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LeuX tRNA-dependent and -independent mechanisms of *Escherichia coli* pathogenesis in acute cystitis

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Summary

Uropathogenic *Escherichia coli* (UPEC) contain multiple horizontally acquired pathogenicity-associated islands (PAI) implicated in the pathogenesis of urinary tract infection. In a murine model of cystitis, type 1 pili-mediated bladder epithelial invasion and intracellular proliferation are key events associated with UPEC virulence. In this study, we examined the mechanisms by which a conserved PAI contributes to UPEC pathogenesis in acute cystitis. In the human UPEC strain UTI89, spontaneous excision of PAI II_{UTI89} disrupts the adjacent *leuX* tRNA locus. Loss of wild-type *leuX*-encoded $tRNA_5^{Leu}$ significantly delayed, but did not eliminate, FimB recombinase-mediated phase variation of type 1 pili. FimX, an additional FimB-like, *leuX*-independent recombinase, was also found to mediate type 1 pili phase variation. However, whereas FimX activity is relatively slow *in vitro*, it is rapid *in vivo* as a non-piliated strain lacking the other *fim* recombinases rapidly expressed type 1 pili upon experimental infection. Finally, we found that disruption of *leuX*, but not loss of PAI II_{UTI89} genes, reduced bladder epithelial invasion and intracellular proliferation, independent of type 1 piliation. These findings indicate that the predominant mechanism for preservation of PAI II_{UTI89} during the establishment of acute cystitis is maintenance of wild-type *leuX*, and not PAI II_{UTI89} gene content.

Introduction

Escherichia coli primarily inhabit vertebrate gastrointestinal tracts as commensal organisms. Yet certain strains of *E. coli* are distinguished as pathogens, in part, by the presence of unique virulence factor genes not found in environmental or commensal strains (Groisman and Ochman, 1994). These pathogen-associated genes are often found clustered together on horizontally acquired genomic islands termed pathogenicity-associated islands (PAI)

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(Hacker *et al*., 1990). This type of horizontal gene transfer enables recipient strains to colonize unique host niches through a number of mechanisms, including host tissue invasiveness (Groisman and Ochman, 1996). Features typical of PAI include: high frequency of insertion at tRNA loci, GC content distinct from the rest of the chromosome, flanking direct repeats and presence of mobility genes (Dobrindt *et al*., 2004; Gal-Mor and Finlay, 2006). Hacker and colleagues have proposed that PAI become fixed into the genome by mutation over time as part of the normal adaptation process of PAI acquisition, where continued mutation and rearrangement eventually lead to genome reduction (Dobrindt *et al*., 2004).

Pathogenicity-associated islands are common among uropathogenic *E. coli* (UPEC) (Blum *et al*., 1995; Brzuszkiewicz *et al*., 2006; Chen *et al*., 2006; Lloyd *et al*., 2007), a genetically heterogeneous pathotype of *E. coli* isolates that are the most common cause of urinary tract infections (Foxman, 2002). However, despite their prevalence, the importance of these PAI in the urinary tract niche remains unclear. This is because many of these islands have become fixed within the genome, either losing the elements necessary for excision or becoming linked to critical genes such that excision would reduce the fitness of the strain (Hacker and Kaper, 1999). An example of the latter is found in the human pyelonephritis UPEC isolate 536, where spontaneous excision of a genomic island, PAI II_{536}, results in deletion of two nucleotides within the adjacent *leuX* tRNA gene, *leuX* $GC_{80\text{-}81}$. This occurs because PAI insertion into the ancestral strain duplicated the 3′ end of the *leuX* tRNA, leaving an intact *leuX* gene at one end of the PAI and a 3′ fragment of *leuX* at the other end. This non-coding 3′ fragment of *leuX* can then undergo mutation without disrupting $tRNA_5^{Leu}$ expression and function. However, upon PAI excision via the direct repeats any mutated sequence within the 3′ end of this *leuX* fragment becomes incorporated into the coding *leuX* gene. In the case of strain 536, a two-nucleotide deletion has occurred near the 3′ end of the non-coding *leuX* fragment (Blum *et al*., 1994). These PAI excision dynamics have important clinical relevance as it has been demonstrated *in vitro* that fluoroquinolone antibiotic exposure can induce PAI excision and fluoroquinolone resistant UPEC isolates commonly lack PAI-encoded virulence factors (Soto *et al*., 2006).

Although *leuX*-associated PAI with synteny to PAI II_{536} are found among many UPEC (Bidet *et al*., 2005), the importance of these genomic islands, independent of *leuX*, in urinary tract disease pathogenesis is largely unproven. The UPEC strain UTI89, a human cystitis isolate, contains a previously uncharacterized 118.9 kb PAI, designated PAI II_{UTI89}, that is closely related to PAI II_{536} (99.1% identical over 97.3 kb) and is also found adjacent to the *leuX* locus. Almost all of the 124 open reading frames found on PAI II_{UTI89} are unique within the UTI89 genome, and many encode putative virulence factor genes that could hypothetically potentiate the invasiveness of UPEC in the urinary tract (Chen *et al*., 2006). These include the genes for P pili (Roberts *et al*., 1994; Winberg *et al*., 1995), the toxins α-haemolysin (Nagy *et al*., 2006) and cytotoxic necrotizing factor 1 (CNF1) (Doye *et al*., 2002; Davis *et al*., 2005), the putative invasin, Hek (Srinivasan *et al*., 2003), and the recently identified contact-dependent inhibition of growth exoprotein, CdiA (Aoki *et al*., 2005). The presence of PAI II_{UTI89} also directly affects the expression of virulence factors

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elsewhere on the chromosome by encoding transcriptional regulators such as PapB, which negatively regulates type 1 pili expression (Xia *et al*., 2000; Holden *et al*., 2006).

Type 1 pili are the major virulence factors in UPEC pathogenesis in a murine model of cystitis (Connell *et al*., 1996; Wright *et al*., 2007). The type 1 pilus-associated adhesin, FimH, mediates UPEC adherence to and invasion into the urinary bladder epithelium (urothelium) (Mulvey *et al*., 1998). These early events of cystitis are critical for the establishment of both acute infection and chronic persistence within quiescent intracellular reservoirs (Mulvey *et al*., 1998; 2001; Mysorekar and Hultgren, 2006). Type 1 pili bind with high affinity to mannosylated glycoprotein receptors present on urothelial cells, such as uroplakin Ia and α3β1 integrins (Zhou *et al*., 2001; Eto *et al*., 2007). After invasion of the urothelium, some of these bacteria proceed to replicate rapidly within the cytoplasm to form intracellular bacterial communities (IBC) (Anderson *et al*., 2003; Justice *et al*., 2004). The biofilm-like organization of the IBC requires the expression of type 1 pili (Wright *et al*., 2007) and appears to provide a protected niche where UPEC can more effectively multiply and survive in the face of vigorous innate immune responses (Mulvey *et al*., 2000; Anderson *et al*., 2003), which are mounted in response to UPEC infection (Mysorekar *et al*., 2002).

Spontaneous excision of PAI II_{536} and the resultant *leuX* $GC_{80–81}$ mutation completely abolishes type 1 pili expression in the UPEC strain 536, and this defect is likely the primary mechanism for loss of urinary tract virulence (Blum *et al*., 1994). Indeed, type 1 pili expression, and therefore virulence in cystitis, is highly dependent on the *leuX*-encoded $tRNA_5^{Leu}$, which translates the rare leucine codon UUG (Ritter *et al*., 1995). Type 1 pili are encoded by the *fim* operon and their expression is phase variable, originating from the *fim* operon promoter, which is contained within the *fimS*-invertible element (Abraham *et al*., 1985). Two tyrosine recombinases, FimB and FimE, are known to control the orientation of the *fimS*-invertible region. FimB has bidirectional activity, but predominantly switches *fim* operon transcription from OFF to ON, while FimE mediates solely ON to OFF phase switching (Klemm, 1986). Additional FimB homologues also mediate type 1 pili phase variation *in vitro* or *in vivo* (Bryan *et al*., 2006; Xie *et al*., 2006). The dependence of type 1 pili expression on $tRNA_5^{Leu}$ was demonstrated to be a direct result of impaired UUG codon translation within the *fimB* recombinase transcript in *leuX* mutants (Ritter *et al*., 1997). Therefore, the regulation of rare codon tRNA expression may provide a mechanism for the fine tuning of virulence gene expression in UPEC as they adapt to diverse niches (Saier, 1995; Piechaczek *et al*., 2000; Dobrindt and Hacker, 2001).

Our goal in this study was to dissect the specific mechanisms by which PAI II_{UTI89} contributes to virulence in a murine model of cystitis. In particular, we hypothesized that the presence of PAI II_{UTI89} genes as a whole, independent of *leuX*, would be important or even necessary for the early events of cystitis, namely urothelial invasion and IBC formation. We discovered that the spontaneous loss of PAI II_{UTI89} in UTI89 disrupts *leuX*, thereby delaying but not eliminating type 1 pili phase switching, *in vitro*. This defect, which was restored by complementation with wild-type (wt) *leuX*, was a result of an apparent loss of $tRNA_5^{Leu}$ function, but not expression. Overexpression of either FimB or FimX, a FimB homologue in UTI89, also restored normal phase switching, *in vitro*. However, we found that FimX is less dependent on $tRNA_5^{Leu}$-mediated translation than FimB and was able to mediate phase

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switching in UTI89 *fimBE* slowly *in vitro*, but rapidly *in vivo* in a murine model of cystitis. Finally, we showed that the absence of PAI II_{UTI89} gene content did not significantly affect the early establishment of cystitis, whereas mutation of *leuX* led to a significant attenuation of UTI89, independent of type 1 piliation, during the early acute stages of UPEC pathogenesis.

Results

PAI II_{UTI89} genes are conserved among lower urinary tract isolates

The prevalence of PAI II_{UTI89}-like islands among UPEC isolates from women with lower urinary tract infection (LUTI) was unknown. Therefore, we surveyed a collection of 163 UPEC isolates from adult women with LUTI by multiplex PCR for the presence of the three PAI II_{UTI89} genes *cnf1, hek* and *hlyA*, which, when found concurrently, are a specific indicator of a PAI II_{UTI89}-like genomic island (Bidet *et al*., 2005). We found that the individual prevalences of these genes in our collection of clinical isolates ranged from 44% to 48% (Table S1), and that all three genes were found concurrently in 43% of isolates. These prevalences were significantly higher ($P < 0.0001$, Fisher's exact test) than those found in the *E. coli* reference collection (ECOR) of mostly commensal isolates (Ochman and Selander, 1984) in which the individual gene prevalences ranged from 8% to 17%. Only six strains (8%) in the ECOR set were positive for all three genes, and one of these was a human cystitis isolate. These findings indicate that at least 43% of these clinical isolates contain a PAI II_{UTI89}-like island, and suggest that PAI similar to PAI II_{UTI89} are evolutionarily conserved among lower urinary tract isolates.

Wild-type leuX , a PAI II_{UTI89}-associated tRNA gene, is important but not essential for fimS *phase switching* in vitro

In contrast to UPEC strain 536 PAI II_{536}, which is unable to express type 1 pili, spontaneous excision of PAI II_{UTI89} does not abolish expression of type 1 pili *in vitro* (G. Anderson and S. Hultgren, unpubl. data). Additional spontaneous PAI II_{UTI89} deletion mutants (*PAI* II_{UTI89}^{-}) were isolated by selection on blood agar plates as described in *Experimental procedures*. Chromosomal sequencing of the *leuX* locus in six non-haemolytic mutants lacking PAI II_{UTI89} revealed that each had an identical single-base-pair deletion at nucleotide 80 in the 3′ end of the *leuX* gene, *leuX* G_{80} (*leuX**), as would be predicted by the genome sequence (Fig. 1). All six clones were found to express type 1 pili as determined by mannose-sensitive haemagglutination (MSHA) titres, but at a markedly delayed rate compared with wt UTI89 (data not shown). The kinetics of type 1 pili expression in one of these UTI89 PAI II_{UTI89} clones, designated TJH1 (*leuX*, PAI* II_{UTI89}^{-}), is shown in Fig. 2A. Reversion of the 3′ end of the *leuX* locus in TJH1 to the wt sequence, TJH1 COMP (*leuX*^{wt}, *PAI* II_{UTI89}^{-}), restored wt piliation (Fig. 2A). Phase assays, which amplify the *fimS*-invertible region and utilize a restriction endonuclease digest of the PCR product to determine the orientation of the *fim* operon promoter, demonstrated that the delay in type 1 pili expression in TJH1 was a result of delayed OFF to ON phase switching (Fig. 2A). A directed mutation of *leuX* in UTI89 mimicking that found in TJH1, UTI89 *leuX* G_{80} (*leuX*, PAI* II_{UTI89}^{+}), also resulted in delayed but not absent expression of type 1 pili, and was complemented by *leuX* expressed *in trans* (Fig. 2B). These data demonstrate that the

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leuX G_{80} mutation causes delayed phase variation in TJH1, while loss of PAI II_{UTI89}-associated genes has no effect.

To explain the differences in type 1 piliation phenotypes between spontaneous PAI II deletion mutants in UPEC strains 536 and UTI89, we hypothesized that a UTI89 strain with a two-nucleotide deletion in *leuX* mimicking that found in 536 PAI II_{536}, UTI89 *leuX* $GC_{80\text{-}81}$, would be completely unable to express type 1 pili. Surprisingly, however, we found that such a mutant, as well as one with a complete *leuX* deletion, expressed type 1 pili with kinetics similar to UTI89 *leuX* G_{80} (Fig. 2C). Furthermore, quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis of these mutant strains indicated that the various single- or double-nucleotide mutants expressed higher levels of $tRNA_5^{Leu}$, relative to the *leuX* null strain, UTI89 *leuX* (Fig. 2D). These data demonstrate that both *leuX* G_{80} and *leuX* $GC_{80\text{-}81}$ strains continue to produce $tRNA_5^{Leu}$, but these mutant tRNA are apparently non-functional, resulting in delayed phase variation and type 1 piliation similar to that seen in a *leuX* null strain. Therefore, *leuX* G_{80} strains are functionally *leuX* null (*leuX*$^-$). Furthermore, the UTI89 *leuX*, phenotype indicates that $tRNA_5^{Leu}$ are not absolutely required for production of type 1 pili in UTI89.

FimX, a fimB homologue in UTI89, has lower UUG leucine codon usage than fimB

An alternative explanation for the ability of TJH1 (*leuX*$^-$*, PAI* II_{UTI89}^-) to express type 1 pili, despite *leuX* mutation, was the presence of additional, *leuX*-independent, *fim* recombinases. Therefore, we analysed the UTI89 genome and identified a FimB homologue, FimX (Fig. 1). FimX shares 49.1% amino acid identity to FimB and orthologues were recently described in two other extraintestinal *E. coli* strains (Bryan *et al*., 2006; Xie *et al*., 2006). We performed a PCR survey of a collection of 61 UPEC isolates, and found that the *fimX* gene was present in 83.9% of these strains including strain 536, but was not detected in the K12 strain MG1655. Interestingly, the usage of the UUG codon, which is translated by the *leuX*-encoded $tRNA_5^{Leu}$, differs significantly among the three *fim* recombinases in UTI89. UUG is used in 23%, 9% and 6% of the leucine codons in *fimB, fimX* and *fimE* respectively (Table S2). This codon usage in *fimB* is significantly higher than that found in either *fimX* or *fimE* ($P < 0.05$ and $P < 0.01$ respectively; binomial test). The leucine codon bias between *fimX* and *fimB* suggested that *fimX* expression may maintain the ability to phase switch in some *leuX* mutant strains despite poor to absent *fimB* translation.

Both FimB and FimX contribute to fimS *phase switching in the absence of wt* leuX

To test whether FimX was capable of mediating *fimS* phase switching, we constructed a double mutant in UTI89, lacking both *fimB* and *fimE*, that was isolated and maintained *in vitro* in the phase OFF position. This mutant, $UTI89_{OFF}$ *fimBE*, had delayed *fimS* phase switching from OFF to ON and type 1 piliation as measured by MSHA *in vitro* that resembled the kinetics of switching in TJH1 (data not shown). This similarity suggested that TJH1 (*leuX*$^-$*, PAI* II_{UTI89}^-) with a *leuX* mutation may functionally be *fimB* null due to the relatively high UUG codon usage of *fimB*. Therefore, we tested the relative contributions of FimB and FimX to *fimS* phase switching in the TJH1 background by constructing *fimB* and *fimX* single-and paired-deletion mutants. By 48 h of static *in vitro* growth, TJH1 *fimX* had a modest but statistically significant ($P = 0.03$, Student's *t*-test) decrease in MSHA compared

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with the parental strain, which was reflected in the amount of FimA (Fig. 3A). In contrast, TJH1 *fimB* had a marked loss of MSHA ($P < 0.0001$) and FimA. Interestingly, TJH1 *fimB fimX* continued to produce a small phase ON population and minimally detectable amount of FimA (Fig. 3A). The origin of the residual phase variation remains unknown, as FimE is thought to have only unidirectional, ON to OFF, phase activity (Klemm, 1986). Surprisingly, these data suggest that although FimX makes a significant contribution to *in vitro* phase switching in TJH1, FimB continues to have recombinase activity.

To test the abilities of FimB and FimX to independently mediate phase switching activity regardless of recombinase expression levels in a *leuX* G_{80} background *in vitro*, we overexpressed either *fimX* or *fimB in trans* in TJH1 *fimB fimX*. Each recombinase was equally capable of restoring *fimS* phase switching and high MSHA in this strain after 24 h growth (Fig. 3B). This finding demonstrates that both FimB and FimX have recombinase activity when overexpressed in a *leuX* G_{80} mutant strain of UTI89, suggesting that increased levels of recombinase transcription are capable of overcoming the functional $tRNA_5^{Leu}$ deficiency in this genetic background.

LeuX-encoded $tRNA_5^{Leu}$-dependent and -independent recombinases

To test whether FimB and FimX recombinase activities are adversely affected by *leuX* mutation when expressed at native levels, we complemented TJH1 *fimX* and *fimB* mutants with a plasmid expressing *leuX* from its native promoter. At 24 h of growth, FimB-mediated type 1 expression in TJH1 *fimX* was enhanced by *leuX*, provided *in trans* (Fig. 3C), whereas there was no positive effect on FimX-mediated phase variation in TJH1 *fimB*. These data are consistent with our hypothesis that FimB expression is more dependent on the *leuX*-encoded $tRNA_5^{Leu}$ than is FimX, likely due to the differences in the UUG codon usage. However, because *fimX* expession *in vitro* appears to be inherently delayed, its contribution to overall *in vitro* phase variation in TJH1 still does not outweigh the role of FimB.

FimX mediates rapid phase switching of type 1 pili in vivo

FimX appears to play a lesser role than FimB in type 1 pili phase switching *in vitro*. We investigated the *in vivo* function of FimX by testing the ability of a phase OFF nonpiliated UTI89 *fimB fimE* mutant ($UTI89_{OFF}$ *fimBE*) to infect the mouse bladder (Fig. 4A), anticipating that the mutant would have a dramatic loss of fitness. However, the phase OFF non-piliated culture was able to colonize and invade the bladder urothelium to the same level as the wt vector control, UTI89/VC ($P = 0.4$, Mann–Whitney test). Individual colonies ($n = 10$) and bacteria from an aggregate sweep of gentamicin-protected $UTI89_{OFF}$ *fimBE* were found to be exclusively in the phase ON orientation (data not shown). These phase ON colonies of $UTI89_{OFF}$ *fimBE* had high MSHA titres also, $1:2^8$ (data not shown). These data indicate that a subpopulation of $UTI89_{OFF}$ *fimBE* underwent rapid *fimS* phase switching *in vivo*, produced type 1 pili and consequently invaded the urothelium. Consistent with these findings, $UTI89_{OFF}$ *fimBE* was able to form IBC morphologically similar to those formed by the wt parental strain at 6 h post infection (hpi). The development of high-density IBC by 6 hpi implies that the phase switch undergone by $UTI89_{OFF}$ *fimBE* occurred

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shortly following inoculation. However, UTI89$_{OFF}$ *fimBE* recovered from the extracellular fraction remained in the OFF orientation (*n* = 10 colonies). UTI89$_{OFF}$ *fimBEX* was found to be dramatically attenuated in its ability to infect the mouse bladder, confirming that FimX was mediating this *in vivo* phase switching (Fig. 4B). Accordingly, the expression of *fimX in trans* restored the virulence of UTI89$_{OFF}$ *fimBEX*.

FimX and FimB both contribute to persistence during cystitis

The ability of FimX in UTI89$_{OFF}$ *fimBE* to rapidly induce *fimS* phase switching from OFF to ON and carry out the UPEC pathogenic cycle in the mouse bladder led us to hypothesize that FimX was the dominant recombinase *in vivo*. This hypothesis was tested by assaying the ability of either FimX or FimB null mutants to compete with wt UTI89, *in vivo*. Approximately equal colony-forming units (cfu) of wt and UTI89 *fimX* or *fimB* were co-inoculated into the bladders of C3H/HeN mice, and cfu were determined at 30 hpi. The medians of the competitive indices for each infection were 0.9 and 0.2 for *fimX* and *fimB*, respectively, suggesting only modest attenuation of these mutants (Fig. 4C). Furthermore, the competitive indices were not statistically different ($P = 0.09$, Mann–Whitney). Therefore, these findings suggest that FimB and FimX have overlapping and compensatory activities during acute cystitis.

Wild-type leuX, but not PAI II$_{UTI89}$ gene content, is necessary for full virulence of UTI89 in urothelial invasion and IBC formation

We then sought to test the individual roles of wt *leuX* and PAI II$_{UTI89}$ in urothelial invasion and IBC formation, key events in early acute infection. We employed the isogenic UTI89 mutants utilized in the *in vitro* studies, TJH1 (*leuX*$^-$, *PAI II*$_{UTI89}$$^-$) and UTI89 *leuX* G$_{80}$ (*leuX*$^-$, *PAI II*$_{UTI89}$$^+$), as well as a directed PAI II$_{UTI89}$ deletion mutant that retained a wt *leuX* locus, called TJH2 (*leuX*$^+$, *PAI II*$_{UTI89}$$^-$). The growth rates of the mutants were comparable to wt in rich and minimal media (data not shown). To compensate for the differences in type 1 pili phase variation among these strains, overexpression of *fimX in trans* was used to direct *fimS* phase switching from OFF to ON, resulting in equivalent MSHA titres and FimA expression of the inoculating strains (Fig. 5A).

Urothelial invasion was determined by *ex vivo* gentamicin protection assays at 1 hpi (Fig. 5B). We found that UTI89 *leuX* G$_{80}$ /*pBAD-fimX* (*leuX*$^-$, *PAI II*$_{UTI89}$$^+$) was consistently attenuated ($P < 0.01$, Mann–Whitney) in its ability to invade bladder urothelium and/or survive intracellularly. However, the gentamicin-protected bladder titres from mice infected with TJH1/*pBAD-fimX* (*leuX*$^-$, *PAI II*$^{UTI89-}$) and TJH2/*pBAD-fimX* (*leuX*$^+$, *PAI II*$_{UTI89}$$^-$) were not significantly different from those infected with wt UTI89 in any experiment. In an independent control experiment, UTI89$_{ON}$ *fimBE* carrying either a control or *fimX* expression vector were shown to have equivalent invasion, suggesting that *fimX* had no detectable type 1 pili-independent effects in this assay (data not shown). IBC formation was assayed qualitatively by confocal microscopy and quantitatively by *lacZ* staining of whole mounted, fixed bladders at 6 hpi (Fig. 5C). All three mutant strains overexpressing FimX produced IBC morphologically similar to UTI89/*pBAD-fimX* (data not shown). However, UTI89 *leuX* G$_{80}$ /*pBAD-fimX* (*leuX*$^-$, *PAI II*$_{UTI89}$$^+$) had decreased numbers of IBC compared with UTI89/*pBAD-fimX* (*leuX*$^+$, *PAI II*$_{UTI89}$$^+$; $P < 0.10$), and TJH1/*pBAD-fimX*

(*leuX*⁻, $PAI\ II_{UTI89}^{-}$) developed fewer IBC than TJH2/*pBAD-fimX* (*leuX*⁺, $PAI\ II_{UTI89}^{-}$; $P < 0.05$). Taken together, these findings strongly indicate that wt *leuX* is necessary for efficient urothelial invasion and/or intracellular survival, as well as IBC formation. Surprisingly, the presence of the pathogenicity island genes, independent of *leuX*, did not enhance IBC formation as TJH2/*pBAD-fimX* (*leuX*⁺, $PAI\ II_{UTI89}^{-}$) formed more IBC than UTI89/*pBAD-fimX* (*leuX*⁺, $PAI\ II_{UTI89}^{+}$; $P < 0.10$), and TJH1/*pBAD-fimX* (*leuX*⁻, $PAI\ II_{UTI89}^{-}$) formed more IBC than UTI89 *leuX* G_{80} /*pBAD-fimX* (*leuX*⁻, $PAI\ II_{UTI89}^{+}$; $P = 0.15$).

Discussion

The acquisition of unique genes by horizontal transfer, often from other prokaryotic species, has promoted tremendous diversity within the *E. coli* species (Feil and Spratt, 2001), enabling many strains to evolve as successful pathotypes (Welch *et al*., 2002). Traditionally, if these genetic elements play a role in pathogenesis, they have been termed pathogenicity-associated islands (PAI) (Hacker *et al*., 1990). These islands, which are often inserted at tRNA loci, appear to become fixed within the genome over time by mutation or further recombination (Hacker and Kaper, 1999). In some cases, this fixation is mediated by disruption of the associated tRNA locus as a consequence of spontaneous excision of the island (Blum *et al*., 1994). Therefore, PAI gene content is not the only mechanism for island conservation in these strains. The higher prevalence of PAI II_{UTI89} genes in lower urinary tract isolates when compared with commensal strains suggests that PAI II_{UTI89}-containing strains may be selected for in colonizing the urinary tract. However, as this correlation does not demonstrate that PAI gene content is operative in this selection, we investigated the consequences of losing this PAI in a murine model of acute cystitis. Surprisingly, we found that *leuX* mutation, and not loss of PAI II_{UTI89} gene content, was the predominant mechanism for loss of virulence during the establishment of acute cystitis by UTI89 after spontaneous excision of this island, independent of any effect on type 1 piliation. These studies also led to the discovery that the *leuX*-independent *fim* recombinase, FimX, rapidly mediates *fimS* phase switching *in vivo*.

In this report, we show for the first time that *leuX* mutation, independent of its effect on type 1 pili-mediated invasion, reduces urothelial invasion and IBC formation in murine cystitis. Examination of the proteome of *leuX* mutants of 536 grown in minimal media demonstrated that *leuX* was necessary for normal expression of a small number of abundant proteins, including iron scavenging effectors (Piechaczek *et al*., 2000). Yet, many of the proteins that were adversely affected were not enriched in UUG codons, suggesting that the effect was at least in part mediated by undetected low abundance transcriptional regulators. *LeuX* is unusual among tRNA genes in that it is regulated by the stress factor RpoH (Dobrindt and Hacker, 2001). Furthermore, *leuX* expression was previously shown to be important for stationary-phase survival in bladder mucus (Dobrindt *et al*., 1998), an important extracellular phenotype of UPEC. In contrast, our studies suggest roles for $tRNA_5^{Leu}$ during invasion, intracellular survival, and rapid intracellular proliferation. As a consequence of *leuX* mutation, the expression of invasins or adhesins that could potentiate the invasion process or effectors necessary for intracellular survival and IBC formation may be adversely affected. Therefore, *leuX* could be important for necessary bacterial stress responses initiated in both the extracellular and intracellular environments of the urinary tract.

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Wild-type *leuX* may also be necessary for continued *fimS* phase switching within the developing IBC, as IBC formation is highly dependent on type 1 pili expression post invasion (Wright *et al*., 2007). *FimS-gfp* transcriptional reporter fusions responsive to Fim recombinase-mediated phase variation are predominantly inactive in early invasive bacteria (P. Seed and S. Hultgren, unpubl. data), suggesting that bacteria may initiate IBC formation starting in the phase OFF orientation. Therefore, the IBC defect due to *leuX* G_{80} may be at least partially due to impaired phase switching of these invasive bacteria from OFF to ON. However, *leuX* mutation could also be affecting the assembly of type 1 pili during rapid replication, as two proteins critical for pili formation, FimG and FimC, have unusually high UUG codon usage: 36% and 31%, respectively (Table S2) (Saulino *et al*., 2000).

We demonstrate that as a consequence of PAI II_{UTI89} excision the recombined *leuX* gene acquires a single-nucleotide deletion, *leuX* G_{80}. This mutation, independent of the presence of PAI II_{UTI89}, delayed but did not eliminate FimB-mediated type 1 pili phase switching from OFF to ON. Moreover, *fimB* overexpression restored OFF to ON phase switching in the UTI89 PAI II_{UTI89} (*leuX* G_{80}) mutant strain, whereas *fimB* overexpression was unable to restore type 1 piliation in 536 PAI II_{536} (Ritter *et al*., 1997). Interestingly, directed mutation of the *leuX* gene in UTI89 mimicking that observed with PAI II excision in 536, *leuX* $GC_{80\text{-}81}$, resulted in a type 1 piliation defect similar to that seen in both *leuX* G_{80} and *leuX* null strains, despite the fact that the various *leuX* G_{80} and *leuX* $GC_{80\text{-}81}$ mutant strains all had higher than wt levels of $tRNA_5^{Leu}$ expression. This suggests that $tRNA_5^{Leu}$ function is completely abrogated in the *leuX* G_{80} and *leuX* $GC_{80\text{-}81}$ mutant strains. Thus, in contrast to strain 536, UTI89 is capable of expressing type 1 pili despite the complete absence of functional $tRNA_5^{Leu}$. While we have observed that wt 536 produces significantly less type 1 pili than UTI89 under optimal conditions (T. Hannan and S. Hultgren, unpubl. data), the specific differences in the requirements of each strain for $tRNA_5^{Leu}$ to express type 1 pili may be accounted for by suppressor mutation(s) found in UTI89 but not 536. Alternatively, additional strain background factors, such as differences in the expression of the *leuZ*-encoded $tRNA_6^{Leu}$ that is capable of 'wobbling' onto and translating the UUG codon, may also explain these differences, which merit further study.

Our studies of *leuX* also revealed the role of an additional Fim recombinase in uropathogenesis. Mounting evidence suggests that UPEC have acquired a battery of recombinases that have type 1 pili phase switching activity (Bryan *et al*., 2006; Xie *et al*., 2006). We found that, in contrast to its orthologue in the UPEC strain CFT073 (Bryan *et al*., 2006), UTI89 FimX can be the sole mediator of *in vivo fimS* phase switching. Furthermore, FimB and FimX are able to significantly compensate for each other during acute cystitis. However, both FimB- and FimX-mediated type 1 pili expression are sensitive to different environmental cues as was evident by comparison of *in vitro* and *in vivo* phase variation. *In vitro*, FimB and FimX produced rapid and slow switching, respectively. In contrast, an environmental signal in the lower urinary tract markedly affects FimX-mediated type 1 pili expression through rapid phase switching in the absence of FimB and FimE. Thus, FimX and FimB may provide important environmentally sensitive regulatory controls for type 1 pili regulation and thereby fine tune the control of type 1 pili expression in non-commensal niches.

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Independent of its relationship to *leuX*, PAI II_{UTI89} genes likely serve some function among uropathogenic *E. coli* strains because they are more frequently present in lower urinary tract isolates than in commensal isolates. Surprisingly, we were unable to demonstrate that PAI II_{UTI89} positively affects the virulence of UPEC in urothelial invasion and IBC formation. In fact, we have shown that deletion of the entire island while leaving *leuX* intact may actually be beneficial to UTI89 in early intracellular stages of cystitis. This could be due to the loss of the PAI II_{UTI89}-encoded transcriptional regulator PapB, which negatively regulates type 1 pili expression (Xia *et al*., 2000). We cannot exclude that this genomic island enhances UPEC virulence in later stages of uropathogenesis such as during extracellular colonization in the face of a host immune response. For example, the ρ-GTPase-activating toxin, CNF1, is found on PAI II_{UTI89} and has been demonstrated to enhance the virulence of UPEC in the urinary tract, possibly by preventing the phagocytosis of UPEC by neutrophils encountered in the extracellular environment (Rippere-Lampe *et al*., 2001; Davis *et al*., 2005). PAI II_{UTI89} gene content may also be selected for in the ascension from the gut to the urinary bladder, as UPEC commonly colonize the periurethral and vaginal epithelium prior to introduction into and ascension of the urethra.

In summary, we have shown a specific role for *leuX* $tRNA_5^{Leu}$-dependent factors in the earliest stages of cystitis, namely adherence, invasion and IBC formation. The effects of *leuX* G_{80} mutation on invasion and IBC formation further support the hypothesis that minor codon usage could serve as a mode of regulation of virulence factor expression *in vivo* (Saier, 1995; Piechaczek *et al*., 2000). Through the elucidation of *in vivo* expressed $tRNA_5^{Leu}$-dependent factors, we anticipate the discovery of essential UPEC virulence determinants for cystitis that would be ideal targets for therapeutic inhibition.

Experimental procedures

Bacterial strains and cultivation

Strains of *Escherichia coli* used in this study are listed in Table S3. Bacteria were routinely cultured in Luria–Bertani (LB) broth containing, where appropriate, kanamycin 50 μg ml^{-1}, ampicillin 100 μg ml^{-1} and chloramphenicol 20 μg ml^{-1}.

Deletion strain construction

Deletion mutations were made using the red recombinase method, as previously described, using pKD4 or pKD13 as a template and the primers as listed in Table S4 (Datsenko and Wanner, 2000; Murphy and Campellone, 2003). PCR was performed with flanking primers to confirm the appropriate deletions. Antibiotic insertions were removed by transforming the mutant strains with pCP20 (Cherepanov and Wackernagel, 1995) expressing the FLP recombinase. The resultant strains subsequently had no additional antibiotic resistance compared with the parental wt strain.

Spontaneous PAI II_{UTI89} deletion mutant derivation

Spontaneous PAI II_{UTI89} deletion mutants were created by aerobically growing a liquid culture of UTI89 in LB broth to stationary phase at room temperature (22°C), growth conditions under which the rate of spontaneous deletion has been shown to be maximal

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(Middendorf *et al*., 2004). The bacteria were then diluted and plated onto trypticase soy agar plates containing 5% sheep blood for screening. Non-haemolytic clones were isolated and confirmed by PCR and sequencing of the scar region, using a three-primer set: PAI II_{UTI89} 5′ FLANK F, 5′ INT R and 3′ FLANK R.

Phase and haemagglutination assays

Phase assays were performed by a modified method of Smith *et al*. (Horcajada *et al*., 2005) using the primers PHASE 1, PHASE 2 and fim#14. The inclusion of primer fim#14 allowed PCR amplification of the *fim* promoter-invertible region in both wt and mutants with *fimB–fimE* deletions. Amplicons were digested with BstUI which singly and asymmetrically cuts within the amplicon, and the products run on a 2% TBE agarose gel. Haemagglutination assays for mannose-sensitive agglutination of guinea pig red blood cells were performed as previously described using 3% red blood cells (OD_{640} = ~1.9–2; Hultgren *et al*., 1986).

qRT-PCR

RNA was collected from 5 ml of 24 h static growth bacterial cultures in LB using disruptor beads and Trizol (Sigma-Aldrich) followed by DNase I treatment (Fisher RQ1) and RNA cleanup (Zymo Research). One microgram of RNA was reverse transcribed using random hexamers and iScript (Bio-Rad). cDNA was diluted fourfold in DEPC-treated water, and 1 μl of each dilution was used per 25 μl of qPCR reaction. Amplicons were detected using the inclusion of 1 μl per reaction (rxn^{-1}) of a 1:3000 dilution of SYBR Green (Invitrogen) in a mixture of 1× APEX Taq polymerase buffer (Genesse Scientific), 1 U of APEX Taq polymerase, 2.5 mM $MgCl_2$ (final) and 0.2 μM primers (IDT). Primers 16s RT L/R and leuX RT L/R were included in the 16s and *leuX* reaction mixtures respectively (Table S4). cDNA for 16s RNA analysis (calibrator) was performed by 1 cycle of 95°C (5 min) then 40 cycles of 95°C (10 s), 55°C (30 s), 72°C (15 s), 80°C (5 s) and plate reading. *leuX* cDNA analysis was performed by 1 cycle of 95°C (5 min) then 40 cycles of 95°C (10 s), 57°C (30 s), 72°C (15 s), 80°C (5 s) and plate reading. Melting curve analysis and gel electrophoresis were used to ensure amplicon homogeneity. Relative quantity (RQ) was derived by the Ct method in comparison with UTI89 *leuX* (ABI User Bulletin #2). Reactions lacking reverse transcriptase (–RT) were performed to ensure the adequacy of DNase treatment.

PCR analysis

Bacterial chromosomal DNA from a library of clinical isolates (Garofalo *et al*., 2007) was isolated using the Wizard genomic purification kit (Promega). Multiplex PCR reactions to determine the presence of *cnf1, hek* and *hlyA* (primers shown in Table S4) in this library were performed as previously described (Johnson and Stell, 2000). The presence of *fimX* was assessed by PCR with prepared genomic DNA from clinical UPEC isolates using the primers *fimX* PCR PROBE #1 and #2 (Table S4).

Mouse infections

C3H/HeN mice were obtained from Harlan (Indianapolis, IN). Bacterial strains were inoculated into LB broth from freezer stock, grown aerobically to mid- to late-log phase and then subcultured 1:100 into fresh media and grown statically at 37°C for 18 h. These

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cultures were spun at room temperature for 7 min at 5500 r.p.m., re-suspended in PBS and then diluted to approximately 2–4 × 10^8 cfu ml^{-1}. Fifty microlitres of this suspension (~1–2 × 10^7 cfu) was inoculated into the bladders of 6- to 8-week-old female mice by transurethral catheterization. All animal studies were approved and performed in accordance with Committee for Animal Studies at Washington University School of Medicine.

Ex vivo *gentamicin protection assay to assess for invasion*

The presence of intracellular bacteria *in vivo* in the murine bladder was assayed by a modified gentamicin-protection assay as previously described (Justice *et al*., 2006).

Competitive infections

The reference strain used was UTI89 att_{λ}::PSSH10-1 (Wright *et al*., 2007), which has a spectinomycin resistance cassette. In the experiments involving the UTI89 *fimB*, the reference and test strains each carried pBAD-*fimX* and were grown in the presence of both chloramphenicol 20 μg ml^{-1} and 0.1% L-arabinose to promote the *in vitro* expression of type 1 pili. This modification was performed to compensate for the poor *in vitro* phase variation and type 1 pili expression of *fimB* null strains. Prior studies have shown that the arabinose promoter has no appreciable activity in the lower urinary tract in the absence of induction (Justice *et al*., 2006). Thus, after inoculation into the murine bladder, FimX expressed from the *ara* promoter was expected to be diluted out due to the absence of arabinose in the bladder thus restoring the wt and *fimB* phenotypes. Mice were inoculated transurethrally with 50 μl of a reference/test strain mixture, amounting to ~1–2 × 10^7 cfu per strain. Bladders were mechanically homogenized in 0.01% Triton X-100 in PBS. Dilutions of homogenates were plated on LB agar, LB kanamycin 50 μg ml^{-1} or LB spectinomycin 50 μg ml^{-1}. Competitive indices were calculated as (cfu_OUT_{test}/cfu_OUT_{ref})/(cfu_IN_{test}/cfu_IN_{ref}).

Visualization of whole mount bladders for IBC formation

Two approaches were used to quantify and/or examine IBC formation. In the first method, infected bladders were stretched and fixed with 3% paraformaldehyde for 45 min at room temperature in the dark. The bladders were permeabilized with PBS containing Triton X-100 0.01% for 15 min. The bladders were rinsed with sterile PBS five times. Bladders were incubated for 5 min at room temperature in a 1:1000 dilution of Topro-3 (Molecular Probes) followed by five PBS washes. The bladders were mounted on glass slides under Prolong antifade/mount (Molecular Probes) and imaged immediately on a Zeiss LSM510 laser scanning confocal microcope. Alternatively, IBC numbers were enumerated by fixation and β-galactosidase staining of whole mount bladders as previously described (Justice *et al*., 2006).

Statistical analysis

Statistical analyses using the non-parametric, Mann–Whitney *U*-test, Fisher's exact test or Student's *t*-test were performed using GraphPad Prism (GraphPad Software) where indicated in the main text. The binomial test was used to compare codon usage between genes using *ad hoc* perl scripts. Statistical significance was defined by attaining *P*-values 0.05.

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Supplementary Material

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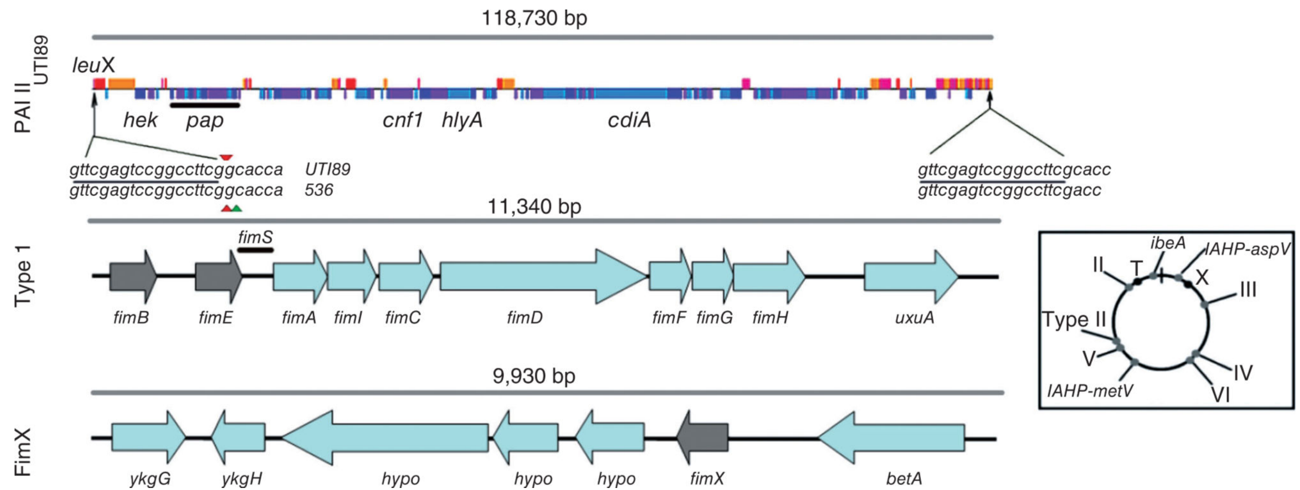

Fig. 1.

Overview of the genomic organization of PAI II_{UTI89}, the *fim* operon (‘Type 1’) and the *fimX* locus (‘FimX’). Open reading frames shown in the PAI II_{UTI89} diagram are depicted either above or below the line, representing forward or reverse strand orientation of the genes, respectively. The direct repeat locations (arrows) are shown flanking the PAI II_{UTI89} region, and the repeat sequences are shown below (underlined text). The small triangles flanking the direct repeat sequence text indicate the nucleotides deleted in the *leuX* gene of either UTI89 or 536 after spontaneous excision of each respective PAI II by recombination of the direct repeats. Recombinase genes in Type 1 and FimX loci are indicated in grey and ‘hypo’ indicates that the gene was annotated as a hypothetical protein. Inset: The relative chromosomal positions of these loci (‘II’, PAI II_{UTI89}; ‘T’, *fim* operon; ‘X’, *fimX* locus) and other UTI89 PAI are shown on the circular map at the lower right. ‘*ibeA*’ indicates PAI containing the invasion of brain endothelium virulence gene (Johnson *et al*., 2007). ‘Type II’ indicates type II secretion system island (Chen *et al*., 2006). PAI containing IcmF-associated homologous proteins are denoted *IAHP* (Das and Chaudhuri, 2003) followed by the tRNA insertion site. Roman numerals indicate similarity to PAI numbered by convention in pyelonephritis strain 536 (Dobrindt *et al*., 2002; Brzuszkiewicz *et al*., 2006).

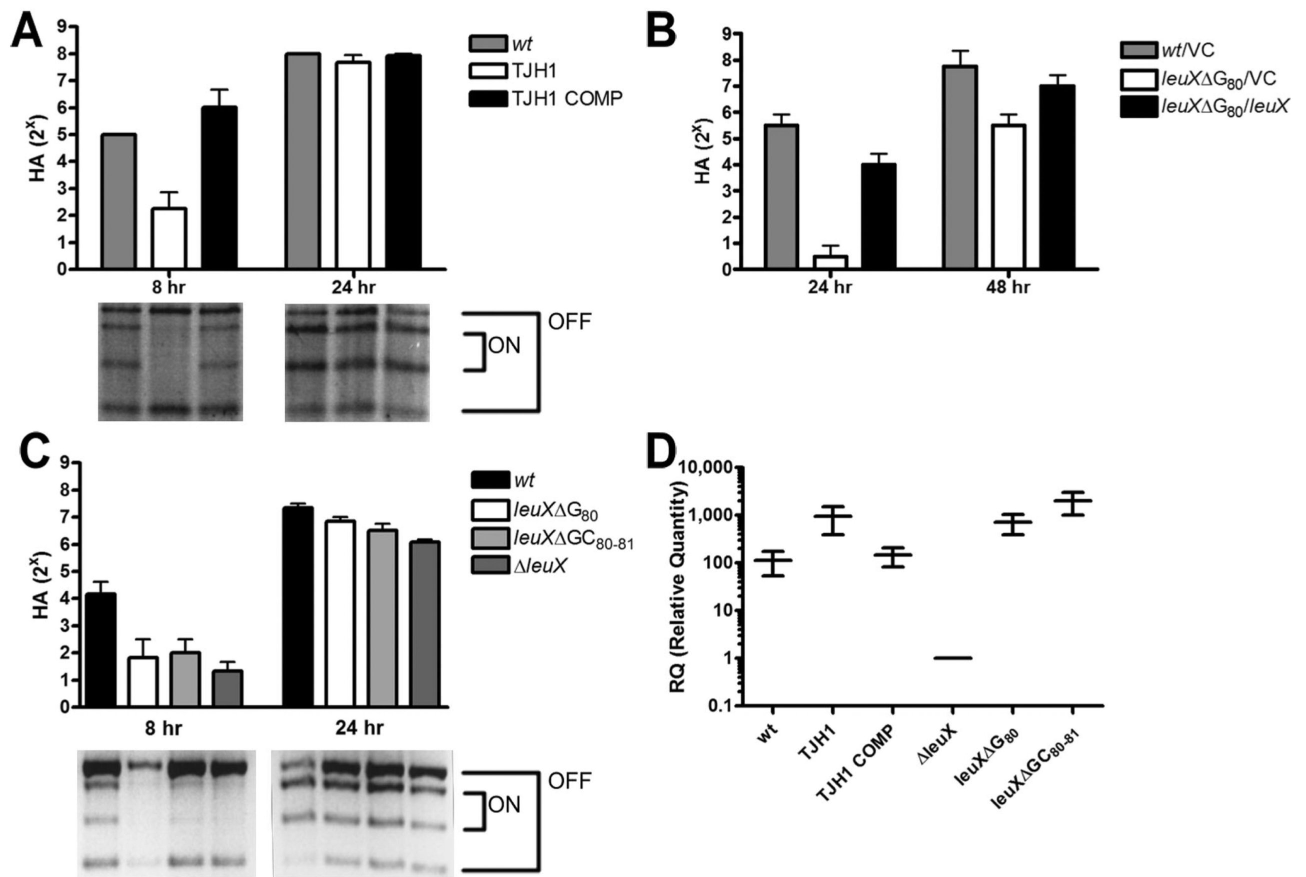

Fig. 2.

Mutation of *leuX* due to spontaneous excision of PAI II_{UTI89} delays type 1 pili expression due to a complete loss of $tRNA_5^{Leu}$ function.

A–C. Type 1 pili expression and *fimS* phase switch orientation in static broth cultures of wt UTI89 (wt) and derivatives grown at 37°C.

A. Mannose-sensitive haemagglutination (MSHA) titres (8 and 24 h) and representative phase assays of wt, TJH1 (*leuX*$^-$, *PAI II*$^-$) and TJH1 COMP (*leuX*$^+$, *PAI II*$^-$).

B. MSHA titres (24 and 48 h) of wt and UTI89 *leuX* *G*$_{80}$, carrying either pBAD33 (VC) or pBAD-*leuX* (*leuX*) *in trans*.

C. MSHA titres (8 and 24 h) and representative phase assays of wt or isogenic *leuX* mutants. All trials in (A), (B) and (C) were performed in duplicate in three independent experiments. Error bars represent standard deviations.

D. Relative quantity (RQ) of *leuX* tRNA in wt or isogenic *leuX* mutants compared with UTI89 *leuX* after 24 h of static growth in broth culture at 37°C. Horizontal bars represent mean values and vertical bars show RQ minimum and maximum range. qRT-PCR was performed on cDNA derived from each strain grown in three independent cultures in two independent experiments. qRT-PCR reactions were performed in duplicate.

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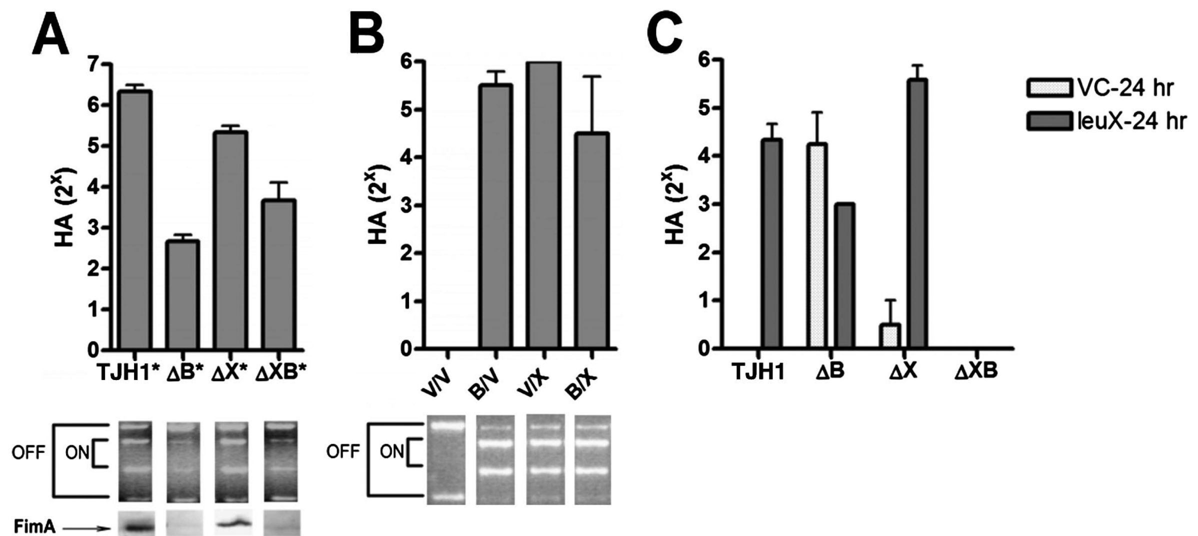

Fig. 3.

Roles of FimB and FimX recombinases in type 1 pili phase switching *in vitro* in TJH1.

A–C. Type 1 pili expression and *fimS* phase switch orientation in static broth cultures of TJH1 (*leuX*$^-$, *PAI II*$^-$) and derivatives grown at 37°C.

A. MSHA titres (48 h) of TJH1 and derivatives B (*fimB*$^-$), X (*fim*X$^-$) or XB (*fimX*$^-$*fimB*$^-$). The asterisk (*) indicates each strain carried pTrc99a as a vector control. Representative phase assays and FimA immunoblot results are shown for each sample.

B. MSHA titres (24 h) of TJH1 XB (*leuX*$^-$, *PAI* $II_{UT189}$$^-$, *fimX*$^-$, *fimB*$^-$) carrying two of the following plasmids *in trans*: (i) control vectors (V), (ii) inducible *fimB* (B) and/or (iii) inducible *fimX* vector (X). Representative phase assays are shown for each sample.

C. MSHA titres (24 h) of TJH1 and derivatives with either pBAD33 (VC) or *leuX in trans*.

All trials in (A), (B) and (C) were performed in duplicate in three independent experiments. Error bars represent standard deviations.

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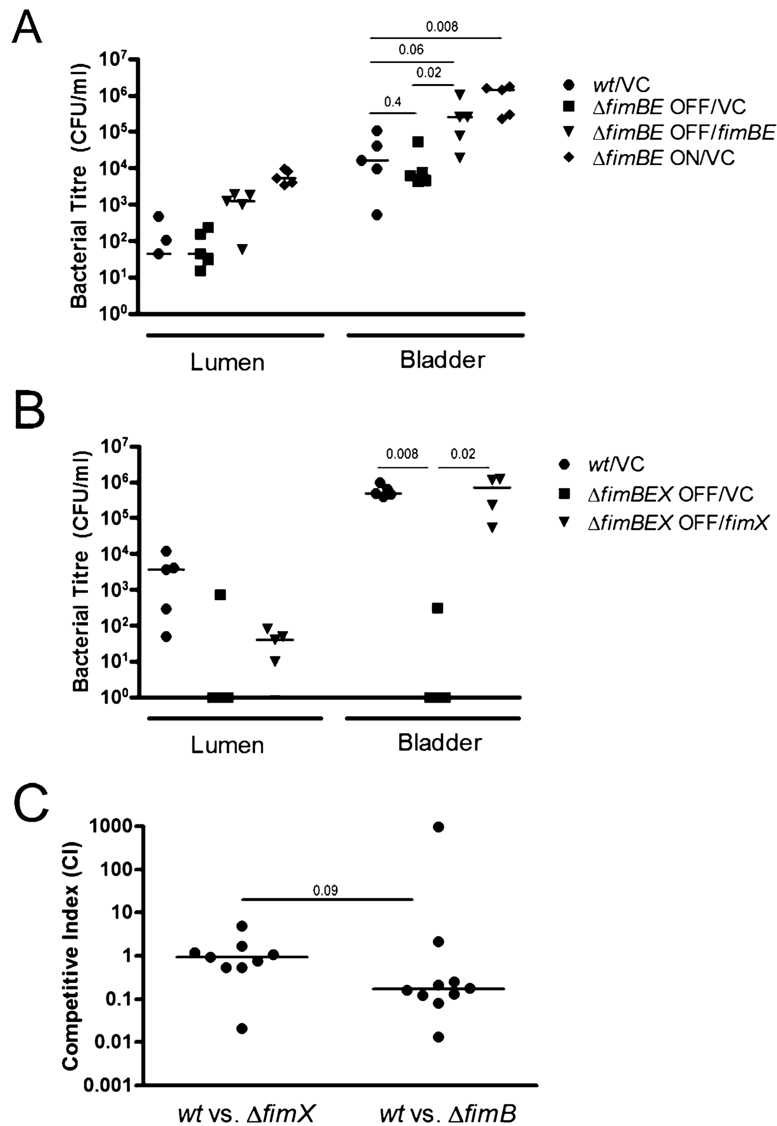

Fig. 4.

FimX-dependent phase variation of type 1 pili *in vivo*.

A and B. *Ex vivo* gentamicin protection assays were performed to distinguish extracellular and intracellular bacteria in infected C3H/HeN female mice at 6 hpi (as per *Experimental procedures*). Luminal contents (Lumen) are obtained from PBS washes of the removed bladder and represent relative luminal colonization. Bladder shows the cfu per ml of intracellular bacteria in the gentamicin-treated bladder homogenate.

A. Mice were transurethrally infected with 10^7 cfu of UTI89 with control plasmid (wt/VC), a UTI89*fimB-fimE–* strain phase OFF *in vitro* with either the control plasmid (*fimBE* OFF/VC) or a plasmid carrying the *fimB* and *fimE* genes (*fimBE* OFF/*fimBE*), or a *fimBE* strain phase ON *in vitro* carrying the control plasmid (*fimBE* ON/VC).

B. Mice were transurethrally infected with 10^7 cfu of wt/VC or UTI89*fimB-fimE-fimX–* strains phase OFF *in vitro* carrying either control (*fimBEX* OFF/VC) or *fimX* (δ*fimBEX* OFF/fimX) vectors *in trans*.

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C. C3H/HeN female mice were transurethrally infected with an equal ratio of UTI89 att$_{\lambda}$::PSSH10-1 (wt, Spec^{R}) and either *fimB* or *fimX* null bacteria (Kan^{R}) for competitive infections. In the wt versus *fimB* null competitive experiment, each strain carried a plasmid with *fimX* under the *ara* promoter and grown in the presence of L-arabinose to induce phase OFF to ON changes, normally requiring native *fimB in vitro*. Colony-forming units (cfu) and competitive indices were scored for each strain as indicated in *Experimental procedures*.

A–C. Bars represent the median value for each group and *P*-values were calculated using the Mann–Whitney *U*-test.

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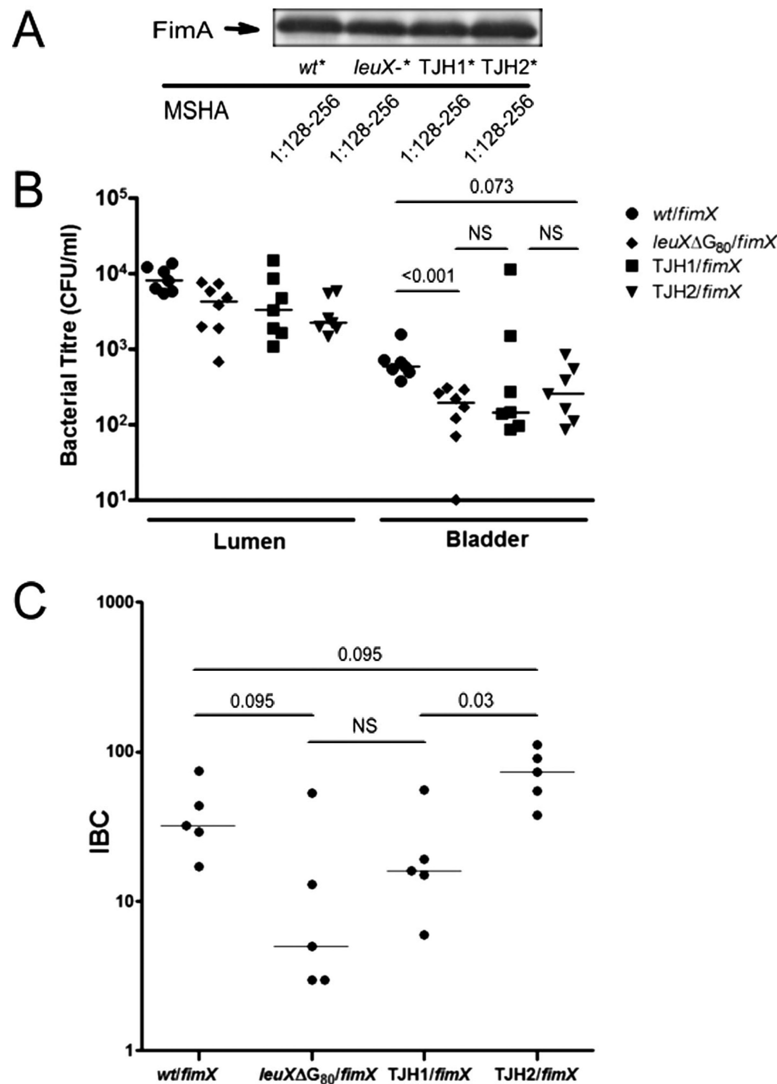

Fig. 5.

Wild-type *leuX*, not PAI II_{UTI89} gene content, is necessary for full UPEC virulence during early acute events in experimental cystitis.

A–C. The individual roles of the *leuX*-encoded $tRNA_5^{Leu}$ and PAI II content in early events in acute cystitis were tested in an experimental cystitis model comparing the isogenic mutants UTI89 *leuX* G_{80} ('*leuX*–', *leuX*$^-$*, PAI* II_{UTI89}^+), TJH1 (*leuX*$^-$*, PAI* II_{UTI89}^-) and TJH2 (*leuX*$^+$*, PAI* II_{UTI89}^-) with wt UTI89.

A. Immunoblot detecting FimA and MSHA in the same inocula of UTI89 (wt) and derivatives used in the experiments displayed in (B) and (C), which were performed on the same day. The asterisks (*) indicate strains carried pBAD-*fimX, in trans*, and were grown statically in 0.1% arabinose in LB/Cm20 for 18 h, thereby overexpressing *fimX* and driving type 1 piliation prior to inoculation into mice.

B. *In vivo* invasion of UTI89 (wt) and derivatives overexpressing *fimX*, measured by *ex vivo* gentamicin protection at 1 hpi. Luminal contents (Lumen) demonstrate the relative presence of each strain in the bladder lumen. Bladder shows the cfu present after gentamicin

treatment of each bladder. These data are from a single experiment representative of three independent experiments, except for UTI89 *leuX* G_{80} ($n = 2$).

C. IBC formation by strains UTI89 and derivatives, all overexpressing *fimX*, at 6 hpi measured by β-galactosidase staining. These data are from a single experiment representative of two independent experiments, except for UTI89 *leuX* G_{80} ($n = 1$).

B and C: Bars represent the median value for each group and *P*-values were calculated using the Mann–Whitney *U*-test.