Polyamine-Mediated Resistance of Uropathogenic *Escherichia coli* to Nitrosative Stress

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Received 19 October 2005/Accepted 13 November 2005

During the course of a urinary tract infection, substantial levels of nitric oxide and reactive nitrogen intermediates are generated. We have found that many uropathogenic strains of *Escherichia coli* display far greater resistance to nitrosative stress than the K-12 reference strain MG1655. By selecting and screening for uropathogenic *E. coli* transposon mutants that are unable to grow in the presence of acidified nitrite, the *cadC* gene product was identified as a key facilitator of nitrosative stress resistance. Mutation of *cadC*, or its transcriptional targets *cadA* and *cadB*, results in loss of significant production of the polyamine cadaverine and increased sensitivity to acidified nitrite. Exogenous addition of cadaverine or other polyamines rescues growth of *cad* mutants under nitrosative stress. In wild-type cells, the concentration of cadaverine produced per cell is substantially increased by exposure to acidified nitrite. The mechanism behind polyamine-mediated rescue from nitrosative stress is unclear, but it is not attributable solely to chemical quenching of reactive nitrogen species or reduction in mutation frequency.

Along with its roles in neurotransmission, vasodilation, cell adhesion, and platelet inhibition, nitric oxide (NO) is also a key player in innate immunity. NO is formed by the action of nitric oxide synthases (NOS), which are induced and activated during the course of infections caused by many different pathogens. NO may then be converted to a variety of other damaging reactive nitrogen intermediates (RNIs) such as peroxynitrite and nitrosothiols. These RNIs can inflict extensive damage on the nucleic acids, proteins, and lipids of invading microbes. Thiols, amines, aromatic residues, heme groups, and iron-sulfur clusters are particularly susceptible to attack (9, 10). NOS activity has been shown to be crucial in control of pathogen load in numerous infections, particularly *Salmonella*, *Leishmania*, and *Listeria* spp. and mycobacteria (9).

Other infections which may be influenced by RNIs are those elicited by strains of uropathogenic *Escherichia coli* (UPEC). These bacteria are the primary causative agents of urinary tract infections, including both bladder and kidney infections (3). Within hours of initiation of a bladder infection, levels of nitrite in the urine increase threefold (24), and eventually gaseous levels of NO within the bladder increase 30- to 50-fold over uninfected controls (17). Much of this increase is likely due to the action of endothelial (e) NOS in the bladder, which has been shown to be upregulated and activated by intraperitoneal injection of *E. coli* lipopolysaccharide (14). The bacteria themselves, which can produce NO through nitrite reductases under conditions of low oxygen tension (7), may also contribute to the high levels of RNIs in an infected bladder. Like mycobacteria and several other pathogens that are known to be kept in check by RNIs, UPEC can persist long-term within a host as an intracellular pathogen and may lie in wait in

quiescent reservoirs between recurrences of acute infection (19).

Considering the high levels of RNIs generated during the course of a UTI, we reasoned that UPEC, like many other pathogens, may have evolved mechanisms to resist the damaging effects of NO and its derivatives. Here we report on a UPEC isolate that has increased resistance to RNIs relative to a nonpathogenic K-12 reference strain. To identify factors involved in resistance of this UPEC isolate to nitrosative stress, we utilized a genetic selection and screening approach that identified a transcriptional regulator of polyamine synthesis. In addition, we demonstrate that polyamines are key factors in mediating RNI resistance and that production of the polyamine cadaverine is upregulated upon exposure to RNIs. To elucidate the mechanism of UPEC resistance to nitrosative stress, we test two mechanisms by which polyamines have been reported to protect against oxidative stress: chemical quenching and protection against mutagenesis.

MATERIALS AND METHODS

Materials. Sodium nitrite, MES (morpholineethanesulfonic acid), polyamines, and antibiotics were obtained from Sigma-Aldrich (St. Louis, MO). IPTG (isopropyl- β -D-thiogalactopyranoside) was purchased from Teknova (Hollister, CA).

Strains and mutagenesis. MG1655 and UTI89 have been described previously (2, 19). UTI89 were transposon-mutagenized according to kit protocol (EZ-Tn5 <R6K γ /KAN-2>Tnp Transposome Kit, Epicenter, Madison, WI). Targeted gene knockouts were created in UTI89 by Lambda Red-mediated linear transformation (8, 20) and verified by PCR of the affected region. Primer sequences are provided in Table 1.

Growth conditions. All growth experiments were performed in 100 mM MES-buffered LB (pH 5), with or without added acidified sodium nitrite (ASN; sodium nitrite in 100 mM MES-buffered LB [pH 5]). Fresh MES-LB starter cultures were grown to stationary phase and then subcultured 1:100 into 6 ml of MES-LB in capped 20-by-150-mm borosilicate glass tubes. Cultures grew with shaking (225 rpm, tilted at a 30° angle) at 37°C, and optical densities at 600 nm (OD₆₀₀) were determined at the indicated times.

Identification of ASN-sensitive UTI89 mutants. Pools of transposon-mutagenized UTI89 mutants were cultured in 3 mM ASN (a concentration that is bacteriostatic for K-12 strains) for 1 h and then treated with 0.5 mg of penicillin

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TABLE 1. Primers used in this study

Primer ^a	Sequence (5' to 3') ^b
CadA	
KO-F	ATGAACGTTATTGCAATATTGAATCACATGGGGGTTTATTTTAAAGAAGATGTGTAGGCTGGAGCTGCTTCG
KO-R	TTATTTTTTGCTTTCTTCTTTCAATACCTTAACGGTATAGCGGCCATCAGCATATGAATATCCTCCTTAG
Ver-F	GCAATATTGAATCACATGGGGG
Ver-R	CAATACCTTAACGGTATAGCGG
CadB	
KO-F	ATGAGTTCTGCCAAGAAGATCGGGCTATTTCCTGTACCGGTGTTGTTGCTGTGTAGGCTGGAGCTGCTTCG
KO-R	TTAATGTGCGTTAGACGCGGTGTGTTATCCATTGAGTGGCTCTGGCGCTCATATGAATATCCTCCTTAG
Ver-F	CCAAGAAGATCGGGCTATTTCG
Ver-R	GTTAGACGCGGTGTGTTATC
CadC	
KO-F	AAAAAATAGGTTATGGCAATTATCATTGCATCATTCCTTTTCGAATGAGTGTGTAGGCTGGAGCTGCTTCG
KO-R	ACCCGGGAGTTACTTATTCTGAAGCGAGAAATTTGTCGAGATAAGGTACACATATGAATATCCTCCTTAG
Ver-F	GGCAATTATCATTGCATCATTCCT
Ver-R	GGGAGTTACTTATTCTGAAGCG
Ldc	
KO-F	ATCGCCACGGTTTGAGCAGGCTATGATTAAGGAAGGATTTTCCAGGAGTGTGTAGGCTGGAGCTGCTTCG
KO-R	GTCTCCTTTATTGTAAACAGCACGTTACTCGCCCGGAAGCCGCTCTGCATATGAATATCCTCCTTAG
Ver-F	GTTCTGAAAAGGGTCACTTC
Ver-R	GGTGAACCTGTTTAAACCC

^a KO and Ver primers are used for knockout and verification of knockout, respectively. F and R refer to forward and reverse primers, respectively.

^b Underlined portions of the primers are not specific to the target gene but allow amplification of the antibiotic cassettes of plasmids pKD3 and pKD4 of the lambda red knockout system (8).

G/ml to kill any dividing bacteria. Mutant pools were subjected to five rounds of this selection, and then screened individually for verification of sensitivity to ASN. Sites of transposon integration were identified by arbitrary PCR and sequencing as previously described (22).

Quantitation of cadaverine. Cadaverine was quantified by using a colorimetric assay as previously described (23, 25), with the following modifications. A total of 200 μ l of total bacterial culture was thoroughly vortexed with 50 μ l of chloroform and then mixed with 400 μ l of 0.5 M K_2CO_3 –5.1 mM 2',4',6'-trinitrobenzylsulfonic acid, and incubated for 5 min at 42°C. Then, 800 μ l of toluene was used to extract the colored product, *N,N'*-bistrinitrophenylcadaverine, which was quantified by measuring the absorbance at 340 nm. Standard curves show that the assay is linear between cadaverine concentrations of 0.1 to 7 mM. When appropriate, titers of the cultures used in the cadaverine quantitation assays were determined to calculate the number of CFU present.

RESULTS AND DISCUSSION

UTI89 is more resistant to ASN stress than MG1655. A well-established system for generating RNIs using ASN was used to examine bacterial RNI resistance in vitro (11, 18). When added to LB buffered to pH 5 with 100 mM MES, sodium nitrite dismutates to nitrous acid, which can then form nitric oxide and other RNIs (1). The pH of the media is not altered by addition of ASN. As shown in Fig. 1, the uropathogen UTI89 and the K-12 reference strain MG1655 grow similarly in unsupplemented MES-buffered LB (pH 5). However, addition of 3 mM ASN to the media reveals a differential phenotype: UTI89 is able to recover from ASN treatment far more readily than the nonpathogenic strain MG1655. In keeping with the reported bacteriostatic nature of acidified nitrite on *E. coli* (16), we found that MG1655 was not killed in ASN but merely unable to grow. Ongoing screens indicate that resistance to 3 mM ASN is not unique to UTI89 and is fairly common among UPEC isolates.

ASN resistance in UPEC requires the *cad* gene cluster. To investigate the mechanism of UTI89 resistance to RNIs, we

performed a genetic selection and screen for loss of the resistance phenotype. Pools of transposon-mutagenized UTI89 were cultured with ASN and penicillin. Because penicillin kills only dividing bacteria, this treatment selected for UPEC mutants which had lost resistance to ASN. Although this initial screen was not saturating, it yielded several mutants with reduced ASN resistance. One transposon insertion which produced sensitivity to ASN was in the gene *cadC*. CadC is the acid-inducible transcriptional activator of the *cad* operon, consisting of *cadB*, which encodes a lysine-cadaverine antiporter, and *cadA*, which encodes lysine decarboxylase (30). Plasmid-

FIG. 1. The effect of 3 mM ASN on growth of *E. coli* strains UTI89 and MG1655. Fresh stationary phase starter cultures were subcultured 1:100 into 100 mM MES-buffered LB (pH 5) with or without added ASN. The cultures were grown with constant shaking at 37°C, and the OD₆₀₀ values were determined at the indicated times.

FIG. 2. CadC expression from pPH2200 complements the mutant phenotype of the *cadC* transposon mutant, and $\Delta cadA$, $\Delta cadB$, and $\Delta cadC$ UTI89 mutants are attenuated for growth in the presence of 3 mM ASN. (A) ODs were determined 24 h after subculture into media containing 3 mM ASN and 1 mM IPTG, plus 50 μ g of ampicillin/ml for the plasmid-bearing strain. (B) ODs were determined 24 h after subculture into medium containing 3 mM ASN. Bars show means $\pm$ the standard deviation of three replicates.

borne *cadC* (pPH2200), (30) complements the mutant phenotype, verifying the identity of the transposon mutation (Fig. 2A). Mutants with targeted deletion of *cadA*, *cadB*, or *cadC* all phenocopy the original transposon *cadC* mutation, demonstrating that the effects of the mutation are due to disruption of function of the *cad* operon and not some other unidentified target of the *cadC* transcriptional activator (Fig. 2B). Deletion of *ldc*, which encodes a second, more weakly expressed lysine decarboxylase in *E. coli* (15), did not affect the resistance of UTI89 to ASN. It should be noted that the actual time to recovery in ASN by wild-type UTI89 was variable between experiments, but the severely attenuated growth of MG1655 and the uncomplemented UTI89 *cad* mutants was invariant.

The lysine decarboxylase *cadA* converts lysine to cadaverine, a polyamine. Other major polyamines include putrescine, spermine, and spermidine. A succinct description of this class of nitrogenous bases was provided by Igarashi and Kashiwagi, who referred to polyamines as “mysterious modulators of cellular functions” (13). Polyamines are required for normal growth of all cell types tested thus far and can affect the function of proteins, DNA, and RNA, but the mechanisms behind most of their effects have not yet been fully elucidated. The most often cited role for cadaverine in bacteria is in acid

tolerance. A low pH environment causes activation of the transcriptional regulator *cadC* (21) and the resultant lysine decarboxylase, *cadA*, consumes a proton while producing cadaverine. The antiporter *cadB* takes up lysine and excretes cadaverine, allowing the consumption of protons (and therefore modulation of pH within the microenvironment) to continue (26). In LB buffered with 100 mM MES at pH 5, the pH of wild-type and $\Delta cadA$ UTI89 cultures varied by at most 0.1 pH unit during the course of the experiments, casting doubt on the possibility that the pH modulating activity of the *cad* operon is crucial in this environment.

Exogenous polyamines promote UPEC growth in ASN. As shown in Fig. 3A, we found that addition of exogenous cadaverine, or other polyamines, can rescue the growth of $\Delta cadA$ (and other *cad* mutant) cultures in the presence of ASN. This contrasts against the role of the *cad* operon in acid tolerance, where the process of cadaverine synthesis—the consumption of a proton—is more important than the cadaverine itself. In control experiments, the addition of exogenous cadaverine in the absence of ASN had only modest effects on the growth of either wild-type UTI89 or the $\Delta cadA$ mutant (Fig. 3B). Our results echo those of Chattopadhyay et al. (5), which showed that polyamines, whether endogenous or exogenous, protect *E. coli* from the toxic effects of oxygen by unknown mechanisms.

It is important to note that polyamines are apparently only one player in ASN stress resistance. As shown in Fig. 4A, the ASN-sensitive strain MG1655 also produces cadaverine, although only approximately half as much as UTI89. Virtually no cadaverine produced by either UTI89 or MG1655 was found associated with bacterial cell pellets, indicating that nearly all of the cadaverine produced by these strains is excreted. Addition of exogenous cadaverine (or spermine, spermidine, or putrescine) does enhance growth of MG1655 in ASN, but to a lesser degree than it affects the UTI89 *cad* mutants. When grown in 3 mM ASN and 3 mM cadaverine, MG1655 entered exponential phase at around the time that UTI89 $\Delta cadA$ entered stationary phase. Other as-yet-unidentified differences between MG1655 and UTI89, which share only ~56% of their protein coding sequences (C.-S. Hung and S. J. Hultgren, personal communication), undoubtedly also affect their differential growth phenotypes in ASN.

ASN treatment stimulates cadaverine production. Because exogenous cadaverine promotes growth in ASN, we wondered whether endogenous production of the polyamine is responsive to ASN stress. We found that exposure to 1 mM ASN (a molarity which slows, but does not halt, bacterial growth) does cause an increase in the amount of cadaverine produced per bacterial CFU in wild-type UTI89 (Fig. 4B). Within 3 h of subculture into ASN-containing media, there is a difference of >5-fold in the amount of cadaverine produced per CFU. We propose that this increase in production on a per-cell basis benefits all bacteria in the culture, since the majority of cadaverine produced appears to be secreted. Similar upregulation of cadaverine synthesis in response to 1 mM ASN is observed in the more ASN-sensitive strain MG1655, further emphasizing that factors other than cadaverine must also influence ASN resistance.

Cadaverine does not rescue by chemical quenching. One possibility for the mechanism of rescue by polyamines is simple quenching: RNIs react with amine groups, which are plentiful

FIG. 3. Rescue of the Δ cadA mutant by polyamines. (A) ODs of cultures with (+) or without (–) 3 mM ASN and with or without the indicated polyamines at 17 h after subculture with UTI89 or the isogenic Δ cadA mutant. This time point captures wild-type UTI89 (+ASN) in exponential growth phase and shows that spermine and spermidine are more effective than either cadaverine or putrescine in rescuing bacterial growth in ASN. Wild-type UTI89 and the polyamine-supplemented Δ cadA mutant all eventually recover fully in the presence of ASN, whereas the unsupplemented Δ cadA mutant invariably fails to multiply appreciably with ASN present. Bars show means $\pm$ the standard deviation of three replicates. (B) In the absence of ASN, wild-type and Δ cadA mutant cultures have very similar growth kinetics, and the addition of 3 mM cadaverine (cad) has little effect on bacterial growth. The datum points show the means of three replicates. For clarity, error bars are not shown, but the standard deviation for all points is less than 0.03 OD₆₀₀ units.

in polyamines, and so perhaps polyamines act as RNI scavengers. This hypothesis does have a precedent: the polyamine spermine has been shown to protect DNA from hydrogen peroxide-induced damage by acting directly as a free-radical scavenger (12). To determine whether cadaverine benefits bacterial growth in ASN by simply quenching the RNIs, the effects of coincubating cadaverine with ASN for 12 h prior to addition of the RNI-sensitive Δ cadA mutant UTI89 bacteria was tested as outlined in Fig. 5A. The cadaverine and ASN concentrations tested were those which have shown biologically relevant effects: 3 mM cadaverine rescues bacterial growth in 3 mM ASN (see Fig. 3A). Rather than enhancing growth of the Δ cadA mutant, as would be expected if RNI quenching occurred, prior coincubation of cadaverine with ASN instead actually reproducibly slowed bacterial growth slightly compared to samples in which ASN was not first coincubated with cadaverine (Fig. 5B). These data indicate that cadaverine does not facilitate bacterial growth in the presence of ASN by quenching RNIs.

This conclusion is in keeping with our observation that MG1655 is able to produce substantial amounts of cadaverine but still remains sensitive to 3 mM ASN.

Cadaverine does not decrease mutation frequency. Positively charged polyamines interact strongly with DNA and have been reported to affect the activity of many DNA-modifying enzymes such as polymerases and topoisomerases (6, 27). Given that RNIs can damage DNA (4, 10), we wondered whether polyamines may serve to reduce the frequency of ASN-induced mutation, as has been shown to occur with paraquat-induced oxidative stress (28, 29). This hypothesis was tested by measuring the frequency of mutation to rifamycin resistance (acquired by mutation of the *rpoB* gene) of wild-type and Δ cadA UTI89 growing in MES-LB (pH 5) with or without 1 mM ASN. This concentration of ASN does slow the growth of the Δ cadA mutant relative to the wild type, but both cultures undergo the same number of doublings and reach equivalent densities at stationary phase within the time course of the

FIG. 4. Quantitation of cadaverine production. (A) Comparison of total cadaverine generated by MG1655, UTI89, and the $\Delta cadA$ UTI89 mutant grown for 3 h in unsupplemented MES-buffered LB (pH 5). (B) Time course of cadaverine produced per CFU of wild-type UTI89 with or without added 1 mM ASN in the media. Bars show means $\pm$ the standard deviation of three replicates. *, Cadaverine levels below the minimal level of detection for the assay.

experiment. Although growth in ASN does increase the mutation frequency of both wild-type and $\Delta cadA$ bacteria beyond that observed without nitrosative stress, the results presented in Fig. 6 demonstrate that both strains experience the same level of mutagenesis. The inability to produce significant amounts of cadaverine did not notably affect mutation frequency. Similar results were obtained in assays for development of auxotrophic mutations (data not shown).

Concluding remarks. One clear lesson from the current polyamine literature is that these small molecules can have maddeningly pleiotropic effects on biological functions. The work presented here represents the first demonstration that, among their various effects, polyamines are also key mediators of resistance to nitrosative stress in bacteria. Under acidic conditions, such as those used in these studies, *cadC* expression and consequently cadaverine synthesis are strongly induced (21). In contrast, none of the other polyamine biosynthetic pathways in *E. coli*, including those for spermidine and putrescine production, are acid inducible (6). As a result, cadaverine is likely the prominent polyamine generated by UTI89 in low-pH environments. This may explain why disruption of the *cad* gene cluster is sufficient to abrogate endogenous polyamine-dependent resistance to ASN and why exogenously added polyamines can overcome this defect. Interestingly, we

FIG. 5. Cadaverine does not rescue growth of $\Delta cadA$ UTI89 in ASN by quenching RNI activity. (A) Diagram of the experimental protocol used. The final concentrations of both ASN and cadaverine in the indicated cultures were 3 mM. (B) $\Delta cadA$ UTI89 grows normally in MES-LB (pH 5) (◆) but fails to multiply in the presence of 3 mM ASN (■). Coincubating ASN with cadaverine for 12 h prior to addition of bacteria did not reduce its inhibitory activity (○) and actually rendered the medium slightly more attenuating to growth of the $\Delta cadA$ mutant than media in which there was no prior coincubation of ASN with cadaverine (▲). These results were consistent in three separate experiments and indicate that the protective effect of cadaverine is not via an RNI quenching mechanism.

FIG. 6. Cadaverine does not abrogate the mutagenic effects of ASN. Wild-type UTI89 and the $\Delta cadA$ mutant were grown in MES-LB (pH 5) with or without 1 mM ASN for 24 h prior to plating on selective (plus 100 μ g of rifamycin/ml) and nonselective LB agar. Mutation frequencies were determined by dividing the number of rifamycin-resistant colonies by the total number of bacteria present in each sample. Bars show the means $\pm$ the standard deviation of three replicates.

have also shown that cadaverine production is actually amplified by exposure to ASN. How ASN stimulates cadaverine production is currently unknown. However, it is likely that this phenomenon further enhances UPEC survival in the face of RNIs generated during course of a UTI.

The mechanism(s) by which cadaverine or any polyamine mediates UPEC resistance to nitrosative stress remains unknown. However, in the present study we have ruled out two prime potential resistance mechanisms known to be applicable to oxidative stress, demonstrating that polyamines do not appreciably quench RNIs and that polyamines do not provide enhanced resistance to the mutagenic effects of RNIs. Whether or not cadaverine plays any role in modulating the expression of stress response or other genes in UPEC after exposure to RNIs remains unknown. As noted previously, the fact that the ASN-sensitive strain MG1655 does produce significant amounts of cadaverine indicates that factors other than polyamine production are at play. We are currently investigating what these other factors might be, as well as whether ASN resistance by UPEC isolates has any correlation to clinical outcome, including persistence and recurrence of the infection.

ACKNOWLEDGMENTS

pPH2200 was made by Eric Olson and kindly provided by Jia Liu (Pfizer).

J.M.B. was supported by National Institutes of Health Genetics Training Grant T32-GM07464. This study was supported by grants DK068585 and DK069526 from the National Institutes of Health.

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