Research Article

Temporal Regulation of *fim* Genes in Uropathogenic *Escherichia coli* during Infection of the Murine Urinary Tract

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Received 9 August 2017; Revised 28 November 2017; Accepted 7 December 2017; Published 27 December 2017

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cademic Editor: Giovanna Franciosa

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Uropathogenic *Escherichia coli* (UPEC) adhere to cells in the human urinary tract via type 1 pili that undergo phase variation where a 314-bp *fimS* DNA element flips between Phase-ON and Phase-OFF orientations through two site-specific recombinases, FimB and FimE. Three *fim-lux* operon transcriptional fusions were created and moved into the clinical UPEC isolate NU149 to determine their temporal regulation in UPEC growing in the urinary tract. Within murine urinary tracts, the UPEC strains demonstrated elevated transcription of *fimA* and *fimB* early in the infection, but lower transcription by the fifth day in murine kidneys. In contrast, *fimE* transcription was much lower than either *fimA* or *fimB* early, increased markedly at 24 h after inoculation, and then dropped five days after inoculation. Positioning of *fimS* was primarily in the Phase-ON position over the time span in UPEC infected bladders, whereas in UPEC infected murine kidneys the Phase-OFF orientation was favored by the fifth day after inoculation. Hemagglutination titers with guinea pig erythrocytes remained constant in UPEC growing in infected murine bladders but fell substantially in UPEC infected kidneys over time. Our results show temporal in vivo regulation of *fim* gene expression in different environmental niches when UPEC infects the murine urinary tract.

1. Introduction

Urinary tract infections (UTIs) remain one of the most common infections of humans in the United States. Approximately 10.5 million office visits are due to UTIs annually, resulting in over 100,000 hospitalizations and an estimated cost of $3.5 billion per year [1–3]. 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 [2].

The ability to bind to uroepithelial cells lining the human urinary tract is generally considered one of the first steps in UPEC initiated UTIs. Type 1 pili facilitate this binding to epithelial cells in the bladder, lungs, intestine, and buccal cells; proximal tubular cells of the kidney; and various inflammatory cells [4–6]. Following adherence of the UPEC cells, bacterial invasion and persistence in target host cells due to the type 1 pili expressed by UPEC can occur [4, 7, 8].

Expression of FimA, the main structural subunit of the type 1 pili encoded by the *fimA* gene [9, 10], is affected by phase variation, a ON-OFF switching process that allows individual cells to alternate between piliated (Phase-ON) and non-type 1 piliated states (Phase-OFF) [11, 12]. This phase switching is due to the inversion of a 314-bp *fimS* DNA element containing the promoter for the *fimA* structural gene [13, 14]. When the *fimA* promoter is aligned in the Phase-ON orientation, transcription of *fimA* occurs. However, when the *fimS* element is in the Phase-OFF orientation, there is no transcription of *fimA*, resulting in a non-type 1 piliated phenotype [15, 16]. The phase switching of the 314-bp *fimS* sequence is controlled by the products of two regulatory genes, *fimB* and *fimE*, located upstream of *fimA* [16]. The *fimB* and *fimE* gene products are site-specific recombinases influencing the positioning of the *fimS* region [16–18]. FimE appears to promote inversion of the promoter-containing *fimS* element from the Phase-ON to Phase-OFF orientation [18, 19], whereas FimB promotes switching in both directions with a slight switching bias toward the Phase-ON orientation [16, 18, 20].
Both of the fim recombinase genes are transcribed independently. The consensus is that there are two fimB promoters [21–23], although one study with an E. coli K-12 strain has indicated a single promoter for fimB [24]. A third potential fimB promoter was also identified in UPEC strains [23] that may be tied to sialic acid concentration in the urinary tract [25], but this has not been confirmed. A single promoter has been identified for fimE [24]. Regulation of the fimB and fimE genes in UPEC cells growing in the human urinary tract and other mammals is still largely uncharacterized.

Inside the urinary tract, UPEC grow in an environment bathed in urine. Human and murine urine typically have a slightly acidic pH and the osmolality can vary [26, 27]. Previous work in our laboratory has demonstrated that pH and osmotic changes in growth media have an effect on fim gene expression [28, 29]. Transcription of fimA, fimB, and fimE were reduced in the bacteria growing in acidic Luria broth (LB) medium. Previously, it was shown that growth of E. coli in medium with a combination of an acidic pH and high osmolality resulted in a significant decline in fimB and fimA transcription compared to growth in neutral pH/low osmolality medium [29].

Although there have been studies that have examined fim gene expression in UPEC colonizing a murine urinary tract, only a limited number of studies had examined the expression of type 1 pili in UPEC growing in vivo [30–33]. More studies have examined positioning of the fimS element in UPEC strains infected murine bladders and kidneys [34–40]. A few studies have examined the expression of fimA in UPEC infecting murine bladders [30, 35]. However, only one study has examined fimB expression in UPEC growing in murine bladders, but this study was limited to a 48 h period and did not examine fim recombinase gene transcription in infected murine kidneys [35].

In order to address whether there is temporal regulation of fim genes in UPEC cells growing in murine urinary tracts, we constructed fimA, fimB, and fimE-lux transcriptional fusions and moved these fusions into a UPEC strain. We used these recombinant UPEC strains to infect murine urinary tracts and then examined the expression of the fim genes over a five-day period. 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. Our results may help us understand the temporal regulation of these adhesion genes in E. coli colonizing the human urinary tract.

2. Materials and Methods

2.1. 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 is a clinical isolate obtained from a patient with cystitis that expresses type 1 pili, but not P pili [30]. The NU149 strain has been used for fim gene transcriptional analyses and type 1 pili expression [24, 28, 31, 41]. 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; chloramphenicol, 12.5 μg/ml; kanamycin, 40 μg/ml; and erythromycin, 150 μg/ml.

2.2. Construction of the fim-lux Reporter Fusions. To create the fimA-lux reporter fusion on the single copy plasmid pPP2-6, pAON-1 plasmid DNA [28] was extracted using a commercial kit (Qiagen). The pAON-1 DNA containing the promoter for fimA was digested with the restriction endonuclease enzymes EcoRI and BamHI to separate the fimA promoter sequence from the pU188 backbone [42] and ligated to pHSS22 plasmid DNA [28] cut with the same restriction endonuclease enzymes described above. A transformation into E. coli DH5α MCR cells was followed by selection on LA plates containing 40 μg kanamycin/ml. One clone, labeled pHD-01, with the proper EcoRI and BamHI restriction endonuclease digestion pattern was chosen for additional processing.

Next, pXen5 [43] plasmid DNA was extracted as described above, cut with BamHI, and ligated to BamHI-cut pHD-01 plasmid DNA created above. The ligation DNA was transformed into E. coli strain DH5α MCR. Plasmid pXen5 contains an erythromycin resistance gene and a promoterless lux operon. The transformants were then plated onto LA containing 40 μg kanamycin/ml incubated at 37°C. All transformants were patched onto LA plates containing 150 μg erythromycin/ml as well as LA containing 40 μg kanamycin/ml. Transformants that were kanamycin-resistant (KanR) but erythromycin-sensitive (ErmR) were monitored for luminescence above background levels and had plasmid DNA extracted as described above. Aliquots of plasmid DNA from several transformants were then digested with the restriction endonuclease to confirm the insertion of the lux operon into pHD-01 plasmid. One clone that showed bioluminescence as well as the proper PstI 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 reporter fusion [28]. The pPP2-6 plasmid has a chloramphenicol resistance gene and an origin of replication to replicate a single copy. Plasmid DNA from pPP2-6 and pHD-02 constructs were digested with the NotI restriction endonuclease, ligated together, and transformed into E. coli DH5α MCR cells. Transformants were plated onto LA containing 12.5 μg chloramphenicol/ml and incubated at 37°C and then screened for bioluminescence as previously described. One transformant that displayed bioluminescence above background levels with the proper NotI digestion pattern named pHD-03 was identified. Plasmid pHD-03 was electroporated into electrocompetent strain NU149 cells by a procedure described by Casali and Preston [44], selecting for transformants with 12.5 μg/ml of chloramphenicol. Transformants were screened for bioluminescence. One NU149/pHD-03 clone was chosen for further analysis.

For construction of the fimE-lux reporter fusion on the pPP2-6, plasmid pMP5-2.17 containing the fimE promoter was used [28]. The pMP5-2.17 plasmid DNA was processed as described above and one clone, labeled pHD-04, was used for further analysis. The fimE promoter DNA from pHD-04
Table 1: Bacterial strains and plasmids used in this study.

| Strains/plasmids | Description | Reference or source |
|------------------|-------------|---------------------|
| **E. coli strains** | | |
| NU149 | Cystitis clinical isolate | [30] |
| DH5α MCR | General cloning strain | Gibco/BBL |
| **Plasmids** | | |
| pAON-1 | fimA-lacZYA locked Phase-ON on pUJ8 | [28] |
| pP5-48 | fimB-lacZYA on pUJ8 | [28] |
| pMP5-2.17 | fimE-lacZYA on pUJ8 | [28] |
| pUJ8 | trp-1-lacZ phoA Ap' | [42] |
| pHSS22 | oriT Km' | [28] |
| pXen-5 | Em', promoterless lux operon | [43] |
| pPP2-6 | PR274 with multiple cloning site and Cm' | [28] |
| pWS414-2 | fimB promoters on pHSS22 | This study |
| pWS414-27 | fimB-lux fusion on pHSS22 | This study |
| pWS414-38 | fimB-lux fusion on pHSS22 | This study |
| 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 pPHSS22 | 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 |

was ligated to pXen-5, resulting in the plasmid named pHD-05. Plasmid DNA from pHD-05 was ligated into the pPP2-6 plasmid that resulted in the pHD-06 plasmid.

Construction of the fimB-lux reporter fusion was done as follows. The pP5-48 plasmid DNA containing the fimB promoter [28] was extracted as described above, leading to creation of the pWS414-2, pWS414-27, and ultimately the pWS414-38 plasmid.

### 2.3. Construction of the ftsZ-lux Reporter Fusion

For the housekeeping gene control used in this study, the ftsZ gene was chosen, which we used previously in other studies [28, 31, 45]. To construct the ftsZ-lux reporter fusion, the ftsZ promoter region was amplified using the primer pair EcFtsZ5 (5'-CAGGAATTCAAACATCGTCAAAGCGGTTGA-3') and EcFtsZ6 (5'-CAAGGATCCAATTCAACCTTC-AATGCGC-3') using DNA sequence obtained from one E. coli genome sequencing project [46] 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 ftsZ PCR product had an EcoRI restriction endonuclease site at the 5' end and a BamHI restriction endonuclease site at the 3' end. This ftsZ DNA was cut with EcoRI and BamHI, ligated to EcoRI and BamHI-cut pHSS22 DNA, and transformed into DH5α MCR cells. 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 BamHI, and ligated to BamHI digested pXen5 DNA, and the ligation mixture was transformed into DH5α MCR cells. Transformants were selected on LA with kanamycin and erythromycin and screened for bioluminescence as previously noted. One plasmid, labeled pHD-08, was created. The pHD-05 plasmid DNA was ligated to pPP2-6 DNA and transformed into DH5α MCR cells, and selection and screening for bioluminescence were done as described above. One of the plasmids, labeled pHD-09, was transformed into E. coli NU149 as described above.

### 2.4. Testing the fim-lux Fusions in Different In Vitro pH Environments

A previous study has shown that acidic pH and high osmolarity environmental cues regulate the expression of type 1 pili in vitro [28] (Schwan et al., 2002). To measure changes following growth in media with different pH and/or osmolarity, LB was buffered using 0.1 M Na₂HPO₄-NaH₂PO₄ buffer and 1% (vol/vol) glycerol as previously described [28]. The media were separated into a pH ranging between 5.0 and 8.0 with 0.5 pH unit increments. Cultures of E. coli NU149 containing fimA-, fimB-, or fimE-lux fusions 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 incubated statically at 37°C until midlogarithmic phase had been reached. Bioluminescence testing was performed as described below.
2.5. In Vitro Bioluminescence Assays. Each culture was incubated at 37°C statically to midlogarithmic phase. A 500 μl aliquot of each culture was tested for bioluminescence using a FB 12 bioluminescence single tube luminometer (Zylux Corporation). The luminescence results were reported as relative luminescence units (RLU) as described previously [47]. A viable count of each culture was calculated by plating aliquots of 10-fold serially diluted bacteria in phosphate-buffered saline (PBS, [48]) onto LA containing 12.5 μg/ml of chloramphenicol and counting the colonies. The RLU values were divided by the viable counts to achieve RLU/CFU for each culture.

2.6. Murine Urinary Tract Infection Model. A murine urinary tract infection model [30] was used to assess the in vivo regulation of the fim-lux reporter fusions in E. coli NU149. The Institutional Animal Care and Use Committee at the University of Wisconsin-La Crosse approved the study design and the animal handling protocols of this study, including the use of isoflurane to anesthetize the mice. All steps were taken to minimize animal suffering throughout the experiment. Briefly, each E. coli NU149 strain with a specific fim-lux promoter reporter plasmid fusion was grown in LB medium at 37°C statically overnight. One ml culture aliquots were pelleted by centrifugation at 6000×g for two minutes and the supernatant was decanted off. Each pellet was suspended in 100 μl of PBS. A 250 μl volume of 10⁸ CFU/ml bacteria was instilled into the urinary bladders of six to twelve female 4- to 6-week-old Swiss Webster mice per time point through a soft polyethylene catheter adapted to a needle. The number of mice represents the aggregate number from at least two batches of inoculations administered on separate days. A larger inoculum volume was used to achieve 80% colonization of the kidneys at most time points and sufficient bioluminescence at the early time points. After 8, 24, 72, and 120 hours after inoculation (hpi), urine was collected to measure the pH and osmolality, and the mice were then euthanized and the bladders and kidneys were removed. Each organ was homogenized in sterile tissue grinders (Kontes) with 1ml of PBS. The homogenized tissues were tested for bioluminescence as described above. Tenfold 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. The plates were incubated overnight at 37°C and the number of colonies per organ homogenate per construct was calculated. The background fluorescence for each organ homogenate was subtracted from the RLU values. Background corrected RLU values were then divided by the number of bacteria determined by the viable bacteria counts, generating corrected RLU/CFU per time point as reported previously [47].

2.7. Measurement of Murine Urine pH and Osmolality. Murine urine was collected and the pH measured with pH strips and the osmolality measured using a Reichert TS 400 total solids refractometer (Reichert Analytical Instruments, Buffalo, NY) to assess the specific gravity. The specific gravity readings were converted to osmolality using a chart [29].

2.8. PCR for fimS Orientation Determination. To determine the orientation of the fimS invertible element, previously described PCR techniques were used and products visualized with FOTO/Analyst PC Image Software [41, 45]. To quantify the percentage of Phase-ON or Phase-OFF bacteria, a standard curve was prepared as described by Teng et al. [49] using locked-ON (DH5α/pAON-1 [41]) and locked-OFF bacteria (NU149 cells passaged five times on agar shown to be 100% Phase-OFF [41]) as PCR templates and the ImageQuant 5.2 software.

2.9. Hemagglutination Assays. The HA assays were performed with 1% guinea pig erythrocytes (Hardy Diagnostics) as previously described [50], standardizing the HA titer to the viable count. The titers represent the geometric means of ten bladder and ten kidney homogenate samples.

2.10. Statistical Analysis. Student’s t-test was used for statistical analysis of the in vitro growth conditions. An ANOVA analysis with a Bonferroni correction was used for in vivo analysis from the murine urinary tract organ homogenates. P values ≤ 0.05 were considered significant.

3. Results

3.1. Evaluation of the fim-lux Fusions in UPEC Growing in Different pH Media. Previously, fim-lacZYA fusions were created and tested in E. coli strains growing under in vitro conditions [28]. Because of the limitations of using lacZYA reporter 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 reporter fusions on pPP2-6 were electroporated into strain NU149, each strain containing a fim-lux reporter 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 lacZ reporter fusions. The lowest level of sensitivity of these fim-lux fusions was from 1.0 to 3.0 × 10⁵ bacterial cells, depending on the fusion tested.

When the E. coli cells were grown to midlog 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, 0.0022 RLU; fimB-lux, 0.0009 RLU; and fimE-lux, 0.001 RLU) (Figure 1). 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, 36-fold, P < 0.006; and fimE-lux, 26-fold, P < 0.00002). When the pH was greater than 7.0, expression of the fimE-lux fusion reached the highest level at pH 8.0, whereas fimA-lux and fimB-lux transcription dropped slightly compared to growth in pH 7.0 LB media. These results confirmed the fim-lacZ fusions results that showed that, in a low pH environment, transcription of all of the fim genes was repressed.
counts in the murine kidneys ranged from 3.30 to 6.05 \times 10^4 at 8hpi, 2.24 to 5.20 \times 10^4 at 72hpi, and 7.05 \times 10^4 \times 10^4 after 120 hpi (Figure 2). The median viable counts in the murine kidneys ranged from 3.30 to 6.05 \times 10^4 at 8hpi, 2.32 to 8.95 \times 10^4 after 24 hpi, 2.08 to 4.30 \times 10^4 at 72 hpi, and 7.05 \times 10^4 to 5.50 \times 10^4 after 120 hpi (Figure 2).

For the fisZ-lux fusion, transcription of fisZ in UPEC infected murine bladders showed minimal variation from 0.038 to 0.056 (Figure 3(a)) that was not significant. Compared to fisZ expression in infected bladders, fisZ transcription in UPEC infected murine kidneys showed less fluctuation among the five-day period after inoculation, ranging from 0.023 to 0.032. These results indicated transcription of fisZ-lux fusion was relatively stable with less than 0.1-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 8 hpi (0.0185 RLU/CFU) and then increased to the highest level (0.305 RLU/CFU) after 24 hpi (Figure 3(b)). Subsequently, fimA transcription fell to 0.313 RLU/CFU and 0.122 RLU/CFU at 72 and 120 hpi, 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 the first 72 hpi (8 hpi, 0.0004 RLU; 24 hpi, 0.0020 RLU/CFU; and 72 hpi, 0.0010 RLU/CFU) but dropped significantly by 120 hpi (0.0001 RLU). At 120 hpi, fimA transcription was barely detectable. However, the variation of fimA expression in NU149 infected kidneys was not significant during the 120 hpi period (P < 0.104). Although these results did not show significant variation in fimA transcription over time, the results did show much higher fimA expression in the UPEC infected bladders than kidneys.

Transcription of fimB in NU149 infected bladders showed a trend similar to the fimA expression results. At 8 hpi, fimB expression was 0.419 RLU/CFU (Figure 3(c)). After 24 hpi, the RLU/CFU decreased to 0.065 and reached the highest level at 72 hpi (1.13 RLU/CFU). This increase in fimB transcription was significant compared to the 8 hpi (2.7-fold, P < 0.002). By 120 hpi, fimB expression slightly dropped to 0.630 RLU.

Unlike the fimA and fimB transcription results in UPEC infected murine bladders, fimE transcription was much lower than either fimA or fimB transcription. At 8 hpi, fimE expression was 0.0072 RLU/CFU (Figure 3(d)). Transcription of fimE rose to 0.0284 RLU/CFU after 24 hpi and then reached the highest level (0.72 RLU/CFU) at 72 hpi before falling to the lowest level (0.00088 RLU/CFU) at 120 hpi (P < 0.0001).
compared to the five-day period after inoculation. In UPEC infected murine kidneys, fimE transcription significantly increased 30-fold from 8 hpi (0.00036 RLU) compared to 24 hpi (0.0076, $P < 0.02$). At 72 hpi, fimE transcription significantly declined (0.0001 RLU/CFU, $P < 0.04$) and remained down after 120 hpi (0.001 RLU/CFU, $P < 0.02$) compared to the 24 hpi result. By comparing the fimE transcription level at 8 hpi to the 120 hpi results, fimE transcription in NU149 infected murine kidneys increased 1.8-fold. These results suggested fimE transcription first increased and then was repressed in both NU149 infected murine bladders and kidneys, but the final level of fimE transcription in NU149 infected murine kidneys increased slightly over the first time point. Thus, the increase in fimE transcription combined with the decrease in fimB transcription suggested the Phase-OFF orientation driven by FimE recombinase activity might be favored over the five-day infection period in the murine kidneys.

3.3. Positioning of the fimS Invertible Element Favors a Phase-OFF Orientation over a Five-Day Period in UPEC Infecting Murine Kidneys. The fim-lux fusion results demonstrated a temporal regulation of fimA, fimB, and fimE within a UPEC strain growing in murine urinary tracts. We could not directly compare fimB to fimE transcript ratios because of the way each fim-lux fusion was created as well as there being potential posttranscriptional modification differences. Within the infected murine bladders, our results suggest but

\[ \text{Figure 2: Viable counts of E. coli strain NU149 containing different fim-lux fusions in infected murine bladders and kidneys after 8, 24, 72, and 120 hours after inoculation (hpi). Murine tissues included bladders (black square) or kidneys (white circle) from the ftsZ (a), fimA (b), fimB (c), and fimE (d) fusion cultures. Five to twelve animals per time point were examined where each symbol represents one mouse. The black horizontal bars represent the median values for each time point and tissue.} \]
do not confirm the ratio of fimB/fimE transcripts may favor fimB transcription. Presumably, more FimB would mean more Phase-ON orientation for the fimS element. However, in murine kidneys, the decline in fimB transcripts combined with an increase in fimE transcripts imply that more Phase-OFF oriented fimS occurs over the five-day period in murine kidneys.

To confirm the orientation of the fimS element from NU149 cells infecting murine bladders and kidneys over a 120 hpi period, a PCR approach used previously [28, 45] was performed. Chromosomal DNA was isolated from several infected bladder and kidney homogenates for each time point, and a multiplex PCR was performed on each DNA sample. The inoculum had predominately Phase-ON oriented fimS (90.8% Phase-ON, 9.2% Phase-OFF; Figure 4). The 8 hpi samples for both infected bladder and kidney samples also showed the position of the fimS element predominantly in the Phase-ON orientation (89.6% Phase-ON and 88.1% Phase-ON, resp.). At 24 hpi the Phase-OFF orientation of the fimS element increased to 16.4% Phase-OFF in infected murine bladder homogenates and 16.8% to 26.2% Phase-OFF in infected murine kidney homogenates compared to the inoculum. By 72 hpi, the Phase-OFF position in the murine bladders averaged 19.1% Phase-OFF. However, the position of the fimS element in 72 hpi infected kidneys showed an average of 77.8% Phase-OFF orientation as compared to the
Figure 4: Determination of the fimS invertible element orientation by PCR on chromosomal DNA isolated from NU149 grown in LB as well as NU149 infected murine bladder and kidney homogenates spanning a five-day infection period. Two random different UPEC infected murine bladder and kidney homogenates were screened for each time point. Multiplex PCRs were set up with INV and FIMA primers to amplify Phase-ON-oriented DNA (ON, 450 bp product) [28], FIME and INV primers to amplify Phase-OFF-oriented DNA (OFF, 750 bp product) [41], and EcFtsZ1 and 2 primers to amplify the ftsZ gene (302 bp product) [45]. Each multiplex was run at least three separate times. The lanes were loaded onto a 1.5% agarose gel as follows: lane 1, NU149 inoculum; lane 2, NU149 infected bladder mouse 1 day 0.33; lane 3, NU149 infected bladder mouse 2 day 0.33; lane 4, NU149 infected kidney mouse 1 day 0.33; lane 5, NU149 infected kidney mouse 2 day 0.33; lane 6, NU149 infected bladder mouse 1 day 1; lane 7, NU149 infected bladder mouse 2 day 1; lane 8, NU149 infected kidney mouse 1 day 1; lane 9, NU149 infected kidney mouse 2 day 1; lane 10, NU149 infected bladder mouse 1 day 3; lane 11, NU149 infected bladder mouse 2 day 3; lane 12, NU149 infected kidney mouse 1 day 3; lane 13, NU149 infected kidney mouse 2 day 3; lane 14, NU149 infected bladder mouse 1 day 5; lane 15, NU149 infected bladder mouse 2 day 5; lane 16, NU149 infected kidney mouse 1 day 5; lane 17, NU149 infected kidney mouse 2 day 5. For each lane, the intensities of the OFF and ON states were quantified using ImageQuant software (Molecular Dynamics) and corrected to the intensity of the ftsZ band. The corrected values for both states were standardized to the respective wild-type band (lane 1).

Table 2: Measurement of hemagglutination (HA) titer for UPEC strain NU149 infecting murine bladders and kidneys over a five-day time period.

| Organ     | Hours after inoculation |
|-----------|-------------------------|
|           | 8          | 24           | 72           | 120          |
| Bladder   | 222.8*     | 119.4        | 114.4        | 104          |
| Kidney    | 207.9      | 111.4        | 27.9         | 0            |

*HA titer represents the geometric mean from 10 different mouse organ preparations.

inoculum lane. Finally, the 120 hpi results demonstrated a 77.2% Phase-ON and 22.8% Phase-OFF orientation for the fimS element within infected bladder homogenates compared to the inoculum. The greatest change in the positioning of the fimS element occurred in 120 hpi infected kidney homogenates where one sample had a 92.6% Phase-OFF and 7.4% Phase-ON orientation whereas the other kidney homogenate demonstrated 100% Phase-OFF and no Phase-ON oriented fimS element.

3.4. Production of Type 1 Pili Is Altered in a UPEC Strain Infecting Murine Bladders and Kidneys over a Five-Day Time Span. Both the fim-lux fusion and positioning of the fimS invertible element results changed in strain NU149 growing in murine bladders and kidneys over a five-day period. To determine if the level of type 1 pili expressed on the surface also changed in NU149 cells infecting murine bladders and kidneys, HA assays were done using guinea pig erythrocytes. A 512 HA titer was observed for the initial inoculum. The results showed that the HA titers from NU149 infected bladder homogenates fluctuated approximately twofold over the five-day infection (Table 2). However, the geometric means of the HA titers in NU149 infected kidney homogenates varied from 207.9 after 8 hpi to an HA titer of 0 after 120 hpi. Clearly, UPEC cells became more non-type 1 piliated over time in infected murine kidneys.

4. 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 osmolality, 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, human bladder urine has a higher pH and lower osmolarity than kidney urine [29]. Murine urine has an even higher average osmolality [27]. In a previous study, fim-lacZYA fusions were used to examine the in vitro effects of pH and osmolality 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 [28]. 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
fusinfecting murine urinary tracts. The mice, which would hold an advantage over quantitative assessments during many time points by merely anesthetizing the animals and loss of bacterial cell viability. Moreover, if an in vivo fusion can be used with an advantage over quantitative real-time polymerase chain reaction analysis. To assess fim gene regulation in vivo, we created a series of fim-lux reporter 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 to determine whether they matched the results using the fim-lacZYA fusions [28]. 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 LB medium. An exception was that transcription of fimE was the highest in pH 8.0 LB media in the present study, whereas the previous study showed optimal expression in pH 7.0 LB media [28].

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 fusion in a UPEC strain colonizing murine urinary tracts over a five-day postinoculation period. 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 transcription, invertible element PCR, and HA titer results were consistent with previous reports that showed the highest level of type 1 pili expression at 24 hpi in NU149 infected murine bladders. In one study, a cystitis isolate maintained the fimS Phase-ON orientation throughout the entire four-day period of bladder infection [37]. Other studies have shown that type 1 pili expression was most important for bacterial growth in the early stage (24 h after infection) of a UPEC infection in murine bladders and the fimS region remained mostly Phase-ON throughout the entire seven-days bladder infection [34, 38, 40]. 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 after inoculation [30, 32].

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 [51, 52]. Human and murine bladder epithelial cells present an abundance of mannose moieties on their glycoproteins that may serve as receptors for type 1 pili [53], so continued production of type 1 pili would be advantageous for UPEC bladder colonization.

Like the fimA expression results from NU149 infected bladders, NU149 cells in infected murine kidneys also expressed the highest level of fimA transcription at 24 hpi and then displayed a significant drop in fimA transcription thereafter. No fimA transcription was detected in some of the 120 hpi infected kidney homogenates, suggesting that type 1 pili expression had been completely shut down in those NU149 infected kidneys. Our PCR results that examined the position of the fimS element as well as HA titer results coincided with the fimA transcription results. Other studies have also observed the loss of type 1 piliated UPEC cells over time in UPEC infected murine kidneys [30, 32, 54, 55].

Besides the differences in transcription of fimA gene observed in UPEC infection of murine urinary tracts, temporal regulation of both fim recombinase genes was also observed. Transcription of both fimB and fimE was the highest after 72 hpi in NU149 infected murine bladders. Nevertheless, relative fimB transcription went up 2.7-fold when comparing the 8 hpi to the 72 hpi time point, whereas relative fimE transcription increased 100-fold when comparing the 8 hpi to the 72 hpi time point, suggesting the temporal regulation of fimB and fimE in UPEC infecting murine bladders appears to favor fimB transcription in the early stage of the infection and switches at 72 hpi to one that more favored fimE transcription later. Thereafter, fimB expression fell by 0.3-fold after 120 hpi but remained at a high level. Conversely, transcription of fimE declined to barely detectable levels by 120 hpi in NU149 infected murine bladders.

Since FimB and FimE have roles in positioning of the fimS region that contains the fimA promoter [17, 18], 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 higher 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 after inoculation may be the result of environmental cues exerting a direct effect on the regulation of the fimA promoter or the expression of alternative site-specific recombinases, such as HbiI, IpuA, IpuB, Ipab, and LeuX [35, 56, 57].

Our infected bladder results were in agreement with the work of many others. These other studies have relied on other UPEC strains like NU14, UT189, and CFT073 as well as different mouse strains like C3H/HeN, C57BL/6, and CBA/J [7, 30, 33, 35–38, 40, 55, 57, 58]. Although there was some degree of bacterial gene expression variability in these other studies, similar trends were observed in our study. Two recent RNA sequencing studies have shown that there is considerable gene expression variability between UPEC strains growing in the urinary tract [59, 60]. In one study, a difference in the percentage of the fimS element in the Phase-ON orientation for a cystitis strain compared to the pyelonephritis strain CFT073 within the urine of infected mice was observed. Initially, the median percentages of invertible elements in the Phase-ON orientation for FII and CFT073 at the 4 h 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 after 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 [37]. 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 [38].

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 120 hpi. Transcription of fimB and fimE was the highest after 24 hpi in NU149 infected kidneys. Both fimB and fimE transcription were lower at the 72 hpi. By 120 hpi, relative fimB transcription dropped 12.4-fold compared to the 72 hpi time point, but fimE transcription had increased, suggesting the NU149 cells in the murine kidneys would have a relative ratio of fimB to fimE transcripts that favored fimE transcription and subsequently non-type 1 piliated cells by 120 hpi.

The question posed is why UPEC cell populations have their fimS element switch to the Phase-OFF orientation and lose their expression of type 1 pili over time in infected murine kidneys. Unlike bladder epithelial cells that have many mannose-containing receptors on their surface [61], kidneys display few of these receptors on their renal glycolipids [62], so expression of type 1 pili may 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 [29], so fim gene expression would be more repressed in this acidified/higher osmolarity urine [28]. Another possibility is that the type 1 piliated UPEC cells are cleared more readily by macrophages that are more immunogenic [69], 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 [63, 64], so non-type 1 piliated bacteria would hide behind their capsules and evade the murine innate defense. Thus, becoming non-type 1 piliated is an advantage for UPEC survival in the kidney, and the low pH/high osmolality environment encountered in the murine kidney would regulate the fim genes to favor a non-type 1 piliated 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 non-type 1 piliated 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.

5. Conclusion

Temporal regulation of the fimA, fimB, and fimE genes occurs in UPEC cells colonizing murine urinary tracts. Over time in murine kidneys, the ratio of fimE to fimB transcription switches to favor fimE, which results in the fimS element flipping to a more Phase-OFF orientation and the loss of type 1 pili on the surface of the UPEC cells within the murine kidneys.

Conflicts of Interest

The authors declare they have no conflicts of interest.

Acknowledgments

This study was funded by a National Institutes of Health Grant AI-065432 to William R. Schwan and a UWL-Research Education Service and Leadership Grant to Hua Ding.
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