of the fifth day post-inoculation infected kidneys homogenates, suggesting that type 1 pili expression had been completely shut down in those NU149 infected kidneys. Other studies have also observed the loss of type 1 piliated UPEC cells over time in UPEC infected murine kidneys (Hagberg *et al*., 1983a; Hagberg *et al*., 1983b; Hultgren *et al*., 1985; Hultgren *et al*., 1986; Schaeffer *et al*., 1987).

Besides the differences in transcription of *fimA* gene observed in UPEC infection of murine urinary tracts, temporal regulation of the two *fim* recombinase genes

was also observed. Transcription of *fimB* and *fimE* was the highest after three days post-inoculation in NU149 infected murine bladders. Nevertheless, the ratio of *fimB* to *fimE* transcription at day 1 was 2.4-fold higher than at day 3, suggesting the temporal regulation of *fimB* and *fimE* in UPEC infecting murine bladders favored *fimB* in the early stage of the infection and switched at day 3 to one that more favored *fimE* before switching back to favoring *fimB*. Thereafter, *fimB* expression fell by 28% after day 5 post-inoculation, but remained at a high level. Conversely, transcription of *fimE* declined to barely dectable levels by five days post-inoculation in NU149 infected murine bladders.

Since FimB and FimE have roles in positioning of the *fimS* region that contains the *fimA* promoter (McClain *et al*., 1991, 1993), the ratio of these proteins would have an indirect influence on *fimA* transcription by altering the orientation of the *fimS* region. More *fimB* and less *fimE* transcription would favor FimB-promoted recombination in the *fimS* region to the Phase-ON orientation, thus in turn leading to type 1 pili expression. A drop in *fimA* transcription at day 3 could be the result of the ratio of FimB to FimE favoring FimE-promoted recombination. On the other hand, the decline in *fimA* transcription at day 5 post-inoculation may be the result of environmental cues exerting a direct effect on the regulation of the *fimA* promoter.

Our infected bladder results were in agreement with the work of others. One group has previously reported a difference in the percentage of the Phase-ON altered in the *fimS* region between a cystitis strain F11 and a pyelonephritis isolate CFT073 within the urine of infected mice. Initially, the median percentages of invertible elements in the Phase-ON orientation for F11 and CFT073 at the day 0.17 time point were 2% and 9.3%, respectively. At day 1, both strains displayed significant divergence in the orientation of

the *fimS* region. Strain CFT073 had an increase in Phase-ON orientation to only 33.6%, while the Phase-ON orientation at the same time point for strain F11 increased to 84.5%. However, at day 2 and day 3 post-inoculation, these two strains displayed a drop to ≤ 2% for strain CFT073 and 61.2% for F11 of the population in the Phase-ON orientation (Gunther *et al*., 2001). Our *fim* gene transcription results in NU149 infected murine bladders were also consistent with a recent study that showed *fimA* and *fimB* transcription declined over time in UPEC infected murine bladders (Crépin *et al*., 2012).

In NU149 infected murine bladders, the temporal regulation of the *fim* genes favored *fimA* and *fimB* transcription in the early stage of the infections. However, in the murine kidneys, transcription of all three *fim* genes appeared to be repressed by day 5 post-inoculation. Transcription of *fimB* and *fimE* was the highest after day 2 post-inoculation in NU149 infected kidneys. Both *fimB* and f*imE* transcription were lower at the day 3 post-inoculation. By day 5 post-inoculation, *fimB* transcription dropped by 75%, but *fimE* transcription had leveled off, suggesting the NU149 cells in the murine kidneys would have a ratio of FimB to FimE that favored FimE and nonpiliated cells by day 5.

A question that is posed is why the UPEC cells lose their expression of type 1 pili in UPEC infected murine kidneys. Unlike murine bladder epithelial cells that have many mannose-containing receptors on their surface, murine kidneys display few of these receptors on their renal glycolipids (Vaisanen-Rhen *et al*., 1985; Virkola, 1987), so expression of type 1 pili would be of little value to the bacteria in this environment. As UPEC strains ascend into the kidneys, the environmental niche they may encounter would have high osmolarity conditions (800 mM NaCl equivalence) in pockets of the kidneys. Kidney urine has a lower pH than urine found in the bladders (Ross & Neely,

1983), so *fim* gene expression would be more repressed (Schwan *et al*., 2002).

To survive in a low pH/high osmotic stressed environment, the EnvZ/OmpR system would be needed by a UPEC strain. Previously, our laboratory showed *fimB* expression was derepressed in an *ompR* mutant UPEC strain in a low pH environment and OmpR was critical for UPEC survival in murine urinary tract (Schwan *et al*., 2002, 2009). Transcription of *ompR* in *E. coli* has been shown to be insensitive to fluctuations in pH (Rentschler *et al*., 2013; Quinn *et al*., 2014), but OmpR protein levels increased in UPEC grown in an acidic pH versus a neutral pH environment (Rentschler *et al*., 2013). Compared to the wild type parent, the decline in *fimB* transcription within UPEC infected kidneys could be the result of more OmpR proteins being translated in this low pH/high osmolarity environment. More OmpR protein would mean a greater opportunity to bind to the second *fimB* promoter and repress *fimB* expression (Rentschler *et al*., 2013). Less *fimB* expression in UPEC infected kidneys would mean the ratio of FimB to FimE would change to favor FimE and a subsequent Phase-OFF orientation of the *fimS* region. With the *fimS* region being switched to a Phase-OFF orientation combined with direct regulation of *fimA* transcription, a loss of type 1 pili over time in UPEC infected kidneys would occur.

Why would nonpiliated UPEC cells be advantageous in UPEC infected kidneys? Type 1 pili are highly immunogenic (Mulvey *et al*., 2000), so nonpiliated bacteria may be hidden from the host immune system that would otherwise opsonize the bacterial cells by antibodies binding to the type 1 pili on the UPEC surface. The murine kidneys are quite vascularized and macrophage-bacteria interactions would occur more often in murine kidneys as compared to bladders. Type 1 piliated bacteria are targeted directly by

macrophages (Bar-Shavit *et al*., 1980; Silverblatt *et al*., 1979), so nonpiliated bacteria would hide behind their capsules and evade the murine innate defense. Thus, becoming nonpiliated is an advantage for UPEC survival in the kidney, and the low pH/high osmolarity environment encountered in the murine kidney would regulate the *fim* genes to favor a nonpiliated phenotype.

Our *in vivo* results provided the evidence that different environmental niches within a UPEC infected murine urinary tract can regulate *fim* gene transcription, favoring expression of type 1 piliated cells in the murine bladders and nonpiliated cells in the murine kidneys. Additional work is needed to investigate and clarify the molecular mechanisms that are shaped by the environment cues in a murine urinary tract that can influence type 1 pili expression.

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APPENDIX A

REAGENTS FOR DNA AGAROSE GEL ELECTROPHORESIS

Appendix A. Reagents for DNA Agarose Gel Electrophoresis

1) 1X TBE buffer

216 g Tris
110 g Boric acid
80 ml 0.5 M EDTA pH 8.0
20 liters distilled deionized H_2O (dd H_2O)
pH to 8.0

2) 0.8 % agarose gel

2.4 g agarose powder
300 ml 1 X TBE
4 µl of a10 mg ethidium bromide μl^{-1} solution in dd H_2O

APPENDIX B

REAGENTS FOR PCR

Appendix B. Reagents for PCR

1) Incomplete Wigler's buffer (Lisitsyn *et al*., 1993)

26.08 ml sterile dd H_2O
16.75 ml Tris HCl pH 8.8
4 ml of a 1 M $(NH_4)_2SO_4$ solution
0.5 ml of a 1 M $MgCl_2$ solution

- ❖aliquot 946.3 ml into 1.5 ml Sarstedt tubes, store at -20 °C

2) Complete Wigler's buffer (Lisitsyn *et al*., 1993)

50 µl of 10 mg ml^{-1} bovine serum albumin solution
3.47 µl β-mercaptoethanol
Both solutions are added to a tube of Incomplete Wigler's buffer

APPENDIX C

REAGENTS FOR MEDIA

Appendix C. Reagents for Media

1) Luria Bertani (LB) broth pH 5.5 (Schwan *et al*., 2002)

1.5 ml 1 M Na_2HPO_4
34.5 ml 1 M NaH_2PO_4
3.6 ml glycerol
7.2 g LB base (Invitrogen)
dd H_2O to 360 ml

- ❖ pH to 5.5 and autoclave 25 min

2) LB broth pH 6.0 (Schwan *et al*., 2002)

4.32 ml 1 M Na_2HPO_4
31.68 ml 1 M NaH_2PO_4
3.6 ml glycerol
7.2 g LB base (Invitrogen)
dd H_2O to 360 ml

- ❖ pH to 6.0 and autoclave 25 min

3) LB broth pH 6.5 (Schwan *et al*., 2002)

10.926 ml 1 M Na_2HPO_4
25.074 ml 1 M NaH_2PO_4
3.6 ml glycerol
7.2 g LB base (Invitrogen)
dd H_2O to 360 ml

- ❖ pH to 6.5 and autoclave 25 min

4) LB broth pH 7.0 (Schwan *et al*., 2002)

20.772 ml 1 M Na_2HPO_4
15.228 ml 1 M NaH_2PO_4
3.6 ml glycerol
7.2 g LB base (Invitrogen)
dd H_2O to 360 ml

- ❖ pH to 7.0 and autoclave 25 min

5) LB broth pH 7.5 (Schwan *et al*., 2002)

29.52 ml 1 M Na_2HPO_4
6.48 ml 1 M NaH_2PO_4
3.6 ml glycerol
7.2 g LB base (Invitrogen)
dd H_2O to 360 ml

- ❖ pH to 7.5 and autoclave 25 min

6) LB broth pH 8.0 (Schwan *et al*., 2002)

33.552 ml 1 M Na_2HPO_4
2.448 ml 1 M NaH_2PO_4
3.6 ml glycerol
7.2 g LB base (Invitrogen)

dd H_2O to 360 ml

- ❖ pH to 8.0 and autoclave 25 min

7) LB broth pH 5.5 + 100 mM NaCl

2.104 g NaCl is added to LB broth pH 5.5 described above.

8) LB broth pH 5.5 + 100 mM NaCl

2.104 g NaCl is added to LB broth pH 5.5 described above.

9) LB broth pH 5.5 + 200 mM NaCl

4.208 g NaCl is added to LB broth pH 5.5 described above.

10) LB broth pH 5.5 + 400 mM NaCl

8.415 g NaCl is added to LB broth pH 5.5 described above.

11) LB broth pH 5.5 + 800 mM NaCl

16.831 g NaCl is added to LB broth pH 5.5 described above.

12) LB broth pH 7.0 + 100 mM NaCl

2.104 g NaCl is added to LB broth pH 7.0 described above.

13) LB broth pH 7.0 + 200 mM NaCl

4.208 g NaCl is added to LB broth pH 7.0 described above.

14) LB broth pH 7.0 + 400 mM NaCl

8.415 g NaCl is added to LB broth pH 7.0 described above.

15) LB broth pH 7.0 + 800 mM NaCl

16.831 g NaCl is added to LB broth pH 7.0 described above.

APPENDIX D

REAGENTS FOR ORGAN HOMOGENATE BUFFER

Appendix D. Reagents for Organ Homogenate Buffer

1) Phosphate buffered saline (PBS)

8.0 g NaCl
0.2 g KCl
1.15 g Na_2HPO_4
0.2 g KH_2PO_4
1000 ml dd H_2O

- ❖ pH to 7.2 and autoclave 25 min