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Adenylate Cyclase and the Cyclic AMP Receptor Protein Modulate Stress Resistance and Virulence Capacity of Uropathogenic *Escherichia coli*

Grant T. Donovan, J. Paul Norton, Jean M. Bower,* Matthew A. Mulvey

Division of Microbiology and Immunology, Pathology Department, University of Utah School of Medicine, Salt Lake City, Utah, USA

In many bacteria, the second messenger cyclic AMP (cAMP) interacts with the transcription factor cAMP receptor protein (CRP), forming active cAMP-CRP complexes that can control a multitude of cellular activities, including expanded carbon source utilization, stress response pathways, and virulence. Here, we assessed the role of cAMP-CRP as a regulator of stress resistance and virulence in uropathogenic *Escherichia coli* (UPEC), the principal cause of urinary tract infections worldwide. Deletion of genes encoding either CRP or CyaA, the enzyme responsible for cAMP synthesis, attenuates the ability of UPEC to colonize the bladder in a mouse infection model, dependent on intact innate host defenses. UPEC mutants lacking cAMP-CRP grow normally in the presence of glucose but are unable to utilize alternate carbon sources like amino acids, the primary nutrients available to UPEC within the urinary tract. Relative to the wild-type UPEC isolate, the *cyaA* and *crp* deletion mutants are sensitive to nitrosative stress and the superoxide generator methyl viologen but remarkably resistant to hydrogen peroxide (H_2O_2) and acid stress. In the mutant strains, H_2O_2 resistance correlates with elevated catalase activity attributable in part to enhanced translation of the alternate sigma factor RpoS. Acid resistance was promoted by both RpoS-independent and RpoS-dependent mechanisms, including expression of the RpoS-regulated DNA-binding ferritin-like protein Dps. We conclude that balanced input from many cAMP-CRP-responsive elements, including RpoS, is critical to the ability of UPEC to handle the nutrient limitations and severe environmental stresses present within the mammalian urinary tract.

Under homeostatic conditions, the mammalian urinary tract is maintained as a sterile environment through the production of antimicrobial peptides and other toxic compounds, the bulk flow of urine, innate immune cell surveillance mechanisms, and nutrient limitations (1–5). However, select microbial pathogens are capable of colonizing and infecting this normally inhospitable niche. Uropathogenic *Escherichia coli* (UPEC) is the major cause of urinary tract infections (UTI) worldwide, affecting millions and requiring billions of dollars for diagnosis and treatment annually (6). To overcome host defenses and effectively colonize the urinary tract, UPEC employs a variety of virulence factors and stress resistance mechanisms, including adhesive and motility organelles that mediate attachment to and invasion of host cells, toxins that disarm innate immune responses, and multiple iron-scavenging proteins (1, 7–9). The ability to sense and prioritize the use of limited carbon sources within the nutrient-poor environment of the urinary tract is also likely critical to the success of UPEC, but our understanding of the impact that bacterial metabolic pathways have on the establishment and progression of a UTI is incomplete.

Within the urinary tract, UPEC relies largely on the catabolism of small peptides and amino acids for survival and growth (4). UPEC strains that are defective in peptide and carbohydrate transport systems, the tricarboxylic acid (TCA) cycle, and gluconeogenesis are unable to effectively colonize the urinary tract (10, 11). Of note, many UPEC isolates are able to utilize the gluconeogenic amino acid D-serine, which is typically present in urine (12, 13). D-Serine not only provides substrates for the TCA cycle and gluconeogenesis but also serves as an environmental cue that can regulate UPEC virulence. Interplay between bacterial metabolism and virulence is also evident by analysis of the QseBC two-component regulatory system, which was recently shown to modulate carbon flux through key metabolic pathways as well as the expression of UPEC-associated virulence factors like type 1 pili and flagella (14, 15). It is likely that numerous other systems help coordinate the expression of virulence and stress resistance factors with the ability of UPEC to sense and respond to the various carbon sources encountered within the host.

Although *E. coli* strains are generalists with the capacity to metabolize myriad metabolites, they preferentially utilize glucose as a primary carbon source. Transitioning into glucose-limiting conditions triggers the activation of the adenylate cyclase CyaA, producing high levels of the second messenger molecule cyclic AMP (cAMP) (16). Binding of cAMP to the transcription factor cAMP receptor protein (CRP) forms the active cAMP-CRP complex, which directly regulates expression of genes necessary for utilization of alternative carbon sources. However, not all genes that are regulated by cAMP-CRP function in bacterial metabolism (17, 18). Disruption of cAMP signaling within the prominent pathogens *Vibrio cholerae*, *Mycobacterium tuberculosis*, *Salmonella enterica* serovar Typhimurium, and *Pseudomonas aeruginosa* atten-

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Address correspondence to Matthew A. Mulvey, mulvey@path.utah.edu.
* Present address: Jean M. Bower, Biotechnology Department, Salt Lake Community College, West Jordan, Utah, USA.
G.T.D. and J.P.N. contributed equally to this article.

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TABLE 1 Bacterial strains and plasmids

Strain or plasmid	Description	Source
Strains		
UTI89	UPEC strain (cystitis isolate, O18:K1:H7)	25
UTI89Δ*cyaA*	UTI89*cyaA*::Clm^r (pKD3)	This study
UTI89Δ*crp*	UTI89*crp*::Clm^r (pKD3)	This study
UTI89Δ*dps*	UTI89*dps*::Kan^r (TT23691)	This study
UTI89Δ*fimH*	UTI89*fimH*::Clm^r (pKD3)	83
UTI89Δ*otsBA*	UTI89*otsBA*::Kan^r (pKD4)	This study
UTI89Δ*rpoS*	UTI89*rpoS*::Kan^r (TT23691)	This study
UTI89Δ*cyaA*Δ*otsBA*	UTI89*cyaA*::Clm^r (pKD3) *otsBA*::Kan^r (pDK4)	This study
UTI89Δ*crp*Δ*rpoS*	UTI89*crp*::Clm^r (pKD3) *rpoS*::Kan^r (TT23691)	This study
UTI89Δ*crp*Δ*dps*	UTI89*crp*::Clm^r (pKD3) *dps*::Kan^r (TT23691)	This study
TT23691	Strain with Kan^r cassette flanked by universal primer sites	84
Plasmids		
pRR48	Amp^r cloning plasmid containing an IPTG-inducible P*tac* promoter upstream of the MCS	26
pKM208	Amp^r plasmid; encodes IPTG-inducible lambda red recombinase	27
pKD3	Template plasmid for gene disruption; contains FRT[a]-flanked Clm^r cassette	28
pKD4	Template plasmid for gene disruption; contains FRT-flanked Kan^r cassette	28
p*crp*	Amp^r plasmid; *crp* (from UTI89) cloned into PstI, KpnI sites of pRR48	This study
p*cyaA*	Amp^r plasmid; *cyaA* (from UTI89) cloned into PstI, KpnI sites of pRR48	This study
p*dps*	Amp^r plasmid; IPTG-inducible expression of *E. coli* Dps	55
p*rpoS*	Amp^r plasmid; *rpoS* (from UTI89) cloned into PstI, HindIII sites of pRR48	This study

[a] FRT, FLP recombination target.

uates virulence through the misregulation of key virulence genes (18–22). Within pathogenic subsets of *E. coli*, the cAMP-CRP complex has been shown to modulate the expression of type 1 pili, major facilitators of bacterial colonization of the bladder mucosa (23). However, the cumulative effects of cAMP-CRP on the virulence potential of UPEC within the urinary tract remain to be elucidated.

Here, we report that the deletion of genes encoding either CyaA or CRP within the UPEC reference strain UTI89 does not affect growth in the presence of glucose but mutants with these deletions are unable to utilize amino acids as the sole carbon source. Furthermore, these mutants are significantly attenuated in the ability to colonize the bladders of mice, dependent upon the presence of intact innate host defenses. In broth culture assays, the *cyaA* and *crp* mutants are both sensitive to reactive nitrogen species and superoxide radicals generated by methyl viologen but highly resistant to hydrogen peroxide (H_2O_2) and acid stress. Resistance of the mutants to H_2O_2 and acid stress is in part attributable to increased translation of the alternate sigma factor RpoS (σ^S) and RpoS-regulated gene products that include catalases and the DNA-binding, iron storage protein Dps. In total, these data indicate that balanced input from cAMP-CRP is critical to the ability of UPEC to catabolize amino acids and appropriately handle harsh environmental stresses, characteristics that are pertinent to bacterial fitness and survival within the urinary tract.

MATERIALS AND METHODS

Bacterial strains and plasmids. All bacterial strains and plasmids are listed in Table 1. The human cystitis isolate UTI89 has been described previously (24, 25). Expression constructs were made using standard molecular biology techniques with the plasmid pRR48 (26). Where indicated, gene expression from the P*tac* promoter in the pRR48 backbone was induced by addition of 0.5 or 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Primers used to construct all plasmids are indicated in Table 2, along with primers used to verify each clone by sequencing. Antibiotics (50 μg/ml kanamycin, 20 μg/ml chloramphenicol, or 100 μg/ml ampicillin) were added to plates and growth media to select for mutants and to maintain plasmids when necessary. Targeted gene knockouts were generated in UTI89 using the lambda Red-mediated linear transformation system (27, 28). Briefly, an antibiotic resistance cassette was amplified from pKD3, pKD4, or the template strain TT23691 with 40-bp overhangs specific to sites at the 5′ and 3′ ends of each target gene. PCR products were introduced via electroporation into UTI89 carrying pKM208, which encodes an IPTG-inducible lambda Red recombinase. Knockouts were confirmed by PCR using primers listed in Table 2.

Mouse infections. Seven- to 9-week-old female CBA/J or C3H/HeJ mice (Jackson Laboratory) were used in accordance with IACUC-approved protocols as previously described (29). Mice were anesthetized using isoflurane inhalation and inoculated via transurethral catheterization with 50 μl of a bacterial suspension containing approximately 1×10^7 bacteria. For these noncompetitive infection assays, UTI89 and isogenic knockout mutants were grown statically for 24 h in Luria-Bertani (LB) broth, pelleted by spinning at 10,000 × *g* for 8 min, and resuspended in phosphate-buffered saline (PBS) prior to inoculation. Bladders were recovered at 6 h or 3 days postinoculation, weighed, and homogenized in 1 ml PBS containing 0.025% Triton X-100. Homogenates were serially diluted and plated on LB agar plates to determine the number of bacteria per gram of tissue. Mouse experiments were repeated at least twice, and the total combined data from at least 11 animals are presented.

Growth assays. Bacteria were grown from frozen stocks at 37°C with shaking overnight in 5 ml of LB broth, 100 mM morpholineethanesulfonic acid (MES)-buffered LB broth (MES-LB broth; pH 5.0), or modified M9 minimal medium (6 g/liter Na_2HPO_4, 3 g/liter KH_2PO_4, 1 g/liter NH_4Cl, 0.5 g/liter NaCl, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, 0.1% glucose, 0.0025% nicotinic acid, 16.5 μg/ml thiamine, and 0.2% casein amino acids). Bacteria were then diluted 1:100 into the appropriate corresponding medium with or without additives as indicated. Growth of quadruplicate 200-μl samples in shaking 100-well honeycomb plates was assessed at 37°C using a Bioscreen C instrument (Growth Curves USA). Stocks of methyl viologen (MV) (also known as paraquat), acidified sodium nitrite (ASN), trehalose, and H_2O_2 were prepared fresh prior to addition to LB or MES-LB broth cultures. Where indicated, IPTG was added to cultures to

TABLE 2 Primer sequences

Primer	Sequence
crp KO[a]	
Forward	GCGCATGGTGCTTGGCAAACCGCAAACAGACCCGACTCTCTGTGTAGGCTGGAGCTGCTTCG
Reverse	CGCGCTACCAGGTAACGCGCCACTCTGACGGGATTAACGACATATGAATATCCTCCTTAG
crp KO confirmation	
Forward	GTATGCAAAGGACGCCACAT
Reverse	TTCGCCAAGCATTAACCCAA
cyaA KO	
Forward	GCGGAATCACAGTCATGACGGGTAGCAAATCAGGCGATACTGTGTAGGCTGGAGCTGCTTCG
Reverse	TACTGCTGCAACAGCGGCGCGTCATGCTCCTGATTGGCAGCATATGAATATCCTCCTTAG
cyaA KO confirmation	
Forward	AACCAGGCGCGAAAAGTGGT
Reverse	CTGAAAGGCGACGAGTGGAT
otsBA KO	
Forward	ATGTCTGTAAAGCGCGTTCTGCGCAACACAATAAGAAATGTGTAGGCTGGAGCTGCTTCG
Reverse	CTACGCAAGCTTAGGAAAGGTAGCAACTTTATCGCGCTGCCATATGAATATCCTCCTTAG
otsBA KO confirmation	
Forward	AGCGAAACGCACTGTCTGAT
Reverse	TTGCCTACGGTGAGTTAAGC
dps KO	
Forward	TTATTCGATGTTAGACTCGATAAACCACAGGAATTTATCCAGGTCGCGAGCACCAAACACCCCCCAAAACC
Reverse	GTGATAGGAACAGCCAGAATAGCGGAACACATAGCTGGTGCTATACTTAGCACACAACCACACCACACCAC
dps KO confirmation	
Forward	GATAGCAGATGGATGCACTA
Reverse	TGACAGTACGCAAAGAGAGC
rpoS KO	
Forward	CCAGCCTCGCTTGAGACTGGCCTTTCTGACAGATGCTTACCACCAAACACCCCCCAAAACC
Reverse	TGCCGCAGCGATAAATCGGCGGAACCAGGCTTTTGCTTGACACACAACCACACCACACCAC
rpoS KO confirmation	
Forward	AATGATGATTGCCGAATGTGACGCTG
Reverse	GCATTGTGTCGTTATGGGCGTAGG
p*crp*	
Forward	CCCCC CTGCAG ATGGTGCTTGGCAAACCGCA
Reverse	CCCCC GGTACC TTAACGAGTGCCGTAAACGA
p*cyaA*	
Forward	CCCCC CTGCAG TTGTACCTCTATATTGAGAC
Reverse	CCCCC GGTACC TCACGAAAAATACTGCTGCA
p*rpoS*	
Forward	CATTC CTGCAG ATGTTCCGTCAAGGGATCA
Reverse	AGTGC AAGCTT TTATTCGCGGAACAGCGCT
pRR48 sequencing primer	
Forward	CTGCTGAAGAGTACTTTGG
Reverse	CCAAAGCTGAAGACATCCAG

[a] KO, knockout.

induce high-level expression of recombinant proteins from p*dps* or p*rpoS*. MacConkey agar and other reagents used in these assays were obtained from Sigma-Aldrich.

Western blots. UTI89, UTI89Δ*cyaA*, and UTI89Δ*crp* were diluted 1:50 from overnight cultures into fresh LB broth and grown with shaking at 37°C until an optical density at 600 nm (OD_{600}) of 0.4 was reached. One milliliter of each culture was pelleted, resuspended in 200 μl B-PER lysis reagent (Thermo Scientific) containing 1 mM phenylmethylsulfonyl fluoride and protease inhibitor cocktail (Roche), and incubated for 15 min at room temperature. Protein concentrations within the lysates were determined using the BCA reagent system (Pierce), and equivalent protein amounts were resolved by SDS-PAGE and transferred to an Immobilon

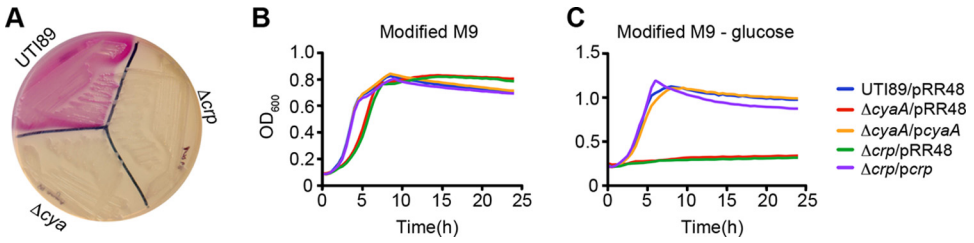

FIG 1 Impaired use of alternative carbon sources by UTI89Δ*cyaA* and UTI89Δ*crp*. (A) MacConkey agar plate streaked with UTI89, UTI89Δ*cyaA*, and UTI89Δ*crp*. Growth of UTI89, UTI89Δ*cyaA*, UTI89Δ*crp*, and complemented mutants in modified M9 medium (B) and modified M9 medium lacking glucose (C). Mutant strains were complemented by uninduced expression of *cyaA* or *crp* from a P*tac* promoter. Strains transformed with the empty vector pRR48 served as controls. Each curve reflects the means of results from a single experiment and is representative of at least three independent experiments performed in quadruplicate.

PVDF-FL membrane (Millipore). Blots were probed using anti-RpoS (Neoclone) and anti-*E. coli* antibodies (Biodesign International) and visualized using enhanced chemiluminescence as previously described (30).

pH stress resistance assays. Bacterial strains from overnight cultures were diluted 1:100 in fresh LB broth and grown with shaking at 37°C for 3 h. LB broth containing 100 μg/ml ampicillin and 0.5 mM IPTG was used for strains carrying plasmids pRR48, p*dps*, or p*rpoS*. Strains were subjected to acid stress (pH 3.5) by the addition of concentrated HCl for 30 min. Bacteria in 1 ml of culture were pelleted at 14,000 × *g* for 5 min and washed in PBS. Surviving bacteria were enumerated by plating serial dilutions on LB agar.

Catalase assays. Overnight bacterial cultures were diluted 1:100 in LB broth and grown with shaking at 37°C to an OD_{600} of 1.0. UTI89/p*rpoS* was grown in broth containing 1 mM IPTG. Bacteria in 1 ml of culture were pelleted, resuspended in 200 μl B-PER lysis reagent (Thermo Scientific), and incubated at room temperature for 15 min. Catalase activity present in the lysates was determined using a Fluoro Catalase kit (Cell Technology) and a Synergy HT multidetection microplate reader (BioTek Instruments, Inc.) according to instructions from the manufacturer.

Trehalose analysis. Chemicals and reagents were purchased from Sigma-Aldrich, except for MSTFA [*N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide], which was purchased from Thermo Scientific, and methoxyamine hydrochloride, which was purchased from MP Biomedicals. Bacterial cultures were grown in modified M9 medium to an OD_{600} of 1.0, pelleted by centrifugation, and frozen. Pellets were suspended in 5 ml of boiling 75% ethanol (EtOH) (aqueous), vortexed, and then incubated at 90°C for 5 min. Cell debris was removed by centrifugation at 5,000 × *g* for 3 min. Supernatants were transferred to new tubes and dried *in vacuo*. Gas chromatography-mass spectrometry (GC-MS) analysis was performed using a Waters GCT Premier mass spectrometer fitted with an Agilent 6890 gas chromatograph and a Gerstel MPS2 autosampler. Dried samples were suspended in 40 μl of a pyridine solution containing 40 mg/ml O-methoxylamine hydrochloride and incubated for 1 h at 30°C. Twenty microliters of each sample was transferred to an autosampler vial and incubated with MSTFA for 30 min at 37°C with shaking. One microliter of sample was injected into the inlet at a 75:1 split ratio. The injector temperature was held at 250°C. The gas chromatograph was obtained using an initial temperature of 95°C for 1 min followed by a 40°C/min ramp up to 110°C, with a hold time of 2 min. This was followed by a second 5°C/min ramp up to 250°C and then a third ramp up to 350°C, with a final hold time of 3 min. A 30-m Restek Rxi-5 MS column with a 5-m-long guard column was employed for analysis. Data were collected using MassLynx 4.1 software. To determine trehalose concentrations specifically, an external calibration curve was developed by performing a 2-fold dilution series starting at 10 μg/μl trehalose in pyridine. This series was analyzed to determine the linear range of analysis, the upper and lower limits of detection and quantitation, and the fragment ion to be utilized for analysis. For quantification, the fragment ion of 331 *m/z* was monitored and the linear range for analysis was determined to be from 100 to 1,000 μg/μl.

Metal stress assay. UTI89/pRR48, UTI89Δ*dps*/pRR48, and UTI89Δ*dps*/p*dps* were grown with shaking overnight at 37°C in LB broth containing 100 μg/ml ampicillin. Nine hundred microliters of each overnight culture was added to a sterile microcentrifuge tube, followed by 100 μl of 0.5 M $CuSO_4$. Tubes were then incubated for 15 min at room temperature, and surviving bacteria were pelleted at 14,000 × *g* for 5 min, washed in PBS, and enumerated by plating serial dilutions on LB agar.

Statistics. Results from *in vivo* mouse assays were analyzed by Mann-Whitney two-tailed *t* tests. Results from the catalase and survival assays were analyzed using two-tailed unpaired *t* tests. Data analysis was performed using Prism 5.0c (GraphPad Software, Inc.). *P* values of less than 0.05 are considered significant.

RESULTS

cAMP-CRP is necessary for lactose and amino acid catabolism by UPEC. Carbon catabolite repression—the preferential use of a carbon source like glucose instead of other secondary carbon sources—is regulated by the generation of cAMP-CRP (16). In the classic example, decreased glucose levels result in enhanced production of cAMP-CRP, which in turn activates the expression of genes needed to catabolize alternate carbon sources like lactose. Using MacConkey agar plates, we established that wild-type UTI89 could consume lactose in the absence of glucose, creating lactic acid and causing the pH indicator neutral red present in the agar to produce a pink color (Fig. 1A). In contrast, the isogenic *cyaA* and *crp* deletion mutants UTI89Δ*cyaA* and UTI89Δ*crp* could not utilize lactose and instead had to ferment available peptone, producing basic ammonia and turning the pH indicator yellow. The ability of the Δ*cyaA* and Δ*crp* mutants to use lactose in these assays was restored by complementation with plasmids p*cyaA* and p*crp*, respectively (data not shown).

Within the urinary tract, UPEC cells are mostly dependent on the catabolism of small peptides and amino acids (10, 12, 13). In modified M9 media containing both glucose and amino acids, wild-type UTI89, UTI89Δ*cyaA*, and UTI89Δ*crp* grew with similar kinetics (Fig. 1B). However, in modified M9 media containing only amino acids as a carbon source, the Δ*cyaA* and Δ*crp* mutants failed to grow unless appropriately complemented with plasmid p*cyaA* or p*crp* (Fig. 1C). The inability of UTI89Δ*cyaA* and UTI89Δ*crp* to catabolize amino acids and other secondary carbon sources such as lactose may affect the fitness of these mutants within the urinary tract.

CRP and CyaA promote UPEC colonization of the bladder. To assess the contribution of cAMP-CRP to UPEC pathogenicity, wild-type UTI89, UTI89Δ*cyaA*, and UTI89Δ*crp* were individually inoculated via transurethral catheterization into adult female

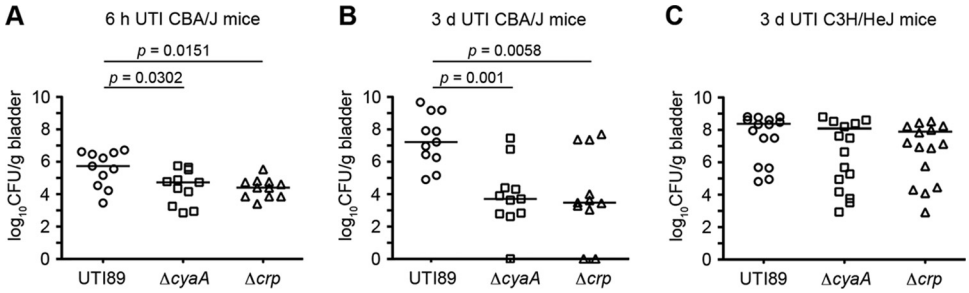

FIG 2 UPEC requires functional cAMP-CRP for virulence in the murine urinary tract. Adult female CBA/J mice (A, B) or C3H/HeJ mice (C) were infected via catheterization with 1×10^7 CFU of wild-type UTI89 or isogenic mutants lacking *cyaA* or *crp*. Graphs show bacterial titers present in the bladder at 6 h (A) and 3 days (B, C) postinoculation. Bars indicate median values for each group; $n \geq 11$ mice. *P* values were determined using Mann-Whitney U tests.

CBA/J mice. In comparison with wild-type UTI89, significantly reduced numbers of both the Δ*cyaA* and Δ*crp* mutants were recovered from bladders at 6 h and 3 days postinoculation (Fig. 2A and B). Interestingly, differences between wild-type UTI89 and the Δ*cyaA* and Δ*crp* mutants were blunted in C3H/HeJ mice (Fig. 2C). Due to defects in Toll-like receptor 4 (TLR4) and possibly other host factors, C3H/HeJ mice have attenuated inflammatory responses and are consequently hypersensitive to UTIs (31–36). In total, these data indicate that cAMP-CRP is critical to the fitness of UPEC within the urinary tract of immunocompetent animals, probably due to regulatory effects of cAMP-CRP on factors that control bacterial resistance to stresses generated by stimulation of host inflammatory cascades. These results prompted us to investigate further potential interplay between cAMP-CRP and stress response mechanisms in UPEC.

UTI89Δ*cyaA* and UTI89Δ*crp* are sensitive to nitrosative stress and methyl viologen but resistant to H_2O_2. Key stresses encountered by UPEC during the course of a UTI include damage elicited by reactive oxygen and nitrogen radicals that can be produced by both host and bacterial cells (37–42). To test the sensitivity of UTI89Δ*cyaA* and UTI89Δ*crp* to nitrosative and oxidative stresses, we utilized acidified sodium nitrite (ASN) and the superoxide generator methyl viologen (MV), respectively (Fig. 3). In these assays, the addition of sodium nitrite to MES-LB broth (pH 5.0) to create ASN results in the production of nitrous acid, NO, and other reactive nitrogen intermediates (43). In MES-LB broth, the Δ*cyaA* and Δ*crp* mutants grew like the wild-type strain, but in the presence of ASN growth of the mutants was markedly attenuated (Fig. 3A and B). Likewise, the addition of MV to LB broth severely impeded growth of both UTI89Δ*cyaA* and UTI89Δ*crp* (Fig. 3D and E). Of note, the mutant cultures did not attain the same optical density as the wild-type pathogen when grown with shaking in nutrient-rich LB broth, but in stationary cultures the mutant and wild-type strains reached equivalent bacterial titers, consistent with results using laboratory K-12 strains (reference 44 and data not shown). While MV impaired growth of UTI89Δ*cyaA* and UTI89Δ*crp*, the same mutants grew remarkably better than the wild-type strain in the presence of H_2O_2 (Fig. 3C). This effect was observed in both MES-LB and regular LB broth cultures, though results were more variable in the latter. Complementation of UTI89Δ*cyaA* and UTI89Δ*crp* with plasmids p*cyaA* and p*crp*, respectively, caused the mutant strains to behave like wild-type UTI89, rendering them sensitive to H_2O_2 and resistant to ASN and MV (Fig. 4 and data not shown).

H_2O_2 resistance correlates with elevated RpoS expression and catalase activity in the absence of cAMP-CRP. cAMP-CRP represses the transcription of the alternate sigma factor RpoS, a master regulator of the general stress response in *E. coli* (45). In

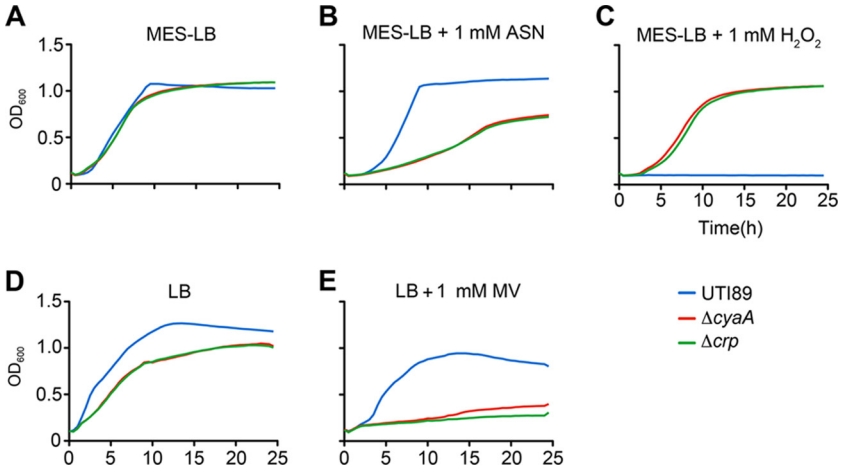

FIG 3 UTI89Δ*cyaA* and UTI89Δ*crp* are sensitive to nitrosative stress and methyl viologen but resistant to H_2O_2. Growth of UTI89, UTI89Δ*cyaA*, and UTI89Δ*crp* in MES-LB broth (A), MES-LB broth + 1 mM ASN (B), MES-LB broth + 1 mM H_2O_2 (C), LB broth (D), and LB broth containing 1 mM MV (E). Growth curves show the means of results from a single experiment and are representative of at least three independent experiments carried out in quadruplicate.

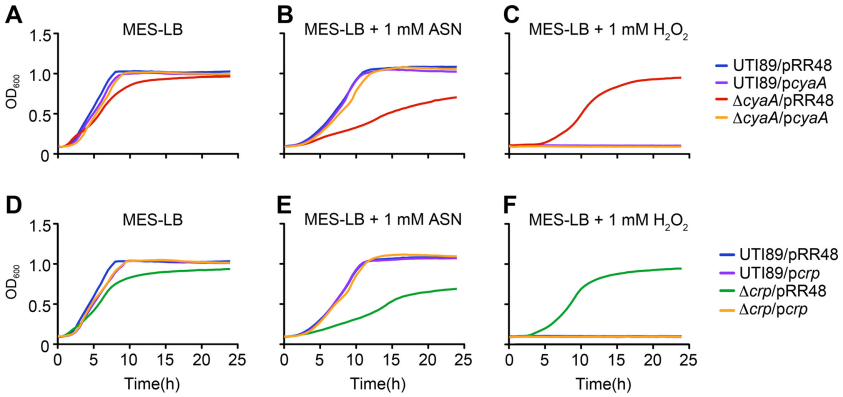

FIG 4 Complementation of UTI89Δ*cyaA* and UTI89Δ*crp* in the presence of nitrosative and oxidative stresses. Graphs show growth of UTI89 versus UTI89Δ*cyaA* (A to C) and UTI89Δ*crp* (D to F) in MES-LB (A, D), MES-LB ± 1 mM ASN (B, E), or MES-LB ± 1 mM H_2O_2 (C, F), all without added IPTG. Strains carried p*cyaA*, p*crp*, or the control plasmid pRR48, as indicated. Growth curves show the means of results from a single experiment and are representative of at least three independent experiments carried out in quadruplicate.

enterohemorrhagic *E. coli* and laboratory K-12 mutant strains that lack cAMP-CRP, RpoS levels are abnormally increased during the exponential growth phase (44, 46, 47). By Western blot analyses, we observed a similar phenomenon, with RpoS levels in UTI89Δ*cyaA* and UTI89Δ*crp* notably increased relative to those of the wild-type strain during exponential growth in broth culture (Fig. 5A). RpoS regulates the expression of various genes that enable bacteria to deal with multiple environmental stresses, including reactive oxygen species like H_2O_2 (45, 48). IPTG-induced expression of RpoS from the plasmid p*rpoS* rescued growth of wild-type UTI89 in the presence of H_2O_2, phenocopying the H_2O_2 resistance seen with the Δ*cyaA* and Δ*crp* mutants (Fig. 5B and C). The high-level resistance of UTI89/p*rpoS*, UTI89Δ*cyaA*, and UTI89Δ*crp* to H_2O_2 correlated with increased catalase activity in these strains (Fig. 5D). These results are in line with previous work showing that RpoS can stimulate expression of stress-responsive catalase genes necessary for the detoxification of H_2O_2 (48, 49).

While these data argue that enhanced expression of one or more RpoS-regulated catalases promotes high-level resistance of UTI89Δ*cyaA* and UTI89Δ*crp* toH_2O_2, it is feasible that other RpoS-regulated genes also contribute to the resistance phenotype of these mutants. To explore this possibility, we investigated two additional loci—*otsBA* and *dps*—known to be regulated by RpoS and previously linked with oxidative stress resistance. The first, *otsBA*, encodes two enzymes used to catalyze the biosynthesis of the disaccharide trehalose, a universal stress protectant produced in abundance by many prokaryotic and eukaryotic organisms (50). In yeast, trehalose protects against oxidative stress caused by H_2O_2 (51, 52). We found that UTI89 mutants lacking cAMP-CRP generate sizeable amounts of trehalose relative to an isogenic Δ*otsAB* mutant or a control mutant strain missing an unrelated gene (Δ*fimH*) (Fig. 5E). However, the addition of exogenous trehalose (up to 3,783 μg/ml) failed to rescue growth of wild-type UTI89 in broth cultures containing 1 mM H_2O_2, and the double deletion mutant UTI89Δ*cyaA*Δ*ostBA* was as resistant to H_2O_2 as UTI89Δ*cyaA* (data not shown). These results indicate that while trehalose levels are greatly elevated in bacteria lacking cAMP-CRP, this phenomenon is likely not essential to the heightened H_2O_2 resistance associated with UTI89Δ*cyaA* and UTI89Δ*crp*.

We next examined Dps, an abundant RpoS- and cAMP-CRP-regulated stationary-phase protein that can protect *E. coli* cells from multiple stresses, including oxidants (44, 46, 53–55). Dps can bind and shield DNA and also has ferritin-like properties, enabling it to sequester and oxidize ferrous ions while detoxifying H_2O_2 in the process (54). In consideration of this information, we hypothesized that forced expression of recombinant Dps would render wild-type UTI89 more resistant to H_2O_2, potentially mimicking UTI89Δ*cyaA* and UTI89Δ*crp*. However, IPTG-induced expression of Dps from plasmid p*dps* had no effect on the growth of either wild-type UTI89 or a Δ*dps* mutant in the presence or absence of H_2O_2 (Fig. 5F and G). Induced expression of Dps did promote survival of UTI89Δ*dps* in a metal ($CuSO_4$) stress resistance assay, confirming the functionality of the p*dps* plasmid (Fig. 5H). In total, these data indicate that increased Dps expression is surprisingly ineffective at promoting H_2O_2 resistance in UTI89.

Acid stress resistance of UTI89Δ*cyaA* and UTI89Δ*crp* is linked with increased RpoS and Dps expression. Within the urinary tract, UPEC will likely come across pH extremes, both within the urine and within host epithelial cells and infiltrating phagocytes (56, 57). In laboratory *E. coli* K-12 strains, cAMP-CRP, RpoS, and Dps can mediate acid stress resistance (58–60). Potential involvement of cAMP-CRP as a regulator of acid stress resistance in UTI89 was assessed using survival assays. Following a 30-min exposure of exponential-growth-phase cultures to acidic (pH 3.5) conditions, we found that the Δ*cyaA* and Δ*crp* mutants had a significant survival advantage over wild-type UTI89 (Fig. 6). In these assays, IPTG-induced expression of recombinant RpoS or Dps was sufficient to enhance survival of the wild-type strain to levels observed with UTI89Δ*cyaA* and UTI89Δ*crp*. To address whether or not RpoS or Dps is necessary for acid resistance, we constructed the mutant strains UTI89Δ*rpoS*, UTI89Δ*dps*, UTI89Δ*dps*Δ*crp*, and UTI89Δ*rpoS*Δ*crp*. In agreement with results observed with other *E. coli* strains (61–63), we found that UTI89Δ*rpoS* is highly sensitive to acid stress (Fig. 6). This sensitivity was reduced nearly 10,000-fold when *crp* was deleted along with *rpoS*. However, the Δ*rpoS* Δ*crp* mutant was still more sensitive than wild-type UTI89 and much more sensitive than UTI89 lacking only *crp*. In contrast, deletion of *dps* had only modest effects on the acid resistance of either UTI89 or UTI89Δ*crp*. Together, these data indicate that the acid stress resistance of UTI89 mutants lacking cAMP-CRP is likely attributable in part to in-

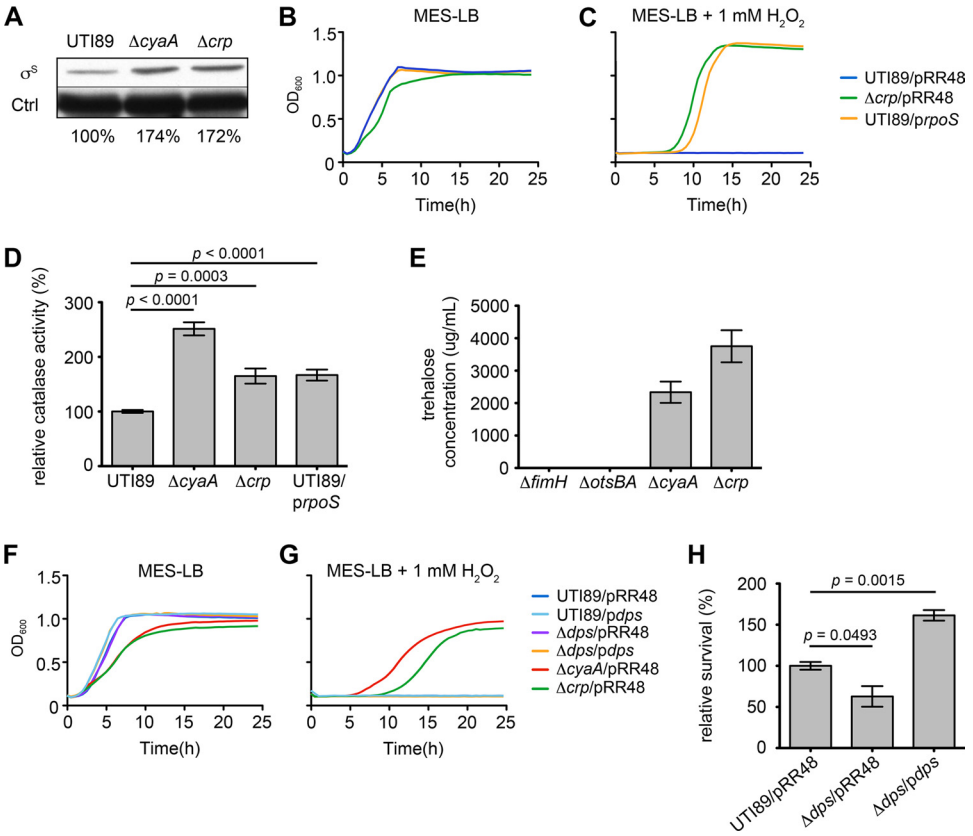

FIG 5 H_2O_2 resistance correlates with increased RpoS expression and catalase activity in UTI89Δ*cyaA* and UTI89Δ*crp*. (A) Western blot of RpoS (σ^S) in UTI89, UTI89Δ*cyaA*, and UTI89Δ*crp* after growth to mid-exponential phase in LB broth. Relative levels of RpoS normalized to loading control (Ctrl) are indicated. (B, C) Curves show growth of UTI89 and UTI89Δ*crp* carrying empty vector pRR48 or p*rpoS*, as indicated, in MES-LB ± 1 mM H_2O_2. (D) Graph of catalase activity in UTI89, UTI89Δ*cyaA*, UTI89Δ*crp*, and UTI89/p*rpoS* following growth to stationary phase in LB broth + 1 mM IPTG. Data are expressed relative to wild-type UTI89 as the means ± standard errors of three independent experiments carried out in triplicate. (E) Levels of trehalose present in UTI89Δ*fimH*, UTI89Δ*otsBA*, UTI89Δ*cyaA*, and UTI89Δ*crp* following growth to stationary phase (OD_{600} = 1.0). The Δ*fimH* mutant carries the same chloramphenicol resistance cassette as the Δ*cyaA* and Δ*crp* mutants and served as the control. (F, G) Graphs show growth of UTI89 and its mutant derivatives (Δ*cyaA*, Δ*crp*, and Δ*dps* mutants) carrying pRR48 or p*dps*, as indicated, in MES-LB ± 1 mM H_2O_2. Each growth curve (B, C, F, and G) shows the means of results from a single experiment and is representative of at least three independent experiments carried out in quadruplicate. Dps and RpoS expression in these assays was induced by addition of 0.5 mM IPTG. (H) Survival of UTI89Δ*dps* complemented with empty vector pRR48 or p*dps* following 15 min of exposure to 0.05 M $CuSO_4$. Results were obtained without addition of IPTG and are presented relative to those of wild-type UTI89/pRR48. The indicated *P* values were determined using two-tailed unpaired *t* tests.

creased cellular levels of RpoS and RpoS-regulated factors like Dps, in addition to other cAMP-CRP-repressible gene products.

DISCUSSION

The misregulation of carbon flux through metabolic pathways can restrict niche availability and alter the virulence potential of *E. coli* and other bacterial species (4, 18, 64–66). cAMP-CRP—a central regulator of carbon metabolism—has been implicated as an important facilitator of host colonization and virulence in many bacterial pathogens, including the uropathogen *Proteus mirabilis* (18, 67). This is not entirely unexpected given the known capacity of cAMP-CRP to modulate far-ranging activities in addition to metabolism (16). Among these is the ability to influence key stress response pathways such as those controlled by OxyR and RpoS (44, 45, 68). Results presented here demonstrate that cAMP-CRP is also critical to the ability of UPEC to effectively colonize the urinary tract.

Earlier work indicated that *E. coli* strains that are deficient in the production of cAMP-CRP express more type 1 pili (23). These filamentous adhesive organelles promote bacterial colonization of the bladder, suggesting that the defects observed with the Δ*cyaA* and Δ*crp* mutants in our *in vivo* assays may be attributable to misregulation of type 1 pilus expression. However, this possibility is countered by recent work showing that elevated levels of type 1 pilus expression actually enhance the ability of UPEC to colonize and persist within the bladder (69). Consequently, we conclude that the inability of the Δ*cyaA* and Δ*crp* mutants to effectively colonize the bladder is mostly due to the effects of diminished cAMP-CRP levels on factors other than type 1 pili.

In our *in vitro* assays, deletion of either *cyaA* or *crp* increased the ability of UPEC to withstand levels of H_2O_2 that prevent growth of the wild-type strain. The Δ*cyaA* and Δ*crp* mutants were also substantially more resistant to acid stress. UPEC likely comes across similar stresses during the course of a UTI, but any increase in stress resistance afforded by the deletion of *cyaA* or *crp* is apparently countered and surpassed *in vivo* by detrimental effects on other systems. For example, the Δ*cyaA* and Δ*crp* mutants are highly sensitive to nitrosative stress and the superoxide generator

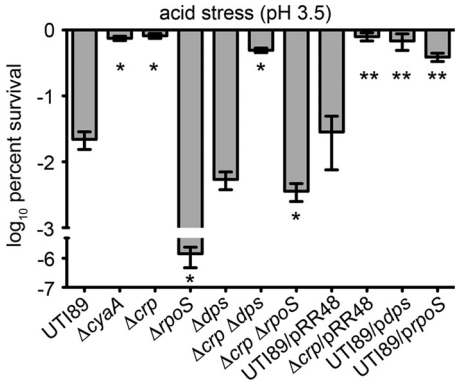

FIG 6 Expression of RpoS or Dps enables wild-type UTI89 to survive low-pH stress at levels similar to those of the Δ*cyaA* and Δ*crp* mutants. After reaching mid-logarithmic growth phase in LB broth, UTI89 and the UTI89Δ*cyaA*, UTI89Δ*crp*, UTI89Δ*dps*, UTI89Δ*rpoS*, UTI89Δ*crp*Δ*dps*, and UTI89Δ*crp*Δ*rpoS* mutants (± pRR48, p*dps*, and p*rpoS*, as indicated) were exposed to acid (pH 3.5) stress for 30 min. Following washes in PBS, numbers of surviving bacteria were determined by dilution plating. Plasmid-containing strains were grown in the presence of 0.5 mM IPTG prior to challenge with pH stress. Data are expressed as the means ± standard deviations of three independent experiments. *P* values of <0.05 are indicated by one asterisk (*) for comparison with UTI89 and by two asterisks (**) for comparison with UTI89/pRR48, as determined by two-tailed unpaired *t* tests.

MV. Both reactive nitrogen and reactive oxygen species like superoxide are abundantly produced in response to a UTI and could compromise the fitness of mutants lacking cAMP-CRP (5, 38, 39, 41, 70). An inability to utilize alternate carbon sources like amino acids may also limit successful colonization of the urinary tract by the Δ*cyaA* and Δ*crp* mutants, as peptides and amino acids are a primary energy source utilized by UPEC during a UTI (10, 12, 13). Furthermore, the massive upregulation of trehalose production by UTI89Δ*cyaA* and UTI89Δ*crp*, while potentially offering a degree of protection under some stressful conditions, may exact a high fitness cost within the nutrient-poor confines of the urinary tract.

Results obtained using C3H/HeJ mice suggest that innate host defenses, and not nutrient availability *per se*, are the primary factors that restrict UTI89Δ*cyaA* and UTI89Δ*crp* from effectively colonizing the urinary tract. C3H/HeJ mice are hyporesponsive to lipopolysaccharide and are therefore unable to mount full-on TLR4-dependent inflammatory responses (32–36, 71, 72). Specific defects associated with C3H/HeJ mice include poor expression of chemokines and greatly reduced infiltration of the bladder mucosa by neutrophils in response to UTI (35, 73, 74). In our assays, wild-type UTI89 and the Δ*cyaA* and Δ*crp* mutants colonized C3H/HeJ mice much better than immunocompetent CBA/J animals, although the bladder-associated titers of the mutant strains were more variable within C3H/HeJ mice (Fig. 2). Significantly, the clear differences in bladder titers observed between wild-type UTI89 and the Δ*cyaA* and Δ*crp* mutants in CBA/J mice were markedly diminished in the C3H/HeJ strain, probably because C3H/HeJ mice present the mutants with a less inflammatory and therefore less stressful environment.

Cumulatively, our data indicate that the effects of cAMP-CRP on multiple metabolic and stress response pathways must be balanced in order for UPEC to effectively colonize the urinary tract. This likely involves input from many cAMP-CRP-responsive regulatory factors, including the alternate sigma factor RpoS. *In vitro*, we found that UPEC mutants lacking cAMP-CRP have elevated levels of RpoS expression, in line with results obtained using other *E. coli* strains (44, 46, 47, 75, 76). The high-level resistance of UTI89Δ*cyaA* and UTI89Δ*crp* to H_2O_2 could be phenocopied by inducing the expression of RpoS in the wild-type pathogen. Other researchers have reported that a laboratory *E. coli* K-12 mutant lacking cAMP-CRP is also highly resistant to H_2O_2 (44). In this case, it was suggested that resistance of the mutant to H_2O_2 was partially attributable to increased RpoS-dependent expression of the DNA binding, ferritin-like protein Dps. We found that IPTG-induced expression of recombinant Dps is not sufficient to rescue growth of wild-type UTI89 in the presence of H_2O_2, suggesting that other, as-yet-undefined factor(s) acting downstream of RpoS mediate H_2O_2 resistance in this pathogen. Chief among the candidate gene products that may promote H_2O_2 resistance are the RpoS-inducible catalases (48, 49), which by inference appear to be upregulated in UTI89Δ*cyaA* and UTI89Δ*crp* (Fig. 5D). Interestingly, in our assays, the UTI89Δ*cyaA* mutant consistently had higher levels of catalase activity than the Δ*crp* mutant, suggesting that cAMP generated by CyaA may boost catalase activity in part via CRP-independent mechanisms.

As with the H_2O_2 sensitivity assays, induced expression of recombinant RpoS increased the acid resistance of wild-type UTI89 to levels observed with the Δ*cyaA* and Δ*crp* mutants (Fig. 6). In this case, overexpression of recombinant Dps gave similar results, suggesting that enhanced production of RpoS in the absence of cAMP-CRP promotes acid stress resistance in UPEC via transcriptional effects on *dps*. However, deletion of *dps* only slightly decreases the acid resistance of either UTI89 or the Δ*crp* mutant. In contrast, deletion of *rpoS* greatly increased the acid sensitivity of UTI89 and, to a far lesser extent, UTI89Δ*crp*. These observations indicate that acid resistance in UPEC does not require Dps, implying the possible involvement of other RpoS-regulated pH stress-responsive genes such as *asr*, *gadA*, and *gadBC* (46, 59). Furthermore, since UTI89Δ*rpoS* is much more sensitive to acid stress than the Δ*crp*Δ*rpoS* double-knockout mutant, we conclude that the absence of cAMP-CRP promotes acid resistance in UPEC via both RpoS-dependent and RpoS-independent mechanisms. The number of cAMP-CRP-repressible genes that could contribute to the observed acid resistance phenotypes independent of RpoS is potentially high (77, 78).

At first glance, the high-level resistance of UTI89Δ*cyaA* and UTI89Δ*crp* to H_2O_2 seems at odds with the increased sensitivity of these mutants to MV. While H_2O_2 and MV both generate oxidative stress, there are appreciable differences in their reactivities, duration of activity, and side effects that may differentially influence their toxicity (79). Methyl viologen is a superoxide generator that reduces diatomic oxygen to form superoxide. Oxidized MV can then be reduced by cellular electron donors, creating a redox cycle that consumes reducing equivalents like NADPH while continually producing superoxide molecules (80). In comparison, the oxidizing effects of H_2O_2 are not regenerated. Within *E. coli*, superoxide dismutase (Sod) enzymes convert superoxide into oxygen and H_2O_2. In turn, catalases convert H_2O_2 into innocuous diatomic oxygen and water. Decreased repression of RpoS within the Δ*cyaA* and Δ*crp* mutants results in increased catalase activity, as reported here (Fig. 5D), and may also stimulate the expression of cAMP-CRP- and RpoS-regulated enzymes like SodB and SodC (81, 82). Consequently, mutants lacking cAMP-CRP are likely better equipped to detoxify both superoxide and H_2O_2, which in turn may drive the redox cycle centered around MV so that reduc-

ing equivalents needed by the bacteria are consumed at a rate that disrupts essential cellular processes. The exact mechanisms by which the Δ*cyaA* and Δ*crp* mutants differentially deal with MV and H_2O_2 require further investigation. However, the opposing effects of these two oxidants on cAMP-CRP-dependent bacterial growth and fitness highlight the complex interplay that is possible between cAMP-CRP and the myriad metabolic and stress response systems that can contribute to the pathogenicity of UPEC.

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