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The Cpx Stress Response System Potentiates the Fitness and Virulence of Uropathogenic *Escherichia coli*

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Strains of uropathogenic *Escherichia coli* (UPEC) are the primary cause of urinary tract infections, representing one of the most widespread and successful groups of pathogens on the planet. To colonize and persist within the urinary tract, UPEC must be able to sense and respond appropriately to environmental stresses, many of which can compromise the bacterial envelope. The Cpx two-component envelope stress response system is comprised of the inner membrane histidine kinase CpxA, the cytosolic response regulator CpxR, and the periplasmic auxiliary factor CpxP. Here, by using deletion mutants along with mouse and zebrafish infection models, we show that the Cpx system is critical to the fitness and virulence of two reference UPEC strains, the cystitis isolate UTI89 and the urosepsis isolate CFT073. Specifically, deletion of the *cpxRA* operon impaired the ability of UTI89 to colonize the murine bladder and greatly reduced the virulence of CFT073 during both systemic and localized infections within zebrafish embryos. These defects coincided with diminished host cell invasion by UTI89 and increased sensitivity of both strains to complement-mediated killing and the aminoglycoside antibiotic amikacin. Results obtained with the *cpxP* deletion mutants were more complicated, indicating variable strain-dependent and niche-specific requirements for this well-conserved auxiliary factor.

Urinary tract infections (UTIs) afflict a large proportion of the human population, representing an enormous health and financial burden worldwide (1). Most UTIs are caused by a genetically diverse group of bacteria known as uropathogenic *Escherichia coli* (UPEC). These pathogens can survive and grow within urine and the lumen of the bladder, but many can also bind and invade uroepithelial cells (2–4). Within the bladder, entry into uroepithelial cells can promote UPEC survival and persistence, rendering the pathogens protected from a variety of stresses and commonly used antibiotics (3, 5, 6). Prior to introduction into the urinary tract, UPEC likely first colonizes the host nasopharynx and gastrointestinal tract, where it does not appear to elicit any overt pathology (7–9). Within these varied host environments, and while in transit between hosts, UPEC will encounter an assorted array of stresses, including reactive nitrogen and oxygen species, nutrient limitation, shearing forces, professional phagocytes, complement and other antimicrobial compounds, competition with other microbes and, potentially, antibiotics (10–15). The ability to deal with these stresses is of paramount importance to the success of UPEC as a pathogen.

The envelope of Gram-negative bacteria interfaces with the extracellular environment, functioning as both a sensor of external conditions and as a selectively permeable physical barrier. Envelope stress response pathways are likely critical to the ability of UPEC to detect and respond to potentially fatal environmental insults during the course of infection. UPEC, as well as other *E. coli* strains, encode a number of envelope stress response systems, including sigma E (σ^E), Rcs, Psp, and the BaeSR and CpxRA two-component systems (16–19). The Cpx system is comprised of the inner membrane histidine kinase CpxA and the cytoplasmic response regulator CpxR (20–22). Autophosphorylation of CpxA in response to envelope stress results in the phosphorylation of CpxR, which then functions as a transcriptional regulator. CpxR controls the expression of protein folding and degrading factors involved in relieving envelope stress and can also regulate biofilm formation (23–26), bacterial adherence (23, 27, 28), motility and chemotaxis (29, 30), type III and type IV secretion systems (31–35) and, possibly, the synthesis of bacterial toxins (27, 36, 37). Studies using *E. coli* K-12 strains like MG1655 and MC4100 have indicated that CpxR may regulate the expression of well over 100 genes (38, 39).

In *E. coli* and other microbes, the Cpx system is subject to negative feedback through CpxP, a small CpxR-regulated periplasmic protein that can bind the sensor kinase CpxA, keeping it in an inactive state (40, 41). CpxR binding sites are situated upstream of the *cpxP* gene within a conserved 146-bp region that separates *cpxP* from the *cpxRA* operon. CpxP is the most highly inducible member of the Cpx regulon so far identified, and it has elevated expression in response to both envelope stress and entry into stationary-phase growth (40, 42). In addition to its role as a negative regulator of CpxA, CpxP also functions as an adaptor protein, interacting with subsets of misfolded periplasmic proteins and delivering them to the protease DegP for degradation (43, 44). In this process, CpxP is degraded along with its misfolded substrate, suggesting a mechanism by which bacteria can post-translationally modulate CpxP levels. By varying the amounts of CpxP within the periplasm, bacteria may be able to fine-tune the Cpx stress response, limiting inappropriate activation of CpxA in the absence of envelope stress and permitting rapid shutoff of the system once the stress is under control (20, 45).

The Cpx system appears to have a key role in regulating the virulence potential of a number of pathogens (17), including *Sal-*

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TABLE 1 Bacterial strains and plasmids

Strain or plasmid	Description	Source or reference(s)
Wild-type strains		
UTI89	UPEC, cystitis isolate (O18:K1:H7)	6, 95
CFT073	Urosepsis isolate (O6:K2:H1)	96
Recombinant strains		
UTI89Δ*cpxP*	UTI89 Δ*cpxP*::clm^r	This study
UTI89Δ*cpxRA*	UTI89 Δ*cpxRA*::clm^r	This study
UTI89Δ*fieF*	UTI89 Δ*fieF*::clm^r	This study
CFT073Δ*cpxP*	CFT073 Δ*cpxP*::clm^r	This study
CFT073Δ*cpxRA*	CFT073 Δ*cpxRA*::clm^r	This study
CFT073Δ*fieF*	CFT073 Δ*fieF*::clm^r	This study
Plasmids		
pKM208	IPTG-inducible Red recombinase expression plasmid, Amp^r	60
pGEN-MCS	High-retention plasmid containing empty multiple-cloning site, Amp^r	61
pJLJ41p	*cpxP* sequence with native promoter from UTI89 cloned into pGEN-MCS; Amp^r	This study
pJLJ42	*cpxRA* sequence with native promoter from UTI89 cloned into pGEN-MCS; Amp^r	This study
pNLP10-*lux*	Low-copy-number cloning vector with promoterless *luxCDABE* operon; Kan^r	39
pJW1-*cpxP-lux*	pNLP10 with P*cpxP*::*luxCDABE*, Kan^r	39

monella spp. (46, 47), *Legionella pneumophila* (31, 48), *Shigella* spp. (33–35), enteropathogenic *E. coli* (32, 49, 50), *Actinobacillus suis* (51), *Haemophilus ducreyi* (52, 53), *Xenorhabdus nematophila* (37, 54), and *Yersinia pseudotuberculosis* (55–57). However, direct evidence that the Cpx system can affect pathogen fitness and virulence *in vivo* within an animal host is limited to only a few studies (47, 50, 53, 54). In UPEC, the Cpx system has been examined primarily with respect to its ability to modulate the expression of P pili, filamentous adhesive organelles that can promote bacterial interactions with host kidney cells (27, 28, 58). Here, by using isogenic deletion mutants, we assessed how components of the Cpx stress response system affect the fitness and virulence of two reference UPEC isolates. Employing *in vitro* assays coupled with *in vivo* mouse and zebrafish infection models, we demonstrate that *cpxP* and *cpxRA* can have profound and sometimes divergent effects on the pathogenic potential of UPEC.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. Targeted gene knockouts were created in the human cystitis isolate UTI89 and the urosepsis isolate CFT073 by using lambda Red-based homologous recombination as previously described (59, 60). Briefly, the chloramphenicol resistance cassette (clm^r) was amplified from the template plasmid pKD3 with flanking 40-bp overhangs specific for the target *cpxP* or *cpxRA* loci. PCR products were electroporated into UTI89 and CFT073 carrying the plasmid pKM208, which encodes an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible lambda Red recombinase. The *yiiP* (*fieF*) gene was knocked out by using a similar approach. Knockout strains were selected on Luria-Bertani (LB) agar plates containing chloramphenicol (20 μg/ml) and verified by PCR using the primers listed in Table 2.

Expression constructs were made using the low-copy-number plasmid pGEN-MCS and standard molecular biology techniques (61). The *cpxP* gene and the *cpxRA* operon were cloned by PCR from the UTI89 chromosome. The primers used (Table 2) to amplify each locus were designed to include 250 bp of upstream and 100 bp of downstream sequences, along with terminal PstI and SalI restriction sites. PCR products were cut using PstI and SalI and ligated into pGEN-MCS to create the CpxP and CpxRA expression constructs pJLJ41p and pJLJ42, respectively.

TABLE 2 Primers used in this study[a]

Primer	Sequence (5′–3′)
cpxP KO	
Forward	ATGCGCATAGTTACCGCTGCCGTCATGGCCT CAACGCTGGGTGTAGGCTGGAGCTGCTTC
Reverse	CTACTGGGAACGTGAGTTGCTACTACTCAATA GCTTCAACCATATGAATATCCTCCTTAG
cpxP confirmation	
Forward	CTATCGTTGAATCGCGACAG
Reverse	GGATGGTGTCTATGGCAAGG
cpxRA KO	
Forward	ATGAATAAAATCCTGTTAGTTGATGATGACC GAGAGCTGGTGTAGGCTGGAGCTGCTTC
Reverse	TTAACTCCGCTTATACAGCGGCAACCAAATC ACCAGCCGTCATATGAATATCCTCCTTAG
cpxRA confirmation	
Forward	ACTGCCAGCGTTGAGGCCATGA
Reverse	GAGTGTAGGCCTGATAAGACGCTATCAGC
fieF KO	
Forward	ATGAATCAATCTTATGGACGGCTGGTCAGTC GGGCGGCTGTGTAGGCTGGAGCTGCTTCG
Reverse	TTATGAAAGCATAGACCGTTTACCCTCCCTG GGTACGACGCATATGAATATCCTCCTTAG
fieF confirmation	
Forward	CCTTGCCATAGACACCATC
Reverse	TCAGGTCAGGCCAAATGG
pJLJ41p	
Forward (PstI)	AATCCTGCAGATTGTTTAAATACCTCCGAGGC
Reverse (SalI)	TAGAGTCGACTACCAGCGCGGCGAGAATAC
pJLJ42	
Forward (PstI)	TGCTCTGCAGTCATTTGCTCCCAAAATCTTTCT
Reverse (SalI)	GCTAGTCGACAGCGGCAAGATCGAAGATTTTT

[a] Added restriction sites underlined. KO, knockout.

Growth curves. Bacteria were grown from frozen stocks at 37°C with shaking overnight in 5 ml of LB broth 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). Cultures were then diluted 1:100 into the indicated medium, and the growth of quadruplicate 200-μl samples in shaking 100-well honeycomb plates at 37°C was assessed using a Bioscreen C instrument (Growth Curves USA). For competition assays, wild-type and mutant strains diluted 1:200 were mixed at a 1-to-1 ratio in 5 ml modified M9 medium and grown with shaking at 37°C. After 2, 4, and 6 h of growth, titers of the mutant and wild-type strains were determined by plating serial dilutions on LB agar with or without chloramphenicol (to distinguish wild-type and mutant strains). Competitive indices were calculated as follows: $\log_{10}[(\text{mutant}_{\text{output}}/\text{wild-type}_{\text{output}})/(\text{mutant}_{\text{input}}/\text{wild-type}_{\text{input}})]$. Media and other reagents used in these assays were purchased from Sigma-Aldrich.

Amikacin susceptibility assays. Bacteria were grown from frozen stocks with shaking at 37°C in 5 ml modified M9 medium with or without 100 μg/ml ampicillin (used to maintain plasmids). Overnight cultures were diluted 1:100 into 1 ml modified M9 medium containing amikacin at concentrations ranging from 1 to 40 μg/ml. Each culture was then grown with shaking at 37°C for 24 h. The MIC of each strain was determined as the lowest concentration of amikacin needed to prevent growth.

***cpxP* promoter activity assays.** Bacteria carrying pNLP10-*lux* or pJW1-*cpxP-lux* were grown from frozen stocks at 37°C with shaking overnight in 5 ml modified M9 medium containing 50 μg/ml kanamycin (39). Overnight cultures were diluted 1:100 into 5 ml modified M9 medium containing 50 μg/ml kanamycin and incubated with shaking at 37°C for 4 h to reach stationary phase (optical density at 600 nm, ≈1.0). Triplicate 100-μl aliquots of each sample were then transferred into a 96-well white, opaque-walled polystyrene microplate (Dynex Technologies), and luminescence was measured immediately with a Synergy HT multidetection microplate reader (BioTek Instruments, Inc.).

Hemagglutination assays. Hemagglutination titers were determined using guinea pig red blood cells (Colorado Serum Company) as described previously (62). Bacteria used in these assays were grown statically from frozen stocks in 20 ml modified M9 medium or LB broth for 48 h at 37°C.

Mouse UTI model. Seven- to 8-week-old female CBA/J mice (Jackson Laboratory) were used, following IACUC-approved protocols as previously described (5, 63, 64). Wild-type and mutant bacterial strains were grown from frozen stocks in 20 ml static modified M9 medium for 24 h at 37°C. Prior to inoculation, bacteria were pelleted by centrifugation for 10 min at 8,000 × *g* and then resuspended in phosphate-buffered saline (PBS). Mice were anesthetized by using isoflurane inhalation and carefully inoculated by transurethral catheterization with 50 μl of a bacterial suspension containing 1×10^7 CFU. For competitive assays, wild-type UTI89 was mixed 1:1 with either the Δ*cpxP* or Δ*cpxRA* mutant prior to inoculation. For noncompetitive assays, each strain was inoculated separately. Bladders were harvested aseptically at 3 days postinoculation, weighed, and homogenized in sterile PBS containing 0.02% Triton X-100. Bacterial titers present in the input pools and in the bladder homogenates were determined by plating serial dilutions on LB agar plates. For competitive assays, LB agar plates with or without chloramphenicol (20 μg/ml) were used to distinguish the wild-type and mutant strains. Competitive indices were calculated as follows: $\log_{10}[(\text{mutant CFU inoculated}/\text{wild-type CFU inoculated})/(\text{mutant CFU recovered}/\text{wild-type CFU recovered})]$; based on this equation, values of less than 0 indicated that the wild-type strain outcompeted the mutant. Experiments were repeated two to three times, and combined data are shown.

Zebrafish infections. Zebrafish used in this study were handled in accordance with IACUC-approved protocols and following standard procedures (www.zfin.org), as previously described (65). *AB zebrafish embryos were collected from mixed egg clutches in a breeding colony that was maintained on a 14-h light/10-h dark cycle. Embryos were grown at 28.5°C in E3 medium (5 mM NaCl, 0.27 mM KCl, 0.4 mM $CaCl_2$, 0.16 mM $MgSO_4$) containing 0.000016% methylene blue as an antifungal agent. At 48 h postfertilization (hpf), embryos were manually dechorianated, briefly anesthetized with 0.77 mM ethyl 3-aminobenzoate methanesulfonate salt (Tricaine; Sigma-Aldrich), and embedded in low-melting-point agarose (Mo Bio Laboratories) without Tricaine. Agarose-embedded embryos were then transferred to E3 medium lacking methylene blue and infected individually with wild-type CFT073 or the *cpx* mutants. Bacteria were grown from frozen stocks, held static in 12 ml modified M9 medium at 37°C for 24 h. One milliliter from each culture was pelleted, washed once with 1 ml PBS, and resuspended in PBS prior to inoculation into either the pericardial cavity or circulation valley by using an Olympus SZ61 or SZX10 stereomicroscope together with a YOU-1 micromanipulator (Narishige), a Narishige IM-200 microinjector, and a JUN-AIR model 3 compressor setup. For each bacterial strain, 500 to 1,000 CFU suspended in 1 nl PBS was injected per fish. Inoculation titers were determined by adding 10 drops (1 nl each) to 1 ml 0.7% NaCl, which was then serially diluted and plated on LB agar plates. Following injection, embryos were carefully removed from the agar, placed individually into wells of a 48-well plate (Nunc) containing E3 medium, and incubated at 28.5°C. Fish viability was assessed at regular intervals for 72 h following injection by monitoring heart beats and blood flow.

Bacterial host cell association and invasion assays. Host cell association and gentamicin protection-based invasion assays were performed as previously described (66, 67). Strains used in these assays were grown at 37°C for 48 h in static LB broth to induce expression of type 1 pili. Human bladder epithelial cells, designated 5637 (HTB-9; ATCC), were grown at 37°C in 5% CO_2 using RPMI 1640 medium (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (HyClone). Bladder cells were infected with a multiplicity of infection of ~15 bacteria per host cell.

Serum resistance assays. Frozen aliquots of pooled human sera, taken from 7 healthy volunteers by using standard protocols approved by the University of Utah Institutional Review Board, were provided by Andrew Weyrich. Care was taken to not freeze and thaw samples multiple times. Bacteria from overnight cultures grown with shaking at 37°C in modified M9 medium were pelleted by spinning at 8,000 × *g* for 5 min, washed twice, and resuspended in PBS to obtain $\sim 1 \times 10^6$ CFU/ml. About 5×10^4 CFU of each bacterial strain was mixed individually with modified M9 medium containing 20% serum, and 200-μl aliquots of each suspension were immediately placed in a 96-well microtiter plate and incubated with gentle shaking for 2.5 h at 37°C. Plates were then placed on ice, and surviving bacteria were enumerated by plating serial dilutions on LB agar. Results were normalized to input titers. Heat-inactivated serum (treated at 55°C for 30 min) was used as a negative control.

Statistical analysis. The Mann-Whitney U test, Wilcoxon matched pair test, log-rank (Mantel-Cox) test, and Student's *t* test were performed using Prism 5.01 software (GraphPad Software). *P* values of less than 0.05 were defined as significant.

RESULTS

The Cpx system modulates UPEC resistance to amikacin. By using lambda Red-mediated linear recombination, the *cpxRA* operon and the *cpxP* gene were individually deleted from two reference UPEC strains, the cystitis isolate UTI89 and the urosepsis isolate CFT073. As the first step in our efforts to phenotypically characterize these mutants, we assessed their sensitivities to the aminoglycoside antibiotic amikacin. Previous studies showed that laboratory K-12 *E. coli* strains lacking *cpxA* or *cpxRA* have increased sensitivity to amikacin, whereas induction of the Cpx pathway or the expression of constitutively active *cpxA* mutants (*cpxA**) provides strains with improved resistance to amikacin (68–70). Resistance has been attributed to the ability of the Cpx system to activate transcription of drug exporters as well as factors that help alleviate the stress of mistranslated proteins that may

TABLE 3 Amikacin MIC assay results[a]

Strain	MIC (μg/ml)
UTI89	18
UTI89Δ*cpxRA*	6
UTI89Δ*cpxP*	28
UTI89(pGEN-MCS)	16
UTI89Δ*cpxRA*(pJLJ42)	30
UTI89Δ*cpxP*(pJLJ41p)	18
CFT073	20
CFT073Δ*cpxRA*	6
CFT073Δ*cpxP*	30
CFT073(pGEN-MCS)	20
CFT073Δ*cpxRA*(pJLJ42)	16
CFT073Δ*cpxP*(pJLJ41p)	20

[a] The assay was repeated three times and the same results were obtained.

accumulate within the bacterial envelope due to amikacin effects on ribosome activity (25, 70, 71).

In agreement with results obtained using K-12 strains (68, 69), we observed that both UTI89Δ*cpxRA* and CFT073Δ*cpxRA* were highly sensitive to amikacin relative to their wild-type counterparts, as determined by MIC assays (Table 3). Plasmid pJLJ42, which carries the *cpxRA* operon under the control of its native promoter, complemented both Δ*cpxRA* mutants, whereas the empty vector pGEN-MCS had no effect (Table 3 and unpublished observations). Deletion of *cpxP*, which leaves CpxA less repressed (40, 45), rendered UTI89 and CFT073 notably more resistant to amikacin (Table 3). Expression of recombinant *cpxP* using plasmid pJLJ41 restored the resistance of the Δ*cpxP* mutants to wild-type levels. Of note, deletion of the gene *yiiP* (*fieF*) located immediately downstream of *cpxP* did not affect the sensitivity of either UTI89 or CFT073 to amikacin (unpublished observations).

These results indicate that the Cpx response in the UPEC isolates operates, not unexpectedly, similarly to the Cpx response in K-12 strains. To further address this point, we utilized a low-copy-number reporter construct containing the *cpxP* promoter fused to a promoterless *luxCDABE* operon (39). In both CFT073 and UTI89, the deletion of *cpxRA* ablated expression of the *cpxP* reporter in early-stationary-phase cultures, whereas deletion of *cpxP* greatly enhanced expression (Fig. 1). These data parallel those reported for similar assays carried out with K-12 strains, supporting models in which CpxP functions in part as a negative regulator of Cpx activation (40, 45).

The Cpx system provides UPEC with a fitness advantage within the bladder. To address whether or not the Cpx system can affect the fitness of UPEC within the urinary tract, we utilized a well-established UTI model system, focusing on bladder colonization by the cystitis isolate UTI89. Adult female CBA/J mice were inoculated via transurethral catheterization with wild-type UTI89, UTI89Δ*cpxRA* or UTI89Δ*cpxP*, and 3 days later bacterial titers within the bladder were determined. In noncompetitive assays, in which equal numbers of the wild-type and mutant strains were inoculated separately into different mice, the Δ*cpxRA* mutant was recovered in significantly lower numbers than wild-type UTI89 (Fig. 2A). In contrast, no significant difference was observed between wild-type UTI89 and the Δ*cpxP* mutant. Similar results were obtained in competitive assays, in which the wild-type strain was mixed 1:1 with each mutant strain prior to inoculation (Fig. 2B and C). These results indicated that *cpxRA*, but not *cpxP*, is required by UTI89 to effectively colonize the bladder.

During the course of a UTI, UPEC comes across a variety of environmental stresses that can potentially limit its survival and growth within the host (10–15). These stresses include reactive nitrogen and oxygen radicals and numerous membrane-damaging substances. In LB broth and modified M9 medium, the *cpxRA* and *cpxP* deletion mutants grew normally, whether on their own in monoculture or in direct competition with the wild-type strains (Fig. 3). Likewise, no defects were observed with the Δ*cpxRA* or Δ*cpxP* mutants when challenged *in vitro* with nitrosative stress (1 mM acidified sodium nitrite), oxidative stress (0.5 or 1 M methyl viologen), or envelope stress generated by addition of 0.1% sodium dodecyl sulfate (unpublished observations). These findings indicate that deletion of *cpxRA* or *cpxP* does not alter the ability of UPEC to handle generalized stresses.

Divergent effects of the Cpx system on host cell invasion by UPEC. Effective colonization of the bladder by UPEC generally requires the expression of functional type 1 pili (63, 72–75). These filamentous adhesive organelles mediate bacterial attachment to and invasion of bladder epithelial cells, promoting the establish-

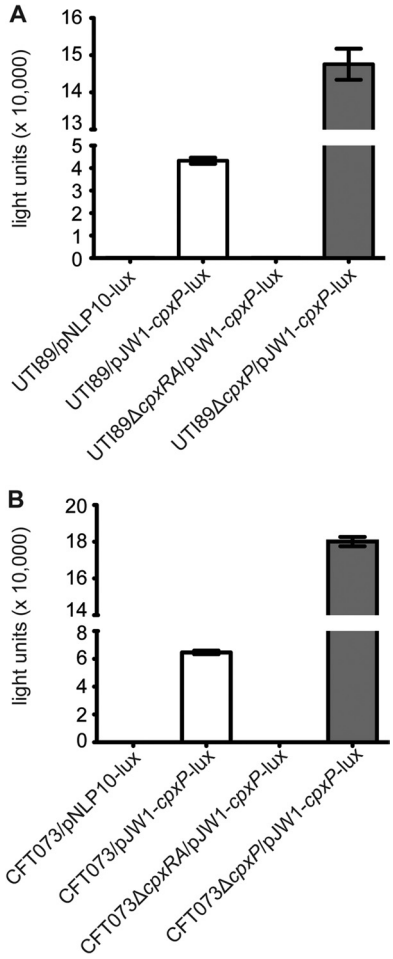

FIG 1 Deletion of *cpxP* enhances Cpx activation in both CFT073 and UTI89. Graphs indicate expression levels (± standard deviations) of the *luxCDABE* operon driven by the *cpxP* promoter in wild-type UTI89 (A) and wild-type CFT073 (B) and their mutant derivatives, following growth to early stationary phase in modified M9 medium. The pNLP1-*lux* plasmid carries a promoterless *luxCDABE* operon. Each graph shows the means ± standard errors of the means of three independent experiments performed in triplicate.

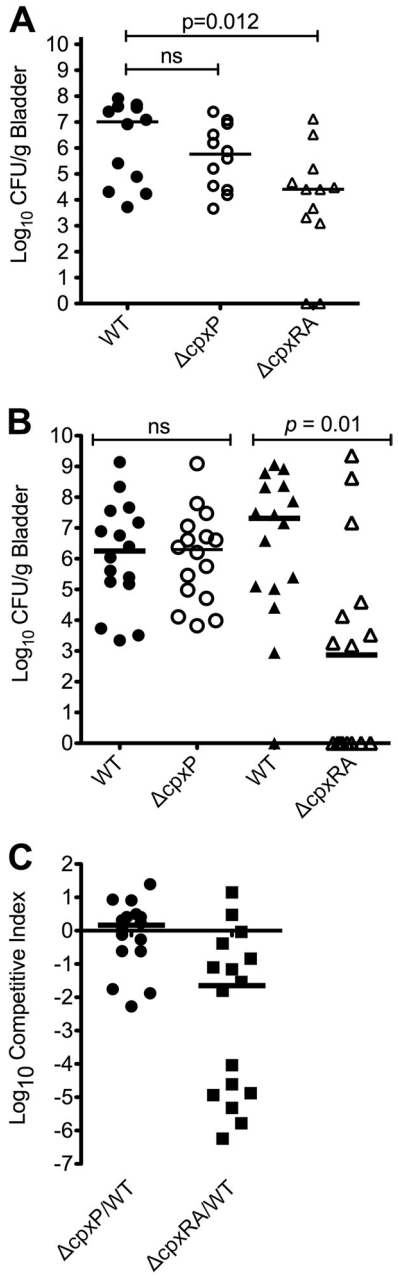

FIG 2 The Cpx system promotes UPEC fitness within the bladder. Adult female CBA/J mice were infected via catheterization with wild-type UTI89, UTI89Δ*cpxP*, or UTI89Δ*cpxRA* in noncompetitive (A) and competitive (B and C) assays. Graphs show bacterial titers present in the bladder at 3 days postinoculation. Bars denote median values for each group ($n \geq 12$ mice). The data in panel B are graphed in panel C as competitive indices. *P* values were determined using the Mann-Whitney U test (A) or Wilcoxon-matched paired signed rank test (B). ns, no significant difference.

ment and persistence of UPEC within the urinary tract (5, 6, 63, 76). In yeast agglutination assays, as well as hemagglutination assays performed using guinea pig red blood cells, we observed no overt differences in the expression of type 1 pili by the Δ*cpxRA* or Δ*cpxP* mutants relative to the wild-type UPEC isolates (unpublished observations). However, UTI89Δ*cpxRA* did show a slight, but significant (~20%), decrease in its ability to adhere to human bladder epithelial cells in culture (Fig. 4A). This reduction in adherence corresponded with a similar (~30%) decrease in host cell invasion by UTI89Δ*cpxRA*, as determined in gentamicin protection assays (Fig. 4B). These modest defects in host cell adherence and invasion could be rescued by complementation of the Δ*cpxRA* mutant with pJLJ42. In contrast to UTI89Δ*cpxRA*, the Δ*cpxP* mutant had no defect in its ability to bind bladder epithelial cells (Fig. 4A), but it was able to invade the host cells at a much higher frequency than either wild-type UTI89 or the Δ*cpxRA* mutant (Fig. 4B). Complementation of UTI89Δ*cpxP* with pJLJ42 reduced the invasion frequencies of this mutant to wild-type levels. Importantly, wild-type UTI89 and the Δ*cpxRA* and Δ*cpxP* mutants grew similarly in the cell culture medium, and all three strains were equally susceptible to killing by gentamicin at the concentration (100 μg/ml) used in these invasion assays.

Cpx components promote UPEC virulence in zebrafish. To assess effects of the Cpx system on UPEC virulence, and not fitness *per se*, we next focused on CFT073 in a zebrafish infection model that was recently developed in our laboratory (65). In this model system, bacteria are microinjected into 48-hpf zebrafish embryos via either a fluid-filled sac surrounding the heart, known as the pericardial cavity (PC), or directly into the bloodstream through the circulation valley. UPEC does not usually spread from the PC, whereas the pathogens rapidly disseminate systemically following inoculation of the bloodstream. At 48 hpf, zebrafish are dependent upon innate host defenses that include phagocytes, antimicrobial peptides, and complement—the same sort of defenses that mammalian hosts employ against UPEC (77–81). The use of zebrafish has proven to be an effective way to identify and functionally define virulence factors of relevance to UPEC and related pathogens that can colonize an assorted array of hosts and host tissues (unpublished observations and references 65 and 82).

Relative to UTI89 and many other UPEC isolates, CFT073 is especially lethal to zebrafish embryos (65). Here, we compared the lethality of wild-type CFT073 to CFT073Δ*cpxRA* and CFT073Δ*cpxP* following inoculation of 500 to 1,000 CFU of each strain individually into the PC or blood. In this infection model, increased bacterial growth correlates with decreased host survival (65). Wild-type CFT073 killed most of the zebrafish embryos within 24 h, irrespective of the site of inoculation (Fig. 5). In comparison to the wild-type strain, the virulence of both the Δ*cpxRA* and Δ*cpxP* mutants was significantly decreased. Virulence defects associated with CFT073Δ*cpxRA* and CFT073Δ*cpxP* were particularly evident following inoculation of the blood (Fig. 5B), which in general appears to be a more challenging and stressful environment than the PC (65). Wild-type CFT073 and the Δ*cpxRA* and Δ*cpxP* mutants grew similarly in modified M9 minimal medium at 28.5°C, the temperature at which the zebrafish embryos are maintained. Plasmid pJLJ42 (*cpxRA*) and pJLJ41 (*cpxP*) rescued the virulence defects associated with CFT073Δ*cpxRA* and CFT073Δ*cpxP*, respectively, but the wild-type strain carrying empty vector was attenuated, complicating interpretation of our *in vivo* complementation assays (unpublished observations).

Strain-dependent effects of Cpx components on serum resistance. Urine, like serum, contains numerous antibacterial factors, including heat-labile components of the complement system that can mediate bacterial opsonization and the formation of membrane attack complexes (14, 83–85). By modulating the composition and resilience of the bacterial envelope, we hypothesized that

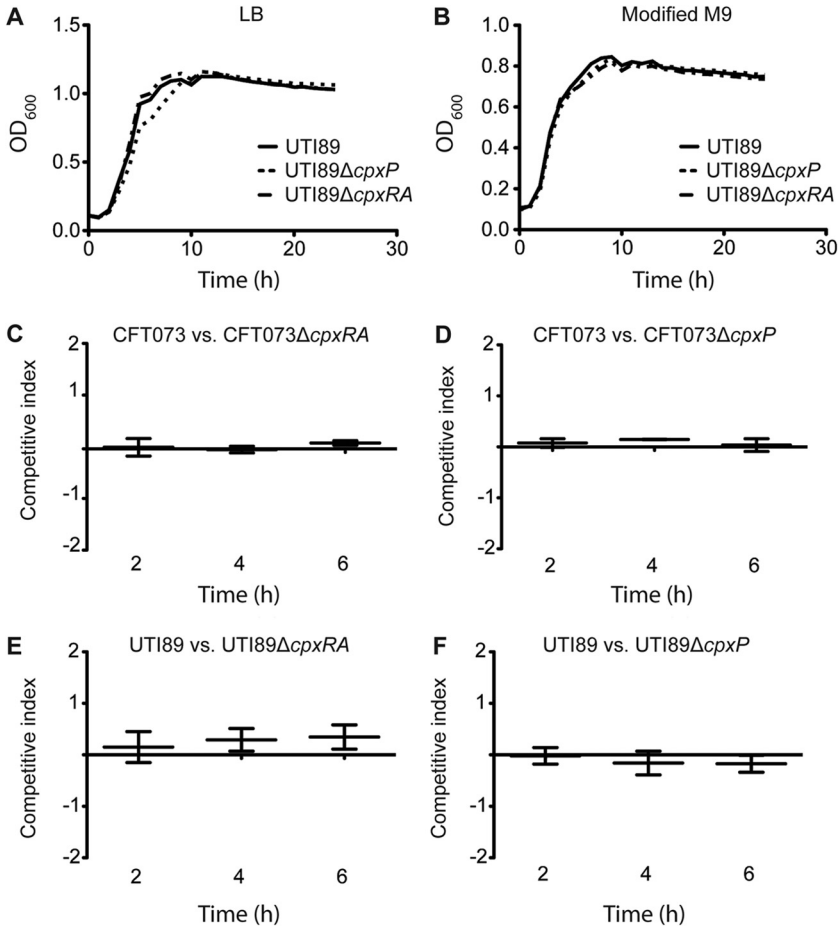

FIG 3 CFT073 and UTI89 mutants lacking either *cpxP* or *cpxRA* grow normally in LB broth and modified M9 medim. (A and B) Growth of wild-type UTI89 and associated Δ*cpxP* and Δ*cpxRA* mutants grown in LB broth (A) and modified M9 medium (B). Graphs are representative of at least three independent experiments performed in quadruplicate. (C to F) Results of competitive growth assays carried out in modified M9 medium with wild-type CFT073 and UTI89 versus isogenic Δ*cpxRA* or Δ*cpxP* mutants, as indicated. Data are presented as box-and-whiskers plots, with means ± the minimum and maximum values from three independent experiments.

the Cpx system can alter the sensitivity of UPEC to serum components. To examine this possibility, serum resistance assays were performed using wild-type UTI89 and CFT073 along with the Δ*cpxRA* and Δ*cpxP* mutants. In these assays, both UTI89Δ*cpxRA* and CFT073Δ*cpxRA* were significantly more sensitive to pooled human sera than their wild-type counterparts (Fig. 6A). UTI89Δ*cpxP* was likewise sensitive, whereas CFT073Δ*cpxP* showed no decrease in serum resistance relative to wild-type CFT073. Serum resistance defects associated with UTI89Δ*cpxRA*, CFT073Δ*cpxRA*, and UTI89Δ*cpxP* were rescued by plasmids carrying CpxRA (pJLJ42) or CpxP (pJLJ41), as appropriate (Fig. 6B). In assays that used heat-inactivated serum, which lacks functional complement, no differences were observed between the wild-type and mutant strains (Fig. 6C). Together, these data indicate that CpxRA, with strain-dependent input from CpxP, can enhance UPEC resistance to serum and, specifically, complement.

DISCUSSION

This study was aimed at delineating the impact of the Cpx envelope stress response system on the fitness and virulence of UPEC. Our results demonstrated that CpxRA and the auxiliary factor CpxP can affect the ability of UPEC to colonize distinct host environments. Employing a well-established mouse UTI model, we found that deletion of *cpxRA* limited the ability of the reference cystitis isolate UTI89 to effectively colonize the bladder, whereas deletion of *cpxP* had only modest effects. In laboratory K-12 *E. coli* strains, CpxP is not an essential regulator of the Cpx system, and instead it appears to modulate how quickly CpxA can be activated or inactivated in response to changing levels of envelope stress (20, 40, 43, 45). Within the bladder, the regulatory effects of CpxP are apparently dispensable to UTI89, at least at the 3-day time point that was analyzed. In contrast, deletion of either *cpxRA* or *cpxP* markedly attenuated the virulence of the urosepsis isolate CFT073 during both localized and systemic infections in zebrafish embryos. These data suggest that CpxP is differentially required by UPEC, depending upon strain background and the host environment. This idea was further supported by *in vitro* assays that showed that the resistance of UTI89 to complement-mediated killing was dependent upon both CpxRA and CpxP, while CFT073 required only CpxRA.

The Cpx system is intercalated within a complex web of signaling cascades and linked up with multiple biosynthetic and metabolic pathways (25, 38, 39, 41, 45, 86–88), making it difficult to

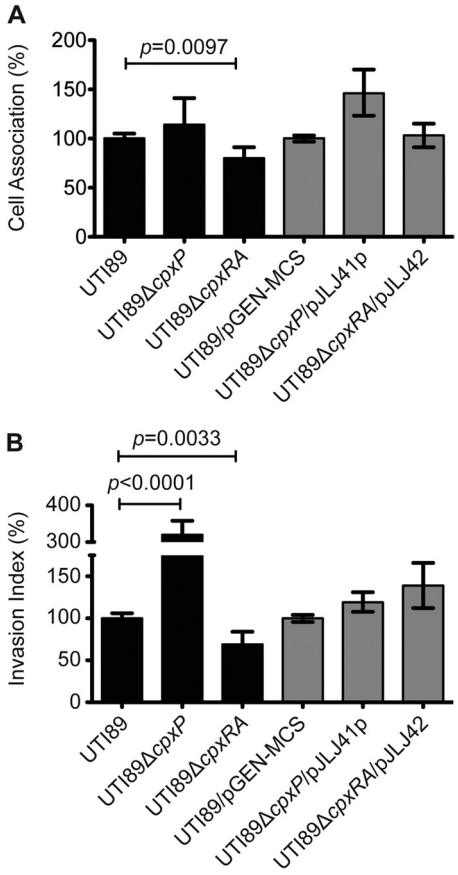

FIG 4 Cpx effects on bladder cell invasion by UTI89. Human bladder epithelial cells were infected with the indicated strains for 2 h, followed by an additional 2-h incubation in medium containing gentamicin (100 μg/ml). Graphs show the total cell-associated bacterial titers prior to addition of gentamicin (A) and for gentamicin-protected, intracellular bacteria (B). Data are expressed relative to wild-type UTI89 (black bars) or UTI89 carrying the control plasmid pGEN-MCS (gray bars) as the means ± standard errors of the means of at least three independent experiments performed in triplicate. The indicated *P* values were calculated using Student's *t* test.

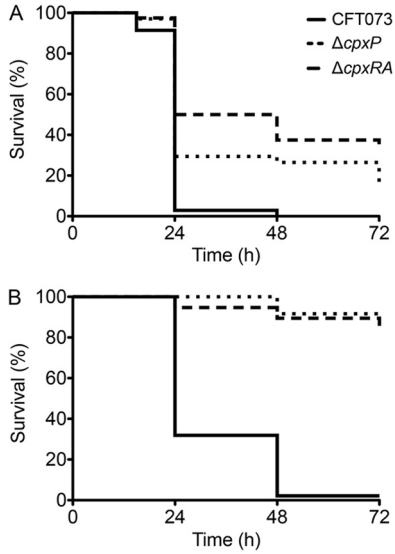

FIG 5 The Cpx system is required for full virulence of CFT073 in zebrafish embryos. The PC (A) or blood (B) of 48-hpf zebrafish embryos was inoculated with 500 to 1,000 CFU of wild-type CFT073, CFT073Δ*cpxP*, or CFT073Δ*cpxRA*, as indicated. Fish were scored for death at 0, 24, 48, and 72 h postinoculation. Data are expressed as the percent survival over time ($n \geq 17$ embryos). $P \leq 0.0008$ for the Δ*cpxP* and Δ*cpxRA* mutants versus control wild-type CFT073, as determined using Mantel-Cox log rank tests.

discern with clarity the specific mechanisms by which Cpx components moderate UPEC stress resistance and virulence phenotypes. It is clear, however, that basic regulation of the Cpx system in UPEC functions similarly to the Cpx system in nonpathogenic K-12 *E. coli* strains. In K-12 strains, CpxP regulates Cpx activation via a negative feedback loop (40), and this also appears to be the case in UPEC (Fig. 1). Furthermore, deletion of *cpxRA* rendered both UTI89 and CFT073 highly sensitive to the aminoglycoside amikacin (at 3 μg/ml), whereas deletion of *cpxP* increased amikacin resistance. These data are in agreement with work carried out in K-12 strains (68–70) and support the notion that activation of the Cpx system safeguards against aminoglycoside antibiotics. Protection is likely afforded by Cpx-mediated upregulation of proteases and other factors that alleviate envelope stress initiated by the mistranslation of inner membrane proteins in the presence of amikacin (70). Cpx activation may also heighten bacterial resistance to antibiotics via effects on the expression of drug transporters (25, 71).

The protective effects of Cpx activation are limited and will not shield against all concentrations and types of antibiotics, including the aminoglycoside gentamicin, used in our host cell invasion assays (70). This means that the slight but significant decrease in host cell invasion by UTI89Δ*cpxRA* and the elevated invasion frequencies seen with UTI89Δ*cpxP* are likely not attributable to Cpx-regulated effects on the susceptibility of UTI89 to gentamicin. Instead, the Cpx system may affect bacterial survival during or immediately after internalization or, alternatively, modulate the efficacy of the invasion process directly by affecting the surface characteristics of UPEC. The latter possibility is buoyed indirectly by observations showing that disruption of the Cpx system can alter bacterial interactions with hydrophobic abiotic surfaces (89). Of note, UPEC mutants lacking either *cpxRA* or *cpxP* were not obviously different from the wild-type strains with respect to motility, biofilm formation in microtiter plate assays, or the expression of curli or type 1 pili (unpublished observations). This indicates that many of the phenotypes commonly associated with UPEC virulence are unaffected by disruption of the Cpx system.

The reduced capacity of UTI89Δ*cpxRA* to bind and invade bladder epithelial cells may contribute to the inability of this mutant to effectively colonize the bladder. However, it is probable that additional CpxRA-regulated activities also play a role. These activities may include Cpx-mediated alterations of the bacterial envelope and peptidoglycan layer that enable bacteria to better deal with antimicrobial peptides and proteins like complement (90). The complement system can mediate bacterial opsonization and the formation of membrane attack complexes, thereby facilitating the clearance of bacteria during both localized and systemic infections (83). The strain-dependent requirement for CpxP in UPEC resistance to complement-mediated killing, as reported here (Fig. 6), highlights the individuality of UPEC isolates, which are often genetically diverse, while also raising questions regarding the functionality of highly conserved proteins like CpxP.

In addition to modulating the activity of CpxA, CpxP can func-

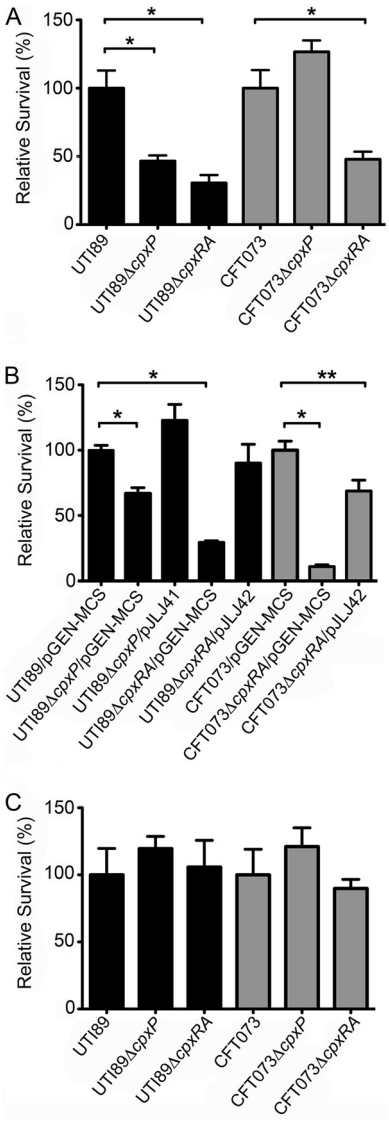

FIG 6 Cpx components have strain-dependent effects on serum resistance. About 5×10^4 CFU of wild-type UTI89 or CFT073 or their mutant derivatives were incubated at 37°C with gentle shaking in modified M9 medium containing 20% human serum (A) or 20% heat-inactivated serum (C). After 2.5 h, surviving bacteria were enumerated by plating serial dilutions. (B) Similar assays with 20% serum were performed using strains carrying plasmids pJLJ41 or pJLJ42 or the control empty vector, pGEN-MCS, as indicated. Data are presented relative to the wild-type strains as the means ± standard errors of the means of at least four independent experiments. In panel B, the control wild-type strains carried pGEN-MCS. *, $P < 0.007$; **, $P = 0.02$ (determined with Student's *t* test).

tion as a periplasmic chaperone and may act as a sensor for metal ions, like zinc and copper (43, 44, 91, 92). UTI89 is apparently more dependent upon one or more of these activities when challenged with complement, whereas CFT073 can make do without CpxP. It is feasible that structural homologues of CpxP, such as Spy and ZraP (92–94), can substitute for CpxP under specific conditions in strains like CFT073. The Cpx system is best known for its effects on the expression of periplasmic chaperones and proteases in response to envelope stress, and the misregulation of these and other factors likely contribute to the myriad defects observed with the Δ*cpxP* and Δ*cpxRA* mutants in our assays.

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