Conditioning of Uropathogenic *Escherichia coli* for Enhanced Colonization of Host

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Running title: Adaptive effects of RNIs and polyamines on UPEC

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ABSTRACT

While in transit within and between hosts, uropathogenic *Escherichia coli* (UPEC) will encounter multiple stresses, including substantial levels of nitric oxide and reactive nitrogen intermediates. Here we show that UPEC, the primary cause of urinary tract infections, can be conditioned to grow at accelerated rates in acidified sodium nitrite (ASN), a model system used to generate nitrosative stress. When inoculated into the bladder of a mouse, ASN-conditioned UPEC are far more likely to establish an infection than non-conditioned bacteria. Microarray analysis of ASN-conditioned bacteria suggests that several NsrR-regulated genes and other stress- and polyamine-responsive factors may be in part responsible for this effect. Relative to K12 reference strains, most UPEC isolates have increased resistance to ASN, and this can be substantially enhanced by addition of the polyamine cadaverine. Nitrosative stress, as generated by ASN, can stimulate cadaverine synthesis by UPEC, and growth of UPEC with cadaverine-supplemented broth, in the absence of ASN, can also promote UPEC colonization of the bladder. These results suggest that UPEC interactions with polyamines or stresses such as reactive nitrogen intermediates can in effect reprogram the bacteria, enabling them to better colonize the host.
INTRODUCTION

The urinary tract is normally a sterile environment, being both hostile and poorly accessible to most microbes. However, roughly half of women in the United States experience a urinary tract infection (UTI) at least once in their lifetime, and one quarter of affected women endure recurrence (22, 25). Greater than 80% of UTIs are due to strains of uropathogenic *Escherichia coli* (UPEC), which are usually presumed to be part-time gut flora that have reached the urinary tract by ascension via the periurethral area (53). Transmission of UPEC among individuals occurs primarily by way of fecal-oral routes and, in some cases, may involve the ingestion of contaminated food products or sexual contact (15, 23, 33, 40, 41, 57). In order to survive and disseminate, UPEC must be able to adapt to multiple environments and stresses both within and outside of the host.

When a UPEC infection occurs, recruitment of nitric oxide (NO)-producing neutrophils to the bladder is an important line of defense (26, 48). Within hours of infection, nitrite levels in the urine increase up to three-fold, and eventually NO within the bladder reaches levels 30- to 50-fold higher than in uninfected controls (39, 48). These high levels of NO are due in part to inducible NO synthase (iNOS) activity, which is upregulated within 6 h after infection (45). A role may also be played by endothelial (e)NOS, which is upregulated and activated in the bladder mucosa by *E. coli* lipopolysaccharide (36), and the bacteria themselves, which can produce NO through nitrite reductases under conditions of low oxygen tension (12). NO is a precursor for a variety of reactive nitrogen intermediates (RNIs), such as peroxynitrite and nitrosothiols, which can inflict extensive damage on nucleic acids, lipids, and proteins. Thiols, amines, aromatic residues, heme groups, and iron-sulfur clusters are particularly susceptible to attack by RNIs, making many key metabolic enzymes targets (17, 18). UPEC may also encounter RNIs outside of the urinary tract, possibly during passage through the upper gastrointestinal tract where nitrate (NO$_3^-$) and nitrite (NO$_2^-$) levels can be very high, or on the surface of meat products, which are often treated with nitrite as a coloring agent and preservative (15, 16, 27, 29, 64).
Adaptive responses that allow a bacterial population to survive one stressful condition can, in some instances, enhance its ability to handle other environmental stresses (2, 5, 31, 32, 42). This cross-protective effect may also potentiate bacterial virulence within a host. Recently, UPEC was found to have the capacity to withstand RNI levels that prevent growth of non-pathogenic E. coli K-12 strains (7, 60). RNI resistance in UPEC is in part controlled by the envelope stress response sigma factor RpoE (σE), the RNA chaperone Hfq, the NO-detoxifying enzyme HmpA, and polyamines (7, 38, 60). Expanding on these findings, we show here that UPEC can transiently adapt to high levels of nitrosative stress via a polyamine-linked mechanism, enabling this pathogen to grow more rapidly upon subsequent exposure to RNIs and to better colonize the urinary tract in a mouse UTI model system.

**MATERIALS AND METHODS**

**Bacterial strains and growth curves.** The K-12 reference strain MG1655 and UTI89, a human cystitis isolate, have been described previously (4, 10, 44). Other UPEC isolates were kindly provided by D. A. Low (University of California, Santa Barbara) and W. E. Hooton (University of Washington School of Medicine). All growth experiments were performed at 37°C in 100 mM morpholineethanesulfonic acid-buffered Luria-Bertani (MES-LB) broth (pH 5), with or without added sodium nitrite or cadaverine (Sigma-Aldrich) as indicated. The cultures in Figure 1B were grown in 5 ml MES-LB broth in loosely capped 20-by-150-mm borosilicate glass tubes with shaking (225 rpm, tilted at a 30° angle) and growth was monitored by optical density at 600 nm (OD<sub>600</sub>) using a Spectronic 20D+ (Thermo). All other growth curves were performed with 200 µl cultures in 100-well honeycomb plates using a Bioscreen C instrument (Growth Curves USA).

**Microarray sample preparation.** Separate colonies of UTI89 grown on LB agar plates from a -80°C freezer stock were used to start overnight shaking cultures in MES-LB broth. Each culture was then diluted 1:100 into 7 ml MES-
LB with or without 3 mM ASN in loosely capped 20-by-150-mm borosilicate glass tubes and grown with shaking at 37°C. Growth was monitored by optical density until OD$_{600}$ = 1.5, at which point cultures were spun down, and pellets frozen at -80°C for at least 12 h. RNA was extracted with hot phenol-chloroform and purified by CsCl centrifugation. cDNA was synthesized, fragmented, and labeled according to the protocol recommended by Affymetrix.

**Affymetrix microarray gene expression analysis.** Fragmented and labeled cDNA (15 µg) was added to 270 µl of hybridization buffer, and hybridized to the Affymetrix GeneChip® *E. coli* Genome 2.0 Array. After 20 h of hybridization at 45°C, the GeneChips® were washed, stained, and scanned according to the standard Affymetrix protocol. The arrays were scanned using an Affymetrix GeneChip Scanner 3000 enabled for High-Resolution Scanning, and the raw images were converted to CEL files using Affymetrix GCOS software. Image processing using the GCRMA method for probe-level data (30) was performed using the Bioconductor Package in the R statistical environment (24). The CEL files were analyzed as a group, background corrected using GCRMA (63), normalized using quantile normalization, and summary measures for probe sets were obtained by median polish. Transcripts were categorized, as in Figure 2, based on literature searches and gene information drawn from EcoCyc (http://ecocyc.org/) (37) and Affymetrix NetAffx (http://www.affymetrix.com/analysis/index.affx).

**Microarray data accession number.** Complete microarray data is available on the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) under accession number [to be determined].

**Quantitative RT-PCR.** Primers for real time (RT)-PCR, which were designed using the D-LUX™ Designer application available on the Invitrogen website, were made to be approximately 50% GC, and to amplify regions 56-65 bp in length. Primers used include norV_289FL (5'-
CGGGAGAACTGATGGCACAAATTCCCG-3'), norV_289FL/307RU (5'- CGAG TCGATAGCGTTGGCAGT-3'), nrdH_153FL (5'- CAACTGCTCAGGGCTTTTC GTCAGTTG-3'), nrdH_153FL/164Rub (5'- CCAGCTAAGATCGC CTGCAA-3'), hmpA_743RL (5'- CGGCCCATAAAGAAATCACCTGCCG-3'), and hmpA_743RL/732FU (5'-AATGTTGCGATGTCGTGAAA-3'). RNA collected for microarray analysis and stored at -80°C was used to make cDNA according to the protocol recommended by Affymetrix. Control reactions containing no reverse transcriptase were also performed. cDNA was used as a template for real time PCR in a Roche LightCycler® 480, using the Roche LightCycler® 480 DNA SYBR Green I Master kit, according to the manufacturer's protocol. In addition to triplicate reactions of experimental cDNA samples, reverse transcriptase-minus controls, and cDNA-minus controls, dilutions of genomic DNA were used to generate a standard curve for each primer set. Quantitation was carried out by the LightCycler® 480 Abs Quant/2nd Derivative Max analysis.

**Mouse infections.** Female CBA/J mice (Jackson Labs), approximately 7-9 weeks old, were used in infection studies according to IACUC-approved protocols. UTI89 from frozen stock was grown shaking overnight at 37°C in MES-LB broth and then sub-cultured 1:100 in 7 ml MES-LB ± 3 mM ASN or 3 mM cadaverine as indicated. These cultures were incubated at 37°C with shaking to OD$_{600}$=1.5. Bacteria were then pelleted for 8 min at 8000xg, washed three times, and resuspended in PBS. Each mouse was briefly anesthetized using isofluorane and inoculated via transurethral catheterization with 50 ul of a bacterial suspension containing approximately $1 \times 10^7$ colony forming units of control, ASN-conditioned, or cadaverine-treated UTI89. Twelve h or 5 d later, bladders were collected, weighed, homogenized, serially diluted, and plated on LB agar plates to enumerate bacterial numbers per gram of tissue. Mouse experiments were repeated at least twice with similar results (total combined data is presented).
Analysis of type 1 pilus expression. Expression of type 1 pili by UTI89 was assessed in two ways. First, the ability of UTI89 to agglutinate *Saccharomyces cerevisiae* was qualitatively determined by mixing 20 µl of each bacterial strain (OD<sub>600</sub> = 1.5) with 200 µl of a 1% suspension of baker’s yeast in PBS on glass slides. In addition, orientation of the *fim* switch was determined using a previously described inverse PCR protocol (8). Chromosomal DNA was prepared from control, ASN-condition, and cadaverine-treated cultures (OD<sub>600</sub> ≈1.5) using the Promega Wizard Genomic DNA kit. 500 ng of each genomic DNA sample was used as template in separate reactions designed to detect the *fim* switch in its on and off orientation. The reactions used the same outside primer (5'-CGACAGCAGAGCTGGTCGCTC-3') with one of two inside primers. The primer 5'-GTAAATTATTTCTTTGTAATTATTTCAACACTCACTCCGC-3' was used to detect *fim* in the off orientation, while its reverse complement was used to detect *fim* in the on orientation. At least three separate cultures were tested for each group, and the ratio of intensities of bands resulting from the PCR reactions was compared to determine relative on and off levels of the *fim* switch.

Statistics. Results from the mouse experiments and in vitro growth assays were analyzed by Fisher’s exact and Mann-Whitney two-tailed *t* tests, respectively, using Prism 5.01 software (GraphPad Software). *P* values of less than 0.05 are considered significant.

RESULTS

Adaptation of UPEC to growth in ASN. Acidified sodium nitrite (ASN) is a widely used system for generating RNIs in vitro (20, 43). When added to LB broth buffered at pH 5 with 100 mM MES (MES-LB), sodium nitrite is converted to nitrous acid, which spontaneously forms NO and other RNIs (3, 62). Upon initial subculture from MES-LB to MES-LB broth with 3 mM ASN, the UPEC cystitis isolate UTI89 has a long lag phase before the start of exponential growth, as shown in Figure 1 (filled triangles). When these bacteria are allowed to reach stationary phase and are then subcultured again into new +ASN broth, the lag
phase is considerably shorter, indicating that the bacteria are now better suited for growth in ASN, either by conditioning or by acquisition of one or more mutations (Figure 1, circles). To distinguish between these possibilities, we subcultured the short lag phase bacteria into broth without ASN, allowed them to reach stationary phase, and again challenged them with subculture into 3 mM ASN. After one round of growth in the absence of ASN, the lag phase returned to the original, longer period (Figure 1, open triangles). Thus, reduction in lag phase is dependent upon previous growth under stress conditions, indicating that adaptation via a conditioning effect, and not by mutation, has occurred.

Enhanced colonization of the bladder by ASN-conditioned UPEC. For some pathogens, increased resistance to certain environmental stresses correlates with enhanced virulence potential (2, 5). Our results indicating that UTI89 can be conditioned to better withstand the stresses generated by in vitro growth in ASN raised the possibility that ASN-conditioned UPEC may also have an increased capacity to colonize the urinary tract. To address this possibility, adult female CBA/J mice were inoculated via transurethral catheterization with UTI89 that had been grown shaking to $OD_{600} = 1.5$ in MES-LB ± 3 mM ASN. Bladders were collected at 12 h and 5 d post-inoculation and bacterial titers were calculated by serial dilution of tissue homogenates. Bacteria grown in the absence of ASN failed to colonize about 50% of the mice, while the ASN-conditioned bacteria effectively colonized all of the animals at both time points examined (Figure 2).

Key virulence factors that enable UPEC to effectively colonize the urinary tract are type 1 pili (a.k.a. type 1 fimbriae, reviewed in (14)). These peritrichously expressed, phase variable multi-subunit filaments promote UPEC adherence to and invasion of bladder epithelial cells by binding host mannose-containing glycoprotein receptors. Differential expression of type 1 pili by ASN-conditioned UPEC could conceivably account for the enhanced capacity of these microbes to colonize the bladder. However, both ASN-conditioned and control bacteria performed equally well in mannose-sensitive, type 1 pili-dependent yeast
agglutination assays. In addition, semi-quantitative PCR analysis of the reversible orientation-dependent genetic switch that controls the phase variable transcription of the fim genes involved in the biogenesis of type 1 pili also demonstrated that ASN-conditioning had no significant effect on type 1 pili expression (data not shown). These results indicate that growth in ASN primes UPEC to better colonize the bladder independent of any overt effects on type 1 pili expression.

Transcriptional profile of ASN-conditioned UPEC. To better understand how UTI89 is able to adapt to growth in ASN, and how this facilitates bacterial colonization of the bladder, gene expression profiles of ASN-conditioned UTI89 were analyzed by microarray. Bacteria from two independent overnight starter cultures were diluted 1:100 into MES-LB broth ± 3 mM ASN and grown to OD\textsubscript{600} = 1.5, at which point RNA was isolated from each culture and processed separately for hybridization to Affymetrix GeneChip® E. coli Genome 2.0 Arrays. These arrays contain probe sets to detect transcripts from the K-12 E. coli lab strain MG1655, the UPEC isolate CFT073, and two enteropathogenic E. coli isolates, O157:H7-EDL933 and O157:H7-Sakai. Though UTI89 is not specifically represented on the array (or any currently available commercial array), our unpublished genomic analysis indicates that only about 271 of the predicted genes in UTI89 are not represented on the chip, making the array a valuable tool for study of this pathogenic isolate.

Analysis of biological duplicates by microarray showed extensive differences in the transcriptome of ASN-conditioned UTI89 relative to untreated controls. Three hundred and four genes were upregulated at least 5-fold in +ASN cultures while 244 genes were downregulated ≥ 5-fold. A summary of these genes by functional category is provided in Figure 3, and the genes included in each category are listed in Supplementary Table S1. Hypothetical and uncharacterized genes constitute the most numerous class affected, with 55 downregulated and 92 upregulated at least 5-fold. The twenty most upregulated and downregulated transcripts detected after growth of UTI89 in ASN are listed in
Tables 1 and 2, respectively. Notably, no change in expression of the major type 1 pilus subunit FimA was detected in ASN-conditioned UTI89, although transcription of some of the minor type 1 pilus subunits was somewhat elevated. Enhanced expression of other major pilus-related genes, including those encoding P pili and curli, was not detected. In contrast, numerous genes involved in the transport and metabolism of carbohydrates were highly altered (Fig. 3), perhaps indicative of a shift in energy utilization strategies in response to RNI stress-induced respiratory chain damage. These changes may also reflect a shift towards metabolic and catabolic pathways (e.g. the acquisition or generation of substrates for the citric acid cycle) that enhance production of reducing equivalents like NADH and NADPH used by many of the proteins implicated in the detection and detoxification of RNIs. The ASN-induced alterations in carbohydrate transport and metabolism did not correlate with any overt changes in either bacterial colony morphology or encapsulation.

Not surprisingly, many of the genes shown previously to be rapidly upregulated in E. coli K-12 strains upon exposure to various inducers of nitrosative stress are also significantly elevated in ASN-conditioned UTI89 (21, 28, 35, 43, 49). These include norVW (encoding flavorubredoxin and its cognate oxidoreductase; up 6198- and 5572-fold, respectively), ytfE (encoding a protein involved in repair of iron-sulfur clusters; up 624-fold), nrdH (encoding a ribonucleotide reductase; up 24.2-fold), hmpA (encoding a NO-detoxifying flavohemoglobin; up 20.9-fold), sufA (encoding an iron-sulfur cluster assembly protein; up 6.4-fold), soxS (encoding a regulator of oxygen stress responses; up 4.2-fold), and others involved in iron utilization, detoxification reactions, and nitrate/nitrite metabolism. Increased expression was verified by quantitative RT-PCR for three of these genes, norV, hmpA, and nrdH, which showed 12717-, 27-, and 8-fold increases, respectively, compared to 6198-, 20-, and 24-fold by microarray. It should be noted that our array experiments were not designed to directly probe the immediate response of UTI89 to RNIs. Instead, we collected RNA once the bacteria had reached OD$_{600}$ = 1.5 to globally assess the status of ASN-conditioned UTI89. In addition to RNIs, these late log-phase bacteria also
likely encounter many other stresses, including nutrient and oxygen limitations. Of note, growth of UTI89 in the presence of ASN to mid-log phase \(\text{OD}_{600} \approx 0.5\), rather than late log-phase \(\text{OD}_{600} = 1.5\), did not significantly enhance bacterial colonization of the bladder (Supplemental Fig. 1). These data suggest that growth to late log phase helps condition UTI89 for survival within the host on top of, and perhaps independent of, ASN effects.

The transcription of several genes that are altered during the transition of *E. coli* from an aerobic to a micro-aerobic environment (47) are differentially expressed in ASN-conditioned UTI89, as are many genes that are regulated by the oxygen- and NO-sensitive transcriptional regulator FNR (11). Several FNR-regulated genes, including *hmpA* and *ytfE*, are also controlled in part by NsrR, a NO-sensitive member of the Rrf2 family of transcription factors (6, 19, 52, 59). Among the top 20 most upregulated genes in ASN-conditioned UTI89 (Table 1), five are repressed by active NsrR. In addition to *ytfE*, these include *yeaR* (encoding a putative tellurite resistance protein; up 1552-fold), *yoaG* (encoding a protein of unknown function; up 394-fold), and *hcp-hcr* (encoding a hydroxylamine reductase and its associated oxidoreductase; up 57.2- and 761-fold, respectively). Transcription of the NsrR-repressed gene *ygbA*, which encodes another hypothetical protein, was also substantially induced (22-fold).

In total, six of the 12 genes/operons shown in previous studies to be repressed by NsrR in *E. coli* were upregulated in our assays (6, 19, 50). Interestingly, expression of these six NsrR regulon members (*yeaR*, *hcp-hcr*, *ytfE*, *yeaR-yoaG*, *ygbA*, and *hmpA*) is also elevated in the UPEC isolate CFT073 and/or the asymptomatic bacteriuria *E. coli* strain 83972 either within the urinary tract of mice or human volunteers or when grown in urine (54, 58). These findings suggest that many, but not necessarily all, members of the NsrR regulon have key roles in the adaptation of UPEC to both RNIs in vitro and to stresses encountered in vivo within the urinary tract. At least one NsrR-repressed gene, *hmpA*, has a clear role in the resistance of UPEC to RNIs in vitro (data not shown and (60)), but deciphering the specific contributions of NsrR and its regulon...
members to UPEC colonization and survival within the host requires further investigation.

*Cadaverine enhances UPEC colonization of the bladder.* By microarray analysis, we found that multiple genes involved in either the transport or metabolism of polyamines were altered by 5-fold or greater in ASN-conditioned UTI89 (see Figure 3 and Supplementary Table S2). Polyamines are ubiquitous polycationic molecules that can modulate myriad cellular functions, including bacterial stress response and virulence cascades (51, 56). Major polyamines produced by bacteria as well as most other forms of life include putrescine, spermidine, and cadaverine. In *E. coli*, putrescine has been shown to significantly enhance the expression of 309 genes, collectively referred to as the polyamine modulon (65). Of these, we found that 111 (36%) are upregulated in ASN-conditioned UTI89 by 2-fold or greater (Supplementary Table S2).

Previously, we reported that exposure of UTI89 to ASN stimulates cadaverine production, causing levels of this polyamine to increase within 3 h nearly 5-fold relative to controls (7). Cadaverine synthesis is controlled by CadC, an acid-inducible transcriptional regulator of the *cad* operon, which consists of *cadB* (encoding a lysine-cadaverine antiporter) and *cadA* (encoding a lysine decarboxylase) (61). Disruption of any of the *cad* genes abrogates cadaverine synthesis by UTI89 and severely attenuates bacterial growth in 3 mM ASN (7). In contrast, the addition of exogenous cadaverine or other polyamines enhances growth of both wild type UTI89 and the *cad* mutants in ASN. This effect was not due to polyamine-mediated quenching of NO radicals or the reduction of mutation frequency. By microarray analysis, transcription of the *cad* genes was not elevated in ASN-conditioned UTI89, suggesting that the *cad* gene products may be transiently induced early on during growth in ASN, but not later as the bacteria approach stationary phase. Interestingly, expression of a CadA homologue, the so-called “constitutive” lysine decarboxylase encoded by *ldcC* as part of the polyamine modulon, was increased 6.2-fold in ASN-conditioned UTI89 (Supplementary Table S2).
These results suggest that polyamines and members of the polyamine modulon, many of which are stress response genes, are important regulators of UPEC RNI resistance and possibly host colonization. To examine this possibility, we first determined if the ability of cadaverine to enhance growth of UTI89 in the presence of 3 mM ASN was unique to this UPEC isolate. Twenty-eight pyelonephritis and 21 cystitis UPEC isolates were sub-cultured into MES-LB broth containing 3 mM ASN ± 3 mM cadaverine, and the time it took each culture to reach OD$_{600}$ = 0.5 was determined. The concentration of cadaverine used in these assays is below levels that are excreted by UTI89 grown in MES-LB broth (7). As shown in Figure 4A, all but seven of the UPEC isolates grew better in the presence of 3 mM ASN than the K-12 reference strain MG1655. The addition of exogenous cadaverine significantly accelerated the growth of all strains, including MG1655, reducing the time required to reach OD$_{600}$ = 0.5 in ASN-containing broth by 7 to more than 41 h (Figures 4A and B). Overall, cadaverine had an equalizing effect on growth of all tested strains, such that they now all reached OD$_{600}$ = 0.5 with ASN present at more similar rates. No significant differences were observed between the cystitis and pyelonephritis isolates in these assays. Notably, 3 mM cadaverine had no effect on UPEC growth rates in MES-LB in the absence of ASN (data not shown and (7)). Considering the effects of cadaverine on UPEC growth in ASN-containing broth and the ability of ASN-conditioned bacteria to better colonize the host, we next asked if growth of UPEC in the presence of 3 mM cadaverine (without ASN) would be sufficient to enhance UPEC colonization of the bladder. Adult female CBA/J mice were inoculated via transurethral catheterization with UTI89 that had been grown shaking to OD$_{600}$ = 1.5 in MES-LB ± 3 mM cadaverine. Bladders were collected at 12 h and 5 d post-inoculation and bacterial titers were calculated by serial dilution of tissue homogenates. As seen with ASN-conditioned UTI89, bacteria grown in cadaverine-supplemented broth were much
more effective at colonizing the bladder than those grown in MES-LB alone (Figure 4C). Greater than 90% of the mice inoculated with cadaverine-treated UTI89 were colonized, while only ~50% of the mice inoculated with control untreated UTI89 harbored the pathogen at the 12 h time point. A similar, although less substantial, difference was observed at 5 d post-inoculation.

**DISCUSSION**

UPEC likely comes across numerous stresses both within the urinary tract and elsewhere while in transit through and between hosts. Transmission via a fecal-oral route alone entails bacterial passage through several diverse and potentially hostile environments, ranging from the oral pharyngeal cavity, stomach, and intestines, to the vaginal and/or periurethral regions prior to ascension into the urinary tract. In addition to RNIs, potential stresses generated at these sites include oxygen radicals, antibacterial peptides, shearing forces, variations in osmolarity and pH, and changes in oxygen and nutrient availability. Results presented here indicate that RNIs, and probably other stresses, encountered by UPEC prior to entry into the urinary tract can significantly impact the outcome of an infection. Our data show that UPEC can transiently adapt to high concentrations of ASN, and that these ASN-conditioned bacteria can better colonize the bladder, possibly via effects on multiple NsrR-regulated genes and other stress- and polyamine-responsive factors.

Relative to a K12 reference strain, most UPEC isolates have heightened resistance to ASN, and this can be substantially augmented by addition of cadaverine (see Fig. 4). RNIs, as generated by ASN, can induce cadaverine production by UPEC (7), and cadaverine on its own in the absence of ASN can also promote UPEC colonization of the bladder. These results suggest that ASN may prime UPEC for increased survival within the urinary tract in part by stimulating polyamine production. Microarray-based experiments described here indicate that a significant fraction of the polyamine modulon is upregulated in
ASN-conditioned UTI89. Many of these genes encode stress response factors that may enable UPEC to better handle the multitude of host defenses in play during the course of a UTI. Polyamines may also increase the virulence potential of UPEC by modifying biofilm formation, affecting the conductivity of outer membrane porins, and/or altering the expression of secreted toxins and iron chelating agents (51, 56). Notably, polyamines are abundant within the intestinal tract (13, 46) and can also be found in substantial quantities within vaginal secretions (9, 34), where they may interact with and phenotypically modulate UPEC prior to entry into the urinary tract. UPEC may also encounter high levels of polyamines within the urinary tract, where urine polyamine concentrations can be elevated as a consequence of pregnancy or UTI (1, 55).

Our data indicate that UTI89 grown to late log-phase in shaking MES-LB broth cultures have only about a 50% chance of effectively colonizing the mouse urinary tract, suggesting that successful infections in this experimental setup are stochastic events, possibly dependent upon random on and off switching of one or more genes within individual bacteria. By maintaining a phenotypically diverse population under relatively hospitable conditions (i.e. aerated MES-LB broth cultures), UPEC may enhance its chances of survival should its environment take a turn for the worse. We suggest that ASN and cadaverine can act as signals, causing a phenotypic shift of the culture population as a whole so that it possibly becomes less diverse, but is overall better suited to handle the rigors of the urinary tract. During a natural infection, UPEC interactions with polyamines or stresses such as RNIs may similarly reprogram the microbes, enhancing their ability to colonize the host. Our microarray analysis indicates that this reprogramming, or conditioning phenomenon, likely involves alteration of multiple regulatory and metabolic pathways, including those involved in stress responses, carbohydrate transport and catabolism, and membrane permeability.

While pre-exposure of UPEC to RNIs and polyamines can facilitate UPEC colonization of the bladder, it is likely that bacterial adaptation to other environmental factors and stressors can have similar or even opposing effects. For example, we found that acclimatization of UTI89 to osmotic stress during...
growth in high salt medium (LB broth with 5% NaCl) renders the pathogen unable to effectively colonize the bladder, in sharp contrast to results obtained with ASN-conditioned UPEC (data not shown). Thus, different stresses encountered by UPEC both within and outside of the urinary tract can affect the establishment and progression of a UTI in profoundly different ways. Ultimately, a greater understanding of how UPEC assimilates and responds to assorted environmental cues and stresses may bring to light novel approaches to prevent and combat UTIs.

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REFERENCES


FIGURE LEGENDS

Figure 1. UTI89 adapts to growth in 3 mM ASN.
(A) Diagram of the experimental protocol used. Colonies of UTI89 grown from a freezer stock were used to start overnight cultures in MES-LB without ASN. For each round shown in the diagram, cultures were grown shaking in tubes at 37°C, and allowed to reach stationary phase before 1:100 subculture into new media with or without ASN as indicated. Culture growth was monitored by optical density at 600 nm (OD$_{600}$).
(B) Growth curves of +ASN cultures indicated in diagram A, demonstrating the difference in lag time between cultures conditioned and unconditioned to 3 mM ASN. Error bars represent standard deviation from the mean of five independent cultures.

Figure 2. Enhanced colonization of the bladder by ASN-conditioned UPEC.
Adult female CBA/J mice were inoculated with $10^7$ CFU of UTI89 that had been grown to OD$_{600} = 1.5$ in MES-LB ± 3 mM ASN. Bacterial titers in bladder homogenates were determined at 12 h or 5 d post-inoculation. Horizontal bars indicate median values for each group. The indicated P values were determined using Fisher’s exact test (n = 13 to 22 mice).

Figure 3. Functional classification of transcripts affected ≥ 5-fold by 3 mM ASN in the growth medium, as determined by microarray analysis. Some transcripts were identified by multiple probe sets as listed in supplementary Table S1, but for clarity each transcript is represented only once in this figure.

Figure 4. Cadaverine stimulates UPEC growth in ASN and colonization of the bladder.
(A) MG1655 (blue) along with 28 pyelonephritis (orange) and 21 cystitis (yellow) isolates were diluted from overnight MES-LB broth cultures into fresh medium containing 3 mM ASN ± 3 mM cadaverine. The top of each bar (-cad) indicates the time required for each individual isolate to reach OD$_{600} = 0.5$ in ASN-
containing broth, while the bottom of each bar (+ cad) denotes the time required to reach the same OD with both ASN and cadaverine present.

(B) Data presented in (A) has been grouped to more clearly show the overall effect of cadaverine (cad) on growth of the UPEC isolates in the presence of 3 mM ASN. The indicated $P$ values were calculated using the Mann-Whitney U test.

(C) Adult female CBA/J mice were inoculated with $10^7$ CFU of UTI89 that had been grown to $OD_{600} = 1.5$ in MES-LB ± 3 mM cadaverine. Bacterial titers in bladder homogenates were determined at 12 h and 5 d post-inoculation. The indicated $P$ values were determined using Fisher’s exact test ($n = 10$ to 22 mice). Horizontal bars in both (B) and (C) indicate median values for each group.
Table 1. 20 most upregulated transcripts in ASN.

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<sup>1</sup> Ratio of expression in +ASN cultures relative to –ASN.

<sup>2</sup> When different probe sets for a single gene yielded different results, all are listed.
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