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DamX Controls Reversible Cell Morphology Switching in Uropathogenic Escherichia coli

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ABSTRACT The ability to change cell morphology is an advantageous characteristic adopted by multiple pathogenic bacteria in order to evade host immune detection and assault during infection. Uropathogenic Escherichia coli (UPEC) exhibits such cellular dynamics and has been shown to transition through a series of distinct morphological phenotypes during a urinary tract infection. Here, we report the first systematic spatio-temporal gene expression analysis of the UPEC transition through these phenotypes by using a flow chamber-based in vitro infection model that simulates conditions in the bladder. This analysis revealed a novel association between the cell division gene damX and reversible UPEC filamentation. We demonstrate a lack of reversible bacterial filamentation in a damX deletion mutant in vitro and absence of a filamentous response by this mutant in a murine model of cystitis. While deletion of damX abrogated UPEC filamentation and secondary surface colonization in tissue culture and in mouse infections, transient overexpression of damX resulted in reversible UPEC filamentation. In this study, we identify a hitherto-unknown damX-mediated mechanism underlying UPEC morphotypical switching. Murine infection studies showed that DamX is essential for establishment of a robust urinary tract infection, thus emphasizing its role as a mediator of virulence. Our study demonstrates the value of an in vitro methodology, in which uroepithelium infection is closely simulated, when undertaking targeted investigations that are challenging to perform in animal infection models.

IMPORTANCE Urinary tract infections (UTIs) are most often caused by uropathogenic Escherichia coli (UPEC) and account for a considerable health care burden. UPEC exhibits a dynamic lifestyle in the course of infection, in which the bacterium transiently adopts alternative morphologies ranging from rod shaped to coccoid and filamentous, rendering it better at immune evasion and host epithelium adhesion. This penchant for morphtype switching might in large measure account for UPEC’s success as a pathogen. In aiming to uncover genes underlying the phenomenon of UPEC morphotypical switching, this study identifies damX, a cell division gene, as a mediator of reversible filamentation during UTI. DamX-mediated filamentation represents an additional pathway for bacterial cell shape control, an alternative to SulA-mediated FtsZ sequestration during UTI, and hence represents a potential target for combating UTI.

The success of a bacterial pathogen depends on its ability to sense its environment and adapt suitably to ensure the best odds of survival. One such adaptation that bestows a selective advantage on the pathogen is its potential for morphological plasticity (1–3). Bacteria have been known to adopt a filamentous morphology when subjected to stresses such as starvation (4), pH change (5), low water activity (6), and exposure to antimicrobial agents (7).

Uropathogenic Escherichia coli (UPEC) has evolved a characteristic lifestyle within the host that enables efficient immune evasion and renders it a successful pathogen. UPEC is the most common cause of urinary tract infections (UTIs) (8), and these bacteria have been observed to undergo morphological differentiation during infection in mice (9) and humans (10). In the course of an acute UTI, the normally rod-shaped UPEC adheres to and invades the superficial cells of the bladder urothelium. Invading UPEC replicates within intracellular bacterial communities (IBCs), consisting mainly of coccoid bacteria (11). Eventually, the intracellular bacterial burden leads to host cell death, at which point UPEC bacteria leave the host cell in the form of motile rods as well as highly filamentous bacteria. The UPEC filaments released into the extracellular milieu possess the ability to revert to the original rod-shaped morphology with the potential to reinitiate a new cycle of infection. Although knowledge of the UPEC infection cycle is derived mainly from murine models of cystitis, IBCs and UPEC filaments have been detected in urine samples from humans with acute UTI (10). The ability to revert to normal rod-shaped morphology is as critical as the ability to form filaments, not just in the case of bacteria like UPEC that adopt filamentation as a virulence strategy but also in cases where filament-
tation is a result of sublethal stress (1). Studies have shown that filamentation of pathogenic bacteria is likely either part of a strategy that helps the pathogen evade the host innate immune response (9, 12) or a stress coping mechanism within a hostile host (13).

The phenomenon of bacterial filamentation is, in essence, a consequence of stalled cell division in the bacterial cell. Cell division, a complex process involving ~30 essential and nonessential genes (14), comprises a sequence of events that commences with the assembly of a membrane-tethered cytoplasmic proto-ring (15). The proto-ring comprises FtsZ, FtsA, and ZipA, which represent the earliest recruits to the septal plane (16), followed by other proteins that build on the proto-ring after a considerable time lag (17). The final step in cell division involves cell wall peptidoglycan remodeling, i.e., degradation of preexisting peptidoglycan and synthesis of septal peptidoglycan (18), which ultimately results in cell wall invagination and the formation of two daughter cells.

While some studies have aimed at identifying genes involved in bacterial filamentation (13, 19–21), this study is the first to systematically analyze the UPEC gene expression profiles at each individual phase of its pathogenesis, with the aim of identifying genes that are critical to its morphological differentiation. Using microarray technology and a previously described (22) flow chamber-based bladder cell infection model, this study not only reaffirms recent reports regarding the importance of a contact surface and fluid flow in UPEC filamentation (23) but also identifies DamX as a key factor that controls reversible switching between normal rod-shaped and filamentous morphology during infection. While previous studies have linked UPEC filamentation with the SOS response and SulA (9, 12, 20), our present study identifies transient DamX overproduction as a key inducer of filamentation during this cycle. Based on the currently available data, we propose that both mechanisms act in concert to create the extensive filamentation observed in vivo but that they are triggered by different mechanisms: the DamX filamentation triggered as a response to the shift from intracellular growth to urine- and liquid shear-exposed surface growth and the SOS filamentation triggered by immune cell attack (12).

RESULTS

Expression analysis revealed differential UPEC transcription profiles during in vitro infection. The infection cycle of the UPEC strain UTI89, originally isolated from a case of cystitis (24), has been well characterized since early reports more than a decade ago of UPEC intracellular behavior during infection (25, 26). Characterization of the genes responsible for progression of the UPEC infection cycle has been limited by the obvious challenges of low bacterial yields from in vitro infection. While previous studies have linked UPEC filamentation with the SOS response and SulA (9, 12, 20), our present study identifies transient DamX overproduction as a key inducer of filamentation during this cycle. Based on the currently available data, we propose that both mechanisms act in concert to create the extensive filamentation observed in vivo but that they are triggered by different mechanisms: the DamX filamentation triggered as a response to the shift from intracellular growth to urine- and liquid shear-exposed surface growth and the SOS filamentation triggered by immune cell attack (12).

A custom-designed UTI89-specific array was used to identify both annotated and unannotated transcripts covering the entire UTI89 genome (accession number NC_007946.1) as well as that of pUTI89 (accession number NC_007941.1). Bacterial cells harvested from the flow chamber apparatus representing the filamentous and reversal phases were isolated from three independent flow chamber experiments and used for hybridization as biological triplicates on the UTI89 array. In order to ascertain the fraction of the sample that was truly filamentous, UTI89 was harvested after growth under two conditions—a reference condition that resulted in rod-shaped UTI89 cells grown in LB culture and a filamentous condition from within the flow chambers during the filamentous phase of infection—and analyzed using flow cytometry. Gating was performed on the culture-grown, normal rod-shaped bacteria, resulting in the populations “P1” and “not P1,” such that rod-shaped bacteria were represented in the former and filaments were represented in the latter populations. Examination of the two populations under the two conditions revealed that ~80% of the flow chamber sample was filamentous, with an FSC-A median (forward scatter area, a measure of cell length) of 2,385, compared to an FSC-A median of ~30 in the nonfilamentous rod-shaped P1 population (Fig. 1a). Since the filamentous phase also contains a small fraction of rod-shaped bacteria, it is possible that expression levels in this phase are underestimated.

Due to the relatively low yield of bacterial mRNA from the intracellular phase, samples were pooled from four parallel flow chambers in three independent experiments to yield biological triplicates. In order to obtain reference conditions that were unbiased toward a particular growth environment, bacteria were cultured to stationary phase in nutrient-rich Luria-Bertani (LB) broth representing the growth medium at the time of infection, as well as sterile human urine with a urine specific gravity (USG; the density) of 1.025, representing the flow medium during in vitro infection. The choices of growth phase and culture medium for the reference condition were admitted challenging, since the UPEC growth phase and nutrient sources are likely subject to frequent changes during intra- and extracellular growth in the flow chamber model.

Gene transcripts from intracellular, filamentous, and reversal phases were filtered on the basis of the P value and log2 fold change in expression, individually, compared to the reference conditions, urine and LB broth. Among the transcripts that crossed the fold change and P value thresholds, those that demonstrated disparate expression trends (up- or downregulated) compared to LB and urine cultures were excluded from further analysis. These transcripts would more likely represent a response to the particular growth environment (LB/urine). Only gene transcripts from each of the three phases that showed consistent trends of differential expression, irrespective of the reference used (LB/urine), were considered further.

A total of 351 transcripts in intracellular phase, 845 in filamentous phase, and 447 in reversal phase were found to display a statistically significant difference in t test results, i.e., P values of ≤0.05 and fold changes of ≥2 log2 compared to the urine cultured reference condition. Among the significant differentially expressed transcripts, 27% of those in the intracellular phase, 33% in the reversal phase, and 75% in the filamentous phase were found to be uniquely differentially expressed during each particular phase of infection. Examination of the significant transcripts revealed that the expression profiles during the intracellular and
Deletion of damX resulted in abrogation of the filamentation phenotype in vitro. The observation that cell division was among the ontologies differentially expressed in the filamentous phase of UTI89 (see Fig. S1c in the supplemental material) was not unexpected, since bacterial filamentation essentially results from an absence of cytokinesis. The array data revealed a cluster of genes upregulated in filaments that included several cell division genes involved in septal ring formation, like zapB (5.2-fold log2 change), ftsA (3.2-fold log2), and cedA (8.4-fold log2), all of which were correspondingly 2- to 6-fold log2 downregulated in the preceding intracellular and subsequent reversal phases (see Fig. S1b). The observation that genes encoding proto-ring components ftsZ, ftsA, and zipA that were upregulated in filaments indicated that a halt in cell division probably occurred downstream of proto-ring assembly. In addition to some essential and nonessential division genes, two genes belonging to the SPOR domain class of proteins (damX and rlpA) were found to be significantly upregulated in filaments (6-fold log2 and 4.2-fold log2, respectively). Conversely, genes encoding other known SPOR domain proteins, FtsN and DedD, were not significantly upregulated during filamentation. Real-time PCR was performed to validate expression levels of several of the cell division genes; data are presented as expression levels in UTI89 filaments relative to expression in a stationary UTI89 urine culture (Fig. 1b).

Based on our findings and previous studies linking cell division genes to a filamentous morphology, sulA (29), cedA (30), and damX (31) were chosen for further exploration in the context of UPEC filamentation. Individual deletion strains for the genes were constructed in UTI89 in order to evaluate their influence on filamentation in the flow chamber infection model. Strains UTI89ΔcedA, UTI89ΔzapB, UTI89ΔsulA, UTI89ΔdamX, and UTI89ΔslmA were all found to demonstrate growth rates comparable to UTI89 wild type when cultured in LB broth and urine (see Fig. S2a in the supplemental material), confirming viability of the respective deletion strains. All five deletion strains were subsequently tested along with the UTI89 wild type for their ability to form filaments and secondary surface colonization, i.e., phenotypes originating from an intracellular reservoir post-gentamicin treatment, in the flow chamber infection model (Fig. 2a). We observed that while UTI89ΔcedA, UTI89ΔzapB, UTI89ΔsulA, and UTI89ΔslmA mutant strains behaved similarly to wild-type UTI89, demonstrating both secondary surface colonization and concurrent filament formation, the UTI89ΔdamX strain was unable to produce both phenotypes.

In order to investigate whether the lack of filamentation was merely due to an inability of strain UTI89ΔdamX to adhere to and invade the bladder epithelial cell line, UTI89ΔdamX mutant cells were tested in a stationary infection setup performed using the bladder epithelial cell line PD07i in multiwell plates. These results showed that the UTI89ΔdamX mutant efficiency equaled that of the wild type in early bladder cell adhesion and invasion in short-term in vitro infection (see Fig. S2b and c in the supplemental material).
material). Additionally, to assess possible intracellular growth defects in the UTI89ΔdamX mutant, we performed a plasmid retention assay using a temperature-sensitive low-copy-number plasmid, pKD46 (32). The percentage of plasmid-retaining bacterial cells at 24 h postinfection (hpi) was found to be on average around 70% in strain UTI89/pKD46 and 72% in strain UTI89ΔdamX/pKD46, demonstrating that both strains underwent similar numbers of intracellular division events (see Fig. S2d) and indicating that damX deletion does not lead to intracellular growth attenuation. We further plotted a standard curve of the percent retention of the pKD46 plasmid along the growth curve, as a function of time, to estimate the average number of intracellular generations. Both the UTI89 and UTI89ΔdamX strains grew to an average of 4 to 5 generations intracellularly (based on the doubling times depicted in Fig. S2a), as depicted in Fig. S2c.

Recent studies have reported that damX, first identified as urf743.3 by Lyngstadaas et al. (31), encodes a SPOR domain-containing protein that is recruited to the septum during cell division (33, 34). SPOR domains are found in many bacterial proteins and are involved in binding to septal peptidoglycan (35–37). Overproduction of DamX has been reported to result in a failure in cell division and a filamentous morphology, while deletion of the damX gene has been associated with bile sensitivity (38) without an discernible impact on cell morphology (34). damX is part of a functionally diverse operon comprised of aroBK, damX, dam, rpe, gph, and trpS. Some previous studies suggested that polarity effects of damX deletion on the downstream dam gene, which encodes the Dam DNA methylase, could account for the phenotypes associated with damX deletion, due to a likely α70 damp1 promoter within the damX open reading frame (39–41). Analysis of dam, aroBK, and damX expression levels in the UTI89ΔdamX mutant relative to the UTI89 wild type revealed no significant effect of damX deletion on dam expression (see Fig. S3a in the supplemental material). In order to confirm with certainty the absence of any polarity issues, we constructed and tested a more subtle deletion mutant, UTI89ΔdamX*, in which the damp1 promoter was left intact. The UTI89ΔdamX* deletion strain replicated the filamentation defect displayed by strain UTI89ΔdamX during the flow chamber-based infection experiments (see Fig. S3b). This finding indicated that the lack of filamentation observed in strain UTI89ΔdamX was more likely attributable directly to the deletion of damX rather than secondary dam-mediated effects.

The UTI89ΔdamX mutant is unable to form filaments in an in vivo mouse model. In order to validate our findings from the in vitro flow chamber model and assess the role of DamX as a mediator of UPEC filamentation within a complex in vivo environment, a mouse cystitis model based on the strain C3H/HeN was chosen for further investigation. C3H/HeN mice are known to be fully immunocompetent, with a wild-type trf-4 gene and a functional PMN (polymorphonuclear leukocyte) response. This mouse strain has been reported to support a robust UPEC pathogenesis cycle in several previous studies (25).

Female C3H/HeN mice were separately inoculated via transurethral catheterization with strain UTI89/pMAN01 or UTI89ΔdamX/pMAN01 subcultured over two rounds of static growth at 37°C in LB medium. The strains were transformed with the pMAN01 plasmid to ensure constitutive expression of gfp, enabling easy microscopic examination of the bacteria. The mouse bladders at 12 and 18 hpi as well as urine collected immediately prior to tissue harvest at 12 h were subjected to microscopic examination for bacterial filaments, exfoliated cells, and IBCs. Alongside this, CFU counts were estimated in bladder homogenates at 18 h, 48 h, and 14 days postinfection. At the 12-h time point, several IBCs were observed in bacteria infected with strain UTI89ΔdamX, although they appeared to be fewer in number than in wild-type-infected bladders (Fig. 3a and b). Inspections of urine specimens at this time point revealed relatively high numbers of exfoliated cells containing IBCs also from the mutant strain infections (Fig. 3h), showing that IBCs are formed by strain UTI89ΔdamX, but they are cleared more rapidly than the wild-type strain.

At 18 hpi, IBCs were largely cleared from bladders infected with strain UTI89ΔdamX, whereas IBCs were still present in wild-type-infected bladders (Fig. 3c and e). The wild-type strain, UTI89wt, exhibited abundant filamentation (Fig. 3d), whereas no filaments were detected in the bladders infected with strain UTI89ΔdamX. Mutant UTI89ΔdamX cells were, however, few and far apart on the bladder lumen wall, making it difficult, based on these inspections alone, to conclude on a possible filamentation defect. By pooling urine samples from strain UTI89ΔdamX-infected mice, much higher numbers (thousands) of bacteria could be analyzed. Strain UTI89ΔdamX in these samples were all found to be normal rod shaped or only slightly elongated (Fig. 3i), which was in stark contrast to the highly filamentous response present in the urine from UTI89wt strain infections (Fig. 3g).

Regarding overall bacterial numbers in the bladders, CFU counts were approximately 2.5 orders of magnitude lower for strain UTI89ΔdamX/pMAN01 than for the UTI89/pMAN01 strain at 18 hpi (Fig. 4), thus indicating a significant defect in

![FIG 2](https://example.com/fig2.png) Strain UTI89ΔdamX was unable to demonstrate secondary infection or filamentation on urine exposure. Flow chamber-based infection of the PD07i bladder cell line was carried out with strains UTI89wt, UTI89ΔslmA, UTI89ΔrapB, UTI89ΔcedA, UTI89ΔcedL, and UTI89ΔdamX. Panels in the top row represent secondary infection and biofilm formation (cloudy appearance) on exposure to concentrated urine, and the bottom row depicts bacteria flushed out of the flow chambers after 22 h of concentrated urine exposure. Scale bars, 20 μm.
progression of infection beyond the initial intracellular infection cycle.

CFU counts measured at 48 h and 14 days postinfection revealed a severely diminished capacity for persistence of strain UTI89/\textit{H9004 damX}/pMAN01 during bladder infection. The CFU detection limit was set at 50 bacteria per bladder, implying that a count of $<50$ bacteria was equivalent to complete clearance of infection. A deletion of \textit{damX} resulted in clearance of the infection at 48 hpi, whereas the UTI89/pMAN01 strain presented high CFU counts in the mouse bladders even at 14 days postinfection (Fig. 4).

Previously, Justice et al. (9) proposed a \textit{sulA}-driven SOS-mediated response as a mechanism underlying UPEC filamentation during the uropathogenic cycle. While our \textit{in vitro} infections failed to show a correlation between \textit{sulA} and UPEC filamentation (Fig. 2), this could have been due to the absence of immune cell attack in this model, which has been hypothesized to underlie the SOS-triggered filamentation (9). To be able to better investigate an \textit{in vivo} \textit{sulA}-filamentation association that perhaps could not be observed in the \textit{in vitro} flow chamber model, we performed infections of the C3H/HeN mouse strain with strain UTI89/\textit{H9004 sulA}/pMAN01. A visual examination of splayed bladders 18 hpi revealed a scenario akin to UTI89/\textit{H9004 damX} strain infection, with very few or no IBCs and no detectable filaments (see Fig. S4a in the supplemental material). Notwithstanding the overall poor bacterial load in these urine samples, our examination of voided urine collected prior to bladder harvest revealed several long filaments along with short rods (see Fig. S4b and c).

\textbf{Variation in \textit{damX} expression levels resulted in reversible filamentation.} In order to prove that transient DamX overproduction is key to reversible UTI89 filamentation, we cloned the UTI89\textit{damX} gene with a strong upstream Shine-Dalgarno sequence in pBAD33, to form pSKdamX, thereby allowing for arabinose-induced expression and subsequent tight repression by glucose addition. Previously, Lyngstadaas et al. (31) reported that overproduction of DamX was detrimental to cell growth. Initial attempts to induce \textit{damX} overexpression during growth in liquid medium proved more challenging than anticipated and did not yield filaments. Unlike the earlier flow chamber experiments that simulated bladder infection, examination of the effect of \textit{damX} overexpression from pSKdamX was performed by seeding UPEC on an abiotic glass surface, for ease of handling. Strain UTI89/pSKdamX was therefore cultured overnight on LB agar plates supplemented with 0.4% glucose to ensure complete repression of
damX expression from pSKdamX and seeded onto sterile glass slides inserted into the flow chamber. After 20 min of seeding, the chambers were exposed to a flow of EZ medium supplemented with 0.2% arabinose. Microscopy images captured at 10-min intervals clearly demonstrated the effect of damX overexpression on UTI89 cell morphology (Fig. 5a to d). Since the morphological plasticity observed during infection entails not only the ability to form filaments but also the ability for those filaments to revert to normal rod-shaped bacteria (25), it was important to demonstrate that termination of damX overexpression with the subsequent addition of 0.4% glucose would facilitate UTI89 filament reversal. This was indeed our observation (Fig. 5e and f). Strain UTI89/pSKdamX exposed exclusively to EZ plus 0.4% glucose was observed in parallel in order to clearly demonstrate that UTI89 filamentation was indeed a result of damX overexpression. As seen in Fig. 5g to l, this control experiment yielded a complete absence of filaments. Concurrently, the vector control strain UTI89/pBAD33 was also tested in the presence of 0.2% arabinose and was found not to form filaments (see Videos S1a to c in the supplemental material).

In light of preexisting knowledge that damX overexpression results in E. coli K-12 filamentation and that the pBAD system is admitted an artificial setup, a comparison of DamX levels between native UTI89 filaments flushed out of the flow chamber model and artificial arabinose-induced UTI89/pSKdamX strain filaments would be critical to validation of this experiment. By Western blot analysis, the DamX level in arabinose-induced UTI89/pSKdamX filaments was found to be elevated and comparable to that of native UTI89 filaments obtained from a flow chamber infection experiment (Fig. 6a). This was in contrast to uninduced UTI89/pSKdamX and rod-shaped UTI89 cells resulting from filament reversal, which contained much lower DamX levels.

Both array and real-time PCR data revealed elevated levels offtsZ expression in native UTI89 filaments. Based on prevailing knowledge linking ftsZ overexpression to a filamentous phenotype, we then proceeded to determine FtsZ levels in these samples by Western analysis. We found that FtsZ levels were elevated in the native filaments (Fig. 6a, lane 4) compared to reverted filaments (lane 5), as expected from our transcriptional data. Concurrently, while we observed an increase in FtsZ levels in the arabinose-induced filaments (lane 2) compared to the preinduction rods (lane 1), there was no discernible drop in FtsZ levels in glucose-induced reverted filaments (lane 3). This suggested that UTI89 filaments were not merely due to ftsZ overexpression and, second, that damX overexpression was not directly linked to ftsZ overexpression. Microscopic examination was performed to confirm the morphology of bacteria flushed out of the flow chambers from each of the five samples used for Western blotting (Fig. 6b).

**DISCUSSION**

While the survival advantage conferred by morphological differentiation on UPEC infection has been recognized (9, 12), the precise mechanisms underlying this phenomenon have not been resolved thus far. This study adopted a novel approach that allowed for transcriptional profiling of distinct phases of UPEC infection and thereby identified a relatively unexplored cell division protein, DamX, as critical to reversible UPEC filamentation.

The understanding of UPEC pathogenesis has grown considerably since the earliest reports detailing the various stages of infection observed in murine cystitis models (11, 25). UPEC has been known to demonstrate a complex infection cycle in the bladder, commencing with adhesion and invasion of the superficial bladder epithelial cells (24), within which the bacteria replicate to form dense intracellular communities of coccoid bacteria, classified as IBCs. The model proposes that a subpopulation within these IBCs then undergoes a process of maturation to form filaments that undergo flux from the infected epithelial cell, and subsequently reverts to normal rod-shaped bacteria that hold the potential to reinitiate a fresh cycle of infection. This cyclic transition between intra- and extracellular localization along with a concurrent transition between rod-shaped, coccoid, and filamentous cell morphologies provides a mechanistic framework for explaining the high degree of recurrences observed among infected individuals (42). Observations from this and previous studies (9) suggest that the filamentous phase of UPEC infection must be
transient and reversible in order to ensure progression of UTIs from the primary episode. In this study, we observed that strain UTI89ΔdamX was incapable of forming filaments either in vitro or in vivo. The mutant strain displayed no reduction in bladder cell adhesion or invasion in vitro. Furthermore, the rate of strain UTI89ΔdamX replication was comparable to that of the wild type both when cultured in human urine and when residing intracellularly in vivo. Indeed, we detected exfoliated cells containing late-stage IBCs in mouse urine after infection by strain UTI89ΔdamX (Fig. 3h). Collectively, our in vitro and in vivo infection data point toward a function for DamX in the late stages of a primary infection cycle, i.e., when filament formation may be required for maintenance and spread of the initial colonization.

While in the extracellular space, facing the combined threats of elimination through bladder voiding (micturition) or phagocytosis by polymorphonuclear leukocytes recruited to the bladder lumen surface at the point of infection, the adoption of a filamentous morphology can be advantageous both in terms of tissue adherence and immune evasion. Such morphological plasticity has been known to offer selective advantages, such as host immune evasion by UPEC (9, 12) and Proteus mirabilis (43), antibiotic resistance in Burkholderia pseudomallei (44), and stress resistance in Caulobacter crescentus (45). By employing an in vitro flow chamber infection model (22) with the ability to recapitulate UPEC behavior reported during in vivo murine infection, UPEC could be harvested from the three main phases of infection—intracellular, filamentous, and reversal. Hence, this study further emphasizes the utility of the flow chamber infection model as a tool allowing for systematic analysis of bacterial gene expression in the course of infection.

Bacterial cells elongate to form filaments when there is a failure to complete cell division after segregation of nucleoids has occurred. Our comparison of UTI89 transcriptional profiles during the three infection phases revealed temporal expression of several genes involved in cell division, indicating a change in the expression levels of cell division genes during filamentation compared to intracellular and reversal phases, during which cell division would be expected to occur more or less normally. Previous fluorescence microscopic inspection of UPEC filaments generated in flow chambers revealed segregated nucleoids and partial septa (22), suggesting that the arrest in cell division occurred at a stage subsequent to proto-ring formation by FtsZ, FtsA, and ZipA. We hypothesize that the elevated expression of transcripts of other divisome proteins (like ZapA, ZapB, ZapC, FtsX, FtsK, and FtsL) observed in the array data, as well as the enrichment of the cell division gene ontology (see Fig. S1b and S2 in the supplemental material) could reflect filaments poised for initiation of cell division as soon as the relevant environmental cue is perceived.

Justice et al. (9) previously suggested that SulA could underlie the process of UPEC filamentation in an immunocompetent host with the ability to mount a host immune response and thereby incite a bacterial SOS response. Such evidence for SulA-mediated filamentation has also been reported in other pathogens, like Listeria monocytogenes (46) and Salmonella enterica serovar Typhi- murium (47), under the onslaught of phagocytic immune cells. In agreement with previous flow chamber model experiments (22), we did not observe filamentation defects in strain UTI89ΔsulA, indicating that this gene is not responsible for the filamentation observed during secondary surface colonization in vivo. Among the candidate mutant strains tested using the flow chamber model in this study, strain UTI89ΔdamX was the only one to display an absence of filamentation as well as secondary surface colonization of the bladder epithelial cells. However, these results do not rule out that recruitment and interaction with neutrophils contributes to the induction of UPEC filamentation in vivo. UPEC may therefore adopt more than one strategy to attain a filamentous morphology, triggered by an SOS stress response during an immune attack as well as a parallel DamX-mediated induction triggered by...
the general physicochemical and hydrodynamic growth conditions present in the bladder.

DamX is recruited to the midcell in an FtsZ-dependent manner (33, 34) and interacts directly with septal peptidoglycan via its C-terminal SPOR domain (36, 37). While deletion of damX was found not to affect cell division in this or previous studies (33, 34), overproduction of DamX has been linked to a filamentous phenotype in the past (31, 48). By using a bacterial two-hybrid screen, DamX has been shown to interact strongly with itself, FtsQ, and another SPOR domain-containing cell division protein, FtsN (33). Z ring maturation and cell division have been described as linear, dependent pathways, where perturbations in early proteins influence assembly of later “downstream” proteins (49), and in this context FtsN is believed to be the final recruit that links Z ring maturation and septal peptidoglycan synthesis, as detailed in a recent review by Weiss (50). Based on reported evidence that FtsN and DamX both localize to the septal peptidoglycan by means of their SPOR domains (34), it is tempting to speculate that DamX at elevated levels could competitively inhibit FtsN self-accumulation and block molecular interactions between essential cell division proteins and/or septal peptidoglycan synthesis. However, findings by Busiek and Margolin (51) demonstrated that FtsN was capable of interacting with FtsA (an essential proto-ring component) in a SPOR-independent manner, and since the FtsN and DamX SPOR domains themselves show <20% identity conservation (35), the mechanism of DamX-mediated filamentation might be more complex than a mere case of competition. An alternative hypothesis is that DamX sequesters FtsQ (33), a Z ring protein (15) known to increase septal peptidoglycan turnover via FtsN-FtsA interactions (52). The precise localization of Z ring components during filament formation and reversal and the role that DamX overproduction plays in this scheme remain subjects for future study that we are currently pursuing.

The dam operon is comprised of seven functionally diverse members, one of which is damX. Despite the positioning of alternative dam promoters within the damX coding region (40), deletion of damX did not have any significant bearing on Dam methylese expression, thereby excluding the possibility that the lack of filamentation observed for strain UTI89 ΔdamX in flow chamber infections was due to a polarity effect on Dam. Studies that have associated deletion of damX with a reduced invasion capacity in S. enterica (53) and induction of the SOS response in E. coli (54) were conducted on damX mutants generated using transposon insertion and therefore likely to have suffered dam polarity effects. The facts that strain UTI89 ΔdamX invaded bladder epithelial cells as efficiently as the wild type but was defective with respect to both secondary surface colonization and filamentation upon exposure to concentrated urine in the flow chamber model suggest a role for DamX in UPEC secondary infection. The exact mechanistic role of DamX-mediated filamentation needs further clarification; however, the defect in secondary surface colonization corresponds with a role of filamentation in urothelial cell adherence under hydrodynamic conditions, as suggested previously (9, 22).

Examination of filamentous strains that were artificially induced from the pSKdamX construct and those native filaments obtained during the course of a flow chamber infection revealed comparable DamX protein levels. DamX levels in filaments (both induced and native) that had reverted to rod shape were also consistent among themselves and significantly lower than their filamentous counterparts. The effective cooption of DamX levels with the corresponding morphological state in each of the UTI89 samples tested further strengthens the postulation of DamX as a mediator of UPEC morphological plasticity. Induction of UPEC filamentation via transient DamX overproduction from plasmid pSKdamX occurred only when seeded on a solid surface, thus implying a role for surface sensing and possibly fluid flow, irrespective of whether that surface is biotic (bladder epithelial cells) or abiotic (glass). This dependency of UPEC filamentation on surface growth has been confirmed in other recent studies of UTI89 grown in flow chambers (22, 23), implying that the process of filamentation does not restrict itself exclusively to the UPEC infection cascade.

We compared the phenotypes resulting from damX overexpression in K-12, for which earlier studies had shown filament formation in liquid culture, to UPEC, for which liquid culture failed to result in filament formation even in concentrated urine. Despite the high degree of sequence conservation between K-12 and UTI89, the prerequisite for a solid surface for filamentation clearly applied only to UTI89 (see Fig. S5 in the supplemental material). The reason for this difference is unknown, but it is tempting to speculate that UPEC-specific surface fimbriae might be implicated in controlling damX induction. Leclerc et al. (53) reported the failure of osmolarity, pH, and oxygen tension as triggers of damX overexpression in their study of S. enterica. Taken together with the surface dependence of stable DamX-mediated filaments observed in our study, upstream activators of damX expression and hence UPEC filamentation could be those involved in surface sensing. However, such a mechanism might seem at odds with the absence of UPEC filaments in bladders derived from infected immunocompromised mice reported by Justice et al. (9). Therefore, we cannot exclude a role of the host immune system in the induction of DamX-mediated filaments.

The Cpx two-component system monitors biogenesis of bacterial surface structures and acts as a sensor of envelope stress, and it has been shown to be important for UPEC virulence (55). Surface structures such as fimbriae are crucial for attachment of UPEC to bladder epithelia (56) as well as abiotic surfaces (57), and UPEC filaments are known to express type 1 fimbriae on their surface, allowing the cells to better adhere to a contact surface in the face of a fluid shear force (22). In this context, it is likely that the Cpx system is activated by the perception of a contact surface by the bacterium (58). Also, in light of studies associating filament formation with decreased antibiotic sensitivity, previous reports of increased antibiotic sensitivity in cpx null mutants (55) might point back to a lack of DamX-mediated filamentation in such mutants.

We report here, for the first time, the involvement of DamX in reversible UPEC filamentation, and we demonstrate a clear association between expression of damX and morphotype switching in UTI89. Our study suggests an alternative explanation for the filamentous response observed in the late and extracellular stages of the IBC cycle. Our data point to DamX as a key inducer of filamentation at this stage of the life cycle, triggered by the drastic changes under growth conditions occurring in the transition from intracellular proliferation to the urine- and liquid shear-exposed surface growth on the bladder lumen. In addition, neutrophil attack most likely contributes to induction of filamentation via an SOS response, triggered by secreted reactive oxygen species (12).

The molecular mechanism of DamX-mediated cell division inhibition is presently unknown and, given the important clinical
implications, further studies aimed at uncovering the DamX morphological switch are warranted.

MATERIALS AND METHODS

Ethics statement. Mice used in this study were handled in accordance with the current guidelines from the National Danish Animal Care Committee. Animal maintenance and experimental protocols were approved by the Ministry of Food, Agriculture and Fisheries, Danish Veterinary and Food Administration (ID 2015-15-0201-00480).

Bacterial strains and growth conditions. UTI89, a human cystitis isolate of the O1K:H7 serotype (24), was used as the model uropathogenic E. coli strain in this deletion. Deletion strains were constructed in UTI89 by the well-established lambda Red recombinase method devised by Datsenko and Wanner (59) using pKD3 and pKD4 plasmids. Strain UTI89ΔdamX was constructed using extended regions of complementarity to damX’ (JM1553 and JM554) and 3’ (JM1555 and JM1556) flanking regions on the UTI89 chromosome. Strain UTI89ΔdamX mutant was constructed using the primers JM1519 and JM991 and confirmed using JM1991 and JM998. All gene deletions were confirmed using gene-specific primers.

Plasmid pSKdamX was derived from a pBAD33 plasmid vector by insertion of a damX insert with the parM Shine-Dalgarno generated by PCR using primers JM1777 and JM600. The vector and insert were digested using XbaI and HindIII fast digest restriction enzymes (Thermo Scientific) and ligated with T4 DNA ligase (Roche), and the sequence was verified. Plasmid pMAN01 was derived from pSC101 plasmid with a constitutively active promoter driving gfp expression. Strains were maintained using 30 µg/ml chloramphenicol (Cml) or 50 µg/ml kanamycin (Kan) as appropriate. All strains and primers are listed in Table S2 in the supplemental material.

UTI89 was cultured at 37°C in Luria-Bertani medium, EZ medium (catalog number m2105; Teknova) without glucose, or in human urine with a USG ranging between 1.025 and 1.030, as suitable. Human urine was filter sterilized, and the USG was measured as detailed previously by Andersen et al. (22). All UTI89 strains were grown in LB medium statically overnight for use in flow chamber and static infection experiments. UTI89 strains used in mouse infections were cultured over two rounds of static growth in LB medium at 37°C as detailed previously (60). Induction and repression of pSKdamX were carried out in EZ medium supplemented with 0.2% arabinose and 0.4% glucose as appropriate.

Cell culture and infection experiments. In vitro infection experiments were performed on PD07t, an immortalized human bladder epithelial cell line kindly provided by David Klumpp (61). The cell line was maintained in EpiLife growth medium (Invitrogen) supplemented with human keratinocyte growth serum (HKGS) and penicillin-streptomycin (Pen/Strep) antibiotics during routine culturing at 5% CO2 and 37°C. EpiLife medium without Pen/Strep was utilized during infection. Static as well as flow chamber-based infection experiments were carried out as explained in detail previously (22). Briefly, urothelial monolayers cultured for 24 h in tissue culture medium were infected for 20 min by detaching the pump and elevating the medium flask to increase hydrostatic pressure, the log2 fold change was calculated (cutoff, ≥2 log fold change), and filtering was based on Student’s t test (significance cutoff, P < 0.05). The statistically significant, differentially expressed transcripts were further examined based on gene ontology information obtained from EcoCyc and literature searches.

Real-time PCR. Primers for real-time PCR, listed in Table S2c in the supplemental material, were designed using Primer3 software. Total RNA was isolated from each infection phase following the same protocol as for
microarray sample preparation. Total RNA from UTI89 wild type and strain UTI89 DamX (see Section S3A in the supplemental material) was harvested from exponentially growing bacteria in culture. One microgram of total RNA was DNase treated for 30 min at 37°C and heat inactivated, then used for cDNA synthesis using a Maxima first-strand cDNA synthesis kit (Thermo Scientific). Real-time quantification was performed using SYBR Select master mix (Life Technologies) as per the recommended protocol. Levels of rrsA were used as an internal control, and real-time PCRs were run in 96-well plates in an Mx3005P QPCR system (Agilent Technologies).

Flow cytometry. Flow cytometry was performed on a BD FACSaria II flow cytometer (Becton, Dickinson and Company). UTI89 cells grown to exponential phase as normal rod-shaped bacteria and filamentous UTI89 obtained from an in vitro flow chamber infection were fixed in 3.7% paraformaldehyde prior to flow cytometry. Data from 10^6 events per sample were collected and analyzed using BD FACS Diva software (Becton, Dickinson and Company). UTI89 from the two conditions was distinguished on the basis of FSC-A and SSC-A on the x and y axes, respectively. Gating was performed using normal rod-shaped bacteria, such that all bacterial events occurring outside gate P1 were designated “not P1” and corresponded to cells longer than normal rod-shaped bacteria.

Microscopy. Each individual phase of UTI89 infection was confirmed by microscopic inspection. All phase-contrast microscopy using UTI89 deletion strains during flow chamber infection was performed using a Leica DMRE fluorescence microscope. Time-lapse imaging of filament induction as well as mouse bladder and urine imaging were performed with the Olympus cellSens Dimension platform v. 1.7 where, using a 40× objective, composite images obtained by collapsing 26 Z-stack slices with a step size of 0.5 μm were captured at 10-min intervals. Contrast adjustment and scale bars were added using ImageJ software.

Western blotting. UTI89 filaments and strain UTI89 pSKdamX samples were obtained by flushing bacteria out of the flow chamber system and snap-freezing. The bacterial cell pellets were resuspended in 1× SDS loading buffer (60 mm Tris-HCl [pH 6.8], 2% SDS, 10% glycerol, 0.005% bromophenol blue, 5 mm EDTA, 0.1 M dithiothreitol) and boiled for 5 min at 90°C with vigorous agitation. Protein from ~10^6 cells was loaded and separated on 4–12% Invitrogen NuPage Novex bis-Tris gels as detailed by Boysen et al. (62). The proteins were transferred onto polyvinylidene difluoride membranes (Amersham Hybond) that were probed with a 1:1,000 dilution of anti-FtsZ (AgriSera), 1:1,000 dilution of anti-DamX (35), or 1:10,000 anti-GroEL antibodies, followed by appropriate secondary antibodies. Band quantification was performed using Image Studio Lite v4 software.

Plasmid stability assay. Strains UTI89/pKD46 and UTI89 DamX/pKD46 were grown overnight with aeration at 30°C in LB plus 100 μg/ml Amp and used to subculture a starter culture in fresh LB plus 100 μg/ml Amp at 30°C to an OD600 of 0.3. The starter culture was diluted to an OD600 of 0.03 in LB at 37°C and sampled every 30 min for CFU plating on LB agar (LA) and LA plus 100 μg/ml Amp plates. Cultures at 30°C in LB plus 100 μg/ml Amp were also used in a flow chamber-based infection of PD071 bladder epithelial cells. Flow chamber-based infection was carried out as before, without any Amp selection. A 100-μg/ml concentration of gentamicin was added 9 hpi, and the infection experiment was terminated after 24 hpi. The chambers were subjected to three short 5-min bursts at a higher flow rate (50 μl/min) to wash away extracellular bacteria and subsequently dismantled to obtain the glass slide with infected bladder cells. The infected epithelial cells were trypsinized and lysed with 0.1% Triton. Intracellular bacteria from infected bladder cells were harvested for CFU plating on LA and LA plus 100 μg/ml Amp plates. CFU counts from LA and LA plus 100 μg/ml Amp plates were used to calculate percentages of plasmid retention over time [100 × ([CFU with LA plus Amp]/[CFU with LA])].

Statistical analysis. All infection and real-time reverse transcription-PCR experiments were conducted on biological triplicates, and mean values were plotted along with standard deviations. Student’s t test was used to calculate P values and determine statistical significance in stationary infections by using GraphPad Prism 5.01 software. A Mann-Whitney unpaired test was used to calculate the significance of mouse CFU counts using GraphPad Prism 5.01 software. P values less than 0.05 were defined significant for all experiments.

Microarray data accession number. Array data have been deposited in ArrayExpress (accession number E-MTAB-3611).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.orglookup/suppl/doi:10.1128/mBio.00642-16/-/DCSupplemental.

REFERENCES


