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Urinary tract infection drives genome instability in uropathogenic *Escherichia coli* and necessitates translesion synthesis DNA polymerase IV for virulence

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Uropathogenic *Escherichia coli* (UPEC) produces ~80% of community-acquired UTI, the second most common infection in humans. During UTI, UPEC has a complex life cycle, replicating and persisting in intracellular and extracellular niches. Host and environmental stresses may affect the integrity of the UPEC genome and threaten its viability. We determined how the host inflammatory response during UTI drives UPEC genome instability and evaluated the role of multiple factors of genome replication and repair for their roles in the maintenance of genome integrity and thus virulence during UTI. The urinary tract environment enhanced the mutation frequency of UPEC ~100-fold relative to in vitro levels. Abrogation of inflammation through a host TLR4-signaling defect significantly reduced the mutation frequency, demonstrating in the importance of the host response as a driver of UPEC genome instability. Inflammation induces the bacterial SOS response, leading to the hypothesis that the UPEC SOS-inducible translesion synthesis (TLS) DNA polymerases would be key factors in UPEC genome instability during UTI. However, while the TLS DNA polymerases enhanced in vitro, they did not increase in vivo mutagenesis. Although it is not a source of enhanced mutagenesis in vivo, the TLS DNA polymerase IV was critical for the survival of UPEC during UTI during an active inflammatory assault. Overall, this study provides the first evidence of a TLS DNA polymerase being critical for UPEC survival during urinary tract infection and points to independent mechanisms for genome instability and the maintenance of genome replication of UPEC under host inflammatory stress.

Introduction

Urinary tract infections (UTIs) are among the most common bacterial infections in humans, affecting about 10 million people annually in the US.[1,2] Understanding conserved molecular mechanisms of UTI pathogenesis may help to identify key bacteria pathways necessary for virulence that, in turn, may provide the basis for the development of new therapeutics, crucial to renew the battle against UTI.

Uropathogenic *Escherichia coli* (UPEC) is the leading etiology of UTIs.[1] UPEC adheres to the urinary tract epithelium, and within the bladder, invades into the epithelium through multiple endocytic pathways and the invagination of fusiform vesicles.[3,4] In some circumstances UPEC rapidly replicates in intracellular bacterial communities (IBC), a biofilm-like state observed in animal and human infections.[3,5] UPEC may also chronically persist in the epithelium (16).

In most hosts, a robust innate immune response is initiated upon the earliest stages of UPEC UTI. During cystitis, the interaction between host toll-like receptor 4 (TLR4) of urinary epithelium and lipopolysaccharide (LPS) of UPEC results in a pro-inflammatory cytokine response with IL-6 and IL-8.[6,7] Antimicrobial peptides and complement may contribute to cell wall stress, while oxidative radicals damage cell structures such as the outer membrane, critical enzymes and the genome of UPEC.[8,9]

The oxidative components of the innate immune response are numerous. Nitric oxide (NO·) produced by inducible nitric oxide synthase (iNOS) in response to UTI inhibits bacterial growth by modifying DNA and proteins.[10] Oxidation may directly damage genomic DNA as well as the dNTPs pool, leading to increased lesions and errors during DNA synthesis.[11,12] These alterations may result in stalling of DNA replication, immediate termination of replication and subsequent cell death. Alternatively, repair of these lesions and errors may require low fidelity bypass synthesis, resulting in mutations, which in turn may promote selective changes such as increased virulence or antibiotic resistance.[13,14]

The most important components of mutational level control entail critical DNA synthesis and DNA repair processes are DNA polymerases. There are five known *E. coli* polymerases where the

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high fidelity DNA polymerase III (Pol III) and DNA polymerase I (Pol I) are essential and responsible for genome replication and Okazaki fragment processing, respectively.[15] DNA polymerases Pol II, Pol IV and Pol V serve as accessory enzymes.[16-18] The primary function for Pol II is remains unclear, but it has been suggested that Pol II may serve as a backup enzyme for Pol III[16] and re-initiates DNA synthesis in UV-irradiated *E. coli* cells.[19] Like Pol III and Pol I, Pol II is considered a high fidelity enzyme due to its proofreading exonuclease activity.[15] On the other hand, Pol IV, encoded by *dinB* (annotated in some UPEC genomes as *dinP*) and Pol V ($UmuD'_2C$) are highly conserved Y-family translesion synthesis (TLS) polymerases with well-established low fidelity synthesis in vitro under laboratory conditions, due, in part, to lack of exonuclease proofreading function.[20,21]

Pol II, Pol IV and Pol V have been previously shown to be SOS-inducible, with increases in the number of molecules upon SOS conditions from 50 to 300, 25 to 2,500 and 0 to 200, respectively.[21,22] In addition to host components and environments that may induce an SOS response, Perez-Capilla and colleagues demonstrated that fluoroquinolones used in the treatment of UTI also induced the expression of Pol IV.[23]

Pol IV and Pol V are necessary for bypassing some errors in the genome that stall replication, but bypass of errors stalling replication can be mutagenic.[24] Overexpression of Pol IV and Pol V significantly increases the number of spontaneous mutations.[25,26] Genetic deletion of *dinB* was shown to result in a modest decrease in the mutation frequency when the target gene was located on episomal DNA (F'), while deletion of *umuDC* coding for Pol V produces no significant decrease in mutation rate under non-stressed in vitro laboratory growth.[18] In vitro, Pol IV has a known role in error-free bypass of cytotoxic alkylating DNA lesions and in error-free translesion synthesis through 8-oxo-guanine lesions, as produced by reactive oxygen species and nitrofuran antibiotics.[27,28] Pol V has a known essential function in the survival of UV irradiation through its synthesis over thymidine dimer photoproducts and abasic sites.[29] However, the relevant in vivo functions of Pol IV and Pol V within a host remain unclear.

Mismatch repair (MMR) and RecA-dependent homologous recombination are additional major mechanisms described in *E. coli* to process damaged DNA, repair errors and repair gaps. MMR is a highly conserved post-replicative system designed to recognize and repair DNA errors as base mispairs, frameshifts and deletions that occur during DNA synthesis and recombination. Depletion, saturation or loss of MMR components in vitro results in increased mutagenesis.[30,31] MMR may be suppressed or saturated under certain physiological conditions such as stationary phase growth and chemical stress.[31,32] These scenarios produce an MMR-deficient phenotype and are associated with increased mutagenesis.[31] Homologous recombination is activated when chromosomal damage cannot be bypassed by synthesis and viability is threatened. DNA lesions blocking DNA replication may lead to the uncoupling of leading and lagging strand synthesis, resulting in exposed single stranded regions of DNA. These regions may be covered with the nucleofilament RecA, which simultaneously cleaves LexA, activating the SOS response and de-repressing a myriad of LexA repressed targets, including three DNA polymerases.[33]

How the integrity of the genome is managed under the relevant physiologic and inflammatory stress conditions present during infection is unclear. In our prior work, we demonstrated that UPEC undergoes an SOS-response during infection.[34] The SOS-response has also been demonstrated to be important for UPEC to persist in the inflamed host,[35] strongly suggesting that host stress components damage the genome and that induction of RecA-dependent SOS pathways was critical for survival during infection.

Here, our aim was to test the hypothesis that inflammation during UTI destabilizes the UPEC genome resulting in: (1) increased acquisition of mutations and (2) a requirement for the TLS DNA polymerases to bypass DNA errors/adducts and assure survival during the host inflammatory response. Through a combination of in vitro (outside the host) and in vivo (inside the host) studies, we demonstrate that the mutability of UPEC is strongly enhanced in vivo during experimental UTI and that genome instability is driven by physical stress in the urinary tract as well as inflammatory stress induced through a TLR4-dependent mechanism. We further demonstrate that during experimental UTI the UPEC TLS DNA polymerase Pol IV is essential for virulence, but despite its lack of proofreading functions, does not contribute to genome instability during infection. Together, these data suggest that UPEC genome instability affects a large proportion of the population during infection and that in vitro studies do not predict the roles of the genome repair processes under physiological host stress.

Results

Mutability of UPEC is strongly enhanced during occupation of the host. To investigate the genome instability of UPEC under host stress conditions, the prototypic UPEC strain UTI89, a K1 human cystitis isolate, was used.[3,36] A series of forward mutagenesis assays were employed to determine in vitro and in vivo mutation frequencies at unlinked, independent alleles (detailed in Materials and Methods). First, the mutation frequency of UTI89 was determined to assess if its constitutive mutation frequency was comparable to that of other *E. coli* strains. It has been shown that some of the natural isolates of *E. coli* have an enhanced mutator phenotype.[37] To exclude UTI89 as a constitutive hypermutator, we compared the in vitro mutation frequencies under non-stress growth conditions of the prototypic UPEC strains UTI89 and NU14 [3] as well as the comparator strain MG1655,[38] a prototypic commensal isolate and found that they were all similar (**Table 1**). Variation in the mutation frequencies at different alleles ranged from ~$0.3/10^8$ for nalidixic acid (nal) resistance, up to $310/10^8$ for *lacI* mutant formation (p-gal growth test).

Next, we determined the frequency of mutations during UTI where UPEC is subjected to host physical and inflammatory stresses. We anticipated that infection would produce a marked increase in genome instability. We infected female C3H/HeN mice via transurethral catheterization to initiate cystitis. At 48 h post-inoculation (hpi), bladder homogenates were plated on both

non-selective and selective media. UTI89 efficiently infected the bladders, reaching an average number of ~10^6 CFU/organ (**Fig. 1A**). Bacteria isolated from the infections where also immediately tested (without intermediate culturing) for a mutability phenotype using three of the forward mutagenesis assays. The mutation frequency was calculated for *lacI* mutants, for 6-azauracil (6-aza) and 5-fluorocytosine (5-FC) resistance (**Fig. 1B–D** and respectively). For all of the markers, we observed that the mutation frequencies were significantly elevated, up to 100-fold compared to the frequencies in the inocula. To determine if the frequency and degree to which genome instability was observed in vivo was specific to one host, the experiments were replicated in female BALB/c mice. As shown in **Figure 1**, the bacterial burden and mutation frequencies for UTI89 at 48 hpi were equivalent in the two mouse strains. These data demonstrate that UTI produces significant genome instability for UPEC.

Induction of the host innate immune response significantly increases the genome instability of UPEC UTI89. We considered that the host environment, which may include physical, chemical and metabolic stresses, may promote mutagenesis, while inflammatory stress could be additive. To model one aspect of physical and metabolic stress for UPEC during its persistence in the urinary tract, UPEC was grown in urine that was collected and pooled from healthy human donors. Overnight growth of UTI89 in human urine resulted in an increase in the mutation frequencies relative to the levels observed in bacteria grown in Luria-Bertani broth (LB) (**Table 2**). Of the multiple phenotypes assessed as measures of mutation frequencies, nalidixic acid resistance was the most dramatically altered, with ~20-fold increase in urine over LB broth. For rifampicin (rif) and 6-aza resistance, we observed 5- and 8-fold increases in mutagenesis, respectively. To exclude the possibility that this phenotype is unique to UTI89, we cultured 12 independent *E. coli* strains (10 UPEC and 2 commensal) in pooled human urine and LB broth. As shown in **Figure 2**, all of the strains had similar mutation frequencies relative to UTI89. In each case, the mutation frequencies were enhanced significantly by growth in human urine. Thus, UTI89 proved to be a representative strain in terms of mutability and did not have a hyper- or hypo-mutable phenotype relative to other *E. coli* in these assays.

Next, we sought to determine the contribution of the innate immune response and induction of inflammation to the development of UPEC genome instability. Female C3H/HeN and C3H/HeJ mice were infected for 48 hpi with UTI89 introduced by transurethral catheterization. C3H/HeJ mice that are hyporesponsive to LPS-TLR4 signaling were used as an inflammation-attenuated strain to assess the role of inflammation as a driver of UPEC genome instability in vivo.[39] As shown in **Figure 3**, infection of C3H/HeJ mice resulted in a mutation frequency approximately 1-log lower than the frequency in C3H/HeN mice. The frequency of mutation for UTI89 in the C3H/HeJ mice animals was similar to the elevated frequency in pooled human urine relative to the inoculum (**Table 2**), again suggesting that the host environment, even in the absence of a strong inflammatory response, still produces significant stress and genome instability for UPEC relative to in vitro growth. However, the combination of host environment and innate immune-driven inflammation produced the greatest degree of genome instability.

Table 1. In vitro mutation frequencies for UPEC UTI89[a]

	Resistant colonies per 10^8 cells				
Strain	**rif^r**	**nal^r**	**6-aza^r**	**5-FC^r**	***lacI***
UTI89	3.0 ± 1	0.3 ± 0.1	130 ± 10	280 ± 20	200 ± 24
NU14	3.3 ± 1	0.4 ± 0.2	120 ± 13	250 ± 25	200 ± 21
MG1655	2.0 ± 1	0.4 ± 0.1	100 ± 5	ND	310 ± 15

[a]Each entry is based on 12 independent cultures. Numbers represent mean mutation frequencies and standard errors (±SE). rif, rifampin; nal, nalidixic acid; 6-aza, 6-azauracil; 5-FC, 5-fluorocytosine; *lacI*, growth on phenyl-β-D-galactopyranoside (as described in Materials and Methods); ND, not determined.

MMR is not a major contributor to genome instability or fitness during acute UPEC UTI. The MMR system, a post-replicative repair system, was investigated as a potential source of the elevated in vivo mutation frequencies observed. MMR detects and repairs most of the typical replication errors and, therefore, was considered a potentially important source of spontaneous mutagenesis in vivo. We hypothesized that, during UTI, the UPEC MMR system may be suppressed or saturated by damage induced through host inflammatory mediators, leading ultimately to high mutation rates. To address this hypothesis, we constructed an MMR-deficient strain of UTI89 carrying a complete deletion of *mutL*. In vitro analyses showed that UTI89 Δ*mutL* strain had an expected strong mutator phenotype (**Table 3**). The largest increase in mutation frequency was observed for rif and nal markers (130- and 60-fold increases in mutation frequency compared to the wt parent, respectively).

We determined the contribution of MMR to UPEC mutability and fitness in vivo by infecting C3H/HeN mice with the *mutL* deletion and wt parent strains. Consistent with a prior study evaluating the fitness of an MMR-deficient UPEC due to inactivation of *mutS*,[40] we found that elimination of MMR did not significantly alter the fitness of UTI89 by 48 hpi (**Fig. 4A**). As shown in **Figure 4B**, UTI89 Δ*mutL,* isolated from the bladder at 48 hpi, had a significantly higher frequency of mutation than MMR-proficient wt isogenic parent. In vivo and in vitro data showed the same pattern and degree of increased mutability in the MMR-deficient strain compared to wt, suggesting that MMR is not suppressed or saturated during UTI and that a loss of capacity by MMR is not the major factor driving high mutability in vivo. Together, these data suggest that MMR does not have a major role in genome instability or in counteracting the host response during acute UTI.

Pol IV is essential for full virulence during UTI but does not drive genome instability during infection. After determining that MMR was not a major contributor to the mutability of UPEC in vivo, we assessed if the accessory DNA polymerases, known to perform critical bypass synthesis on genomic lesions and errors but lacking proofreading functions, may be involved in producing the >100-fold increase in mutation frequency observed in vivo. We also hypothesized that the TLS DNA polymerases may be essential for fitness and virulence during infection to bypass lesions and errors produced by host inflammatory

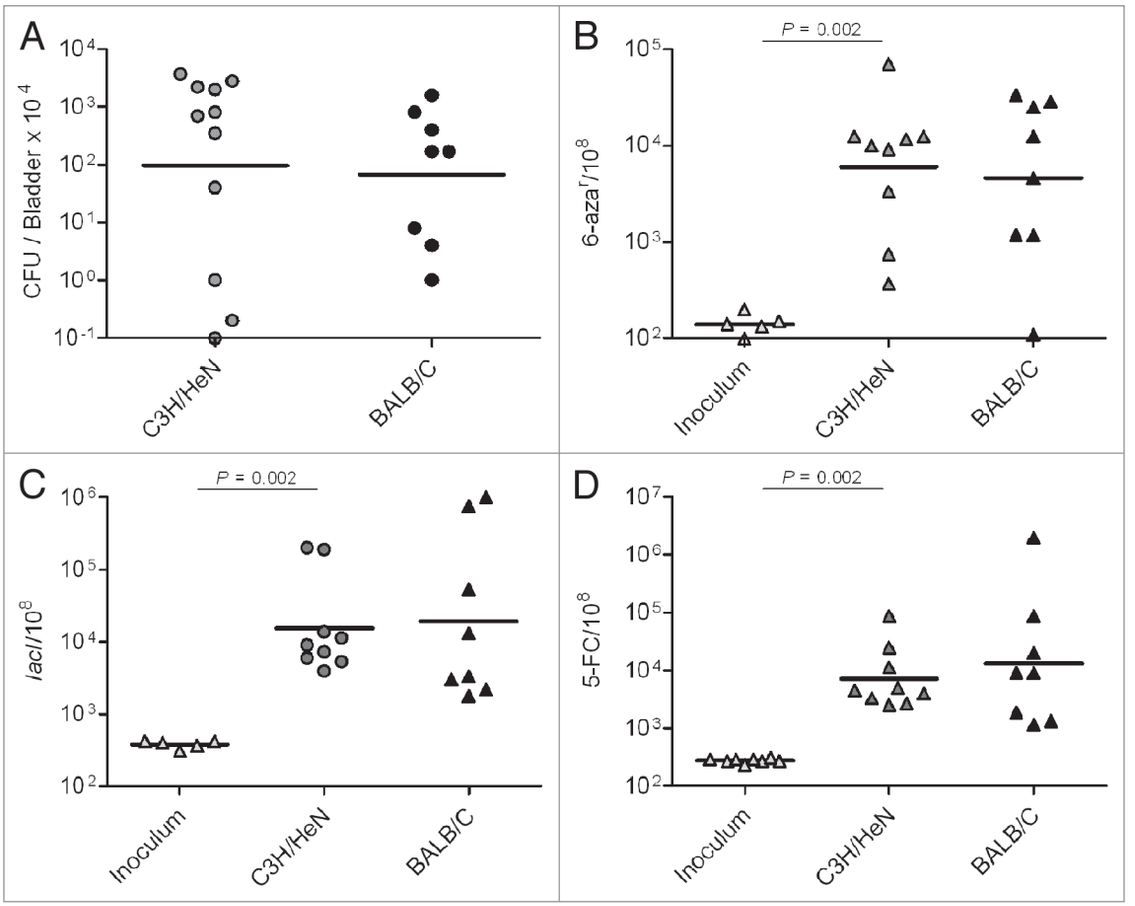

Figure 1. UTI promotes higher mutation frequencies in UTI89. (A) UTI89 infection rates (CFU/Bladder x 10^4 at 48 hpi). (B–D) mutation frequency per 10^8 cells measured for: 6-aza (B), *lacI* (C) and 5-FC (D) markers.

Table 2. Growth in normal human urine increases the mutability of UPEC UTI89 in vitro[a]

		Resistant colonies per 10^8 cells		
Strain	**Medium**	**rif^r**	**nal^r**	**6-aza^r**
UTI89	LB	3.8 ± 1	0.4 ± 0.2	85 ± 13
UTI89	Urine	20 ± 4*	8.0 ± 4*	680 ± 140*

[a]Mutation frequencies data are represented as mean and standard error, based on 8 independent cultures. *$p < 0.05$ compared to growth in LB broth calculated using the non-parametric Mann-Whitney test.

mediators. We focused on the roles of the TLS polymerases Pol IV and Pol V. Each has be en previously shown to be highly induced during the SOS response,[41,42] and prior work has shown the SOS response to be activated during UTI.[34]

To test the role of Pol IV and Pol V in the mutability and survival of UTI89 in vivo, we created single and double deletion mutants in the *dinB* and *umuDC* genes coding for Pol IV and Pol V, respectively. In addition, we created a deletion in *impCA* alone or in combination with other polymerase deletion mutations. The *impCA* open reading frames are present in a 1,911 nt region (sequence 54,600–56,511) of the UTI89 plasmid, pUTI89 and the predicted proteins present some homology to UmuD'$_2$C (1e-30; coverage 61%).

First, we confirmed that all of the single and multi-gene deletion mutants did not have growth defects in vitro (data not shown). We subsequently tested the mutation frequencies of the polymerase mutants in vitro. The mutants were grown in vitro under non-stressed (LB broth) and stressed (human urine) conditions. We observed that the mutation frequencies for all of the *dinB* deficient strain grown in LB broth media were significantly decreased, compared to the isogenic wt parent, as measured by 6-aza resistance (**Fig. 5**). No alteration in the mutation frequencies was observed due to deletions in *umuDC* or *impCA* alone (data not shown). Furthermore, deletion of *umuDC* or *impCA* in the Δ*dinB* background did not produce any significant alteration in the mutation frequencies of the single Δ*dinB* mutant. Although strains carrying the single *dinB* deletion or in combination with other mutations grown in vitro in urine trended toward lower mutation frequencies, these changes were not significant (wt vs. Δ*dinB,* p = 0.2). Thus, under laboratory, non-stressed growth conditions, UPEC Pol IV alone produces a modest but significant increase in the mutant frequency.

To investigate the role of the accessory TLS DNA polymerases in the survival and mutability of UTI89 during UTI, we conducted 48 h infections of C3H/HeN and C3H/HeJ mice with the series of UTI89 derivatives carrying single and combination

mutations in the TLS DNA polymerase genes. As shown in **Figure 6A**, the loss of Pol IV (*dinB* deletion) alone or in combination with other mutations resulted in a significant loss of fitness relative to the isogenic wt strain. In contrast, mutations in *umuDC* or *impCA* without mutation in *dinB* did not significantly alter the fitness of UPEC during experimental UTI. Single copy chromosomal complementation of Δ*dinB* restored the in vivo loss of fitness (**Fig. 6A**).

Next, the mutation frequencies of the mutant and wt strains recovered from infected bladder homogenates at 48 hpi were measured. The overall mutation frequency for all of the strains was significantly increased during infection relative to the inoculum (**Fig. 6B**). However, single and combined mutations in the polymerase genes did not produce statistically significant alterations in the mutation frequencies relative to wt during experimental UTI. There was a trend toward increased mutagenesis among all three strains carrying a deletion in *dinB*. In contrast, the single copy *dinB* complemented strain had a frequency of mutation restored to wt levels (**Sup. Fig. 1**). These data suggest that although Pol IV is required for the full fitness of UPEC during UTI, Pol IV, despite lacking proofreading function, produces high-fidelity repair of the genome during infection. This may suggest that the specific lesions for which Pol IV is required to bypass are either correctly repaired or are otherwise lethal. Alternatively, Pol IV may be required to stall the replication complex, allowing other factors sufficient time for DNA replication and repair.

To confirm that the role of Pol IV in UPEC virulence during UTI was not bacterial or mouse strain specific, several additional experiments were performed. First, we constructed a complete deletion of the *dinB* open reading frame in the prototypic and well-characterized urosepsis isolate CFT073.[43] C3H/HeN mice were infected by transurethral catheterization, and at 48 hpi, bacterial CFU were measured in bladder homogenates. As shown in **Supplemental Figure 2**, CFT073 Δ*dinB* had a significant loss of fitness relative to the wt parent strain, thus demonstrating that Pol IV is important for virulence in independent UPEC isolates. Second, C57BL/6 mice were infected by transurethral inoculation with wt UTI89 and the Δ*dinB* isogenic derivative. As shown in **Supplemental Figure 3A**, UTI89 Δ*dinB* retained the significant loss of fitness previously observed in C3H/HeN mice and further showing that the effect is not host-dependent. The mutation frequency for the 6-aza marker was increased relative to in vitro levels, further confirming our observations from C3H/HeN mice (**Sup. Fig. 3B**). Together, these data demonstrate that Pol IV is important for survival for UPEC in the host environment during experimental UTI, while not contributing to genome instability.

Pol IV is required to maintain UPEC virulence under TLR4-dependent inflammatory stress in vivo. We hypothesized that Pol IV would be required under inflammatory stress and that, with reduced inflammatory stress, a Pol IV mutant would not be significantly attenuated. To address this hypothesis, isogenic derivatives of UTI89 carrying combinations of mutations in *dinB*, *umuDC* and *impCA* were used to infect LPS hypo-responsive mice, C3H/HeJ. As shown in **Figure 7A**, each of the strains had no loss of fitness in this host after 48 hpi, in sharp contrast to the attenuation of Δ*dinB* in the C3H/HeN background where TLR4 signaling is intact. As anticipated, the frequency of 6-azauracil resistance was lower in the C3H/HeJ mice for all strains, measured at approximately 1-log higher than the frequency in vitro (**Fig. 7B**) and approximately the same frequency as observed after growth in human urine from non-infected individuals (**Table 2**).

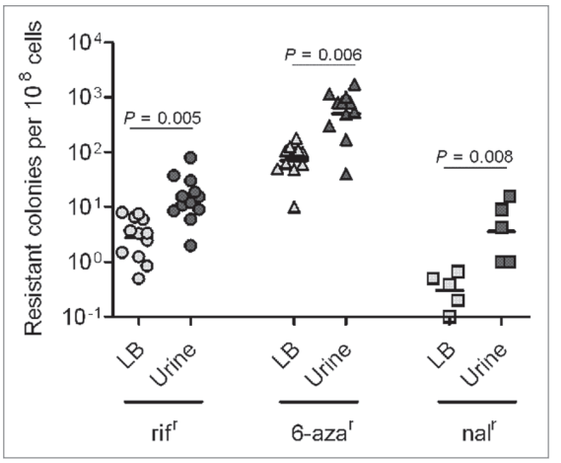

Figure 2. Growth under urine stress increases mutability for multiple *E. coli* strains. *E. coli* strains UPEC: CI5, E80, DS17, CFT073, GR12, PY2, UTI89, r-UTI89, NU14, EC45 and K-12: MG1655 and KA796 were grown overnight in LB broth and urine, in parallel, at 37°C. Mutation frequencies plotted as resistant colonies per 10^8 cells. Each plotted point is based on three independent cultures.

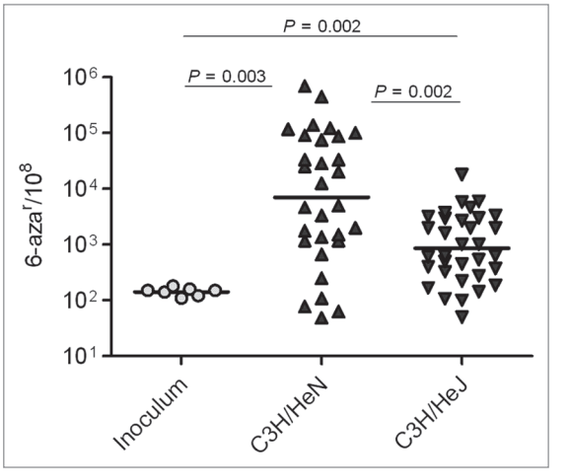

Figure 3. Host environment and innate immune response stimulate progressive increases mutation frequencies. Mutation frequencies are shown as number of 6-aza^r colonies per 10^8 cells.

Table 3. The UTI89 MMR mutant has enhanced mutability in vitro[a]

	Resistant colonies per 10^8 cells		
Strain	**rif^r**	**nal^r**	**6-aza^r**
UTI89	4 ± 1	0.4 ± 0.2	52 ± 13
UTI89 Δ*mutL*	530 ± 4	240 ± 4	461 ± 140

[a]Each entry is based on 12 independent cultures. Numbers represent a mean of mutant frequencies and standard error (±SE).

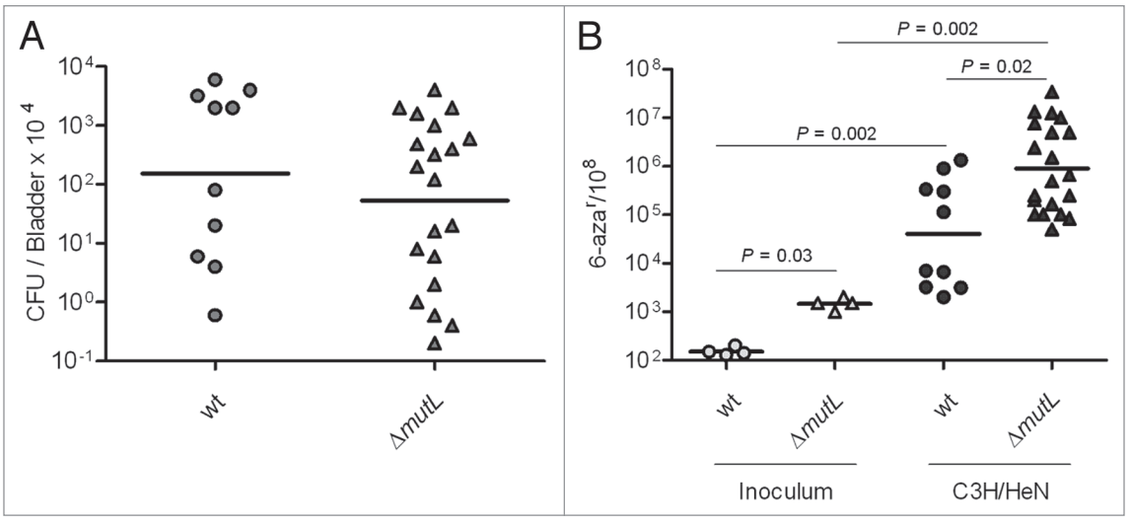

Figure 4. MMR is not suppressed or saturated during UTI. (A) Infection rates (CFU/Bladder x 10^4) of UTI89 and UTI89 Δ*mutL* strains. (B) Mutation frequencies per 10^8 cells measured for 6-aza^r marker.

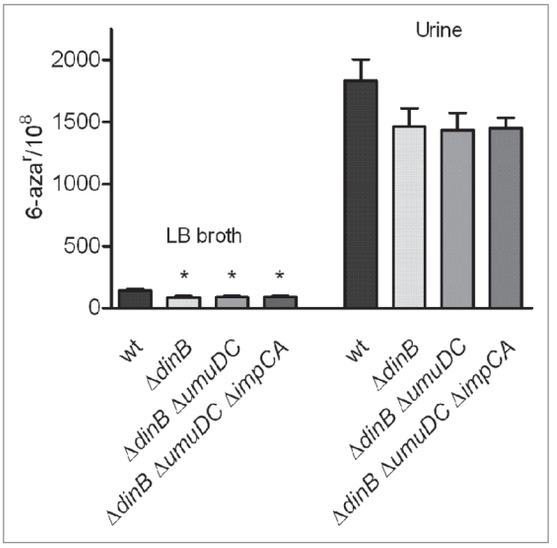

Figure 5. Mutation frequencies of UPEC UTI89 derivatives lacking genes encoding for TLS DNA polymerases. Strains were grown over night in LB broth and urine, in parallel, at 37°C. Mean mutation frequencies for 6-aza^r are based on eight independent cultures. Error bars show standard error (SE). Statistically significant differences ($p < 0.05$) in mutation rates frequencies compared to wt UTI89 are indicated by a star (*). p values were calculated using the non-parametric Mann-Whitney test.

Discussion

Our work on the genome instability of UPEC during UTI provides new insights into the plasticity of the pathogen genome under host stress and essential mechanisms required to maintain DNA integrity during infection and, thus, virulence. First, we demonstrate that the urinary tract environment, even in the absence of a significant innate immune response, stimulates a marked increase in mutation frequency. Our data indicate that growth in urine alone is a significant stimulus for mutagenesis. Urine has high osmolarity, elevated urea, low pH and low nutrient availability, each of which may contribute to the stress that ultimately drives pathways involved in UPEC mutagenesis. Preliminary studies supplementing urine with a simple carbon source such as glucose only partially lowers the frequency of mutations for 6-aza (~0.5-log; Gawel and Seed, unpublished), suggesting that nutrient deprivation is only partially causal for inducing mutagenic stress pathways.

We also have demonstrated that the innate immune response, as triggered by the LPS-TLR4 pathway, results in an environment that significantly increases mutagenesis above the level of urine alone. The innate immune response to UPEC UTI is complex and differs in the bladder and kidneys. In the bladder, the major site of infection in our experimental model, a TLR4 response upregulates iNOS expression, resulting in increased nitric oxide.[10,44] Concurrently, IL-6 and IL-8 are produced to recruit neutrophils into the urinary tract. Neutrophils have been previously shown to hone in on extracellular UPEC while also migrating to cells containing intracellular UPEC.[45] Phagocytosis of UPEC may provide a physical stress on UPEC that promotes mutagenesis while the neutrophil oxidative burst produces oxidative radicals with well-known consequences on DNA modification, introducing errors and lesions.[12] In the subepithelium, mast cells and macrophages are recruited, providing additional inflammatory and phagocytic challenges for UPEC. These current studies are not yet sufficient to determine if the measured genome instability arises because of enhanced expression of bacterial mutagenic factors, suppression of bacterial error-correcting mechanisms in the presence of host-induced DNA damage, or employment of low-fidelity DNA repair mechanisms to bypass host-incited DNA damage other than tested thus far. It seems likely that host factors such as oxidative radicals directly produce genome damage, necessitating repair and resulting in the introduction of mutations.

We hypothesized that one principle source of mutations in the UPEC genome during host stress may arise from suppression

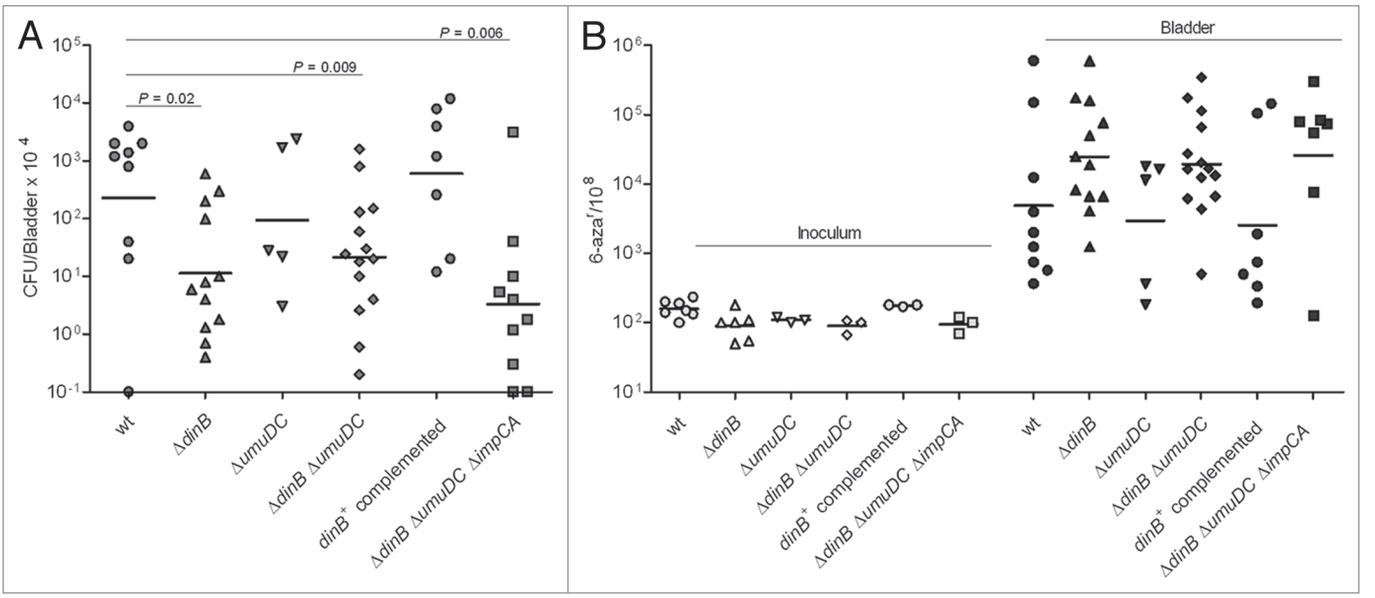

Figure 6. DNA polymerase IV improves survival of UTI89 strain in vivo but does not contribute to genome instability. (A) Infection rates (CFU/Bladder x 10^4) of UTI89 and its mutant derivatives. (B) Mutation frequencies per 10^8 cells measured for the 6-aza^r marker. The mutation frequencies were statistically different ($p < 0.05$) between the inocula and the bacteria recovered from infected bladders for all comparisons.

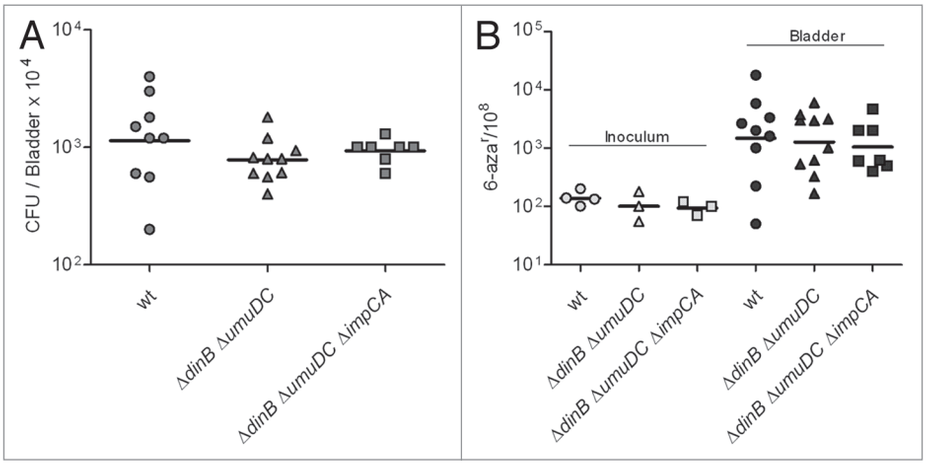

Figure 7. The Δ*dinB* mutant is not attenuated in the LPS hyporesponsive TLR4-host. (A) Infection rates (CFU/Bladder x 10^4) of UTI89 and its mutant derivatives. (B) mutation frequency per 10^8 cells measured for 6-aza^r marker. The mutation frequencies were statistically different ($p < 0.05$) between the inocula and the bacteria recovered from infected bladders for all comparisons.

or saturation of MMR. Experimental testing of this hypothesis, however, demonstrated that MMR is active during infections, significantly reducing the frequencies of mutations incurred to a degree similar to non-stress UPEC in vitro. Based on the >1-log increase in mutation frequency in the MMR mutant (Δ*mutL*), we conclude that numerous post replication errors require MMR for correction during UTI. However, the relative increase in frequency of mutations between wt and the MMR mutant is not greater in vivo than in vitro. Thus, while the MMR system appears to be important for maintaining genome stability in vitro and in vivo, the host environment and inflammatory response does not necessitate exceptional requirements for the MMR system.

Previously, SulA, a component of the SOS regulon, was shown to play an essential role in UPEC virulence in the mouse model of human UTI.[34] More recently, Li et al. confirmed the importance of the SOS response for UPEC during UTI by showing the attenuation a RecA-deficient strain of UTI89 during murine UTI.[35] Expression of the TLS polymerases IV and V is greatly enhanced as part of the SOS response,[41,42] and the polymerases have important roles in long-term survival and stationary phase mutagenesis in vitro,[22,46] raising the possibility that these polymerases were not only responsible for enhanced mutation frequencies measured during UTI but also were critical factors necessary for mitigating genomic injury sustained during infection. Numerous prior studies have demonstrated that each of these polymerases

yield low fidelity synthesis during processing undamaged and damaged templates due to that lack of exonuclease functions.[24] In addition to evaluating the potential roles of Pol IV and Pol V in genome instability and fitness of UPEC during UTI, we considered the possibility that additional homologs may be present and thus responsible for any observed phenotypes. In *Salmonella typhimurium*, a plasmid-borne homolog of Pol V called SamAB has been described.[47] A BLAST analysis of the UTI89 genome for Pol V homologs suggested such a factor, annotated as encoded by the open reading frames *impCA,* may be present on the megaplasmid. We did not find any in vitro and in vivo genetic evidence for an active role of Pol V or ImpC/ImpA proteins in pathogenicity and mutability of UPEC UTI89 strain. While no major role for Pol V was observed, we cannot fully exclude that substitution for Pol V by another accessory polymerase does mask any observation of an in vivo function.

In contrast to the lack of an apparent requirement for Pol V and ImpCA in vivo during UTI, we discovered a novel in vivo phenotype for Pol IV during UTI. The loss of Pol IV in UTI89 and CFT073 resulted in the attenuation of both strains during UTI in multiple host backgrounds. Interestingly, the mutation frequencies of Pol IV mutant were not significantly different than wt. The Pol IV-deficient strain trended toward having higher mutation frequencies than wt UTI89, opposite of what was expected were Pol IV contributing to mutagenesis during UTI. In our in vitro studies, we also observed a modest anti-mutagenic effect with the loss of *dinB* in UTI89 when grown in rich LB medium. However, this phenotype was abolished when the mutant was propagated in human urine, yielding the same mutation frequencies as wt (**Fig. 5**). This may indicate that Pol IV is recruited for translesion synthesis over different errors or lesions in vitro and in vivo and that the bypass of these damages is low and high fidelity in vitro and in vivo, respectively. As working model we propose, that certain lesions or errors introduced into the genome during host initiate stress conditions may predominate and not only require Pol IV for bypass but result in error-free synthesis. This may indicate the nature of the host response and lesions induced in vivo for which Pol IV is so important in maintaining viability and thus virulence. Consistent with this idea, we found that the Pol IV mutant was no longer attenuated in mice with an LPS hypomorphic phenotype, whereby LPS-TLR4 signaling is known to induce the innate immune response of which an oxidative stress response is part. These data together indicate a unique and important role for Pol IV in the maintenance and fidelity of DNA synthesis of UPEC under inflammatory stress.

The alternative explanation for the loss of fitness in the *dinB* mutant fitness under inflammatory stress conditions during UTI may be due to a requirement for DinB as a brake on DNA synthesis, allowing other repair factors sufficient time to resolve DNA damage and errors, thus serving as a specific checkpoint to secure genome stability.[48] Thus, the loss of *dinB* would be expected to have the phenotypes we observed in vivo, namely a survival defect and enhanced mutagenesis. This is also consistent with our measurements of DinB-independent mutagenesis during UTI that was enhanced with inflammation.

Parallel to our report, Gutierrez et al. recently showed that the absence of DinB resulted in loss of fitness in competive infections with wt UPEC CFT073 in a murine model of bacteremia,[49] consistent with our demonstration of a role of DinB in UTI pathogenesis. In that report, the authors demonstrate that the attenuated phenotype of the *dinB* mutant was restored when the *yafP* gene, encoding for an N-acetyltransferase, was also deleted. Guttierez et al. concluded, that YafP enzyme may play a role in the activation of numerous mutagenic compounds for which DinB is required to process the lesions resulting from these derivatives. However, it must be pointed that the *dinB-yafP* two gene organization in the UPEC strain CFT073 is rather unique to only about 40% of studied by them *E. coli* strains. Nevertheless, majority of *E. coli* strains (including UPEC UTI89 strain and the commensal K-12 MG1655 strain) have a four gene operon structure (*dinB-yafN-yafO-yafP*). Therefore we may expect that in our experimental system the phenotype for *yafP* may differ from the prior report due to (1) the differencies in type of stress associated with bladder infection vs. extraintestinal and (2) the structure of the *yaf* operon in used model isolates CFT073 and UTI89. Future studies will elucidate the broader role of the *yaf* operon in extraintestinal virulence among diverse strains and the interactions of its gene products with DinB.

If Pol IV, Pol V, ImpCA or the loss of MMR functionality do not act to enhance in vivo mutagenesis, under what processes does enhanced mutagenesis arise? There several additional processes through which their imbalance, dysfunction and inactivation may result in mutagenesis. One may predict the source is in one of three systems: (1) alterations in 3'→5' proofreading by the major and accessory polymerases; (2) inhibition or failure of base-excision repair (BER) or nucleotide-excision repair (NER); and (3) alteration in dNTP pools such as inhibition of MutT, involved in sanitation of damaged dNTPs.[50] Future studies will determine the relative activities of these systems in vivo and their contributions to mutagenesis and pathogen adaption.

One intriguing idea is that under inflammatory stress, UPEC engages essential systems to manage specific genome integrity, without which viability is threatened, while concurrently activating mutagenesis as an evolved stress response through which adaptive mutations may be generated. This concept may imply that mutagenesis is not necessarily the essential consequence of repairing otherwise fatal damage. Instead, stress-induced mutagenesis may be activated through a portion of the population resulting in an infrequent selective advantage through rare beneficial mutations that arise to improve the phenotype of UPEC either as a pathogen or as a commensal, the state in which the organisms most commonly exists. In terms of adaptation, prior studies of UPEC have demonstrated pathoadaptive changes among UPEC in the *E. coli* pangenome. One example is the FimH lectin adhesin of type 1 pili, a critical virulence factor for UPEC. FimH of UPEC has an increased affinity for monomannosylations present on apical proteins of the urinary tract such as uroplakins while FimH of commensal bacteria have greater affinity for tri-mannosylations more likely to be present in non-urinary tract niches.[13,14,51]

Table 4. *E. coli* strains used in this study

Strain	Description	Source
UTI89	UPEC	3
CFT073	UPEC	43
J96	UPEC	55
CI5	UPEC	56
E80	UPEC	57
DS17	UPEC	58
GR12	UPEC	55
PY2	UPEC	57
rUTI89	UPEC	57
NU14	UPEC	3
EC45	UPEC	57
MG1655	K-12	59
KA796	K-12	60
UTI89 Δ*dinP*::*kan*	Δ*dinP*	This study
UTI89 Δ*dinP*	Δ*dinP*	This study
UTI89 Δ*umuDC*::*cam*	Δ*umuDC*	This study
UTI89 Δ*umuDC*	Δ*umuDC*	This study
UTI89 Δ*impCA*::*cam*	Δ*impCA*	This study
UTI89 Δ*dinP*-Δ*umuDC*	Δ*dinP*, Δ*umuDC*	This study
UTI89 Δ*dinP*-Δ*umuDC*-Δ*impCA*	Δ*dinP*, Δ*umuDC*, Δ*impCA*	This study
UTI89 Δ*mutL*::*kan*	Δ*mutL*	This study
UTI89, *proAB*::*cam*	Δ*proAB*	This study
UTI89, *proAB*$^+$, *dinP*$^+$, complemented	*dinP*$^+$	This study
UTI89, *proAB*$^+$, complemented	*proAB*$^+$	This study
CFT073 Δ*dinP*::*cam*	Δ*dinP*	This study

In conclusion, we have shown that the interaction of UPEC with the host results in highly enhanced genome instability, independent of the TLS polymerases and the post-replicative MMR system. Our data suggest that the UPEC genome has significant plasticity during infection and the host, particularly during inflammatory responses, may be a major driver in diversity among these pathogen-commensal organisms. Furthermore, we have demonstrated a major role for the TLS DNA polymerase IV in mitigating host inflammatory stress during UTI. Given the conservation of Pol IV among prokaryotes in particular, the TLS DNA Polymerases may have important global roles in the maintenance of genome integrity and tolerating host inflammatory assaults.

Materials and Methods

Strains. Bacterial strains and oligonucleotides used in this study are listed in **Table 4 and Supplemental Table 1**, respectively. All deletion mutants were constructed by the red recombinase method entailing direct replacement of gene(s) of interest with an antibiotic resistant cassette flanked by FLP recombinase recognition sequences[52] or by generalized transduction using P1 phage. The chromosomal complementation of the *dinB* deletion was performed using a two-step procedure. First, a complete deletion of *proAB,* a linked locus to *dinB,* was made in UTI89 (wt) and UTI89 Δ*dinB* using red recombinase and resulting in *proAB*::*cat*. Next, each strain was subjected to generalized transduction using P1 phage particles derived from wt UTI89 and selecting for *proAB*$^+$ on MM medium lacking proline. The reconstitution of *dinB* was confirmed by PCR.

Media. Minimal medium (MM) and Luria-Bertani (LB) broth solid and liquid media were standard recipes as described.[53] Human urine was collected and pooled from at least 3 healthy donors. Minimal medium (MM) with Vogel-Bonner salts[54] was supplemented with 5 μg/ml of nicotinic acid and 0.4% glucose. Solid media contained 1.5% agar. Where required, antibiotics and/or selection agents were added to the following final concentrations: ampicillin (amp), 100 μg/ml; chloramphenicol (cam), 20 μg/ml; kanamycin (kan), 50 μg/ml; rifampicin (rif), 100 μg/ml; 6-azauracil (6-aza), 40 μg/ml; 5-fluorocytosine (5-FC), 40 μg/ml; nalidixic acid (nal), 40 μg/ml. P-gal solid MM media contained 1.5% agar supplemented with 5 μg/ml of nicotinic acid and 800 μg/ml phenyl-β-D-galactopyranoside as a carbon source.

Experimental murine UTI. Mice (6–8-week-old female) were obtained from Charles River Laboratories through the NCI—Frederick resource. Bacterial strains were prepared for infection as previously described.[61] Mice were inoculated with 1–2 x 10^7 CFU by transurethral catheterization in a volume of 50 μl.

Mutation frequency measurements. For in vitro tests, we used 6–12 independent LB or urine cultures (3 ml each) initiated from single colonies (one colony per tube) and grown over night to saturation at 37°C while rotating on a rotator wheel. The 100 μl of 10^6-fold diluted cultures were plated on LB and MM plates to determine the total cell count. The 100 μl of the undiluted cultures were plated on selective plates to determine the number of resistant (rif^r, nal^r, 6-aza^r, 5-FC^r, respectively) or *lacI* mutant colonies. UTI89 *mutL* (MMR-mismatch repair deficient) cultures were 10-fold diluted prior plating on LB/rif or MM/6-aza plates due to high mutation rates observed in MMR deficient background.

In vivo mutation frequencies were determined by homogenizing bladders in PBS containing Triton-X 100 (0.02%) and plating homogenate on MM/6-aza, MM/5-FC or MM/p-gal plates. Total cell counts were determined on LB or MM plates in parallel. Cultures were growth at 37°C. Mutation frequencies were calculated as the number of mutants per plate divided by the total number of cells.

The forward mutagenesis assays employed differ in reporter/marker gene and its location on chromosome. The rifampicin resistant and nalidixic acid resistant mutants result from forward mutation(s) occurred in *rpoB* and *gyrA* marker genes, respectively. The 6-azauracil resistant and 5-fluorocytidine resistant mutants arise due to mutation(s) in *upp* or/and *codA* genes of pyrimidine salvage pathway. Colonies growing on P-gal as a carbon source were formed due to forward mutation(s) appeared in *lacI* repressor allele of *lac* operon.

Statistical analyses. The statistic analyses (average frequencies; standard error (SE) or non parametric Mann-Whitney U-test) were determined by using the statistical software program Prism (GraphPad). Statistical significance was defined by attaining p-value ≤ 0.05. Horizontal lines present on the graphs indicate the geometric mean for each group.

Ethics statement. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Animal studies were performed under protocols reviewed and approved by the Duke University Institutional Animal Care and Use Committee (Protocol #: A143-09-05). Urethral catheterization of the animals and the initiation of the infections were carried out under isoflurane anesthesia to minimize trauma to the animals.

Urine specimens from adults providing written informed consent were de-identified and collected in accordance with Protocol #Pro00026252, reviewed and approved by the Duke University Institutional Review Board.

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Note

Supplemental materials can be found at:
www.landesbioscience.com/journals/virulence/article/16143

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