POSTTRANSCRIPTIONAL REGULATION OF THE ENVELOPE IN *ESCHERICHIA COLI*

by

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STATEMENT OF DISSERTATION APPROVAL

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ABSTRACT

Prokaryotes make extensive use of posttranscriptional regulation to modulate diverse cellular processes such as central carbon metabolism, stress response pathways, and virulence determinants. Posttranscriptional regulation in *Escherichia coli* is mediated via two broadly characterized methods. The first utilizes small noncoding RNAs (sRNAs) which bind target mRNA transcripts to alter their stability and translation. Nearly all characterized sRNAs function jointly with an RNA chaperone protein, Hfq. The second method employs mRNA-binding proteins which directly mediate translational inhibition or activation upon mRNA targets. Posttranscriptional regulation by both methods was recently demonstrated important to pathogenesis by several bacterial organisms. This study addresses the role of posttranscriptional regulation in uropathogenic *Escherichia coli* (UPEC), the organisms responsible for the majority of urinary tract infections. Specifically, deletion of Hfq, an RNA chaperone required for many sRNA-mRNA interactions, strongly reduced infection in murine models of cystitis and pyelonephritis and virtually eliminated formation of UPEC intracellular bacterial communities (IBCs). The *hfq* mutant experienced severe sensitivities to membrane disrupting agents such as polymyxin B, reactive oxygen species (ROS) and reactive nitrogen species (RNS) during in vitro models of host innate immune function. These phenotypes mirrored those of a σ^E-deleted UPEC, suggesting Hfq's involvement in posttranscriptional regulation of virulence was largely exerted at the bacterial envelope. In addition, RNS-treatment of

UPEC resulted in posttranscriptional downregulation of CpxP, a periplasmic regulator of the Cpx envelope stress response pathway. This downregulation was dependent on carbon storage regulator A (CsrA), a protein posttranscriptional regulator, as overexpression of CsrB, an sRNA antagonist of CsrA function, was sufficient to prevent as well as overcome downregulation of CpxP by RNS. Overexpression of CpxP in the presence of RNS proved beneficial to growth, however, suggesting CpxP downregulation by urinary RNS may not just disrupt UPEC's envelope, but impair the Cpx pathway involved in its repair. Anti-nitrotyrosine immunoblotting and mass-spectrometry indicate nitrosation of CsrA at tyrosine 48, a residue immediately adjacent to the domain implicated in RNA interaction, possibly altering CsrA's binding properties. These results demonstrate posttranscriptional regulation assisting virulence, but also imply manipulation by the host to deter growth.

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CHAPTER 1

INTRODUCTION

Worldwide, urinary tract infections (UTI) rank among the most common human infections, second only to respiratory infections (1, 2). Strains of uropathogenic *Escherichia coli* are the causative agent of more than 80% of UTIs and also represent a significant proportion of nosocomial infections (3, 4). Though not commonly lethal, the sheer number of UTIs represent an enormous financial and health burden worldwide (1, 3). UTIs are strikingly predominant in women, likely attributable to anatomical differences such as reduced urethral length and proximity of the urethral meatus to the perineum, a possible staging point for infection (5, 6). Moreover, while approximately 50% of women will experience at least one UTI in their lifetime, the rate of recurrence is high, with one in four women having recurrent or relapsing infections (7). Interestingly, within individual patients, recurrent infections tend to be caused by UPEC strains that are phenotypically and/or genotypically identical to the strain responsible for the initial acute infection (8-10). In certain instances, UTI recurrence or relapse with identical strains has occurred months-to-years subsequent to the initial infection (11).

The incorrigible lifestyle of UPEC

UPEC have evolved numerous virulence mechanisms that facilitate infection of the normally sterile urinary tract (12). As summarized in Fig. 1.1, factors and events associated with increased UPEC survival within the urinary tract include, but are not limited to, the expression of adhesive organelles, biofilm formation, flagella, and the activation of numerous stress response pathways. Crosstalk among host and bacterial factors dictates the course of disease, ultimately leading to the eradication or further dissemination of the pathogens, or, alternatively, to a sort of détente in which UPEC can persist within host tissues for long periods without eliciting overt damage or inflammatory reactions.

Key virulence factors encoded by virtually all UPEC isolates are type 1 pili, which are phase-variable polymeric fibrous adhesive organelles expressed on the bacterial surface (13, 14). Located at the distal tip of each type 1 pilus is an adhesin, FimH, which binds mannosylated glycoprotein receptors on bladder epithelial cells (see Fig. 1.1). FimH receptors include uroplakin Ia (UPIa) and $\alpha_3\beta_1$ integrin complexes (15). UP1a expression is limited primarily to terminally differentiated bladder epithelial cells where it associates with other uroplakin proteins to form hexagonal complexes that coat nearly the entire lumenal surface of the bladder (16, 17). $\alpha_3\beta_1$ integrins, on the other hand, are more broadly distributed throughout the bladder epithelium and other tissues, where they act as key signaling and adherence factors that regulate the formation of focal adhesions and other cellular processes (15, 18-20). Binding of FimH initiates a cascade of intracellular signaling events culminating in internalization of UPEC via an actin- and microtubule-dependent zipper-like mechanism (21) (see Fig. 1.1).

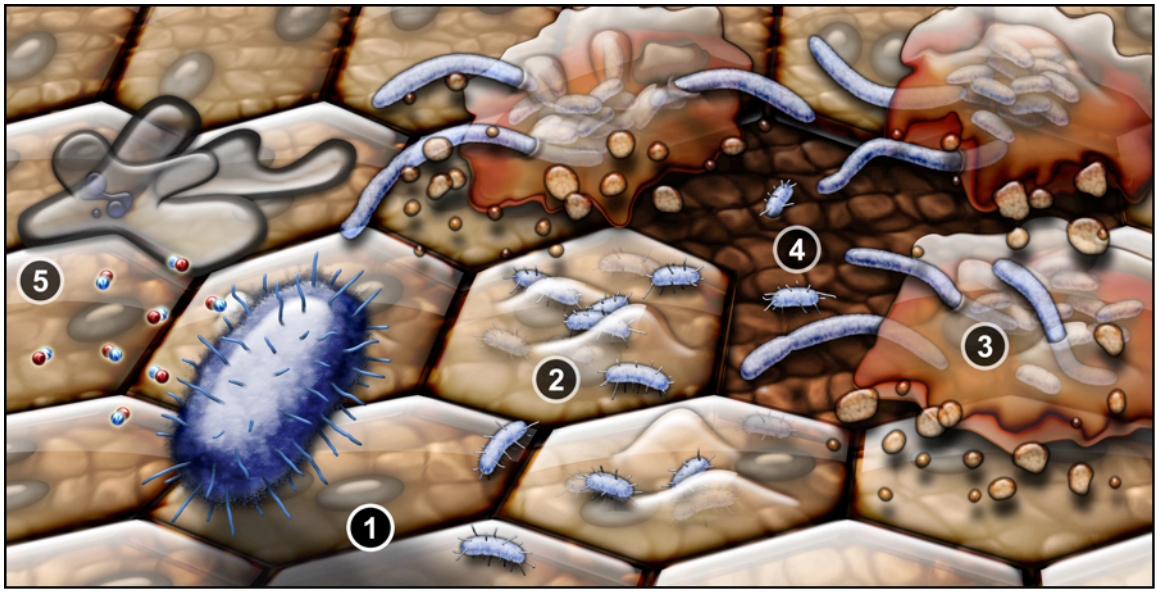

FIG. 1.1 Overview of UPEC infection in the bladder. UPEC are shown in blue (not to scale), terminally differentiated bladder epithelial cells are presented as the large hexagonal structures, with small immature bladder cells visible beneath. (1) UPEC adhering to bladder epithelial cells via type 1 pili (short rods). Once cellular attachment is made, UPEC can invade host cells via a zipper-like mechanism (2). Epithelial cells eventually exfoliate in response to infection (3) enabling UPEC to colonize the underlying immature cell layers (4). Host defenses such as polymorphonuclear leukocytes and nitric oxide (NO, red and blue spheres) (5) help resolve the infection.

Once within a host cell, UPEC may replicate, forming large biofilm-like inclusions termed intracellular bacterial communities (IBCs) or "pods" (22-29). Alternately, UPEC may remain intracellular, bound within a late endosomal compartment in a more quiescent state, or the pathogens may traffic back out of the host cell (20) (see Fig. 1.1). Intracellular multiplication of UPEC is seemingly dependent upon the abundance of actin within the target host cell. This conclusion is based in part on the observation that intracellular bacterial replication occurs rampantly in the actin-poor, terminally differentiated superficial bladder epithelial cells, while UPEC growth is severely restricted within the immature, actin-rich underlying cells (25) (Fig. 1.1). The ability of UPEC to invade and persist quiescently within the immature cells of the bladder

for many weeks to months may help explain the remarkable predilection for UTIs to recur. Quiescent reservoirs of UPEC within immature bladder cells may undergo resurgence as the occupied host cells terminally differentiate, a process accompanied by dramatic redistribution of cellular actin filaments (25).

Aggregation of UPEC into biofilm-like communities, including IBCs or extracellular aggregates, is known to positively influence the infection process, and factors that enhance biofilm formation, such as the autoaggregation surface protein antigen 43, improve UPEC persistence in the bladder (30). Aggregation and biofilm formation may concentrate nutrients and enable UPEC to better resist antimicrobial factors, including antibiotics (31). While sessile activities associated with biofilm formation have marked influence on the UPEC infectious cycle, flagella-mediated motility has also been shown to confer a competitive advantage. Specifically, motility facilitates UPEC ascension into the upper urinary tract, as demonstrated in several studies in which flagellated UPEC were observed to have a significant advantage over non-motile competitors during UTI (24, 32-34).

Host defenses against UPEC include the presence of high solute concentrations within the urine, which are generally inhibitory to bacterial growth, and the generation of high shear forces associated by the regular flow of urine, that work to remove non-adherent or loosely adherent bacteria (35). These largely passive methods of maintaining environmental sterility are enhanced by the production of many anti-bacterial compounds, including small cationic peptides known as defensins that are capable of disrupting bacterial membrane integrity (36), and iron sequestration factors such as lactoferrin that deprive invading bacteria of essential iron. Soluble factors such as

secretory immunoglobulin A (sIgA) and Tamm-Horsfall protein also bind bacteria, preventing adherence to host cells and assisting the removal of microbes from the urinary tract with the flow of urine (37, 38). The normally long-lived bladder epithelial cells can themselves be sacrificially shed via an apoptotic-like mechanism to facilitate removal of bound and internalized bacteria (27, 39, 40). However, bladder cell exfoliation may also provide UPEC with access to deeper layers of the bladder epithelium.

UPEC must also deal with infiltrating neutrophils and other immune effector cells that act to eliminate pathogens by phagocytosis and by the release of numerous anti-bacterial compounds, including reactive nitrogen species (RNS) and reactive oxygen species (ROS) (Fig. 1.1.5). RNS and ROS can covalently modify and damage lipids, proteins, and nucleic acids (RNA and DNA), disrupting components both within the bacterial cytosol as well as the bacterial envelope, including the inner and outer membranes and the intervening periplasmic space (26, 41-44). Additional sources of RNS and ROS include bladder epithelial cells, which contribute RNS during the course of a UTI (45) and UPEC themselves under anaerobic or microaerophilic conditions where RNS forms as a metabolic by-product during reduction of abundant urinary nitrate to nitrite (46). Nitrite itself is also a reactive end product of nitric oxide (NO) generation. Urine nitrite levels, which can exceed 500 μM, are frequently used as a diagnostic indicator of UTI (46). Of note, UPEC isolates are often able to resist RNS levels that prevent growth of standard laboratory *E. coli* K12 strains (44, 47, 48).

Nitrosative stress responses

E. coli and other bacterial species employ multiple oxidative and nitrostive stress sensing and response systems. Among these is the prototypical SOS stress response pathway, which is activated by DNA damage caused by various agents, including oxygen radicals and RNS such as S-nitrosothiols (49). Interestingly, the SOS response can also be activated by polyamine compounds (50, 51), and polyamines such as cadaverine can enhance the resistance of UPEC to RNS (47, 52). Multiple redox-sensitive pathways and factors, such as the transcriptional regulators SoxR, OxyR, and NorR, recognize and respond to oxidative and nitrosative stresses by upregulating flavorubredoxins, oxidoreductases, iron-transporters and catalases, which act to detoxify nitrosative and oxidative radicals (53-59). Virtually all of the RNS- and ROS-responsive systems described to date are cytosolicly localized.

The bacterial envelope represents the principle interface between a bacterium and its environment. It is here that early detection of, and first responses to, environmental and host-generated stresses are most likely to occur. Due to the highly ionic nature of many RNS and ROS, the bacterial envelope is only variably permeable to these radicals. RNS- and ROS-mediated damage to lipids and proteins within the envelope is therefore are plausible, especially in the face of host inflammatory responses. Considering these facts, I hypothesized that one of the earliest bacterial responses to nitrosative stress is activation of envelope stress response pathways.

To address this hypothesis I generated short-lived GFP promoter fusion reporter constructs to assess activation of a canonical envelope stress response pathway, the Cpx system (Fig. 1.2). This system is composed of three principle components: the integral

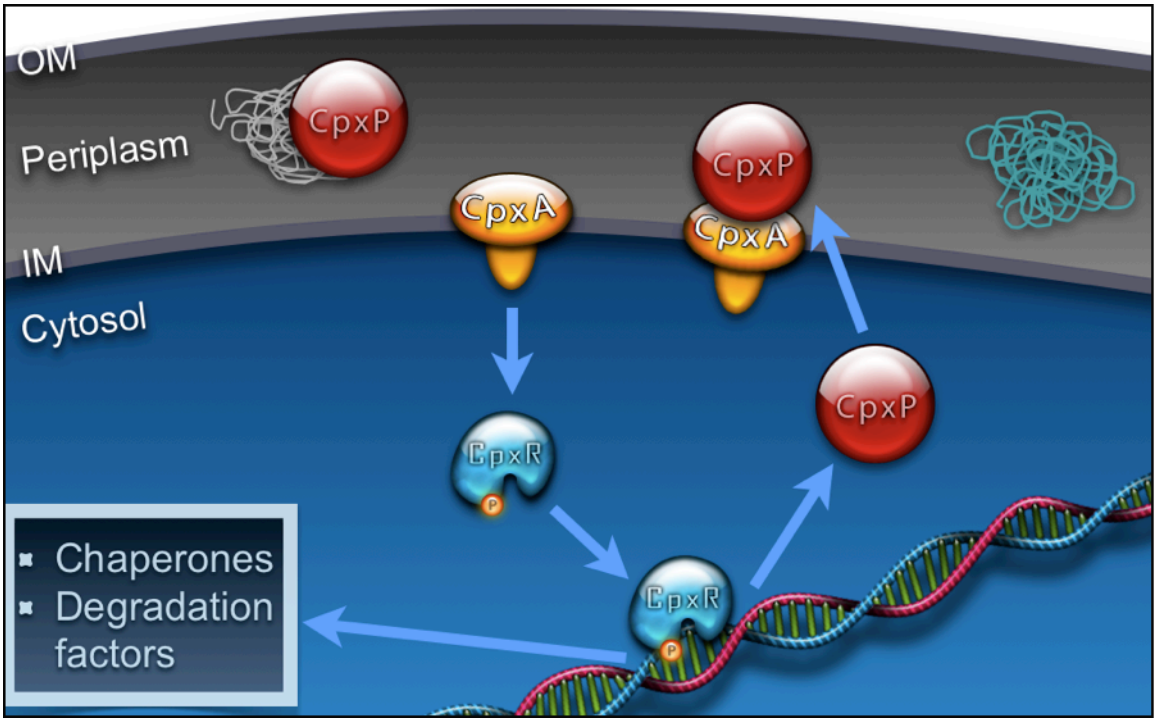

FIG. 1.2. Schematic representation of Cpx pathway activation. Misfolded periplasmic proteins (squiggly lines) cause the periplasmic adaptor protein, CpxP, to dissociate from the sensor-kinase CpxA, resulting in CpxA activation and subsequent phosphorylation of the response regulator CpxR. Acting as a transcription factor, CpxR modulates the expression of over 100 genes, including *cpxP*. As periplasmic stress is alleviated, CpxP binds once again binds and inhibits CpxA as part of an autoinhibitory mechanism.

inner-membrane histidine kinase CpxA; the cytosolic transcription factor CpxR; and the periplasmic adaptor CpxP. Under noninducing conditions, CpxP binds to the periplasmic region of CpxA, keeping CpxA in an inactive state. Under inducing stresses, such as the generation of misfolded pilin subunits within the periplasm, alkaline pH, and/or bacterial adherence to abiotic surfaces, CpxP dissociates from CpxA, allowing CpxA to phosphorylate the cytosolic response regulator CpxR. Once phosphorylated, CpxR can mediate transcriptional activation or repression of a broad set of genes including periplasmic disulfide bond catalysts, isomerases, chaperones, and proteases (60). CpxA, R, and P are also all upregulated downstream of Cpx pathway activation, although only

CpxP expression is entirely dependent upon the CpxR transcription factor (46). Because of this last property, CpxP reporter constructs are often used as sensitive indicators of Cpx activation (61, 62). Using a similar approach, I exploited a *cpxP* promoter fusion to assess activation of the Cpx pathway in response to nitrosative stress, with the hypothesis that RNS would induce misfolding of periplasmic proteins.

Chapter 2 of this dissertation describes results from my study of the Cpx pathway in UPEC under nitrosative stress. Using Cpx reporter constructs, I demonstrate the proper induction of these reporters under Cpx-inducing conditions, but also observed that, contrary to my hypothesis, CpxP expression under nitrosative stress was completely abolished. Notably, RNS-mediated repression of other Cpx gene products, aside from CpxP, was not observed. RT-PCR and microarray analysis indicated that repression of CpxP expression in response to RNS occurred via a posttranscriptional mechanism. Coincident with the ablation of CpxP expression, I also observed massive upregulation of several cytosolic nitrosative stress response genes and multiple genes associated with motility. These results indicate potentially important functional links among the Cpx system, nitrosative stress responses, motility, and posttranscriptional regulators of gene expression in UPEC.

Posttranscriptional regulation in *E.coli*

In assessing potential mechanisms for the posttranscriptional control of CpxP expression in the presence of RNS, I became intrigued with the general mechanisms of posttranscriptional regulation (PTR) in bacteria. In particular, how do pathogens employ PTR to modulate virulence? In UPEC, what specific virulence determinants are regulated

by PTR and what are the functional consequences of this regulation? These questions form the core of what this dissertation addresses.

Several PTR mechanisms have been well characterized in *E.coli* and related species. These mechanisms fall broadly into two categories: 1) those involving interactions between regulatory proteins like CsrA with target mRNAs and 2) those where small noncoding RNAs (sRNAs) interact with specific mRNA transcripts. Both cases may involve translational repression or activation and may also affect message stability. Translational repression can result from occlusion of the Shine/Dalgarno sequence, abrogating ribosome binding. Conversely, posttranscriptional regulatory factors may promote relaxation of secondary structures within target transcripts, opening up ribosome binding sites, thereby promoting translation. Alterations to message stability occur when Hfq, in conjunction with sRNAs, protects or reveals RNase degradation sites within the mRNA (see reviews (63-66)).

Protein-based posttranscriptional regulation: CsrA

The PTR factor carbon storage regulator A, or CsrA, is a global regulator of glycogen biosynthesis and central carbon metabolism in *E. coli*, with noted homologs extending to Gram-positive organisms (67-70). CsrA is capable of translational activation, RNA stabilization, and translational inhibition, mediated by CsrA interactions with consensus ruACArGGAuGU motifs in target transcripts (71). CsrA can have broad effects leading, for example, to increased stabilization and/or translation of mRNA transcripts associated with glycolysis and the glyoxalate shunt (69, 72), acetyl-coenzyme A synthesis (73), and the *flhDC* master regulator of motility (74). Alternately, CsrA can downregulate metabolic processes such glycogen synthesis and gluconeogenesis (69, 75,

76). Furthermore in several Gram-negative bacterial pathogens CsrA regulates virulence determinants such as biofilm production (77, 78) and attachment (78), extracellular amyloid-fibrils termed curli (79), and motility (46). The ability of CsrA to regulate virulence determinants and carbon metabolism along with biofilm formation and motility has prompted speculation that this posttranscriptional regulator acts to modulate key physiological changes in bacterial pathogens as they switch from acute to chronic/persistent phases of an infection cycle (80).

Interestingly, CsrA activity is itself posttranscriptionally regulated, with two genomically-encoded sRNAs, CsrB and CsrC, acting as antagonists (81, 82). Both sRNAs are almost entirely composed of variable CsrA-binding sites, with the stoichiometry of CsrB-to-CsrA binding calculated at 18:1 (83). These binding sites are believed to serve as molecular decoys to titrate CsrA dimers away from target transcripts. CsrB and CsrC are activated by the transcriptional regulator UvrY, a component of the BarA-UvrY two-component system that respond to glucose and the glycolytic end-products acetate and formate (84-86). An autoinhibitory loop is formed as CsrA induces glycolysis, producing acetate and formate, which results in activation of UvrY and increased production of the CsrB sRNA (81).

Noting the large upregulation of motility-associated genes concomitant to posttranscriptional downregulation of CpxP under nitrosative stress, I hypothesized that posttranscriptional regulator CsrA may be responsible. This possibility was tested, as described in Chapter 2, by inducing overexpression of the CsrA antagonist CsrB. These experiments demonstrate that antagonization of CsrA function is sufficient to alleviate CpxP repression under nitrosative stress. To investigate the possibility of CsrA binding

the *cpxP* leader region, I determined the 5' untranslated region (UTR) of the *cpxP* transcript. Within the 5' UTR is a region with some homology to the known CsrA-binding consensus. I hypothesize that modification of CsrA or one or more of its targets in the presence of RNS leads to repression of CpxP translation. Key amino acid residues affected by RNS include cysteines and tyrosines. CsrA lacks cysteines, but contains two conserved tyrosine residues, Tyr48 and Tyr61. Immunoblots using nitrotyrosine-specific antibody and tandem mass-spectrometry of purified, RNS-treated CsrA indicated that Tyr48 of CsrA could be nitrated. Tyr48 is adjacent to Ile47, a residue shown to be critical to the ability of CsrA to bind RNA targets (87). In silico analyses indicate nitration of Tyr48 would introduce substantial steric hindrance within an alpha-helix containing Ile47, possibly altering the RNA-binding properties of CsrA. This may represent a mechanism whereby the binding specificity of CsrA may be altered, and may contribute to the posttranscriptional repression of CpxP expression under nitrosative stress.

The ability of RNS to cause posttranscriptional downregulation of CpxP raised the question of whether this phenomenon was beneficial or detrimental to UPEC. In in vitro assays, I found that overexpression of CpxP in UPEC in the presence of RNS enhanced the growth of UPEC, while a *cpxP* null mutant grew similar to the wild type strain. These results suggested that CpxP levels were modulatory, rather than essential, to UPEC survival and growth in the presence of RNS. However, in a murine model of cystitis, the *cpxP* null mutant was significantly less fit than the parent wild type strain, at least in competition assays. Of note, *cpxR* and *cpxA* null mutants were similarly disadvantaged in vivo in competition assays. These data indicate that the Cpx system can assist growth in the murine urinary tract or in nitrosative stress conditions. This implies

that the downregulation of CpxP by RNS may be a host mechanism to disrupt a canonical bacterial envelope stress response pathway, thereby inhibiting the infection process. This conclusion, however, remains open to interpretation since RNS-mediated attenuation of CpxP expression may have alternate outcomes under varying environmental conditions. One particularly intriguing possibility is that RNS generated by UPEC themselves may be employed as a means to posttranscriptionally regulate the Cpx system.

Small-noncoding RNAs and the RNA chaperone Hfq

The second class of PTR employs sRNA base-pairing with mRNA transcripts to facilitate translational activation, repression and/or to alter message stability. These base-pairing interactions often occur through multiple, nonadjacent regions of 2-8bp (88, 89). sRNAs may inhibit translation by blocking the ribosome binding site (RBS) within the 5'-UTR. A prime example of this is the OxyS sRNA , which binds and inhibits translation of *flhA* mRNA (90). Activation of translation can occur when an sRNA disrupts an incipient auto-inhibitory RNA duplex in the 5' leader region of a transcript, such as occurs with the RprA and DsrA sRNAs as they interact with transcripts encoding the stationary-phase sigma factor RpoS (91-94). sRNAs may also affect translation of multiple targets. For example, DsrA not only activates translation of *rpoS* transcripts, but also represses translation of *hns* transcripts, which code for a histone-like protein (95, 96). Analysis of the differential binding and action of DsrA on the *rpoS* and *hns* tanscripts revealed that DsrA could assume multiple conformations for achieving different base-pairing interactions with its targets (95, 96). sRNAs are genomically encoded and transcribed normally from individual promoters, typically resolving in a rho-independent terminator (89). Although most sRNAs are unique genes with independent

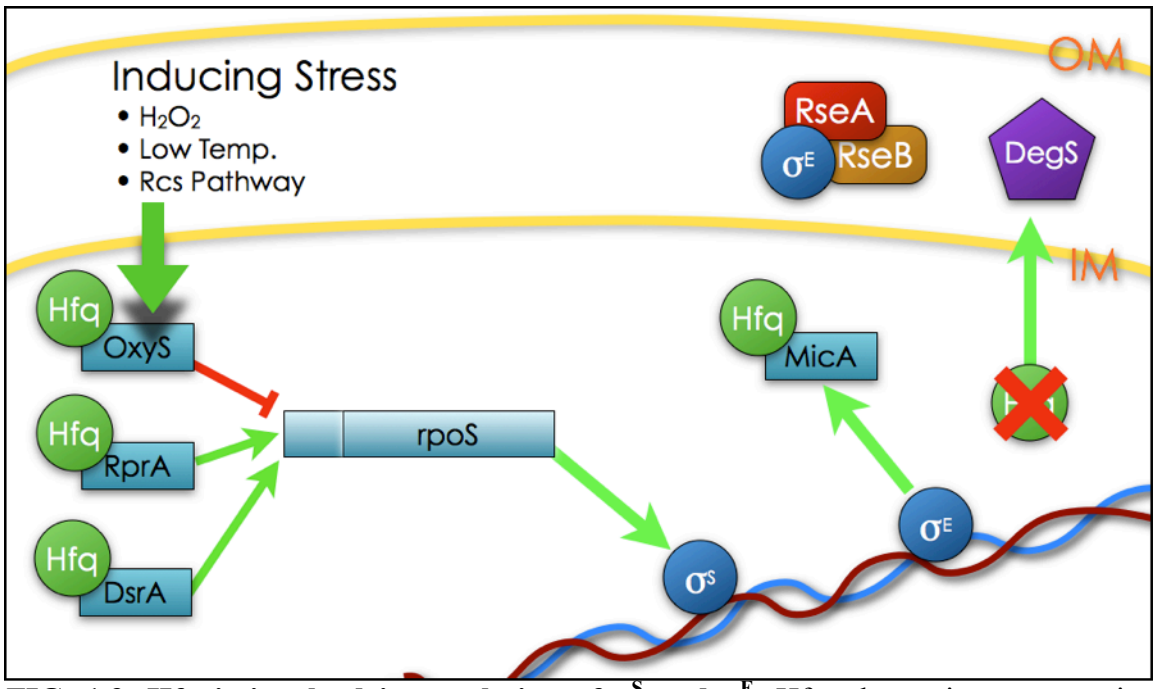

FIG. 1.3. Hfq is involved in regulation of σ^S and σ^E. Hfq, shown in green, assists sRNA (shown in cyan) in interactions governing translation of the *rpoS* gene, coding for the σ^S transcription factor. Deletion of *hfq* results in constitutive activation of the periplasmic degradation factor DegS which degrades RseA and RseB leading to constitutive activation of σ^E.

promoters, at least one sRNA, SgrS, exists that is derived from an existing mRNA, transcribed opposite of the protein coding strand, (97).

Of all the confirmed sRNAs in the literature, over half require a protein co-factor, Hfq, to mediate their effects. Hfq, which is highly conserved amongst bacteria and archaea, was originally characterized as a host factor required for Qβ phage replication (98, 99). Forming homohexameric complexes, Hfq has noted structural similarity to eukaryotic Sm-like proteins that are involved in RNA splicing (100-103). Hfq non-specifically binds RNAs at A/U-rich sites (104), facilitating interactions between mRNAs and sRNAs (105). Hfq is involved in the pathogenesis of many organisms, including *Brucella abortus* (106), *Pseudomonas aeruginosa* (107), *Listeria monocytogenes* (108),

Vibro cholerae (109), *Legionella pneumophila* (110), and *Salmonella typhimurium* (111), although it is dispensable for infection by the Gram-positive *Staphylococcus auerus* (112). In K12 laboratory *E. coli* strains, Hfq acts as a pleiotropic regulator (113), controlling the expression of at least two major bacterial sigma transcription factors (see Fig. 1.3). The stationary-phase or general stress-response sigma factor σ^S, encoded by the *rpoS* gene, is regulated by three Hfq-dependent sRNAs: DsrA, RprA, and OxyS (114). σ^S assists bacterial adaptation to oxidative, hyperosmotic, pH, nutritional, and UV radiation stresses (115). Many phenotypes associated with *hfq* deletion have previously been attributed to effects on RpoS expression (116). Hfq is also involved in the regulation of *rpoE*, which encodes the envelope stress sigma factor σ^E (109, 111, 117-120). Loss of *hfq* results indirectly in constitutive activation of the σ^E regulon due to upregulation of the periplasmic protease DegS, which degrades the the σ^E sequestration factor RseA (111). An *hfq* mutant may thus be anticipated to have phenotypes resulting from effects on either the σ^S or the σ^E stress response pathways.

Control of message stability and translation by sRNAs represents a potentially fast-acting method for modifying protein and mRNA levels, permitting highly specific fine-tuning of gene expression above and beyond transcriptional control. sRNAs tend to be degraded with their mRNA interaction partners, providing an auto-inhibitory mechanism, and further assisting energetic efficiency in the bacterium. Energy- and time-intensive translation is not required for sRNAs to function, enabling them to be produced and act rapidly. In this respect, sRNAs function ideally as rapid response systems, and have been found, not surprisingly, to regulate many stress-response and bacterial envelope proteins. Indeed, approximately half of the known Hfq-dependent sRNAs

characterized affect translation of envelope proteins. The OmrA/OmrB sRNAs regulates curli expression by directly interacting with the RBS of the *csgD* transcript (121). Porins or outer membrane proteins (OMPs), the predominant envelope proteins, are frequently the target of sRNA regulation (122-125). For example, expression of the highly abundant OMP, OmpA, is regulated by the sRNA MicA (126-128), while OmpC is controlled by the sRNA OmpW (118), OmpF by the sRNA MicF (129-131), and YbfM, an OMP of unknown function, by the sRNA MicM/RybB/SroB (132). These porins tend to be beta-barrel structures believed to function as solute channels through the outer membrane, although several also serve as phage receptors (133). At least two of these sRNAs, MicA and RybB, also fall under control of the σ^E transcriptional regulator (118). In addition, at least one two-component signal transduction system, the PhoPQ pathway, which responds to low-levels of extracellular divalent cations, is translationally regulated by the sRNA MicA (134).

After addressing the involvement of the protein-mediated class of posttranscriptional regulation in UPEC, as exemplified by CsrA, I turned my attention to the possible involvement of sRNAs as mediators of UPEC fitness and virulence. To broadly test the role of sRNAs in UPEC pathogenesis, I deleted *hfq* from a reference UPEC isolate (UTI89) and tested its fitness and virulence potential in a range of in vivo and in vitro assays, as described in Chapter 3. Although the UPEC *hfq* mutant grew normally during in vitro growth analyses, it was highly defective in bladder colonization and completely unable to colonize the kidneys. In addition, the *hfq* mutant was virtually incapable of forming IBCs. These experiments indicate the necessity of Hfq, and hence, sRNAs during the infection process.

How does Hfq, and thus sRNAs, influence UPEC virulence? These observed defects in infection in vivo could be attributable to disregulation of many specific virulence determinants, including disruption of envelope integrity, improper expression of the σ^S response regulator, and/or the disturbance of one or more stress response pathways. As detailed in Chapter 3, in vitro assays indicate that disruption of *hfq* in UPEC has diverse pleiotropic effects, leading to decreased biofilm formation, reduced motility, and increased bacterial susceptibility to RNS, ROS, and envelope-disrupting cationic peptides like polymyxin B. Considering the impact that Hfq has on σ^E and σ^S, the phenotypic comparisons were made among *hfq*, *rpoS* (σ^S) and *rpoErseABC* (σ^E) deletion mutants. Although I observed some phenotypic overlap among these mutants, the *hfq* mutant was dramatically more sensitive to polymyxin B and RNS, indicating observed phenotypes associated with disruption of *hfq* were not solely due to disregulation of σ^E and σ^S. Moreover, the *hfq* mutant uniquely exhibited highly altered lipopolysaccharide, the predominant surface molecules. In total, these results demonstrate the importance of Hfq to UPEC infection, while highlighting the distinct contribution that Hfq has on bacterial phenotypes independent of the σ^S and σ^E stress response systems.

Summary

Taken together, the data presented in this thesis begin to delineate the critical importance of PTR in regulating the fitness and virulence potential of UPEC. The protein-mediated arm of PTR, CsrA, downregulates CpxP expression under nitrosative stress, which in turn may have direct bearing on the fitness of UPEC within the host. Although this work only begins to address indirectly the contribution of CsrA to UPEC

virulence, the results suggest that this posttranscriptional regulator will be a central player in the pathogenesis of UTIs. The pleiotropic effects of Hfq on UPEC virulence and fitness within the host and in in vitro assays likewise highlight the critical involvement of sRNAs as important virulence factors with wide-ranging targets. Cumulatively, this work demonstrates that PTR can vitally impact UPEC virulence mechanisms, suggesting novel means by which these pathogens can sense and respond to the numerous stresses encountered within the host environment.

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CHAPTER 2

CSRA-MEDIATED POSTTRANSCRIPTIONAL REGULATION OF THE CPX PATHWAY IN UROPATHOGENIC *ESCHERICHIA COLI* UNDER NITROSATIVE STRESS

Abstract

During the course of a urinary tract infection, strains of uropathogenic *Escherichia coli* (UPEC) can elicit a number of host inflammatory responses, including the production of reactive nitrogen species (RNS). These radicals can react with and damage membrane proteins and other components of the bacterial envelope, potentially affecting envelope stress response pathways. To address this possibility, I employed promoter-fusion reporter constructs to assess activation of the Cpx two-component envelope stress response system. I found that exposure of UPEC to RNS generated by acidified sodium nitrite (ASN) abrogated expression of a key Cpx regulon member, CpxP, by a posttranscriptional mechanism involving the global regulator CsrA. In vitro growth assays as well as in vivo competition experiments using a murine infection model indicated that the Cpx system, including CpxP, can positively affect the fitness of UPEC in the face of RNS and other stresses encountered within the host environment. In total,

these data indicate that RNS-mediated attenuation of CpxP expression via CsrA may factor in as a host defense within the urinary tract and at other sites of infection, but this phenomenon may also represent a mechanism by which bacteria themselves can modulate the Cpx system through the generation of endogenous RNS.

Introduction

Urinary tract infections (UTIs), which include cystitis and pyelonephritis, currently rank among the most common of infectious diseases within the human population (1). The vast majority of UTIs are caused by strains of uropathogenic *Escherichia coli* (UPEC) that can bind and invade host cells within the urinary tract (2, 3). The infection process can stimulate a number of antimicrobial and proinflammatory host responses, including the generation of nitric oxide (NO) and other reactive nitrogen species (RNS). Nitrite (NO_2^-) is a significant RNS present during a UTI, reaching concentrations in excess of 500 μM in the urine of infected patients (4). The activity of host nitric oxide synthases, as well as nitrate-reducing uropathogenic bacteria like UPEC, are likely sources of nitrite within the urinary tract during a UTI (5-7). Acidification of nitrite in low pH environments can result in formation of additional RNS, including nitric oxide (NO), nitrogen dioxide (NO_2), and nitrous acid (HNO_2) (8, 9). In the lab, acidified sodium nitrite (ASN) is a widely used system for generating RNS in vitro. During the course of a UTI, RNS like those produced via ASN are thought to contribute to the antibacterial characteristics of urine (4).

RNS damage nucleic acids, lipids, and proteins and can inactivate or alter enzymatic processes, metabolic pathways, and signal transduction cascades (10-16). In Gram-negative bacteria, RNS have been shown to stimulate a number of cytoplasmic stress responses, including the SOS pathway (17, 18), the OxyR transcription factor (19, 20), and the SoxRS two-component regulatory system (21). RNS effects on the bacterial envelope, which in Gram-negative bacteria is comprised of an inner and outer membrane and the intervening periplasmic space, are less well defined.

The bacterial envelope interfaces with the external environment and functions as a selectively permeable physical barrier. Recent work using atomic force microscopy indicates that exposure of *E. coli* to NO can severely compromise the integrity of the envelope (22). By reacting with and damaging components of the bacterial envelope, RNS may trigger the activation of stress response pathways that specifically recognize and respond to envelope stress. In UPEC and in other *E. coli* strains, envelope stress is sensed by one or more of several identified systems: these include the σ^E and Rcs pathways along with the BaeSR and Cpx two-component systems (23-25).

The Cpx system is comprised of the inner membrane histidine kinase CpxA and the cytoplasmic response regulator CpxR (26). In response to envelope stress CpxA phosphorylates 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 (27, 28), bacterial adherence (27, 29, 30), motility and chemotaxis (31), type III and type IV secretion systems (32-36), and, potentially, bacterial toxins such as α-hemolysin and cytotoxic necrotizing factor 1 (29,

37). Data from Lin and co-workers have suggested that CpxR can directly control nearly 100 genes in the *E. coli* K12 reference strain MG1655 (38). CpxR appears to have a key role in regulating virulence in a number of pathogens (23), including *Salmonella* species (39, 40), *Legionella pneumophilia* (32), *Shigella sonnei* (34-36), enteropathogenic *E. coli* (33), *Actinobacillus suis* (41), *Haemophilus ducreyi* (42), *Xenorhabdus nematophila* (43), *Yersinia pseudotuberculosis* (44), and potentially UPEC (29, 30, 45).

In *E. coli* and other microbes the Cpx system is subject to negative feedback through CpxP, a small CpxR-regulated periplasmic protein that is proposed to bind the sensor kinase CpxA, keeping it in an inactive state (26, 46). CpxP is the most highly inducible member of the Cpx regulon so far identified and has elevated expression in response to both envelope stress and entry into stationary phase growth (46, 47). In addition to its role as a negative regulator of CpxA, CpxP also functions as an adaptor protein, interacting with a subset of misfolded periplasmic proteins and delivering them to the protease DegP for degradation (48, 49). In this process, CpxP is degraded along with its misfolded substrate, suggesting a mechanism by which bacteria can post-translationally modulate CpxP levels. To date, stimuli that explicitly inhibit CpxP expression have not been reported.

Here, I sought to determine if RNS, which are generated in copious amounts during the course of a UTI, could influence the Cpx pathway. my results reveal an unexpected mechanism whereby nitrosative stress represses CpxP expression in a posttranscriptional manner involving the global regulatory factor CsrA (Carbon Storage Regulator). Possible consequences of this inhibitory pathway are discussed in light of

data showing for the first time that CpxP, as well as CpxR and CpxA, significantly enhance the fitness of UPEC within the urinary tract.

Results

Cpx reporter constructs

The *cpxRA* operon and *cpxP* are separated on the *E. coli* chromosome by a well-conserved 146 bp segment containing two putative CpxR binding sites (Fig. 2.1.a). CpxP is among the most highly inducible members of the Cpx regulon, and in *E. coli* K12 strains the transcription of *cpxP* is almost entirely dependent upon CpxR (47, 50). To assess RNS effects on the Cpx stress response system, I created a transcriptional fusion using the 146 bp intergenic region upstream of the *cpxP* start site linked to a reporter gene encoding a destabilized variant of Green Fluorescent Protein (GFP-ASV). In *E. coli*, this GFP variant has a half-life of approximately 120 min (51), allowing us to follow expression levels of this reporter with a fine degree of temporal resolution.

The *cpxP-GFP* reporter construct (pJLJ5) was transformed into the human cystitis isolate UTI89, and in control experiments was shown to be responsive to stimuli known to activate the Cpx pathway. Specifically, GFP-ASV expression under control of the *cpxP* promoter was elevated in response to overexpression of either the PapE pilin subunit or the outer membrane lipoprotein NlpE, as determined by Western blot analyses (data not shown and Fig. 2.2.a). Expression of *cpxP-GFP* was not observed in a mutant lacking *cpxR* (Fig. 2.1.b). As expected of CpxP itself (31, 47), expression of the *cpxP-GFP* reporter was also increased as UTI89/pJLJ5 entered into late log-phase growth (see

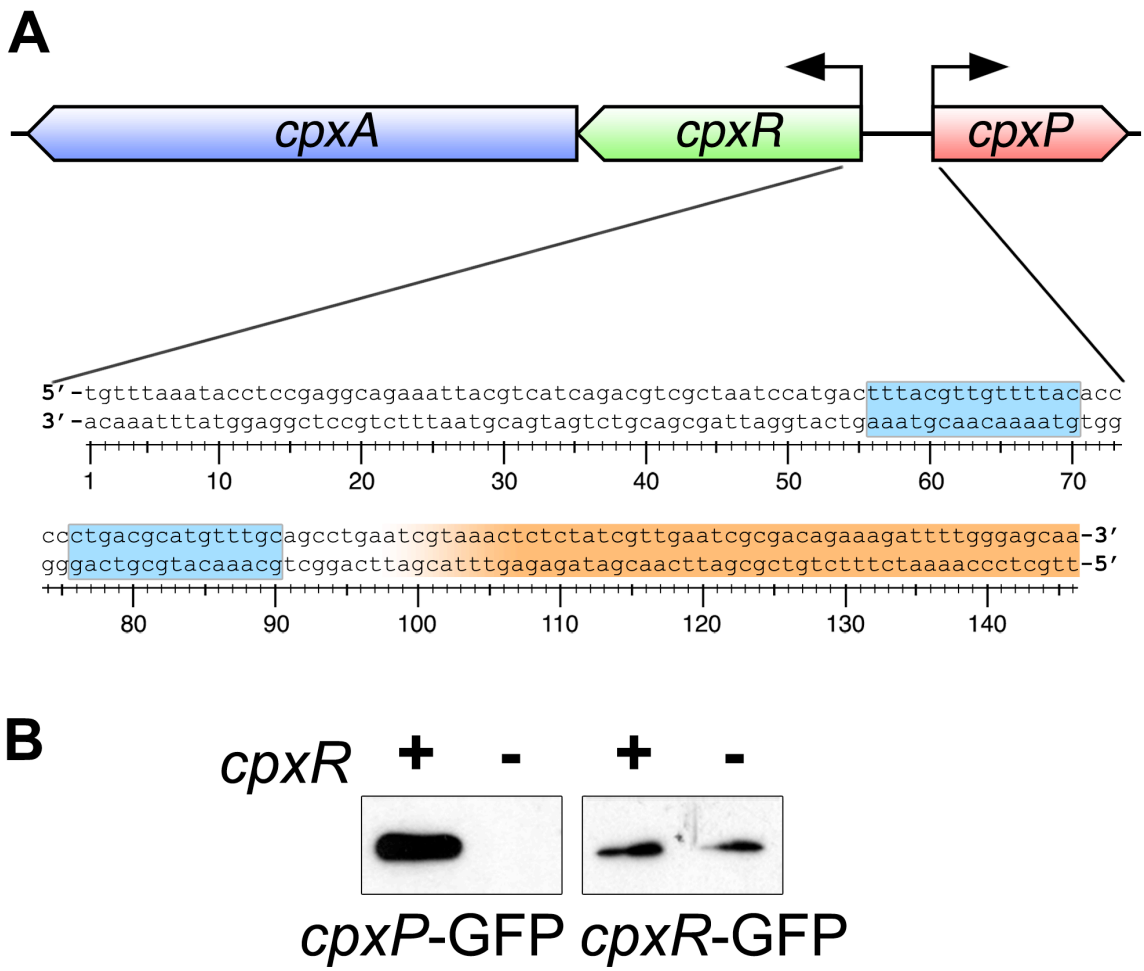

FIG. 2.1. The Cpx gene cluster and CpxR-dependent expression of the *cpxP*-GFP reporter. (A) Organization of the cpx genes, with the 146 bp intergenic region from which *cpxRA* and *cpxP* are divergergently transcribed shown in detail. Potential CpxR binding sites are shown in sky blue boxes, while the orange-shaded region indicates the putatitive 5'-UTR for *cpxP*. (B) Image shows Western blots, probed with anti-GFP antibody, used to examine the expression of *cpxP*-GFP (pJLJ5) and *cpxR*-GFP (pJLJ10) reporters in late log-phase cultures (=0.8) of wild-type UTI89 and an isogenic *cpxR* knockout mutant.

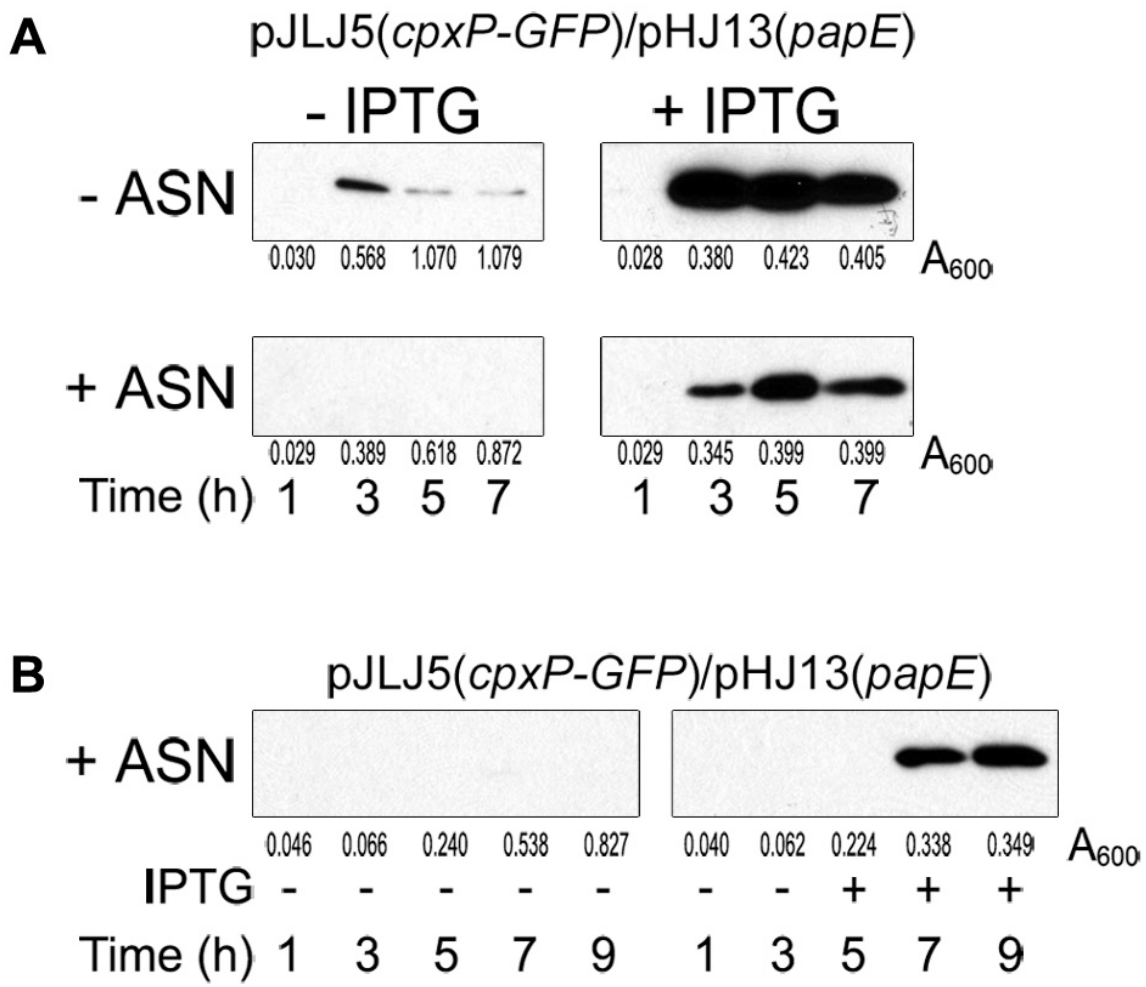

FIG 2.2. PapE overexpression counters ASN effects on CpxP expression. Western blots probed with anti-GFP antibody were used to detect expression of the *cpxP*-GFP reporter (pJLJ5) with or without IPTG-mediated induction of PapE expression (pHJ13) ± the addition of 1 mM ASN. (A) IPTG was added, as indicated, to induce PapE expression coordinate with 1 mM ASN at the 1 h time point, leading to enhanced *cpxP*-GFP expression. All images shown were obtained from a single blot using equivalent exposure. (B) ASN was added to cultures at the 1 h time point, while the addition of IPTG for induction of PapE expression was delayed until the 5 h time point. These experiments were repeated at least three times with similar results.

controls in Fig. 2.3.b). Reversing the orientation of the 146 bp *cpxP* promoter region in front of *gfp-ASV* created a putative *cpxR* reporter construct (pJLJ10), which served as an additional control in these studies. Expression of *cpxR-GFP* was only slightly diminished in the Δ*cpxR* mutant (Fig. 2.1.b), consistent with previous observations showing that, in contrast to *cpxP*, *cpxR* transcription itself is just partially regulated by CpxR. GFP expression was not detected under any of the tested conditions in UTI89 carrying a promoterless-*GFP* construct (pJLJ1, data not shown). Of note, for comparative analysis of Western blots in this study, I used equivalent film exposure times and ensured that equal amounts of protein from each sample were loaded onto the gels as appropriate (see Experimental Procedures).

RNS effects on CpxP expression

To assess the effects of nitrosative stress on the Cpx pathway, overnight cultures of the recombinant UTI89 strains grown in MES-buffered LB broth (MES-LB, pH 5) were diluted into fresh media and grown shaking for 1 h prior to the addition of 1 mM ASN to generate RNS. Exposure to ASN markedly slowed the growth of the bacteria and caused them to enter stationary phase at a lower density (Fig. 2.3.a). As anticipated, CpxP expression, as monitored using the *cpxP-GFP* reporter construct, was induced as UTI89 entered late log-phase growth in the absence of ASN (Fig. 2.3.b). However, in contrast to my predictions, CpxP expression in the presence of ASN was completely abolished. Under the same conditions, CpxR expression, as discerned using the *cpxR-GFP* reporter construct, was not notably affected by the presence of ASN (Fig. 2.3.c). Expression levels of GFP-ASV driven by a *lac* promoter were also unfazed by ASN (data

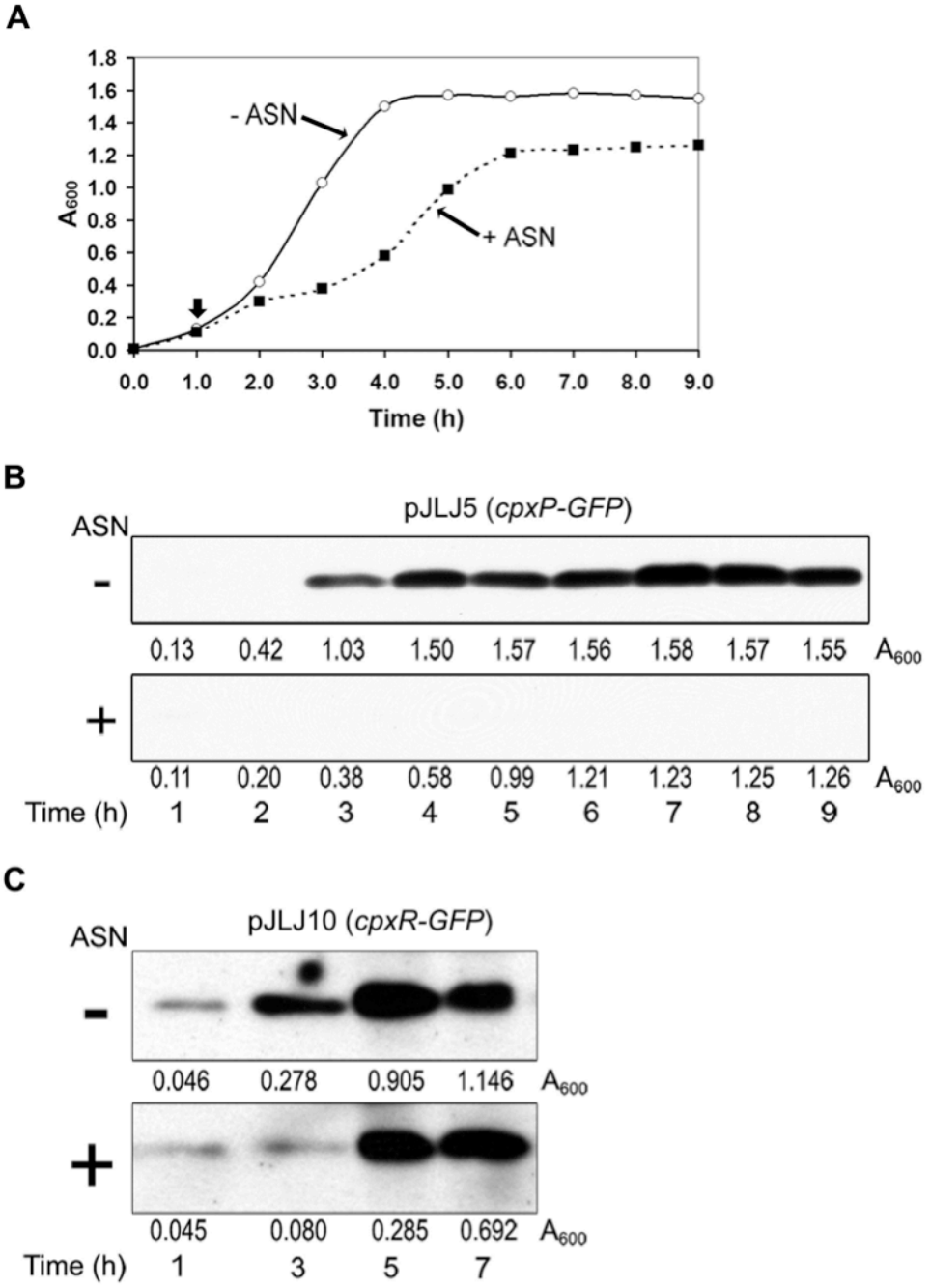

FIG. 2.3. Growth of UPEC in the presence of RNS abrogates CpxP expression. (A) Growth curves of UTI89/pJLJ5 (*cpxP*-GFP reporter) grown shaking in broth ± 1 mM ASN, which was added at the 1 h time point (thick arrow). (B) Western blots, probed using anti-GFP antibody, show levels of *cpxP*-GFP expression over time in UTI89/pJLJ5 ± ASN. (C) Western blots showing levels of the *cpxR*-GFP reporter in UTI89/pJLJ10 during growth ± ASN. These experiments were repeated three or more times with similar results.

not shown). Thus, loss of GFP-ASV expression by bacteria carrying the *cpxP-GFP* reporter was not simply due to enhanced proteolysis of GFP-ASV upon exposure to ASN. Rather, ASN had a seemingly specific inhibitory effect on expression of the *cpxP-GFP* promoter fusion.

Once inhibited by exposure to ASN, expression of the CpxP did not recover for up to 24 h later (data not shown). However, forced expression of PapE, a strong inducer of Cpx activation (52), was able to prevent complete abrogation of CpxP expression in the presence of ASN (Fig. 2.2.a), and could even resurrect CpxP expression at late time points following exposure to ASN (Fig. 2.2.b). Overexpression of another potent inducer of the Cpx pathway, NlpE (53), had a similar antagonistic effect (data not shown), indicating that ASN-mediated downregulation of CpxP expression can in effect be overridden under some conditions if the Cpx pathway is sufficiently activated by other stimuli.

Growth phase- and RelA-dependent effects on CpxP
expression in the presence of RNS

While exposure of early growth phase UTI89 cultures to ASN had a profound and lasting inhibitory effect on CpxP expression, the addition of ASN to mid-log or stationary phase cultures was seemingly inconsequential (Fig. 2.4). Coincident with bacterial entry into stationary phase is the accumulation of the alarmone guanosine tetraphosphate (ppGpp), a global transcriptional regulator that is synthesized by the *relA* gene product (54). As amino acids are depleted, increased ppGpp levels can drastically alter the transcriptional profile of the bacterial cell as part of the stringent response, diminishing

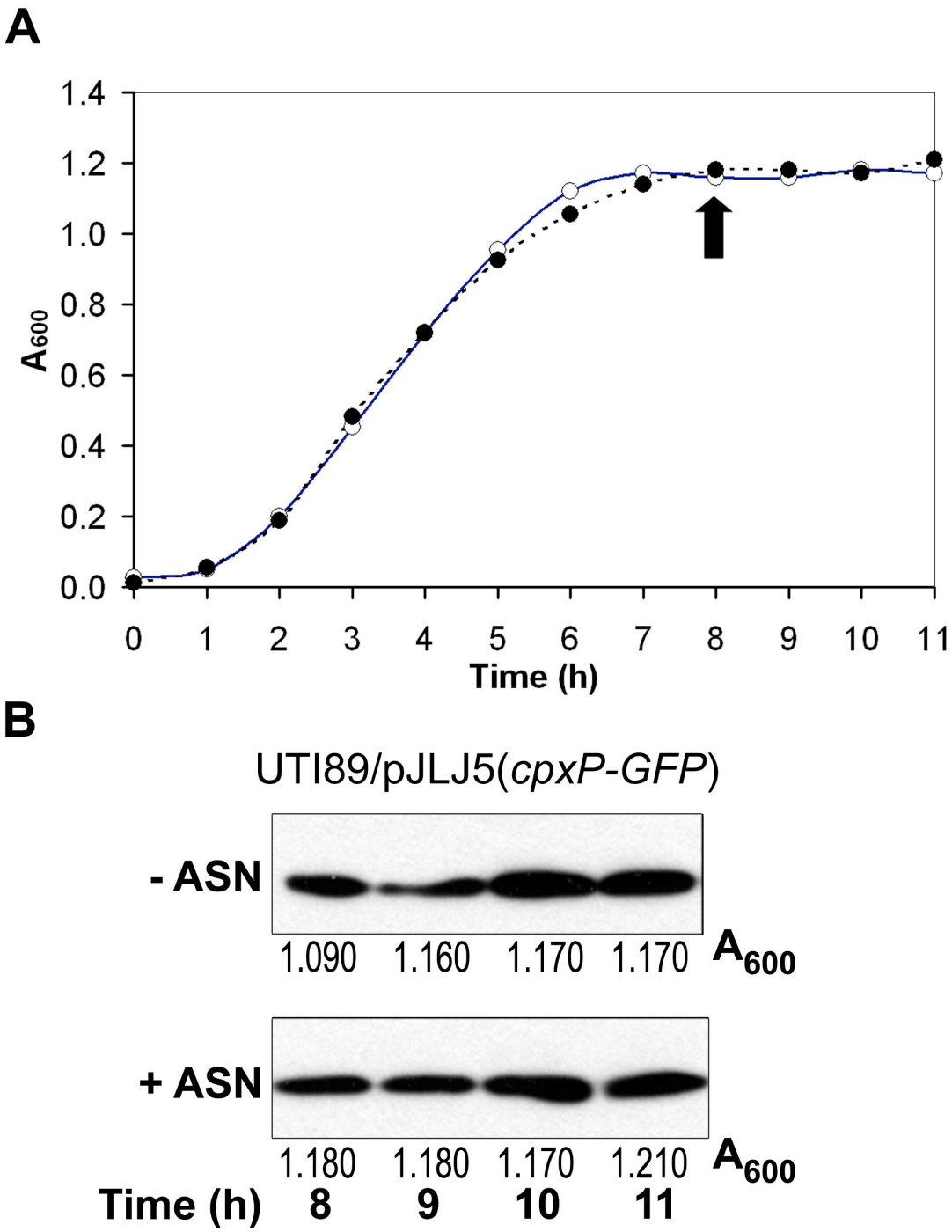

FIG 2.4. ASN effects on CpxP expression are limited in stationary phase cultures. (A) Representative growth curves of UTI89/pJLJ5 grown shaking in MES-LB in the presence (dotted line) or absence (solid line) of 1 mM ASN, which was added at the 8 h time point (arrow). (B) Western blots using anti-GFP antibody show expression of the *cpxP*-GFP reporter in the stationary phase cultures ± ASN. These experiments were repeated three times with similar results.

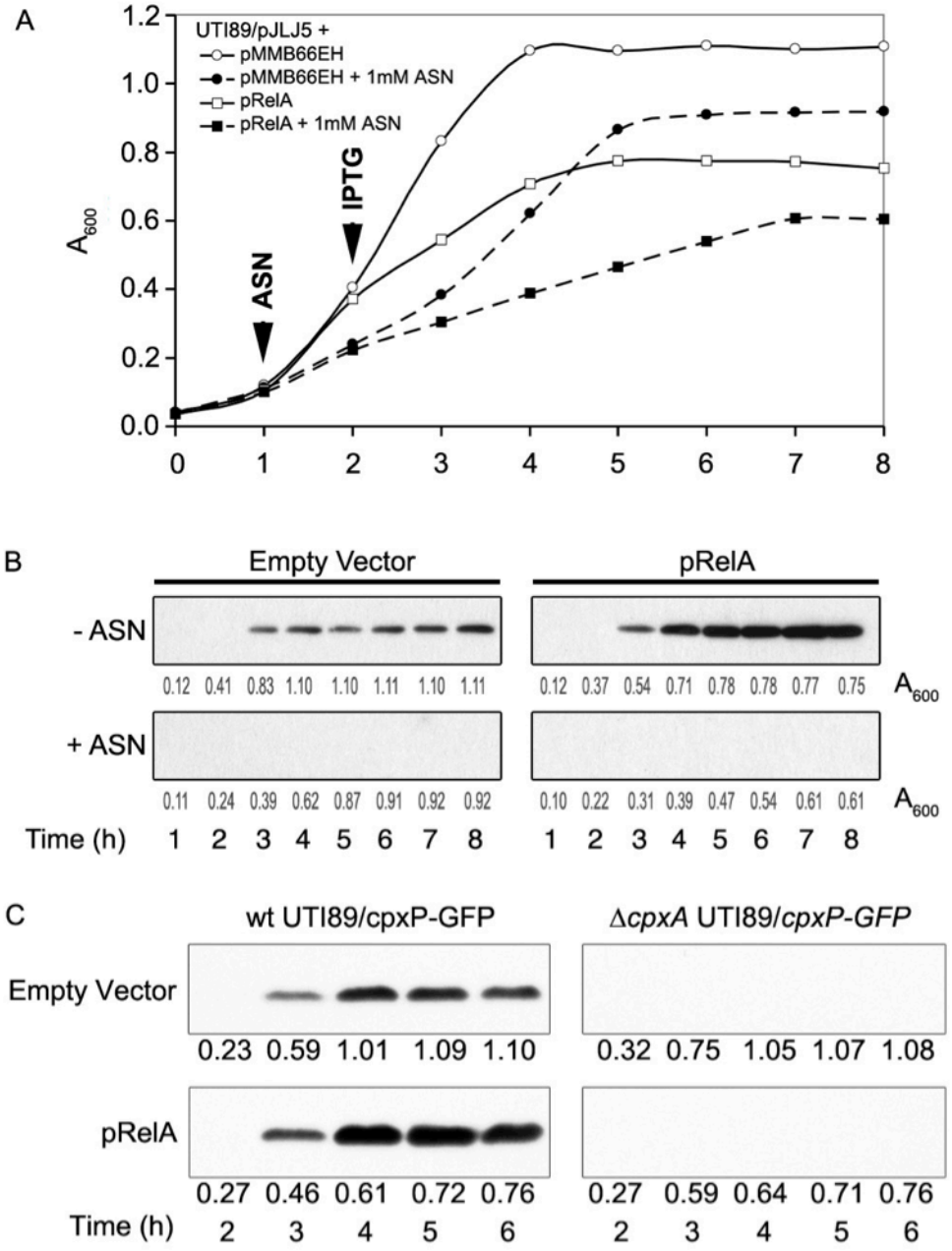

FIG. 2.5. RelA and CpxA effects on CpxP expression in UTI89. (A) Curves show growth of UTI89/pJLJ5 (*cpxP*-GFP reporter) carrying either pRelA (for IPTG-inducible expression of RelA) or the empty vector control pMMB66EH. ASN was added to the indicated cultures (dotted lines) at the 1 h time point, while IPTG was added to all samples at 2 h. (B) Westen blots probed with anti-GFP antibody showing levels of *cpxP*-GFP expression at hourly time points in the samples indicated in (A). (C) Western blots showing levels of *cpxP*-GFP expression over time in wild-type UTI89 versus an isogenic Δ*cpxA* mutant, either without (empty vector control) or with (pRelA) overexpression of RelA, which was induced at the 2 h time point. Results shown are representative of experiments repeated at least three times.

the expression of genes required for growth and proliferation while activating many genes involved in virulence and survival pathways (55). To test the capacity ppGpp levels to affect the Cpx pathway, and specifically ASN-mediated downregulation of CpxP, I employed an IPTG-inducible RelA expression construct (56). Overexpression of RelA drove UTI89 to enter stationary phase growth early (Fig. 2.5.a), and stimulated *cpxP*-GFP expression by as much as four fold over controls (Fig. 2.5.b). However, induced expression of RelA did not overcome the inhibitory effects of ASN on CpxP expression.

CsrA-mediated downregulation of CpxP by RNS

Semiquantitative reverse-transcription PCR (RT-PCR) was performed to assess *cpxP* message levels in UTI89 cultures grown to late log-phase (=0.8) in the presence or absence of 1 mM ASN. Amplification of 16S RNA served as a positive control in these assays. As shown in Figure 2.6.a, growth in ASN had little effect on transcription of either native *cpxP* or 16S RNA. Message levels of the *cpxP-GFP* promoter fusion was similarly unaffected by ASN, while *cpxR* was slightly elevated. These data indicate that ASN-mediated downregulation of CpxP expression likely occurs via a posttranscriptional mechanism.

Microarray analysis of UTI89 cultures grown to OD600=0.8 with or without ASN present corroborated these results, indicating that *cpxP*, *cpxR*, *cpxA*, and 16S RNA expression changed in response to ASN by only 0.84-, 1.45-, 1.50-, and 0.9-fold, respectively. In sharp contrast, the expression of many genes like *norV*, *narK*, *yeaR*, *yoaG*, which are known to be responsive to nitrosative stress based on previous work from my lab and others (57-61), were massively upregulated by growth of UTI89 in

ASN, with some being activated by greater than 1,000-fold. A summary of the top 100 most highly expressed genes according to functional category is presented in Fig. 2.6.b, and the genes represented in this graph are listed in Table 2.3. Nearly half of these genes (colored blue in Fig. 2.6.b) are involved in bacterial motility, and can be positively controlled either directly or indirectly by a master regulator of motility, the multimeric DNA-binding FlhDC protein complex (62).

By microarray analysis, expression levels of the genes encoding FlhDC were found to be upregulated about two-fold in the presence of ASN. As with other transcriptional regulators, even small changes in the abundance of FlhDC can potentially have sizeable effects on the expression of FlhDC-regulated genes (63). The *flhDC* transcript is bound and stabilized by CsrA, a global regulatory protein (64). CsrA is antagonized by two small noncoding RNA (sRNA) CsrB and CsrC, which sequester and thereby inactivate multiple CsrA homodimers (65, 66). Expression of CsrB is controlled by the BarA-UvrY two-component system, which itself is regulated by CsrA as part of an autoregulatory circuit (67). Recently, the BarA sensor kinase was shown to be responsive to formate and acetate, end products of glucose metabolism that can accumulate in growth media as bacteria transition into stationary phase (68).

CsrA recognizes consensus binding sites in the 5'-untranslated region (UTR) of many transcripts and can either activate, as in the case of *flhDC*, or inhibit translation of these target messages (64, 69). The remarkable induction of FlhDC-regulated genes in UTI89 in the presence of ASN (Fig. 2.6.b) suggested the potential involvement of CsrA, which I hypothesized could also negatively affect *cpxP* translation in my assays. As

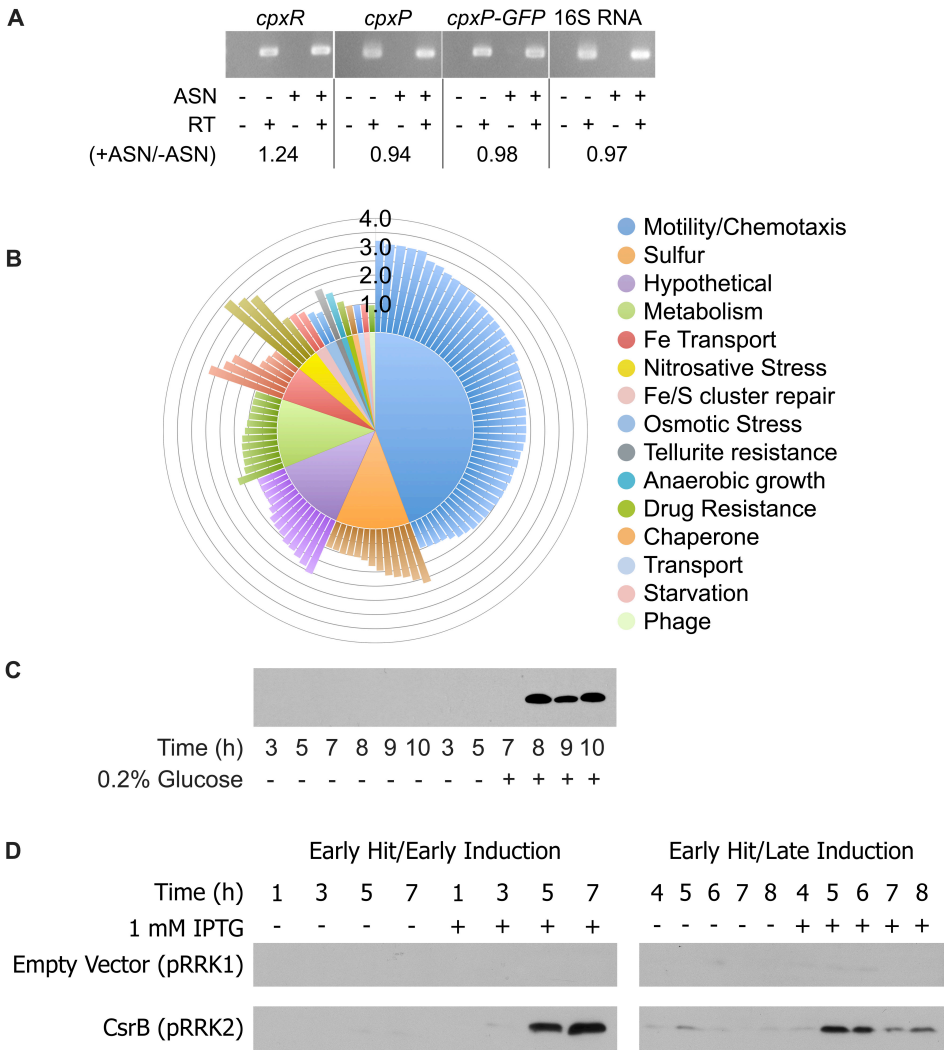

FIG. 2.6. Posttranscriptional control of CpxP expression by CsrA in the presence of ASN. (A) RT-PCR was performed on total RNA isolated from UTI89/pJLJ5 cultures grown to OD600=0.8 ± 1 mM ASN and primers specific for *cpxR*, *cpxP*, *gfp*, and 16S RNA. Mean integrated intensity rations of RT-PCR products from the ASN-treated versus nontreated samples (+ASN/-ASN) are shown, 1.0 indicating no difference in transcript levels between the +ASN and –ASN samples. (B) Pie-chart showing the top 100 upregulated genes in UTI89 grown to OD600=0.8 in the presence of 1 mM ASN, as assessed by Affymetrix *E.coli* 2.0 microarrays. Genes were sorted and color-coded by category, as indicated on the right. The outer bars in the graph represent fold-change differences between the ASN-treated and nontreated samples, shown on a $\log_{10}$ scale. (C) Western blot analysis of *cpxP*-GFP expression in UTI89/pJLJ5 grown in the presence of 1 mM ASN, with glucose added at the 7 h. (D) Western blots show *cpxP*-GFP expression in UTI89/pJLJ5 carrying either an empty vector control (pRRK1) or the IPTG-inducible CsrB expression construct pRRK2. All samples were treated with ASN at the 1 h time point, leading to abrogation of *cpxP*-GFP expression. This effect was countered by inducing CsrB expression with addition of IPTG at either the 1 or 4 h time points.

indirect support for this possibility, I found that the addition of 0.2% glucose to stationary phase cultures that were grown in the presence of 1 mM ASN resulted in the restoration of *cpxP*-GFP expression (Fig. 2.6.c). This effect may be explained by the ability of glucose to indirectly activate the BarA-UvrY two-component system, resulting in elevated CsrB levels and subsequent inhibition of CsrA (68). However, this interpretation is clouded by the fact that glucose can stimulate CpxP expression via the phosphotransacetylase-acetate kinase pathway and the generation of acetyl phosphate, which can directly activate CpxR (70-72). As a more direct test of CsrA involvement, I utilized an IPTG-inducible CsrB expression construct. Induced expression of CsrB either coordinate with the addition of ASN in early growth phase, or later as UTI89 entered late log phase growth, rescued expression of the *cpxP*-GFP reporter (Fig. 2.6.d). Together, these data indicate that CsrA can negatively affect the translation of *cpxP* in the presence of nitrosative stress.

Overexpression of CpxP enhances bacterial growth in ASN

The physiological consequences of RNS effects on the Cpx pathway, and CpxP specifically, were addressed using isogenic mutants of UTI89 lacking *cpxP*, *cpxR*, or *cpxA*. In control experiments, I noted that the Δ*cpxR* and Δ*cpxA* mutants were hypersensitive to amikacin, while the Δ*cpxP* mutant displayed enhanced resistance to this antibiotic (Fig. 2.7). These mutant phenotypes were rescued by complementation with plasmids encoding either *cpxRA* or *cpxP*, as appropriate. These results mirror those from previous studies in which K12 *E. coli* strains having mutated *cpx* genes displayed altered growth phenotypes in the presence of amikacin (73). Growth of UTI89 mutants lacking

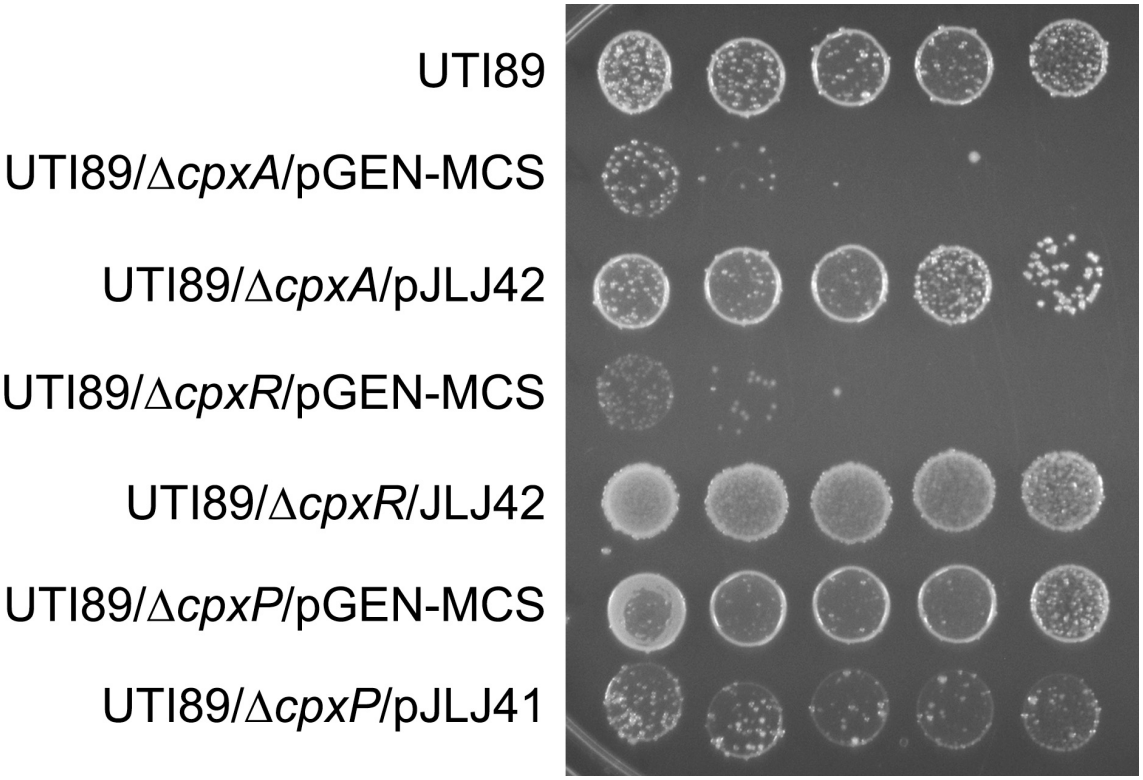

FIG 2.7. Trans complementation of the *cpx* mutants. Wild-type UTI89 and the *cpx* null mutants (Δ*cpxA*, Δ*cpxR*, and Δ*cpxP*), each carrying either the empty vector control pGEN-MCS or complementation plasmids, were grown overnight shaking in LB broth in the presence of ampicillin, as needed, to better maintain plasmid selection. Serial dilutions of the overnight cultures were spotted onto LB agar plates containing 3 $\mu g \cdot ml^{-1}$ amikacin, and incubated for 24 h at 37°C. The image shows bacterial growth on the amikacin plates, with dilutions starting at 10^{-2} on the left and ending at 10^{-6} on the right. Plasmid pJLJ41 encodes *cpxP* downstream of its native promoter, while pJLJ42 encodes the *cpxRA* operon under control of its native promoter. Due to polar effects, the expression of *cpxA* is likely disrupted in the Δ*cpxR* mutant, and so pJLJ42 was used for complementation of both the Δ*cpxR* and Δ*cpxA* mutant strains.

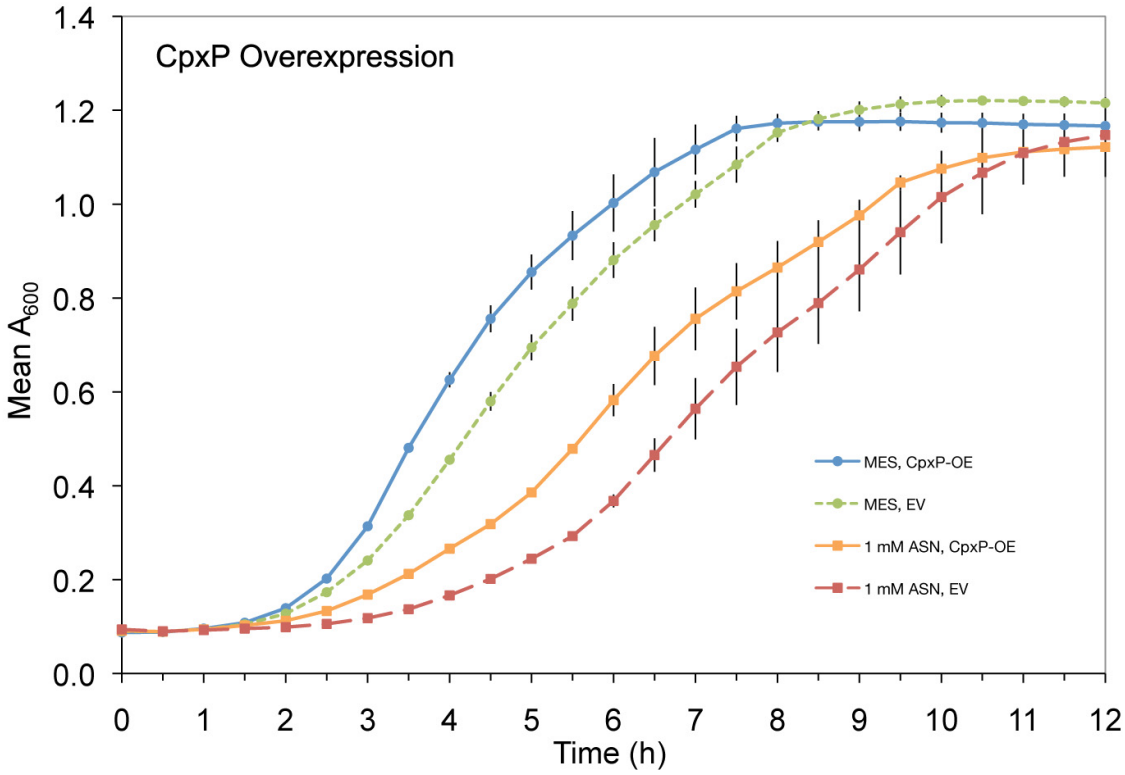

Fig. 2.8. Overexpression of CpxP enhanced UPEC growth in the presence of RNS. Curves indicate the growth of UTI89 carrying either an empty vector control (EV, pRR48; dotted lines) or the IPTG-inducible CpxP expression construct pRRK12 (OE; solid lines). All samples were grown in the presence of 1 mM IPTG ± 1 mM ASN, as indicated. Lines depict mean values from quadruplicate samples ± S.D.

any one of the *cpx* genes in LB or MES-LB broth ± 1 mM ASN was not notably different from the wild-type control strain (data not shown), suggesting that the Cpx system is not essential for UPEC resistance to RNS. However, induced overexpression of CpxP did enhance UPEC growth in the presence of ASN, enabling this pathogen to reach mid-log (=0.5) about 1 h ahead of controls (Fig. 2.8). This lead was reduced to 30 min in the absence of ASN.

Cpx confers a competitive advantage to

UPEC within the urinary tract

While deletion of the *cpx* genes had no detrimental effect on growth of UTI89 in my in vitro assays, I reasoned that phenotypes associated with these mutants may become more apparent within the urinary tract where the pathogens are likely to encounter much more stringent environments replete with RNS plus numerous other antimicrobial factors. To address this possibility, I employed a well-established mouse UTI model system in which $1X10^7$ CFU of bacteria were inoculated into adult female CBA/J mice via transurethral catheterization. After 3 d, bacterial numbers present in the bladders were determined by plating tissue homogenates. When wild-type UTI89 and each of the *cpx* mutants were inoculated by themselves into separate mice, no significant differences in the numbers of bacteria recovered 3 d were later observed (data not shown). However, when wild-type UTI89 was mixed 1:1 with each *cpx* mutant prior to inoculation, the wild-type strain was able to effectively outcompete each mutant (Fig. 2.9). These results demonstrate that the Cpx system can provide a clear competitive advantage to UPEC during the course of a UTI, supporting the possibility that dysregulation of the Cpx pathway by RNS may modulate bacterial fitness within the host environment.

Discussion

While the Cpx stress response pathway is known to modulate a number of virulence- and stress-associated phenomena in UPEC in vitro (23, 74), this is the first study to show that Cpx components significantly affect the fitness of UPEC within the

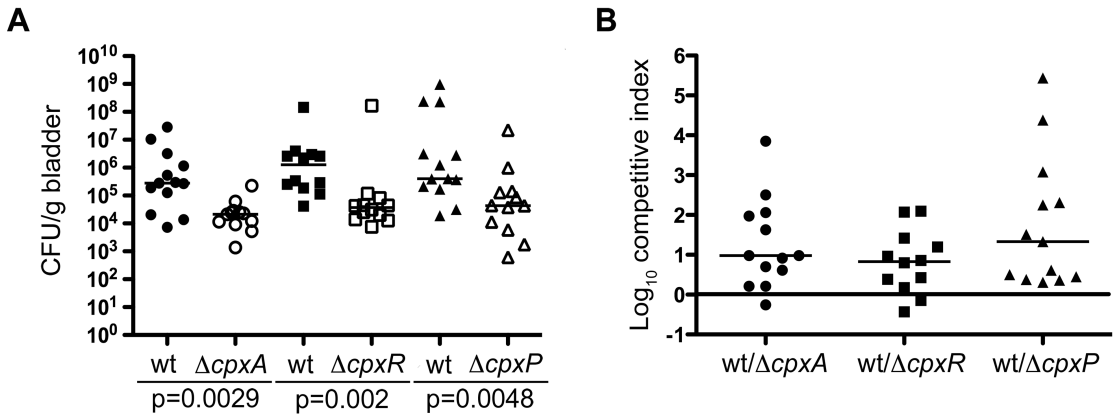

Fig. 2.9. Competitive advantage of wild-type UTI89 over isogenic cpx mutants within the bladder. Adult female CBA/J mice were inoculated with a 1:1 mixture of wild-type UTI89 with each of the individual *cpx* null mutants, Δ*cpxA*, Δ*cpxR*, or Δ*cpxP*. After 3 d, wild-type and mutant titers present within the bladder were determined. Data are presented as (A) total CFU/g bladder tissue and as (B) competitive indices, with values >0 indicating that the wild-type strain outcompeted the mutant within the host. The horizontal bars indicate median values for each group. *P* values were determined using Mann-Whitney U tests (*n* = 12-13 mice).

host during the course of a UTI. The diminished fitness of the *cpxP* null mutant is particularly intriguing considering its role as an auxiliary factor that is not strictly required for activation of the Cpx system (46, 47). Raivio and colleagues have suggested that the primary function of CpxP within the periplasm is to adjust the sensitivity of the CpxA histidine kinase to appropriate levels as environmental conditions vary (75). Accordingly, it was reported that deletion of *cpxP* leaves CpxA in a more active state, and consequently less responsive to Cpx-inducing cues (46, 48, 75). Therefore, modulation of CpxP levels within the periplasm likely provides UPEC and other bacteria with a means to fine-tune Cpx responses. The modulatory effects of CpxP may become more critical in rapidly changing and hostile environments as encountered within the host.

CpxP levels can be adjusted at the transcriptional level via activation of CpxR (46), or posttranslationally within the periplasm by proteolysis mediated by DegP (48, 49). Results presented here indicate a third mechanism involving translational repression of *cpxP* transcripts in the presence of RNS. Posttranscriptional control of mRNA transcripts in bacteria can be mediated by regulatory sRNA molecules or by RNA-binding proteins like CsrA. The RNA chaperone Hfq often promotes interactions between sRNA molecules and specific transcripts, usually resulting in accelerated decay or repressed translation of the target message (76). I recently reported that Hfq is a key regulator of multiple fitness and virulence phenotypes associated with UPEC, including resistance to high levels of RNS (77). However, Hfq was not required for RNS-mediated abrogation CpxP expression in UPEC as observed in this study (data not shown). Rather, my data implicate CsrA, a global regulatory protein that binds as a dimer to the 5'-UTRs of target transcripts, often occluding the Shine-Delegarno sequence (66, 78-84).

CsrA recognizes the consensus sequence RUACARCGAUGU in target transcripts (85), and can act to either promote or repress translation. Key systems that are negatively controlled by CsrA include glycogen synthesis, gluconeogenesis, and biofilm development (84, 86-89). CsrA positively affects glycolysis, acetate metabolism, and motility, with the latter being mediated via translational effects on the master regulator of motility, FlhDC (64, 87, 90). The ability of CsrA to enhance translation of the *flhDC* mRNA likely contributed to the high abundance of motility-associated genes that I detected by microarray analysis as being highly upregulated in UTI89 grown in the presence of ASN. Stimulation of these motility genes by ASN may represent a defense

TABLE 2.1. Strains and plasmids used in this study

Strain or plasmid	Description	Source
Strains		
UTI89	UPEC cystitis isolate	(104, 105)
MM530	UTI89 *ΔcpxA::clm*	This study
MM639	UTI89 *ΔcpxP::clm*	This study
MM427	UTI89 *ΔcpxR::clm*	This study
TT23216	*Salmonella* template strain containing a clm^R cassette flanked by universal primer sequences	J. Roth, (77)
MM173	UTI89/pJLJ5	This study
MM495	UTI89/pJLJ10	This study
MM1097	UTI89/pRR48	This study
MM1095	UTI89/pRRK12	This study
MM371	UTI89/pGFP(ASV)	This study
MM638	UTI89/pKM208	This study
MM527	UTI89/pJLJ5/pHJ13	This study
MM503	UTI89/pJLJ5/pLD404	This study
MM531	UTI89 *ΔcpxA::clm*/pJLJ5	This study
MM444	UTI89 *ΔcpxR::clm*/pJLJ5	This study
MM315	UTI89/pMMB66EH	This study
MM316	UTI89/pMMB66EH/pJLJ5	This study
MM511	UTI89/pJLJ5/pMMB66EH	This study
MM512	UTI89/pJLJ5 /pRelA	This study
MM641	UTI89 *cpxA::clmR*/pJLJ5 /pMM66EH	This study
MM533	UTI89 *cpxA::clmR*/pJLJ5/pMMBRelA	This study
MC4100	Laboratory K12 *E. coli* strain	*Coli* Genetic Stock Center
Plasmids		
pACYC177	Parent of pJLJ1, pJLJ5 and pJLJ10; p15A ori; Kan^r Amp^r	New England Biolabs
pGFP(ASV)	Expresses GFP(ASV) from P_{lac} promoter; Amp^r	Clontech
pJLJ1	Control vector carrying promoterless *gfp(ASV)*; Kan^r	This study
pJLJ5	Vector for expression of GFP(ASV) from the *cpxP* promoter region; Kan^r	This study
pJLJ10	Vector for expression of GFP(ASV) from the *cpxR*A promoter region; Kan^r	This study

TABLE 2.1. Continued

Strain or plasmid	Description	Source
Plasmids		
pRR48	*lacI*^{q}/p$_{tac}$ expression vector; Amp^{r}	S. Parkinson, (103)
pRRK12	IPTG-inducible CpxP expression construct; Amp^{r}	This study
pRRK1	Parental plasmid for pRRK2, derived from pRR48 by mutation of the Shine-Dalgarno sequence; Amp^{r}	This study
pRRK2	Encodes IPTG-inducible CsrB; Amp^{r}	This study
pLD404	Vector for constitutive expression of NlpE; Amp^{r}	(53)
pHJ13	Encodes IPTG-inducible PapE; Amp^{r}	(45)
pKM208	IPTG-inducible Red recombinase expression plasmid; Amp^{r}	(107)
pJLJ41	Expresses CpxP under its native promoter; Amp^{r}	This study
pJLJ42	Expresses CpxRA under its native promoter; Amp^{r}	This study
pGEN-MCS	Parent plasmid for pJLJ41 and pJLJ42, contains *hok sok* post-segregation killing system and two par loci; Amp^{r}	-102
pMMB66EH	Empty-vector control vector for pRelA; Amp^{r}	(113)
pMMBRelA	IPTG-inducible RelA expression construct; Amp^{r}	(56)

TABLE 2.2. Primers used in this study

Primer[a]	Sequence	Construct/ Gene
Plasmid construction		
MP141 – F	AACTGGATCCTCATTGTTTAAATACCTCCG	pJLJ5
MP142 – R	TGATGGTACCTTGCTCCCAAAATCTTTCGT	pJLJ5
MP149 – F	CGCTCGGATCCCATCATTTGCTCCCAAAATCTT	pJLJ10
MP150 – R	AACCGGGTACCTGTTTAAATACCTCCGAGGCA	pJLJ10
CpxP for (pst1)	AATCCTGCAGATTGTTTAAATACCTCCGAGGC	pJLJ41
CpxP rev (salI)	TAGAGTCGACTACCAGCGCGGCGAGAATAC	pJLJ41
CpxRA for (pst1)	TGCTCTGCAGTCATTTGCTCCCAAAATCTTTCT	pJLJ42
CpxRA rev (salI)	GCTAGTCGACAGCGGCAAGATCGAAGATTTTT	pJLJ42
P222 - F	GTGAGCGGATAACAATTTACTGCAGTGTGAAATGCTGC AGGATCCG	pRRK1
P223 - R	CGGATCCTGCAGCATTTCACACTGCAGTAAATTGTTATC CGCTCAC	pRRK1
P216 - F	GGGGTACCCCAACTGCCGCGAAGAATAGCA	pRRK2
P224 - R	CAATGCATTGGTTCTGCAGTTTTGTCTGTAAGCGCCTTG TAA	pRRK2
P322 - F	CGGGATCCCAGATTTTGGGAGCAAATGATGCG	pRRK12
P323 - R	GGGGTACCAAGCAGCAGGCAAATTGAGGATAAA	pRRK12
RT-PCR		
MP22 - F	TCATTAAGCCACGCTGCTGA	cpxP
MP23 - R	ACTCAATAGCTTCAACGATGAA	*cpxP*
MP24 - F	ATGACCAGCCACACTGGAACT	16S rRNA
MP25 - R	AGTATCAGATGCAGTTCCCAG	16S rRNA
MP43 - F	AGGGTGAAGGTGATGCAACA	*gfp*
MP44 - R	CTCAAGAAGGACCATGTGGT	*gfp*
MP45 - F	AAAGTCATGGATTAGCGACGT	*cpxR*
MP46 - R	TCAGCACTAAGGCATCAACTT	*cpxR*

TABLE 2.2. Continued

Primer[a]	Sequence	Construct/ Gene
Knockout construction		
MP47 - F	ATGCGGCGTAAACGCCTTATCCTGCCTACAAATGCGGAG TCACCAAACACCCCCCAAAACC	*cpxA*
MP48 - R	AAACCTTGCGTGGTCGCGGCTATCTGATGGTTTCTGCTT CCACACAACCACACCACACCAC	*cpxA*
MP63 - F	CTCTCTATCGTTGAATCGCGACAGAAAGATTTTGGGAGC AACACCAAACACCCCCCAAAACC	*cpxP*
MP64 - R	GAAGACAGGGATGGTGTCTATGGCAAGGAAAACAGGG TTTCACACAACCACACCACACCAC	*cpxP*
MP143 - F	ATGAATAAAATCCTGTTAGTTGATGATGACCGAGAGCTG ACTTCCCTATTTGTGTAGGCTGGAGCTGCTTCG	*cpxR*
MP144 - R	TCATGAAGCAGAAACCATCAGATAGCCGCGACCACGCA AGGTTTTAAACCACATATGAATATCCTCCTTAG	*cpxR*

[a] F and R refer to forward and reverse primers.

strategy for UPEC, enabling these pathogens to better evade RNS generated within the host or other environments.

The microarray results suggested CsrA activity may be of significant consequence to UPEC in the face of RNS. To test this possibility, I tested the ability of CsrA to modulate CpxP levels. Although I was able to construct a *csrA* null mutation for this purpose, this mutant strain proved to be exceptionally difficult to work with, forming large insoluble aggregates that have made subsequent analyses problematic. Consequently, I resorted to alternate approaches. CsrA is antagonized by the sRNA CsrB, the transcription of which is promoted by activation of the BarA-UvrY two-component system, which responds to glucose and, more specifically, the glucose metabolic end products formate and acetate (65, 67, 68). The BarA-UvrY system, in turn, is positively

regulated by CsrA, creating an autoregulatory loop (67). In my assays, ASN-mediated attenuation of CpxP translation was countered by the induced expression of CsrB, as well as addition of the BarA activator glucose.

These observations link the regulation of the Cpx system to a number of pathways that are integral to bacterial fitness in general, and to UPEC virulence in particular. Specifically, the BarA-UvrY system has been implicated as a virulence determinant for UPEC within the urinary tract (91), and the capacity of CsrA to control biofilm formation and carbon utilization pathways has implicit relevance to the survival of UPEC within the host (92-94). Recently, it was reported that UPEC isolates are often much better equipped to handle RNS, being able to survive and adapt to high levels of ASN (2, 57, 95). Part of this adaptive response involved upregulation of the polyamine cadaverine, which can facilitate UPEC growth in the face of RNS (2, 57). Interestingly, FlhDC acts as a positive regulator of *cadA*, which encodes a lysine decarboxylase involved in cadaverine biogenesis (96), suggesting another link between CsrA and the fitness and virulence potential of UPEC.

The ability of CsrA to repress CpxP expression in the presence of ASN is also likely pertinent to the pathogenesis of UTIs. On one hand, CsrA-mediated effects on *cpxP* translation may act as part of an adaptive response, altering activation levels of CpxA and perhaps enabling UPEC to better deal with the effects of nitrosative stress on the bacterial envelope. Alternatively, dysregulation of the Cpx system due to RNS may represent a specific detriment with which UPEC must deal. RNS-mediated downregulation of CpxP expression in conjunction with additional stresses and antimicrobial factors encountered

within the host may act to restrict UPEC growth and dissemination. This scenario is supported by my observations that a *cpxP* null mutant is significantly disadvantaged within the host urinary tract, at least when in competition with the wild-type pathogen. In the end, it is likely that RNS-mediated attenuation of CpxP expression will have variable effects on UPEC fitness depending on the cumulative input of multiple signaling systems and environmental cues.

Whether or not CsrA abrogates CpxP expression by directly interacting with *cpxP* transcripts or by other less direct means remains to be tested. Using a primer walking technique, I determined that the 5'-UTR of *cpxP* extends at least 45 bp upstream of the translation start site (see Fig. 2.1.a and data not shown). Within this region there is potentially one degenerate CsrA binding site overlapping the Shine-Delgarno sequence. It is feasible that modification of CsrA in the presence of RNS alters its binding specificity, enabling CsrA to recognize an alternate repertoire of mRNA transcripts. In support of this possibility, I have found by both Western blot analysis and mass spectroscopy that a highly conserved tyrosine residue (Y47) within CsrA becomes nitrated when purified CsrA is exposed to ASN (unpublished observations). Y47 is located within a loop region surrounded by residues known to mediate CsrA-RNA interactions (81, 97). I am currently working to understand the functional significance of Y47 nitration. However, it is noteworthy that there is a growing body of evidence that tyrosine nitration in eukaryotic systems can modify protein function and alter signaling cascades (98-101). Since UPEC can produce their own RNS via nitrite reductases under conditions of low oxygen tension, as often encountered within host niches like the bladder (7), it may be that these bacteria

can also employ RNS as signaling molecules, with CsrA and, indirectly, *cpxP* being functionally relevant targets.

Experimental procedures

Plasmid constructs

Plasmids and primers used in this study are listed in Tables 2.1 and 2.2, respectively. To create the *cpxP-gfp* promoter fusion, the 146 bp region between the divergent start sites of *cpxR* and *cpxP* from the *E. coli* strain MC4100 was amplified by PCR using primers MP141 and MP142 (Table 2.2). The PCR product was digested with BamH1 and Asp718 and ligated into the multiple cloning site of pGFP(ASV) (Clonetech). The resulting plasmid was digested with BamH1 and BsiW1 to generate a ~900 bp fragment containing the *cpxP* promoter upstream of the *gfp*(ASV) gene. This fragment was ligated into the BamH1 and Ban1 sites of the low copy number plasmid pACYC177 (New England Biolabs) to create pJLJ5. To construct pJLJ10, the 146 bp *cpxP* promoter region in pJLJ5 was replaced with the same sequence in the opposite orientation. As a control, plasmid pJLJ1 was constructed by ligating the promoterless *gfp* (ASV) gene from pGFP(ASV) into the BamH1 and Ban1 sites of pACYC177. In control experiments, *E. coli* strains carrying pJLJ1 did not express any detectable GFP under any conditions tested. Plasmid pJLJ41 was made by first amplifying *cpxP* off the UTI89 chromosome along with 250 bp of upstream and 100 bp of downstream sequences using primers CpxPfor(pst1) and CpxPrev(salI). The resulting PCR product was digested and ligated into PstI and SalI sites within the high-retention, low copy number plasmid

pGEN-MCS (102). pJLJ42, carrying *cpxRA* plus 250 bp of upstream and 100 bp of downstream sequences, was similarly constructed using primers CpxRAfor(pst1) and CpxRArev(salI).

Plasmids pHJ13 (encoding IPTG-inducible PapE), pLD404 (for constitutive expression of NlpE), pMMBrelA (for IPTG-inducible expression of RelA), and the expression construct pRR48 have been described previously (45, 53, 56, 103). To create pRRK12, the *cpxP* sequence from UTI89 was amplified using primers P322 and P323, which introduced flanking 5'-BamH1 and 3'-Kpn1 restriction sites used to ligate *cpxP* downstream of the *tac* promoter in pRR48. Plasmid pRRK1 was constructed using primers P222 and P223 to mutate the Shine-Dalgarno sequence within pRR48 to a PstI site (Site-Directed Mutagenesis II Kit, Stratagene). The *csrB* coding sequence from UTI89 was amplified using primers P216 and P224 and ligated into PstI and BamHI sites within pRRK1 to create pRRK2.

Bacterial strains and growth conditions

Strains used in this study are listed in Table 2.1. Plasmids were introduced into UTI89, a human cystitis isolate (104, 105), by electroporation. Targeted gene knockouts were created in UTI89 using linear transformation and lambda Red-dependent recombination essentially as described (106, 107). Primers were designed to amplify the chloramphenicol (*clm*) resistance gene from strain TT23216 with appropriate flanking sequences having homology to regions within and around *cpxR*, *cpxA*, or *cpxP*. All gene knockouts were verified by PCR of the affected genomic regions.

TABLE 2.3. Top one hundred upregulated microarray genes

Gene	UTI89 Locus Tag	Function	Ratio[a]	Probe set[b]
ygbD	C3073	Flavorubredoxin oxidoreductase	2,554.8	1766754_s_at
flgB	C1198	Flagellar biosynthesis	1,612.4	1762105_s_at
norV	C3072	Flavorubredoxin	1,253.1	1768449_s_at
			723.4	1767683_at
			53.9	1768764_s_at
flgE	C1201	Flagellar biosynthesis	1,246.4	1760787_s_at
flgC	C119	Flagellar biosynthesis	1,227.1	1768120_s_at
flgD	C1200	Flagellar biosynthesis	1,158.2	1760643_s_at
flgG	C1203	Flagellar biosynthesis	1,155.5	1767435_s_at
fliM	C2145	Flagellar biosynthesis	617.0	1761189_s_at
fliL	C2144	Flagellar biosynthesis	551.8	1768910_s_at
ycdO	C1081	Hypothetical protein	548.7	1764800_s_at
			206.6	1763371_at
fliN	C2146	Flagellar biosynthesis	406.9	1765241_s_at
flgA	C1197	Flagellar biosynthesis	358.0	1763116_s_at
fliA	C2123	Flagellar biosynthesis	317.6	1763490_s_at
flgH	C1204	Flagellar biosynthesis	286.3	1768045_s_at
fliK	C2143	Flagellar biosynthesis	205.5	1759897_s_at
flgJ	C1206	Flagellar biosynthesis	202.0	1763207_s_at
flgF	C1202	Flagellar biosynthesis	182.8	1761549_s_at
fliF	C2138	Flagellar biosynthesis	175.3	1763750_s_at
fliO	C2147	Flagellar biosynthesis	164.8	1761160_s_at
flgK	C1207	Flagellar biosynthesis	153.0	1761245_s_at
fliI	C2141	Flagellar biosynthesis	152.1	1766980_s_at
yoga	C1994	Hypothetical protein	146.7	1762744_s_at
ycdB	C1082	Hypothetical protein	137.8	1768768_at
fliJ	C2142	Flagellar biosynthesis	136.9	1760976_s_at
flgI	C1205	Flagellar biosynthesis	112.7	1765040_s_at
cysI	C3127	Sulfite reduction	112.5	1768272_s_at
			76.8	1767086_at
fliC	C2124	Flagellar biosynthesis	99.1	1765832_s_at
c2201	C1993	Hypothetical protein	92.8	1762869_s_at
fliG	C2139	Flagellar biosynthesis	81.8	1766530_s_at
Flip	C2148	Flagellar biosynthesis	79.7	1767430_s_at
fliD	C2125	Flagellar biosynthesis	79.5	1764241_s_at
tar	C2089	Chemotaxis	74.7	1768914_s_at
year	C1995	Putative tellurite resistance protein	74.1	1768944_s_at

TABLE 2.3. Continued

Gene	UTI89 Locus Tag	Function	Ratio[a]	Probe set[b]
Aer	C3510	Chemotaxis	69.9	1766701_s_at
flhA	C2082	Flagellar biosynthesis	69.7	1767873_s_at
Tap	C2088	Chemotaxis	67.4	1766991_s_at
c3225	C3031	Hypothetical protein	62.4	1768634_s_at
flhB	C2083	Flagellar biosynthesis	59.4	1766226_s_at
cysJ	C3128	Sulfite reduction	55.9	1765526_s_at
fliZ	C2122	Flagellar biosynthesis	51.2	1760453_s_at
cheA	C2091	Chemotaxis	47.9	1766750_s_at
aceA	C4574	Isocitrate lyase	46.6	1763981_s_at
ybdB	C0599	Hypothetical protein	43.8	1761866_s_at
cysH	C3126	Sulfite reduction	43.3	1760382_at
			25.5	1759826_s_at
fdnI	C1692	Formate dehydrogenase	42.1	1760891_s_at
fliS	C2126	Flagellar biosynthesis	40.9	1764033_s_at
flgL	C1208	Flagellar biosynthesis	40.8	1768710_s_at
cysP	C2758	Sulfite reduction	39.5	1764785_at
			11.0	1762292_s_at
Nark	C1420	nitrite extrusion protein	37.7	1764323_s_at
fliH	C2140	Flagellar biosynthesis	36.8	1760053_s_at
motA	C2093	Chemotaxis	35.6	1763982_s_at
fdnH	C1691	Formate dehydrogenase	34.3	1763995_s_at
cheR	C2088	Chemotaxis	33.5	1760164_s_at
cheW	C2090	Chemotaxis	30.6	1759302_s_at
cheY	C2086	Chemotaxis	29.4	1764851_s_at
c2419	C2178	Hypothetical protein	28.5	1763274_at
cysA	C2755	Sulfate transport	28.4	1762301_at
cheB	C2087	Chemotaxis	26.6	1765173_s_at
fliQ	C2149	Flagellar biosynthesis	26.6	1766705_s_at
iscR/yfhP	C2853	Fe-S cluster-containing transcription factor	25.9	1764175_s_at
cysD	C3123	Sulfate adenylyltransferase	22.5	1765655_s_at
cheZ	C2085	Chemotaxis	22.1	1765504_s_at
yghK	C3391	Glycolate permease	21.8	1759891_s_at
Flit	C2127	Flagellar biosynthesis	19.7	1762151_s_at
yncE	C1671	Hypothetical protein	19.6	1759697_s_at
astD	C1941	Succinylglutamic semialdehyde dehydrogenase	19.0	1761460_s_at
entB	C0597	Isochorismatase	18.4	1765518_s_at

TABLE 2.3. Continued

Gene	UTI89 Locus Tag	Function	Ratio[a]	Probe set[b]
yhjC	C4053	Transcriptional regulator	18.0	1767782_s_at
betB	C0341	Betaine aldehyde dehydrogenase	17.6	1766956_at
fdnG	C1689	Formate dehydrogenase	16.7	1761354_s_at
marB	C1751	Multiple antibiotic resistance protein	16.7	1760768_s_at
chuS	C4027	Heme/hemoglobin transport protein	15.7	1763313_s_at
cysN	C3122	ATP-sulfurylase	14.9	1763768_s_at
yhjH	C4057	Chemotaxis	14.8	1765046_s_at
beta	C0340	Choline dehydrogenase	13.2	1766741_at
gltB	C3649	Glutamate synthase	13.2	1768316_at
sitA	C1339	Fe Transport	13.1	1761573_at
chuX	C4035	Hypothetical protein	12.7	1769108_s_at
flhE	C2081	Flagellar biosynthesis	11.6	1767174_at
aceB	C4573	Malate synthase A	11.3	1761179_at
flgM	C1196	Flagellar biosynthesis	11.1	1768555_s_at
cysU	C2757	Sulfate transport system permease	10.9	1759413_at
cysC	C3121	Adenylylsulfate kinase	10.8	1761476_s_at
ymdA	C1167	Hypothetical protein	10.6	1764274_s_at
iscS/yfhO	C2582	Cysteine desulfurase	10.5	1759704_s_at
fhuF	C5073	Ferric iron reductase protein fhuF	10.5	1761858_at
dnaJ	C0017	Chaperone	10.5	1769019_s_at
yjcZ	C4704	Hypothetical protein	10.4	1763969_s_at
motB	C2092	Chemotaxis	10.4	1767171_s_at
ycjX	C1592	Hypothetical protein	10.3	1768270_s_at
yiaK	C4117	Oxidoreductase	10.1	1759230_s_at
nrdH	C3033	Glutaredoxin-like protein	9.9	1764080_s_at
cstA	C0600	Carbon starvation protein A	9.6	1766639_s_at
acs	C4659	Acetyl-CoA synthetase	9.2	1761238_s_at
cyoC	C0454	Cytochrome subunit	9.0	1767399_s_at
tsr	C5058	Chemotaxis	9.0	1762388_s_at
yeaJ	C1982	Hypothetical	9.0	1767540_s_at
cyoE	C0451	Cytochrome subunit	8.8	1759601_s_at
c2436	C2188	Putative pesticin receptor	8.8	1767260_at
ompT	C0566	DLP12 Prophage	8.8	1765378_s_at

[a] Ratio of the expression in cultures containing ASN to the expression in cultures not containing ASN.
[b] When different probe sets for a single gene yielded different results, all probe sets are listed.

Except where noted, UTI89 and its derivatives were cultured from -80°C frozen stocks in Luria-Bertani broth buffered at pH 5.0 with 100 mM morpholineethanesulfonic acid (MES-LB). Kanamycin (50 $\mu g \cdot ml^{-1}$), ampicillin (50 $\mu g \cdot ml^{-1}$), and/or chloramphenicol (20 $\mu g \cdot ml^{-1}$) were included when appropriate to maintain plasmid selection. After an overnight incubation shaking at 37°C, bacteria were subcultured 1:100 into fresh MES-LB. Sodium nitrite and isopropyl β-D-1-thiogalactopyranoside (IPTG) were each added to a final concentration of 1 mM as indicated. Bacteria used for RT-PCR, microarray, and Western blot analyses were grown shaking at 225 rpm at 37°C in 5 ml cultures within 120x17 mm screw-capped polystyrene conical tubes (Sarstedt). Growth was monitored by determining the optical density at 600 nm using a Spectronic 20D+ (Thermo). Other growth curves were obtained using shaking 200-ml cultures in 100-well honeycomb plates and a Bioscreen C instrument (Growth Curves USA). Antibiotics, sodium nitrite, and MES were purchased from Sigma-Aldrich, while IPTG was from Teknova.

To test the sensitivities of the *cpx* mutants and complemented strains to amikacin, serial dilutions of overnight bacterial cultures (grown in LB broth in the presence of ampicillin to maintain plasmid selection) were plated as 5 ml drops on LB agar containing 3 $\mu g \cdot ml^{-1}$ amikacin. After a 24 h-incubation at 37°C, plates were photographed using a GelDoc XR instrument (BioRad).

Western blot analysis

Bacterial samples were pelleted at 12000·g for 2-3 min at 4ºC, lysed in Bacterial Protein Extraction Reagent (Pierce), sonicated for 1 min, heated at 100ºC for 5 min, and stored overnight at -20ºC. Equivalent amounts of protein (20 µg as determined by BCA assay, Pierce) from each sample were resolved in 10% acrylamide using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently transferred to PVDF membranes (Millipore) for Western blot analysis. All antibody incubations were performed in 1% BSA, 1% powdered milk, 0.1% Tween-20 in Tris-Buffered Saline (pH 7.4). Blots were incubated with mouse anti-GFP (1:10,000; Santa Cruz) for 45 min at room temperature, followed by a 1-h incubation with secondary anti-mouse HRP-conjugated IgG antibody (1:10,000; Amersham Biosciences). Blots were then washed, developed using the SuperSignal West Pico or SuperSignal West Femto Chemiluminescent kit (Pierce), and exposed to CL-XPosure Film (Pierce). To ensure that equivalent amounts of protein from each sample were loaded, duplicate gels were stained using GelCode Blue (Pierce) and/or blots were re-probed using goat anti-*E. coli* antisera (1:1,000; BioDesign).

Microarray sample preparation

Four separate colonies of UTI89 grown on LB agar plates from a freezer stock were used to start overnight shaking cultures in LB-MES broth. These were then diluted 1:100 into 5 mL LB-MES and grown shaking ± ASN at 37ºC in duplicate 120x17 mm screw-capped conical tubes until the OD600 was 0.8, at which point the bacteria were quickly pelleted and frozen at -80°C for at least 12 h. RNA was extracted using hot

phenol-chloroform and purified by CsCl gradient centrifugation (108). Synthesis of cDNA and subsequent fragmentation and labeling were performed according to Affymetrix protocols.

Microarray gene expression analysis

Fragmented and labeled cDNA (15 μg) was mixed with 270 μl hybridization buffer and hybridized to Affymetrix GeneChip *E. coli* 2.0 genome arrays at 45ºC for 20 h. The GeneChips were then washed, stained, and scanned using Affymetrix protocols and an Affymetrix GeneChip 3000 device with high-resolution scanning enabled. Raw images were converted to CEL files with Affymetrix GCOS software and image processing using the GCRMA method for probe-level data (109) was carried out using the Bioconductor Package in the R statistical environment (110). CEL files were analyzed as a group, background corrected using GCRMA (111), and normalized using quantile normalization. Median polish was used to obtain probe set summary measures. Organization of transcripts was based on available gene data from Affymetrix NetAffx (http://www.affymetrix.com/analysis/index.affx), the annotated UTI89 genome (105), and EcoCyc (http://ecocyc.org/, (112)).

Microarray data accession number

Complete microarray data have been deposited in the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/).

RT-PCR analysis

Total RNA was collected from bacterial cultures (grown ± ASN to an of 0.8) as described above. M-MLV reverse transcriptase (Ambion, Austin, TX) was used to reverse transcribe 1 μg of total RNA using random hexamer primers (Invitrogen) followed by PCR with gene-specific primers listed in Table 2.2 under the RT-PCR heading. Reactions set up without reverse transcriptase were used as controls. Amplicons ranging in size from 340 to 570 bp were resolved using 2% agarose gels, stained with ethidium bromide, and imaged using a GelDoc system (Bio-Rad Laboratories). Integrated band intensities were quantified using ImageJ software (NIH).

Mouse infections

Seven to 8 week old female CBA/J mice (Jackson Labs) were used in accordance with IACUC-approved protocols. Wild type UTI89 and the *cpx* knockout strains were grown from frozen stocks in 20 ml static LB broth at 37ºC for 24 h. Bacteria were pelleted by centrifugation for 8 min at 8,000·g and then resuspended in phosphate-buffered saline. Mice were briefly anesthetized using isofluorane inhalation and inoculated via transurethral catheterization with 50 ml of a bacterial suspension containing ~1 X 10^7 CFU total bacteria. For competition assays, wild type UTI89 and each *cpx* mutant strain were mixed 1:1 prior to inoculation. After 3 d, bladders were collected, weighed, and homogenized in PBS containing 0.02% Triton X-100. Homogenates were serially diluted and plated on LB agar plates ± chloramphenicol to determine the number of bacteria present per gram of tissue and to distinguish wild type UTI89 from the *cpx* knockout mutants. Competitive indices were calculated as Log_{10}

[(wild-type CFU recovered/mutant CFU recovered)/(wild-type CFU inoculated/mutant CFU inoculated)], such that values of greater than 0 indicate that the wild type strain outcompeted the mutant. Mouse experiments were repeated twice with similar results (total combined data is presented in Fig. 2.9).

Statistics

Results from the mouse experiments were analyzed by Mann-Whitney U tests using Prism 5.01 software (Graphpad Software). *P* values of less than 0.05 were considered significant.

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CHAPTER 3

IMPACT OF THE RNA CHAPERONE HFQ ON THE FITNESS AND VIRULENCE POTENTIAL OF UROPATHOGENIC *ESCHERICHIA COLI*

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Impact of the RNA Chaperone Hfq on the Fitness and Virulence Potential of Uropathogenic *Escherichia coli*▽†

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Hfq is a bacterial RNA chaperone involved in the posttranscriptional regulation of many stress-inducible genes via small noncoding RNAs. Here, we show that Hfq is critical for the uropathogenic *Escherichia coli* (UPEC) isolate UTI89 to effectively colonize the bladder and kidneys in a murine urinary tract infection model system. The disruption of *hfq* did not affect bacterial adherence to or invasion of host cells but did limit the development of intracellular microcolonies by UTI89 within the terminally differentiated epithelial cells that line the lumen of the bladder. In vitro, the *hfq* mutant was significantly impaired in its abilities to handle the antibacterial cationic peptide polymyxin B and reactive nitrogen and oxygen radicals and to grow in acidic medium (pH 5.0). Relative to the wild-type strain, the *hfq* mutant also had a substantially reduced migration rate on motility agar and was less prone to form biofilms. Hfq activities are known to impact the regulation of both the stationary-phase sigma factor RpoS (σ^S) and the envelope stress response sigma factor RpoE (σ^E). Although we saw similarities among *hfq*, *rpoS*, and *rpoE* deletion mutants in our assays, the *rpoE* and *hfq* mutants were phenotypically the most alike. Cumulatively, our data indicate that Hfq likely affects UPEC virulence-related phenotypes primarily by modulating membrane homeostasis and envelope stress response pathways.

Small noncoding regulatory RNAs (sRNAs) can modulate the translation and stability of specific target mRNAs in prokaryotes and can thereby impact multiple aspects of bacterial cell physiology. In *Escherichia coli*, more than 60 sRNAs have been conclusively identified, representing 1 to 2% of the number of known protein-encoding genes in this organism (20). Interactions between most sRNA molecules and mRNAs occur through multiple regions of homology of 2 to 8 bp, typically within the 5′ ends of target transcripts (21). In many cases, these RNA-RNA interactions require Hfq, a protein originally identified as a host factor needed for Qβ bacteriophage replication (18, 19). Hfq assembles into homohexameric rings, which are structurally similar to those formed by the Sm and Sm-like proteins that comprise the core of splicing and mRNA degradation complexes in eukaryotic and archaeal cells (21, 52, 56). By binding single-stranded AU-rich regions, Hfq can stabilize sRNA molecules as well as stimulate the formation of sRNA-mRNA pairs. In most cases, these Hfq-mediated interactions have an inhibitory effect on either the translation or the stability of the target mRNA.

A number of sRNA molecules that bind Hfq are key regulatory elements in bacterial stress responses (20). Among these are sRNAs that help control the expression of the sigma factor RpoS (σ^S), a master regulator of the general stress response in *E. coli* and many other gram-negative bacteria. RpoS, which is also known as the stationary-phase sigma factor, regulates the expression of numerous genes that promote bacterial survival in the face of various environmental stresses, including nutrient limitation, UV radiation, hyperosmotic shock, temperature extremes, acidic pH, and oxidative stress (23). The sRNA OxyS, which is expressed in response to oxidative stress, represses RpoS translation (59, 60), while the sRNAs DsrA and RprA enhance RpoS expression (31, 47). Very little RpoS is synthesized in an *hfq* mutant (35, 41), and many of the phenotypic effects observed with an *hfq* knockout have been attributed to defects in RpoS expression (36).

Recent work has revealed that the deletion of *hfq* also impacts the envelope stress response sigma factor, RpoE (σ^E) (25, 46, 54, 13, 16). RpoE is activated in response to extracytoplasmic stresses, like heat shock or misfolded outer membrane proteins (OMPs), and regulates the expression of about 100 genes. These include the sRNAs MicA, which inhibits OmpA expression, and RybB, which inhibits both OmpC and OmpW expression. Together with other RpoE regulon members, these sRNAs in association with Hfq help maintain envelope integrity by coordinating the expression of OMPs and other bacterial envelope components (5, 25, 54, 55). Interestingly, RybB also represses RpoE translation, creating an autoregulatory loop (54). The deletion of *hfq* causes strong activation of RpoE, probably due to diminished RybB activity, coupled with misregulated OMP expression and increased envelope stress. This stress, in turn, likely stimulates the activation of the periplasmic protease DegS and the subsequent degradation of the RpoE sequestration factor RseA (16).

Stress tolerance is central to the ability of many bacterial pathogens to successfully colonize hostile host environments. Considering the roles of Hfq and sRNAs as key regulators of stress response pathways in laboratory *E. coli* K-12 strains (20), we were interested in understanding how Hfq might contribute to the virulence of uropathogenic *E. coli* (UPEC) bacteria.

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
Strains		
E. coli		
UTI89	Wild-type cystitis isolate	38
MM835	UTI89 Δ*hfq*::Clm^r	This study
MM793	UTI89 Δ*rpoS*::Kan^r	This study
MM836	UTI89 Δ(*rpoE-rseABC*)::Clm^r	This study
MM837	UTI89 Δ*hflX*::Clm^r	This study
ES707	UTI89 with pKM208	This study
MM807	UTI89 with pACYC177	This study
MM808	UTI89 Δ*hfq*::Clm^r with pHfq	This study
MM810	UTI89 Δ*hfq*::Clm^r with pACYC177	This study
MM803	UTI89 Δ*hfq*::Clm^r with pKM208	This study
MM905	UTI89 Δ(*rpoE-rseABC*)::Clm^r with pACYC177	This study
MM906	UTI89 Δ(*rpoE-rseABC*)::Clm^r with pJLJ41	This study
MM904	UTI89 Δ*rpoS*::Kan^r with pACYC177	This study
MM 903	UTI89 Δ*rpoS*::Kan^r with pRpoS4	This study
Top10	Ultracompetent strain	Invitrogen
Salmonella		
TT23216	Strain with Clm^r cassette flanked by universal primer sites	John Roth
TT23691	Strain with Kan^r cassette flanked by universal primer sites	John Roth
Plasmids		
pKM208	IPTG-inducible lamda Red recombinase expression plasmid; Amp^r	40
pCR2.1	High-copy-no. cloning vector	Invitrogen
pACYC177	Low-copy-no. vector containing Amp^r and Kan^r cassettes	New England Biolabs
pHfq	*hfq* cloned from UTI89 with native promoter ligated into pACYC177 backbone; contains Amp^r cassette	This study
pRpoS4	*rpoS* cloned from UTI89 with native promoter ligated into pACYC177 backbone; contains Amp^r cassette	This study
pJLJ41	*rpoE-rseABC* from UTI89 with native promoter ligated into pACYC177 backbone; contains Kan^r cassette	This study

These bacteria are the primary cause of urinary tract infections (UTIs), including both cystitis (bladder infection) and pyelonephritis (kidney infection) (17). UTIs are among the most common infections, representing an enormous financial and health burden worldwide (17, 29). The successful colonization of the urinary tract requires that UPEC overcome a barrage of innate host defenses, including the shear flow of urine, the synthesis of soluble and tissue-associated antibacterial molecules, the influx of neutrophils, the exfoliation and clearance of infected host epithelial cells, and the generation of reactive nitrogen species (RNS) and reactive oxygen species (ROS) (6, 32, 39, 42). To counter these defenses, UPEC encodes numerous virulence factors, including various adhesins, toxins, iron chelators, capsule-forming polysaccharides, and flagella (6, 26, 42). The ability of UPEC to invade host epithelial cells, multiply intracellularly, and form biofilms also enhances UPEC virulence and persistence within the urinary tract (6, 37, 38, 50).

Here, we employ a mouse UTI model system to show that Hfq is critical to the ability of UPEC to effectively colonize and persist within the urinary tract. Using in vitro assays, we demonstrate that Hfq affects a number of virulence-related UPEC phenotypes, including biofilm formation, motility, and resistance to RNS, ROS, and the antimicrobial peptide polymyxin B. In addition, we show in comparative analyses that *hfq*, *rpoS*, and *rpoE* UPEC mutants have partly overlapping, yet distinct, phenotypes.

MATERIALS AND METHODS

Strains and plasmids. Bacterial strains used in this study are listed in Table 1. Mutants were constructed with the human cystitis isolate UTI89 (9, 38) by using the lambda Red recombinase method as described previously (11, 40). Antibiotic resistance cassettes were amplified from *Salmonella* strain TT23216 or TT23691 chromosomal templates by PCR using primers listed in Table S1 in the supplemental material. TT23216 and TT23691 (strains containing either a chloramphenicol or a kanamycin resistance cassette flanked by "universal ends" for use in generating knockouts by lambda Red-mediated recombination) were kindly provided by John Roth (University of California, Davis). All primers were designed with overhangs specific for the first 40 bp within or surrounding the 5′ and 3′ ends of the target UTI89 genes. PCR products were introduced by electroporation into UTI89 carrying pKM208, which encodes IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible lambda Red recombinase. Knockouts were verified by PCR using flanking primers specific for each targeted gene.

Primers used for cloning *hfq*, *rpoS*, and *rpoE-rseABC* from UTI89 are listed in Table S1 in the supplemental material. The *hfq* gene, along with 300 bp of upstream sequence, was amplified using whole-colony PCR with primers P112 and P113. The PCR product was ligated into pCR2.1 (Invitrogen), sequenced, and subsequently subcloned into the low-copy-number plasmid pACYC177 (New England Biolabs) by using BamHI and XhoI restriction sites to create pHfq. The *rpoS* gene plus 200 bp of upstream sequence was similarly cloned using primers P180 and P181, creating pRpoS4. The *rpoE* and *rseABC* genes, in addition to 350 bp of upstream and 100 bp of downstream sequences, were cloned using primers F_rpoE and R_rseC. The PCR product was digested and ligated directly into pACYC177 using BamHI and PstI restriction sites to make

pJLJ41. In complementation experiments, pACYC177 served as an empty-vector control.

Mouse infections. Cultures of UTI89 and the *hfq* knockout mutant (UTI89 Δ*hfq*) from freezer stocks were grown in 20 ml of Luria-Bertani (LB) broth for 48 h at 37°C without shaking. Just prior to infection, bacteria from these cultures were pelleted and resuspended in phosphate-buffered saline (PBS). Seven-week-old female CBA/J mice (Jackson Laboratory) were briefly anesthetized with isoflurane and inoculated transurethrally with 50 μl of the bacterial suspension (approximately 10^8 bacteria, as determined by plating) as described previously (37). At days 1, 3, and 5 postinoculation, the mice were sacrificed and the bladder and left kidney of each animal were harvested aseptically, weighed, and homogenized in 1 ml of PBS containing 0.025% Triton X-100. Homogenates were serially diluted and plated onto LB agar plates to determine bacterial titers. Mouse experiments were repeated twice, with similar results.

The formation of intracellular bacterial communities (IBCs) by UTI89 and the Δ*hfq* mutant was quantified as reported previously (28). Bladders from 7-week-old female CBA/J or C3H/HeN mice (Harlan) were recovered 6 h postinoculation with equal numbers of CFU of either wild-type UTI89 or the Δ*hfq* mutant, halved, splayed, and fixed in 10% neutral buffered formalin for 30 min. Bladders were rinsed twice with wash buffer (0.01 M $MgCl_2$, 0.02% octylphenoxypolyethoxyethanol [Igepal], and 0.01% sodium deoxycholate in PBS) and incubated overnight at 4°C in *lacZ* stain buffer (5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 1 mg of X-Gal [5-bromo-4-chloro-3-indolylphosphate]/ml in wash buffer). Stained bladder halves were then washed in PBS, mounted under coverslips, and viewed using bright-field optics under an Olympus BX51 microscope. All mouse experiments were performed under accredited conditions using Institutional Animal Care and Use Committee-approved protocols.

Growth assays. Cultures of UTI89 and its derivatives were grown overnight at 37°C in 5 ml of LB broth, 100 mM morpholineethanesulfonic acid (MES)-buffered LB (LB-MES; pH 5.0), or M9 minimal medium (6 g of Na_2HPO_4/liter, 3 g of KH_2PO_4/liter, 1 g of NH_4Cl/liter, 0.5 g of NaCl/liter, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, 0.1% glucose, 0.0025% nicotinic acid, and 16.5 μg of thiamine/ml in H_2O) in loosely capped 20- by 150-mm borosilicate glass tubes with shaking (225 rpm, with the tubes tilted at a 30° angle). Each culture was diluted to an A_{600} of ~1.0 and then subcultured at 1:100 in LB, LB-MES, or M9 medium. Growth in LB with or without 1 mM methyl viologen (MV) and in LB-MES with or without 1 mM sodium nitrite (Sigma-Aldrich) was assessed. All growth curves were generated from quadruplicate 200-μl cultures in 100-well honeycomb plates at 37°C with shaking by using a Bioscreen C instrument (Growth Curves USA). Overnight cultures of strains carrying pACYC177, pHfq, pRpoS4, or pJLJ41 for complementation experiments were grown in the presence of 100 μg of ampicillin/ml or 50 μg of kanamycin/ml to maintain the plasmids, but the antibiotics were not added to media used in the subsequent assays.

Biofilm assays. In vitro biofilm formation assays were performed as described previously (34). Briefly, 5-ml cultures of UTI89 and its derivatives were first grown overnight with shaking at 37°C in M9 medium. These bacteria were diluted 1:100 in M9 medium, and quadruplicate 100-μl samples in 96-well pinch-bar flat-bottomed polystyrene microtiter plates with lids (NUNC) were incubated without shaking for 48 h at 37°C. Planktonic bacteria were then removed by inverting and shaking the plates vigorously and washing them twice with double-distilled water. Crystal violet (150 μl of a 0.1% solution in water; Sigma-Aldrich) was added to each well, and the plates were incubated at room temperature for an additional 10 min. After the removal of the crystal violet, the wells were washed twice with double-distilled water and air dried at room temperature. Dimethyl sulfoxide (200 μl; Sigma-Aldrich) was added to each well, and the plates were again shaken vigorously for 15 min at room temperature. A 150-μl aliquot from each well was transferred onto a fresh microtiter plate, and A_{562} readings were taken using a Synergy HT multidetection microplate reader (Biotek Instruments, Inc.).

Agglutination, cell association, and invasion assays. The ability of UTI89 and its derivatives to agglutinate *Saccharomyces cerevisiae* cells was qualitatively determined by mixing 20 μl of each bacterial strain (from overnight static cultures) with 200 μl of a 1% suspension of baker's yeast in PBS on glass slides. Hemagglutination assays were carried out using guinea pig red blood cells (Colorado Serum Company) according to established protocols (48). Bacterial host cell association and invasion assays were performed using human bladder epithelial cells (designated 5637 cells) and the A498 human kidney cell line (American Type Culture Collection) as described previously (14).

Motility assays. Prewarmed motility agar plates, containing 0.2% agar (EMD Chemicals) in LB broth, were inoculated (on the surface) with 1 μl of bacteria from overnight cultures that had been grown with shaking and subsequently diluted to an A_{600} of ~1.0. Plates were incubated at 37°C, and bacterial spreading (swarming) was measured at 2-h intervals and photographed using a Nikon D80 digital camera.

Polymyxin B sensitivity assays. Bacterial cultures, grown with shaking overnight at 37°C, were diluted in LB broth to an A_{600} of ~1.0 and subcultured at 1:100 in 5 ml of LB broth containing 0, 1, or 5 μg of polymyxin B (Sigma-Aldrich)/ml. These cultures were incubated at 37°C with shaking at 225 rpm for 1.5 h, and bacterial titers were then determined by plating serial dilutions of each sample.

LPS profiling. Lipopolysaccharide (LPS) profiling was performed as described previously (3, 24). Briefly, bacterial cultures were grown to stationary phase in LB broth at 37°C and normalized to an A_{600} of 1.0. Bacteria from 1 ml of each sample were pelleted and resuspended in 250 μl of water prior to the addition of 250 μl of bacterial lysis buffer (1% sodium dodecyl sulfate, 50 mM Tris-Cl [pH 7.0], 10 mM EDTA). After boiling for 5 min, samples were incubated with proteinase K (1.5 mg/ml; Sigma-Aldrich) for 3 h at 37°C. LPS extracts were subsequently resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 12.5% polyacrylamide gels, which were then stained using the SilverSnap stain kit II (Pierce).

Statistics. Mann-Whitney U and two-tailed unpaired *t* tests were performed using Prism 5.01 software (GraphPad Software). *P* values of less than 0.05 were considered significant.

RESULTS

The disruption of *hfq* attenuates UPEC colonization of the urinary tract. To assess the role of Hfq in cystitis and pyelonephritis caused by UPEC, adult female CBA/J mice were inoculated via transurethral catheterization with a wild-type UPEC cystitis isolate, UTI89, or the *hfq* knockout mutant UTI89 Δ*hfq*. At 1, 3, and 5 days postinoculation, the bladders and kidneys were collected and homogenized and bacterial titers were determined by serial dilution and plating of the tissue homogenates. As shown in Fig. 1, the *hfq* mutant was severely impaired at all time points in its abilities to colonize and persist within both the kidneys and the bladder relative to the wild-type UTI89 strain. However, in vitro, UTI89 Δ*hfq* grew similarly to the wild-type strain in both LB broth and M9 minimal medium (data not shown). Together, these results indicate that Hfq contributes significantly to the fitness of UPEC within the urinary tract.

In vitro assays revealed no defects in the ability of UTI89 Δ*hfq* to interact with host cells: the *hfq* mutant agglutinated yeast and red blood cells normally and was able to bind to and invade both bladder and kidney epithelial cells at wild-type levels in cell culture-based assays (data not shown). Within the superficial epithelial cells that line the luminal surface of the bladder, UTI89 and other UPEC isolates are able to multiply rapidly, forming large cytosolic inclusions referred to as pods, or IBCs (2, 27, 15, 38). UTI89 bacteria within IBCs naturally express LacZ, enabling these inclusions to be visualized and enumerated in whole-mount bladder preparations when stained using X-Gal (28). By 6 h post-transurethral inoculation of adult female CBA/J mice, we detected at least a few IBCs in most of the UTI89-infected bladders but no IBCs were detected in the majority of bladders infected with UTI89 Δ*hfq* (Fig. 1C). Similar results were obtained using C3H/HeN mice. Importantly, the *hfq* mutant was able to express wild-type levels of beta-galactosidase (LacZ) activity, and no X-Gal staining of mock-infected bladders was detected (data not shown). These data, coupled with our observations that UTI89 Δ*hfq* bound to and invaded host cells normally, indicate a defect in the ability of the *hfq* mutant to multiply intracellularly.

Hfq affects UPEC growth at low pH and resistance to RNS and ROS. Within bladder epithelial cells, UPEC is initially

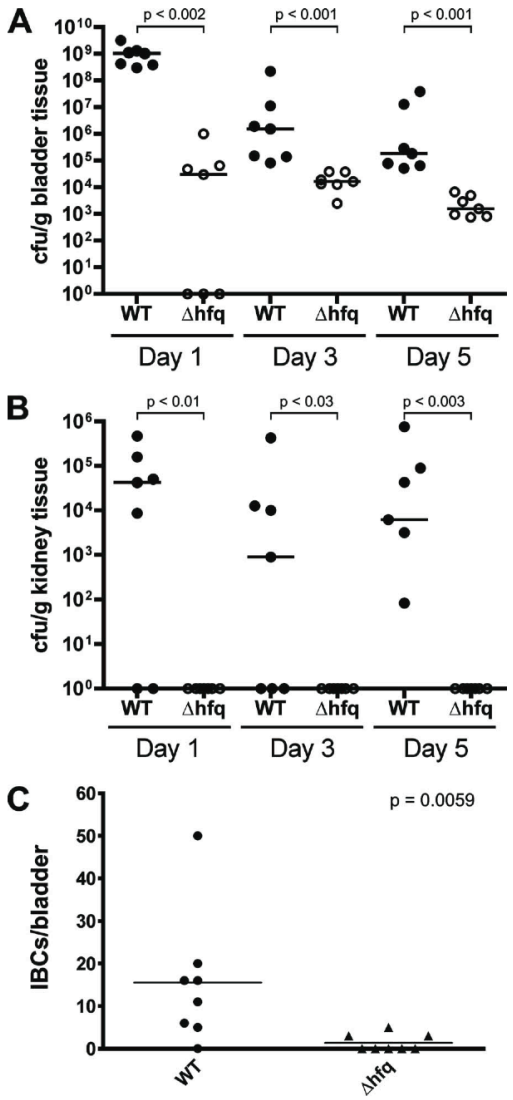

FIG. 1. Hfq is required for effective UPEC colonization of the urinary tract. (A and B) Adult female CBA/J mice were infected with 10^8 CFU of UTI89 or UTI89 Δ*hfq* via transurethral catheterization. Bacterial titers in bladder (A) and kidney (B) homogenates were determined at the indicated times postinoculation. Horizontal bars indicate median values for each group. WT, wild type. (C) The graph shows total numbers of IBCs per bladder at 6 h postinoculation of CBA/J mice with either UTI89 or UTI89 Δ*hfq*. Bars denote mean values. The indicated *P* values were determined using the Mann-Whitney U test ($n = 7$ or 8 mice per group).

trafficked into acidic, late-endosome-like compartments where bacterial replication can be initiated prior to the development of IBCs (15). UPEC may encounter similarly acidic environments within urine during the acute phase of a UTI. In addition, UPEC must deal with high levels of ROS and RNS that are generated within the urinary tract during infection. UPEC isolates like UTI89 often have much greater resistance to these radicals than laboratory *E. coli* K-12 strains (7, 42, 53). A role for Hfq during bacterial growth under acidic conditions was assessed using LB-MES (pH 5.0), while the contribution of Hfq to UPEC RNS and ROS resistance was tested using acidified sodium nitrite (ASN) and MV, respectively. When added to LB-MES (pH 5.0), sodium nitrite is converted into nitrous acid, which spontaneously decomposes to form NO and other RNS (57). MV, on the other hand, generates superoxide radicals (22). We found that the growth of UTI89 Δ*hfq*, which grew normally in LB broth (pH 7.0), consistently lagged behind that of the wild-type strain by more than an hour when the strains were grown in LB-MES (pH 5.0) (Fig. 2A). The relative growth of UTI89 Δ*hfq* in 1 mM ASN was even more severely impaired (Fig. 2B), and in the presence of 1 mM MV, the *hfq* mutant barely grew at all (Fig. 2C). Controls for these and other in vitro assays described in the following sections included UTI89 Δ*hfq* strains complemented with pHfq (a low-copy-number plasmid for the expression of *hfq* from its native promoter) or the empty vector pACYC177. Possible polar effects resulting from *hfq* deletion in UTI89 were controlled for by disrupting *hflX*, which is immediately downstream from and in frame with *hfq*. The *hflX* gene encodes a GTP-binding protein of unknown function. In all assays, the *hflX* mutant and UTI89 Δ*hfq* complemented with pHfq behaved like the wild type (data not shown). Cumulatively, these data indicate that the Hfq RNA chaperone enhances bacterial growth under acidic conditions and that Hfq has an especially important role in UPEC resistance to both ROS and RNS, possibly via indirect effects on the expression of stress-responsive genes.

Effects of Hfq on UPEC motility. The disruption of *hfq* impairs the motility of at least two pathogens, *Salmonella enterica* serovar Typhimurium and *Pseudomonas aeruginosa* (46, 49). Within the urinary tract, motility gives UPEC a survival advantage, enhancing bacterial colonization and persistence (58, 30). In consideration of these data, we wished to determine if Hfq affected the motility of UTI89. By light microscopy, we observed that both wild-type UTI89 and UTI89 Δ*hfq* LB broth cultures contained numerous motile microbes, indicating that Hfq is not an absolute requirement for UTI89 motility. However, on motility agar plates, UTI89 Δ*hfq* showed greatly reduced outward migration (swarming) in comparison with the wild-type parent strain (Fig. 3). This motility defect was eliminated by complementation with pHfq but not with the empty-vector control pACYC177 and was not observed with UTI89 Δ*hflX* (data not shown). All of the strains tested eventually spread across the agar plates, forming concentric rings, like wild-type UTI89, that were characteristic of motile, chemotactic bacteria. These results confirm our microscopic observations that *hfq* is not required for UTI89 motility while also suggesting a role for Hfq as a modulator of UPEC motility rates and/or chemotaxis.

Hfq functions in biofilm formation. Pathogenic *E. coli* strains and other bacteria are more prone to form biofilms when nutrient levels are suboptimal, indicating that biofilm formation may act as an adaptation to nutrient-poor environments, as found within the urinary tract (12, 51). Recently, it was noted that UPEC strains that cause relapsing UTIs in women are generally better able to form biofilms than other UPEC isolates in microtiter plate-based assays, suggesting a

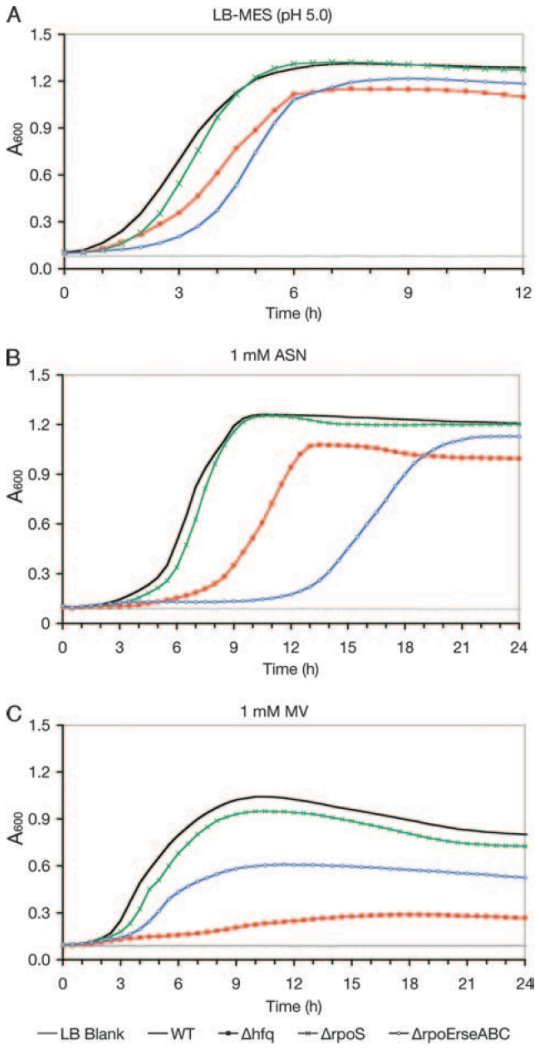

FIG. 2. UPEC resistance to low pH, RNS, and ROS is differentially affected by Hfq, RpoS, and RpoE. Overnight cultures of wild-type UTI89 (WT), UTI89 Δ*hfq*, UTI89 Δ*rpoS*, and UTI89 Δ(*rpoE-rseABC*) were diluted to an A_{600} of 1.0 and subcultured at 1:100 in LB-MES (pH 5.0) (A), LB-MES–1 mM ASN (B), or LB broth–1 mM MV (C). Cultures were grown in plate format, and absorbance measurements were obtained using a Bioscreen C instrument (Growth Curves USA). Each growth curve represents the means of results for quadruplicate samples, and each experiment was repeated three or more times, with similar results.

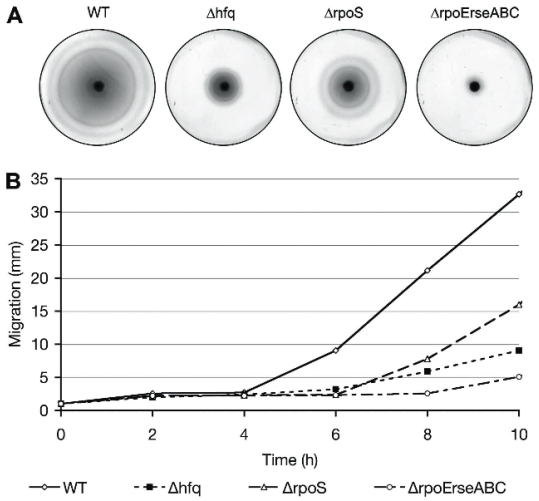

FIG. 3. Effects of *hfq*, *rpoS*, and *rpoE-rseABC* disruption on UPEC motility. (A) Images show the spread of UTI89 (the wild type [WT]) and its derivatives at 37°C 8 h after inoculation onto motility agar plates. (B) The change over time in the diameter (in millimeters) of the area covered by each bacterial strain as it spread across the motility plate is represented in the graph. These experiments were repeated three times, with similar results.

role for biofilm formation in the establishment and persistence of UPEC within the host (50). The involvement of Hfq in the development of biofilms by UPEC was tested by growing UTI89 and its mutant derivatives at 37°C in M9 minimal medium in 96-well polystyrene microtiter plates. After 48 h, all planktonic bacteria were removed and any remaining bacterial biofilms were stained and quantified using crystal violet. In these assays, UTI89 Δ*hfq* showed about a threefold reduction in biofilm formation relative to the wild-type strain (Fig. 4). Biofilm formation was restored to wild-type levels by complementation with pHfq, and no problems with UTI89 Δ*hflX* were observed (data not shown). Notably, the biofilm deficiency seen with the *hfq* mutant was not attributable to growth defects in M9 medium or to any inherent inability of UTI89 Δ*hfq* to retain crystal violet.

Hfq is required for UPEC resistance to polymyxin B. Antimicrobial peptides such as defensins and cathelicidins are important components of the host defense against uropathogenic bacteria (61). These cationic peptides can associate with bacterial membranes, perturbing the integrity of the envelope and potentially disturbing other bacterial components. The capacity of Hfq to influence RpoS and RpoE envelope stress response pathways led us to hypothesize that an *hfq* deletion mutant might be compromised in its ability to deal with the membrane-disrupting activities of antimicrobial cationic peptides. To test this possibility, we employed the cationic peptide polymyxin B, which has been used clinically as a bactericidal antibiotic. Equal numbers of CFU of UTI89 and its derivatives were incubated for 90 min with shaking in LB broth alone or LB broth with polymyxin B added to a final concentration of 1 or 5 μg/ml. The strains grew to similar titers in the absence of polymyxin B, reaching about 10^9 CFU/ml (Fig. 5). However, relative to the wild-type strain, UTI89 Δ*hfq* showed pronounced sensitivity to 1- and 5-μg/ml concentrations of polymyxin B. Complementation with pHfq restored the growth of UTI89 Δ*hfq* to wild-type levels, while UTI89 Δ*hflX* behaved like the wild type in these assays (data not shown). These results indicate a critical role for Hfq in the resistance of UPEC to antimicrobial peptides.

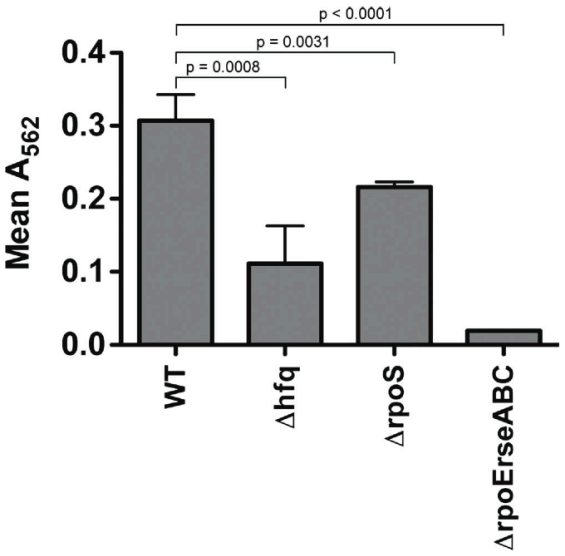

FIG. 4. Effects of *hfq*, *rpoS*, and *rpoE-rseABC* disruption on biofilm formation by UTI89. The wild-type (WT) UTI89 strain and its derivatives were grown in static M9 medium on flat-bottomed microtiter plates at 37°C for 48 h. Added crystal violet that was retained within wells by bacterial biofilms was eluted into dimethyl sulfoxide. The optical density (A_{562}) of this solution correlated with the level of biofilm formation. The graph shows the means for each sample set ± standard deviations, with indicated *P* values determined by two-tailed unpaired *t* tests. Of note, planktonic wild-type and mutant bacteria stain equally well with crystal violet in Gram staining assays.

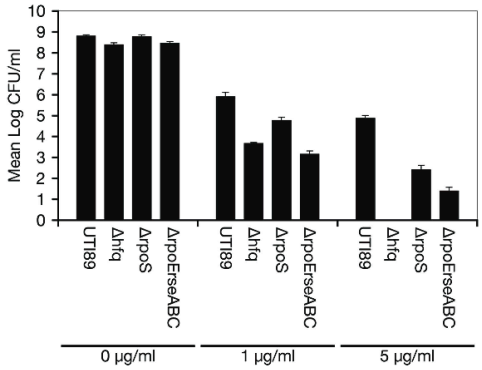

FIG. 5. UTI89 Δ*hfq* is hypersensitive to the cationic peptide polymyxin B. Equal numbers of CFU of UTI89 and its mutant derivatives were subcultured in LB broth with or without 1.0 or 5.0 μg of polymyxin B/ml, as indicated. After shaking at 37°C for 1.5 h, bacterial titers (in CFU per milliliter) in triplicate samples were determined by plating serial dilutions. The graph shows the means for each sample set ± standard deviations. These experiments were repeated three times, with similar results.

Phenotypic overlap among *hfq*, *rpoS*, and *rpoE* mutants. In light of previous observations indicating that many of the phenotypic effects observed with an *hfq* knockout in *E. coli* K-12 may be attributed to defects in RpoS expression (35), we disrupted the *rpoS* gene in UTI89 to create UTI89 Δ*rpoS* for comparison with UTI89 Δ*hfq*. Interestingly, in our assays we found that UTI89 Δ*rpoS* behaved remarkably like the wild-type UPEC strain in its abilities to grow in acidic medium (pH 5.0) and to handle RNS and ROS (Fig. 2). Although worse off than the wild-type strain in other assays, UTI89 Δ*rpoS* was still more motile and more resistant to polymyxin B than UTI89 Δ*hfq* (Fig. 3 and 5). The *rpoS* mutant, which grew normally in M9 and LB media, was also diminished in its capacity to form biofilms in vitro but was still somewhat better at forming biofilms than UTI89 Δ*hfq* (Fig. 4). The latter result correlates with earlier work implicating RpoS as an important factor during biofilm development by *E. coli* (1). The complementation of UTI89 Δ*rpoS* with the plasmid pRpoS4, encoding RpoS under the control of its native promoter, restored the wild-type phenotype in all assays (data not shown). Together, these data indicate that the aberrant phenotypes associated with UTI89 Δ*hfq* are not solely a consequence of diminished RpoS expression.

Because Hfq can affect RpoE- as well as RpoS-mediated stress responses, phenotypic comparisons between UTI89 Δ*hfq* and an *rpoE* mutant, UTI89 Δ(*rpoE-rseABC*), were also made. The *rpoE* mutant strain was constructed so that the *rseABC* genes, which are adjacent to *rpoE* and regulate RpoE activation, were also disrupted. In contrast to results with laboratory *E. coli* K-12 strains (8), the deletion of *rpoE* in UTI89 was not lethal. Using in vitro assays, we found that UTI89 Δ(*rpoE-rseABC*) had substantial defects in motility, biofilm formation, and growth in low-pH medium, as well as increased sensitivity to RNS, ROS, and polymyxin B (Fig. 2 to 5). In control assays, the *rpoE-rseABC* mutant grew normally in both LB and M9 media, and complementation with a plasmid carrying *rpoE-rseABC* under the control of the native promoter restored the wild-type phenotype (data not shown). In total, these results show that UTI89 Δ(*rpoE-rseABC*), rather than UTI89 Δ*rpoS*, was phenotypically the most similar to the *hfq* mutant, indicating a possible link between Hfq effects on the bacterial envelope and the multiple phenotypic defects we found to be associated with UTI89 Δ*hfq*.

DISCUSSION

The RNA chaperone Hfq contributes to the fitness and virulence of several pathogens, including the gram-negative bacteria *Brucella abortus* (45), *P. aeruginosa* (49), *Vibrio cholerae* (13), *Legionella pneumophila* (33), and *Salmonella* serovar Typhimurium (46), as well as the gram-positive organism *Listeria monocytogenes* (10). Although direct comparisons have not been made, it appears that the spectrum and severity of mutant phenotypes observed upon the deletion of *hfq* can vary significantly among the different pathogens so far analyzed. For example, both *Salmonella* serovar Typhimurium and *B. abortus hfq* mutants are unable to multiply well within host cells, while *hfq* is dispensable for the normal intracellular growth of *L. monocytogenes* (10, 45, 46). Here, we have shown that *hfq* is required for UPEC to effectively colonize and persist within the urinary tract. As seen with *Salmonella* serovar Typhimurium and *B. abortus* (45, 46), the deletion of *hfq* appears to attenuate the intracellular growth of UPEC, interfer-

ing with the ability of this pathogen to form IBCs. The disruption of *hfq* also diminished the capacity of UPEC to tolerate RNS, ROS, and the antibacterial cationic peptide polymyxin B and to grow in acidic medium (pH 5.0). Furthermore, the *hfq* mutant had reduced motility and chemotaxis and was significantly impaired in its ability to form biofilms, a trait that has previously been associated with decreased bacterial persistence within the urinary tract (50).

During the course of an infection, Hfq likely synergizes with multiple signaling pathways and sigma factors, including RpoE and RpoS, in order to facilitate the resistance and adaptation of UPEC to hostile host environments. Accordingly, in our assays we saw significant phenotypic overlap among the *hfq*, *rpoS*, and *rpoE-rseABC* mutants, with UTI89 Δ*hfq* and UTI89 Δ(*rpoE-rseABC*) being the most alike. In a laboratory *E. coli* K-12 strain, as well as in *Salmonella* serovar Typhimurium and *V. cholerae*, the deletion of *hfq* has been shown to induce RpoE activation (13, 16, 25, 46, 54). Strikingly, in *V. cholerae*, enhanced RpoE activation accounts for the effects on nearly half the genes that are upregulated in an *hfq* mutant (13). RpoE activation in the absence of Hfq likely occurs in *E. coli* due to increased envelope stress resulting from the aberrant expression of OMPs and other factors, as well as the inability of the Hfq-dependent sRNA RhyB to effectively inhibit RpoE translation (54). Enhanced RpoE activation probably helps ameliorate some of the deleterious effects of *hfq* disruption, but the overstimulation of RpoE may also contribute to these problems by further disturbing the balance of factors involved in membrane repair and maintenance. This possibility has not yet been rigorously tested with UPEC, although the effects of RpoE inactivation have been partially explored. In particular, the deletion of DegS, a protease that indirectly activates RpoE by freeing it from RseA repression, was found to attenuate UPEC virulence in a mouse UTI model (43). Similarly, the disruption of *degP*, *skp*, or *surA*, all of which are members of the RpoE regulon, also decreases UPEC virulence in mice (28, 44). Interestingly, it has been shown previously for *Salmonella* serovar Typhimurium that RpoE can positively regulate *hfq* expression indirectly via the transcriptional activation of another alternate sigma factor, RpoH (σ^H) (4). A similar relationship between RpoE activation and enhanced Hfq expression in UPEC may account for some of the phenotypic overlap seen between the *rpoE-rseABC* and *hfq* knockout mutants in our assays.

Cumulatively, our results indicate a functional link between Hfq, envelope stress, membrane homeostasis, and the virulence potential of UPEC. This connection is further supported by observations that the disruption of *hfq*, but not *rpoS* or *rpoE*, causes marked alterations in the LPS profile of UTI89 (see Fig. S1 in the supplemental material). This effect in turn may significantly influence the overall fitness of UPEC and susceptibility to antibacterial cationic peptides and other stresses encountered within the host. The capacity of Hfq to affect LPS biogenesis and multiple virulence-related phenotypes in UPEC probably reflects the ability of this chaperone to interact with a wide range of different regulatory RNAs, many of which remain to be operationally defined.

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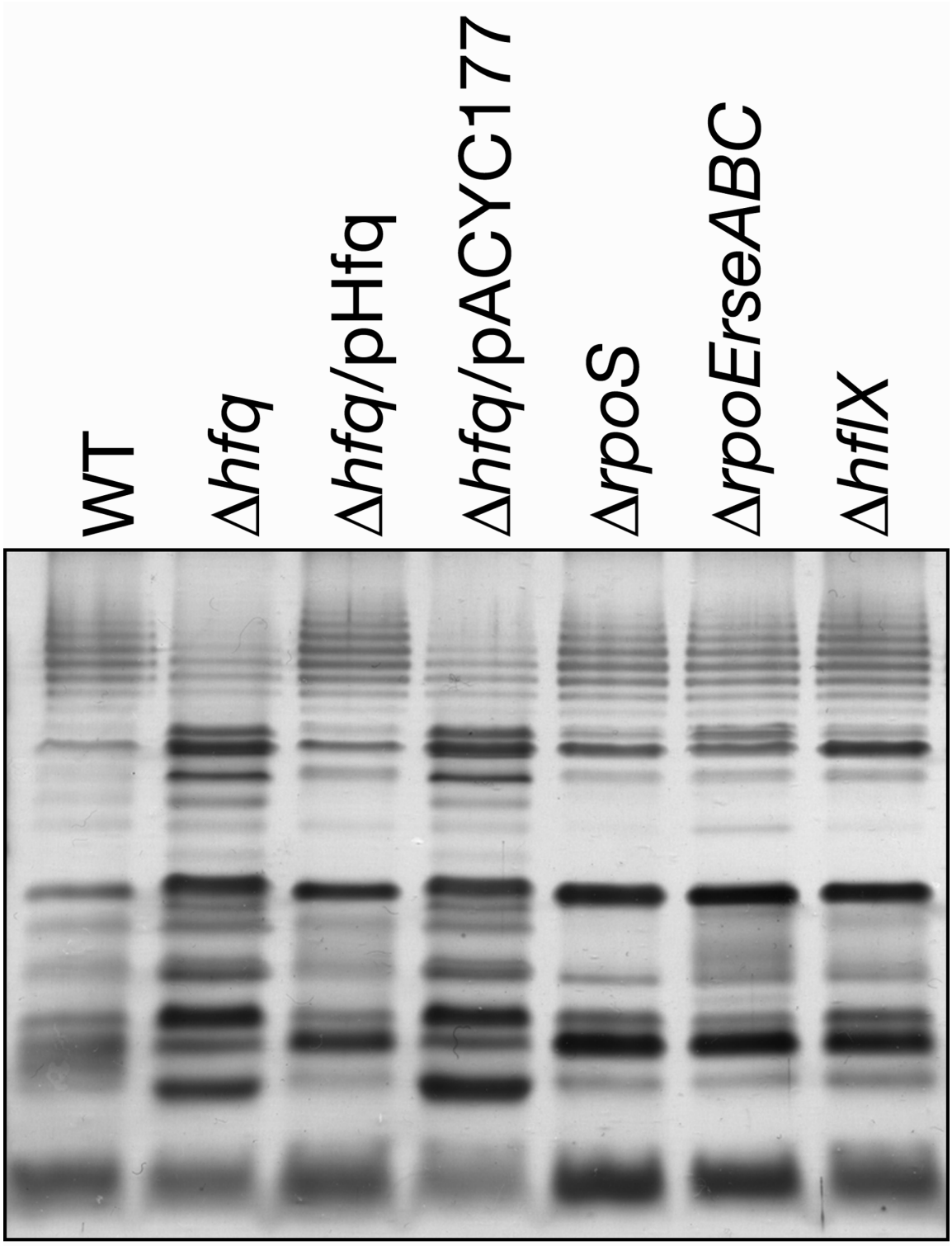
SUPPLEMENTAL TABLE 3.1. Primers used in this study.

Oligonucleotide	5'-3' sequence [a]
Chromosomal mutations	
P82 Hfq_KO_F	ATGGCTAAGGGGCAATCTTTACAAGATCCGTTCCTGAACGCACCAAACACCCCCCAAAACC
P83 Hfq_KO_R	TTATTCGGTTTCTTCGCTGTCCTGTTGCGCGGAAGTATTCCACACAACCACACCACACCAC
P84 sigmaE_KO_F	TCACTGGCTCGCGTCTTCCGAAGATGTTTCAAATCGCACACACCAAACACCCCCCAAAACC
P85 sigmaE_KO_R	GTGGAAATTTGGTTTGGGGAGACTTTACCTCGGATGAGCGCACACAACCACACCACACCAC
P99 rpoS_uniKO_F	CCAGCCTCGCTTGAGACTGGCCTTTCTGACAGATGCTTACCACCAAACACCCCCCAAAACC
P100 rpoS_uniKO_R	TGCCGCAGCGATAAATCGGCGGAACCAGGCTTTTGCTTGACACACAACCACACCACACCAC
P106 hflX_KO_F	ATCCTGCGTTCCCCGCTGATCTATTTAGAGGGTTATACGCCACCAAACACCCCCCAAAACC
P107 hflX_KO_R	ACGCCAGAGGATATGACTCCACGCTTCAGACGCTACGCCGCACACAACCACACCACACCAC
Construction of pHfq	
P112 hfq_cloning_F	CCTTCCATTCGTTGCGTGGGTTATCGCCAGAT
P113 hfq_cloning_R	AATGCTTCGACACCGGCGGAAGAGACCAGA
Construction of pRpoS4	
P180 rpoS_cloning_F	AAAGCCAGCCTCGCTTGA
P181 rpoS_cloning_R	GAGTGCCTACGCCCATAA
Construction of pJLJ41	
F_rpoE	TCATAGGATCCCGGTTTGGTCAGCATAACATCA
R_rseC	CTTAGCTGCAGCACTACAACATTGAGGCGAGG

[a] Underlined sequences allow amplification of antibiotic resistance cassettes flanked by universal primer sites in strains TT23216 and TT23691.



SFig 3.1. Disruption of *hfq* alters the LPS profile of UPEC. Image shows silver-stained SDS-PAGE LPS profiles of wild type (WT) UTI89 and its derivatives.

CHAPTER 4

DISCUSSION

UPEC are adaptive pathogens

UPEC are versatile pathogens capable of colonizing multiple environments within the host, including the alimentary canal, perineum, urethra, bladder, ureters and kidneys. UPEC can also persist intracellularly within phagocytes or within bladder epithelial cells, where they may set up long-term residence (1). As the local environment changes, UPEC must rapidly adapt to survive. Stress conditions, such as changes in pH, osmolarity (2, 3), mechanical forces (4), disruptive host proteins, and chemical stresses such as reactive nitrogen species (RNS) (5-7) and reactive oxygen species (ROS) (8,9), are all known to induce corresponding changes in bacterial behavior as the microbes attempt to cope with these insults. These stresses may impact protein folding in the periplasm where repeating subunits of surface organelles, such as pili fibers, curli, and flagella, are assembled in tightly regulated processes involving numerous chaperones and other co-factors (10-13). Intimate control of envelope constituents is required, therefore, to prevent misfolding and aggregation of transported subunits. A portion of this critical control falls into the realm of posttranscriptional regulation (PTR), with small noncoding RNAs (sRNA) mediating both the decay of outer membrane protein (OMP) transcripts (14-21) and regulation of

the critical σ^E envelope stress response pathway (17, 22, 23). In addition, UPEC employ motility to ascend the urinary tract, and generate both extra- and intracellular biofilms as part of their pathogenic lifestyle (24, 25). Both motility and biofilm production are regulated centrally by PTR, namely through the protein-RNA mediator CsrA (26-29).

In employing the naturally high resistance of UPEC to nitrosative stress to test for activation of the Cpx envelope stress response pathway, I uncovered a posttranscriptional mechanism for downregulation of CpxP. CpxP is an accessory adaptor protein component of the Cpx system that works to ameliorate envelope stress. Seeking to determine the mechanism of this downregulation phenomenon, I examined the involvement of both major arms of PTR in UPEC. First, the protein-mediated arm of PTR was investigated for its role in the downregulation of CpxP by modulating CsrA activity by way of an endogenous inhibitory pathway and by using an sRNA antagonist to CsrA, namely, CsrB. Second, PTR mediated by sRNAs was globally approached by deletion of the RNA chaperone Hfq, a factor required by the majority of sRNAs to mediate interaction with target mRNAs (30-33). Although the nature and severity of the defects varied between these two PTR categories, regulation of key envelope processes was common to both.

The Protein-mRNA mediated arm of PTR: CsrA

In this work I demonstrate that CsrA contributes to stress resistance by modulating an envelope stress response pathway, the Cpx two-component system. As discussed in Chapter 2, nitrosative stress conditions, rather than increasing cellular levels of CpxP, resulted in its ablation. This downregulation could be overcome by adding

exogenous glucose. Adding exogenous glucose is a pleiotropic treatment, as carbohydrate transporters and metabolic pathways are highly upregulated and activated to deal with this preferred nutrient source. However, glucose metabolism produces the byproducts formate and acetate, which in turn activate the BarA/UvrY two-component system (34). The BarA/UvrY system then upregulates expression of the sRNAs CsrB and CsrC, which directly antagonize CsrA activity. To implicate CsrA directly in the downregulation of CpxP in RNS, I induced CsrB from an IPTG-inducible construct and demonstrated that CpxP expression could be restored despite prior treatment with RNS (see Figure 4.1.). Thus, antagonization of CsrA activity, either by glucose addition or ectopic expression of CsrB could prevent downregulation of CpxP in nitrosative conditions. These data imply action by CsrA in the nitrosative downregulation of CpxP.

How does CsrA regulate CpxP?

In light of the inferred regulation of CpxP by CsrA, the next question becomes: "How does CsrA regulate CpxP?" At the time of this writing there are no reports in the literature regarding regulation of the Cpx system by CsrA. The posttranscriptional regulation of CpxP by CsrA, specifically under nitrosative conditions, thus suggests a novel mechanism. Although there is a degenerate CsrA-binding site within the *cpxP* 5' UTR composed of the bases cgACAgaaAgaU (capitalization indicates bases matching consensus), this region lacks multiple conserved bases critical for CsrA interaction according to the SELEX-derived CsrA-binding consensus ruACArGGAuGU (35). This suggests that if CsrA is regulating *cpxP* translation directly, it does so under binding parameters different from other RNA substrates. Alternatively, but not exclusively, CsrA

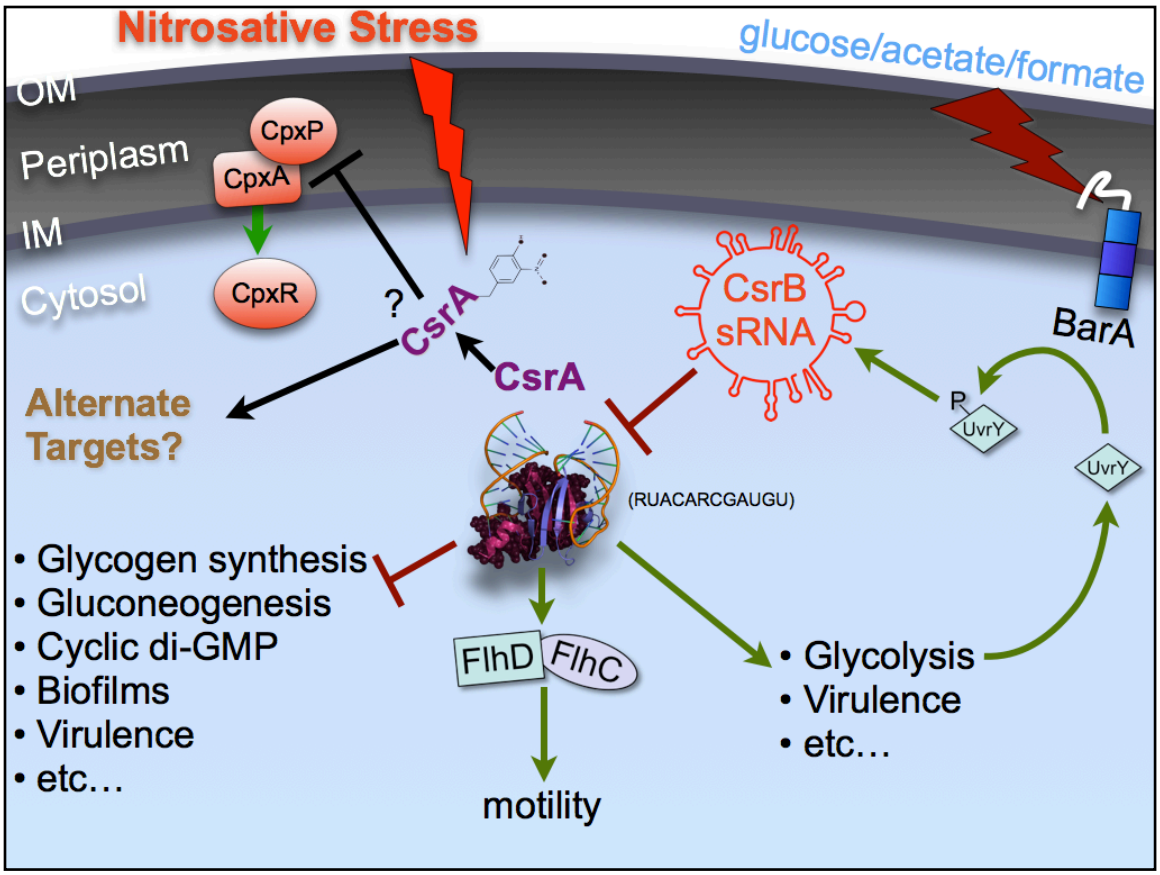


FIG. 4.1. Schematic representation of CsrA regulon. The posttranscriptional regulation of the Cpx system by CsrA under nitrosative stress conditions.

may act indirectly on *cpxP* translation via one or more secondary factors, which in turn regulate translation of the *cpxP* transcript.

Nitrosative stress may result in nitration of proteins at cysteine and tyrosine residues (36, 37). Since CsrA lacks cysteine residues, I investigated the possibility of tyrosine nitration via an anti-nitrotyrosine antibody on RNS-treated CsrA. CsrA in UPEC contains two tyrosine residues, Tyr48 and Tyr63. Immunoblotting indicated the nitration of CsrA during in vitro nitrosation assays (see Chapter 2).Tyr63 is present at the C-terminus of CsrA in an unstructured region with little homology to CsrA from other

species (38). In silico modeling of Tyr48 nitration reveals possible steric hindrance imposed on the C-terminal alpha-helix (see Fig. 4.2.). Though Tyr48 itself does not appear to be directly involved in the RNA binding interface, and in fact is present on the opposite face of the alpha-helix involved in RNA interaction, the adjacent Ile47 residue is functionally essential (38). Nitration of Tyr48 may result in translocation of the alpha-helix where Ile47 resides, altering or abrogating RNA-binding.

Functional consequences of CsrA nitration

This nitration event could foreseeably result in several non-exclusive possibilities. Nitration of CsrA may alter its stability or dimerization kinetics. Nitration may also affect the binding repertoire, either altering CsrA's binding partner interactions or abolishing them altogether.

Once nitrated, is nCsrA's stability affected? Recently, ArgR, an *E. coli* negative regulator of arginine biosynthesis, was shown to degrade after in vitro tyrosine nitration (39). This raises the possibility that nitration of CsrA's Tyr48 or Tyr63 residues may result in CsrA degradation. However, my CpxP reporter assays seem to argue against rapid CsrA degradation. I observed abolishment of CpxP-GFP-ASV expression for at least 8 hours subsequent to application of nitrosative stress (Chapter 2, Figure 2.2.). As mentioned in Chapter 2, this abolishment of the reporter could be seen for as long as 24 h posttreatment. Thus the downregulation of the reporter by CsrA is sustained over many hours. If nCsrA were degraded rapidly, this would require constant resupply of CsrA to maintain CpxP downregulation, even if CsrA functions indirectly. However, the microarray analysis from the ASN-treated UPEC isolates evidenced *csrA* transcript levels

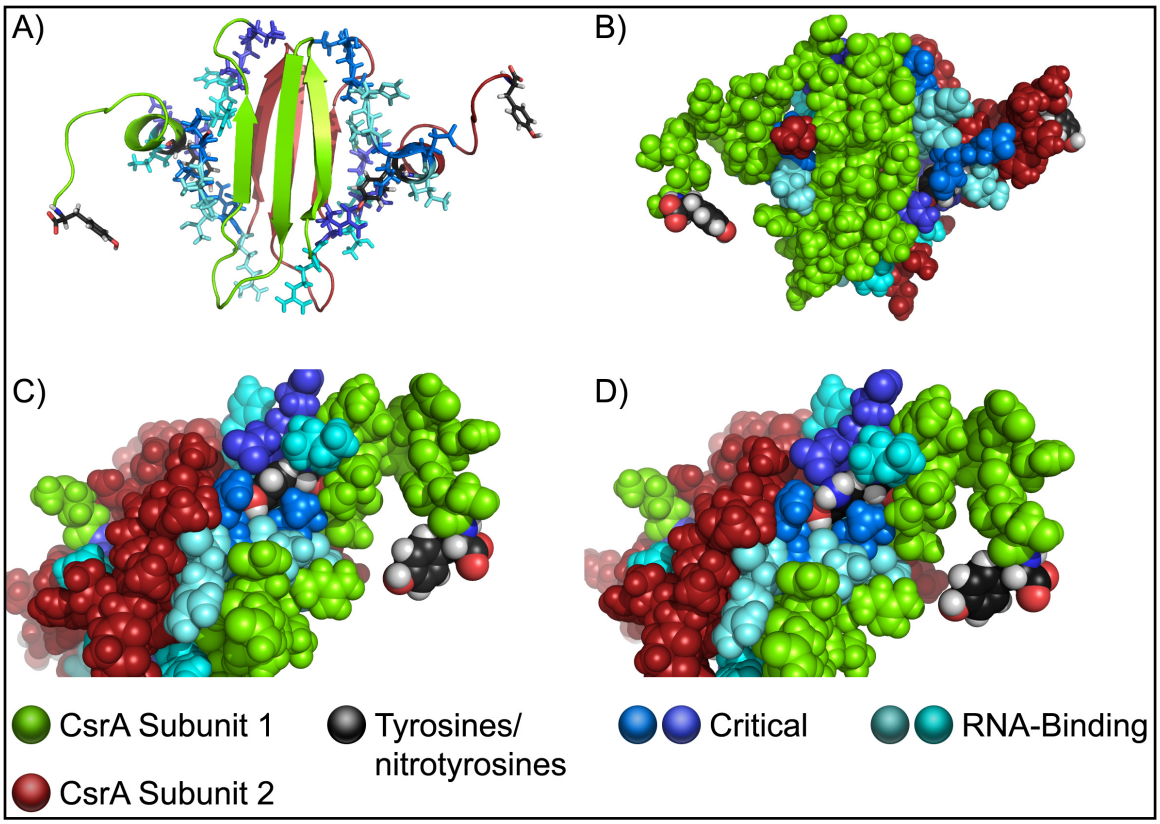


FIG. 4.2. Modeling tyrosine nitration of CsrA. MacPymol (36) was used to model *Escherichia coli* CsrA, PDB entry 1Y00 (37). Tyrosine residues are shown in black. Functionally required residues are shown in blue while cyan indicates RNA-binding residues. A) Cartoon model of CsrA dimer. B) Space-filling model of A. C) Tyr48 shown in center amidst required residues. D) Nitrated Tyr48.

less than 50% of the untreated UPEC (0.412-fold change). Therefore, it is unlikely that nCsrA is rapidly degraded in UPEC, although slow degradation exceeding 24 h)may occur.

Alternatively, CsrA nitration may be transient. Under this scenario, nCsrA may degrade faster than CsrA but remain in check because of the transient nature of nitration. If CsrA nitration is reversible, can UPEC utilize nitration as a posttranslational switch like phosphorylation? What cellular pathways contribute to denitration?

Perhaps CsrA nitration alters or abrogates its regular binding repertoire. The biological possibility of nitration altering protein function is well established. Tyrosine nitration acts as a gain-of-function modification in several eukaryotic systems (36, 37, 40, 41). Numerous examples of protein nitration modulating protein function have also been described in mitochondria, as reviewed by Koeck et al. (42). The possibility of nCsrA exhibiting modified function certainly exists.

Modified functions for nCsrA may include deactivation or alteration of binding partners or kinetics. For instance, nCsrA may recognize a different subset of mRNA transcripts. The latter option seems more feasible considering expression of CsrB was sufficient to alleviate repression of CpxP in the presence of nitrosative stress. Therefore, even if nCsrA has altered binding characteristics, CsrB is nevertheless capable of titrating CsrA away. Future RNA immunoprecipitation and cross-linking studies will likely address if nCsrA binds an alternate repertoire of RNA or protein targets. Most importantly, these studies will clarify if nCsrA can bind the *cpxP* leader region and influence its translation. It will be interesting to see what new targets, if any, nCsrA may be interacting with.

Considering CsrB's activity repressing CsrA despite nitrosative treatment, the nature of its RNA-binding becomes more intriguing. CsrB is known to bind CsrA with a stoichiometry of 1:18 through many CsrA-binding sites of variable nucleotide composition (43). The known variability of these sites within CsrB, as well as its apparent ability to antagonize nCsrA, suggests that modification of CsrA binding specificity under different environmental conditions may be accounted for and held in

check by the variable binding loops of CsrB (and CsrC). The diversity of CsrB's binding sites for CsrA may indicate many altered binding conformations for CsrA, possibly under multiple conditions. If so, the variability of CsrB's sites may evidence an evolutionary adaptation to host-supplied antagonization of CsrA.

Activating the BarA/UvrY pathway, and CsrB, by adding glucose, and artificial CsrB overexpression both alleviate CpxP downregulation in ASN. This implicates CsrA activity in the process. A novel CsrA-binding site within the *cpxP* leader region may allow CsrA binding under noncanonical conditions. The possibility CsrA may be nitrated at a tyrosine residue adjacent to the alpha-helix may enable this noncanonical binding. Nitrated CsrA may also exhibit altered stability and dimerization characteristics. Nitrated CsrA may bind other noncanonical targets, influencing their translation. This last possibility demands further investigation to determine binding kinetics, targets, and other possible downstream effects.

PTR by Hfq

I have shown that Hfq-mediated regulation in UPEC is extremely advantageous. Loss of the RNA chaperone Hfq results in significant colonization defects in murine models of cystitis and pyelonephritis despite normal in vitro growth. Intracellular bacterial communities (IBCs) were virtually absent from an Δ*hfq* mutant despite normal adherence to, and invasion of, host cells. Although the Δ*hfq* mutant exhibited similar in vitro growth characteristics to the parental strain, the mutant had marked defects in biofilm formation, motility, and resistance to ROS, RNS, and cationic peptides.

Additionally, the *hfq* deletion mutant exhibited a notably abnormal lipopolysaccharide (LPS) profile. Prior literature suggests phenotypes observed in *hfq* deletion strains are attributible to effects on the σ^S or σ^E stress-induced alternative sigma factors (44-46). I demonstrated that phenotypes observed from the Δ*hfq* mutant are not exclusively attributable to loss of σ^S or σ^E function by including UPEC *rpoS* and *rpoErseABC* gene deletion mutants as controls. This suggests Hfq is performing additional roles in UPEC pathogenesis beyond those mediated by these stress-responsive alternative sigma factors, and implies the involvement of sRNAs in the overall pathogenesis of UPEC beyond those known to regulate σ^S or σ^E. My studies have globally implicated Hfq, and thus sRNAs, in UPEC pathogenesis. Further studies will delineate which sRNAs are responsible for which aberrant Δ*hfq* UPEC phenotypes.

More specifically, these data demonstrate the great control Hfq exerts on UPEC's envelope. As mentioned above, the *hfq* mutant's LPS profile contains LPS constituents of decreased molecular weight via polyacrylamide gel electrophoresis. Presumably, this results from defects in polysaccharide biosynthesis, transport, polymerization, or turnover. The *hfq* null UPEC was highly sensitive to the cationic peptide polymixin B, demonstrative of envelope damage or compromised envelope maintenance or stress response pathways. Together, these data imply the necessity of Hfq for proper envelope maintenance by demonstrating envelope alterations in its absence. This hypothesis is enhanced by the reduced motility and defective in vivo IBC formation seen with the *hfq* null mutant, since assembly of the flagella and biofilms are both processes requiring numerous periplasmic chaperones and transport complexes through the envelope.

LPS alteration: symptom or cause?

LPS is a significant envelope component, both in physical abundance, but also in imparting structure and survival of environmental conditions (as reviewed by (91)). Perturbations to UPEC LPS could have profound effects on envelope stability or resistance to environmental insults. The foremost question is why Hfq —and likely sRNAs — would regulate LPS? Quick posttranscriptional regulation mediated via Hfq may alter LPS presentation and benefit the bacterium if the new surface presentation diminishes host recognition. In this respect, Hfq functions with sRNAs to effect prompt changes in UPEC antigenicity. Alternatively, the LPS alterations in Δ*hfq* UPEC may make it more antigenic or disrupt LPS stability, facilitating removal of the pathogen from the urinary tract. The latter option is perhaps more plausible considering the significant colonization and IBC-formation defects observed in Δ*hfq* UPEC.

The next question becomes: “How does Hfq regulate LPS presentation?” The mechanism by which Hfq alters the LPS profile is currently unknown, as is how abnormal LPS in the *hfq* mutant relates to the other observed envelope phenotypes. Do the alterations to LPS evident in the *hfq* null UPEC generally destabilize the envelope, leading to the other observed phenotypes, such as reduced motility and sensitivity to cationic peptides? Or does misregulation of some prior envelope component or pathway lead to LPS abnormality concomitant to the other phenotypes? In other words, does LPS alteration cause or result from any of the envelope deficiencies seen in the *hfq* null mutant? The following are speculations on the matter:

The altered LPS profile of the Δhfq mutant is attributable to aberrant production, processing, polymerization of surface sugar molecules or turnover. Production defects could result in decreased LPS components (lipid A, core LPS, or O-antigen polymerized LPS) at any stage since lack of necessary precursors would be followed by an absence of modified, transported, and polymerized products. Processing and transportation defects might resemble O-antigen polymerization defects as they would segregate lipid A and core LPS from the polymerization apparatus in the periplasm, resulting in truncated LPS of reduced molecular weight. Defects in LPS turnover would manifest on an LPS profile as LPS constituents of the same molecular weight with greater or lesser abundance. The *hfq* mutant LPS profile clearly showed accumulation of LPS core and lipid A with polymerized LPS of reduced molecular weight. This suggests the defect or defects responsible are subsequent to production, and may include trafficking of lipid A and the core region to the outer membrane, polymerization, or perhaps enhanced degradation of long-chain polymerized LPS.

The reduced molecular weight LPS in the Δ*hfq* mutant UPEC could also be explained by aberrant LPS modification. At least one sRNA, MgrR, regulates LPS modification via immediate downstream targets. The Hfq-dependent MgrR sRNA represses expression of the Ca^{2+}-induced phosphoethanolamine transferase protein EptB (47) (see Fig. 4.3.). EptB adds phosphoethanolamine modifications to LPS (49,50), which, if present in *E.coli* LPS, could serve to increase its molecular mass. Given MgrR sRNA translationally inhibits EptB, loss of either Hfq or MgrR should derepress EptB, leading to higher molecular weight species of LPS, opposite of observed Δ*hfq* LPS

profiles. Therefore there must be other sRNAs or Hfq effects responsible for the modified LPS observed in the *hfq* null mutant besides MgrR regulation of LPS modification by EptB.

Other sRNAs regulate expolysaccharide and cellulose production, which may be represented on an LPS profile. MicA and CyaR are Hfq-dependent sRNAs which translationally inhibit expression of outer membrane protein OmpX (51, 52) (see Fig. 4.3.). Deletion of *Escherichia coli* OmpX increases exopolysaccharide (EPS) production threefold, whereas overexpression of OmpX has no effect on EPS levels (53). Loss of Hfq should result in inability of MicA and CyaR to inhibit *ompX* translation, the consequence being normal or greater OmpX expression. However, the presence of OmpX only appears to maintain normal levels of EPS, and even enhanced levels of OmpX have no effect on EPS, as noted above (53). So, while MicA and CyaR sRNAs can influence EPS levels, the current body of literature suggests they only have power to increase EPS levels through OmpX, not decrease them, as may be seen in the *hfq* null LPS profile.

As an interesting aside, OmpX assists in virulence for *Yersinia pestis* and UPEC by promoting adhesion to cellular surfaces (92-94). In *Escherichia coli*, deletion of *ompX* upregulates type 1 pili production dramatically while reducing motility rates through agar (53). My *hfq* mutant clearly had reduced motility rates relative to wild type, like an *ompX* mutant, but also reduced type 1 pili, as evidenced by hemagglutination and cell adherence assays (see Chapter 3). Alleviation of *ompX* repression by the Hfq-dependent sRNAs MicA and CyaR in an *hfq* null mutant would be anticipated to increase motility and reduce type 1 pili, although only one of these is seen. Therefore, the motility and type 1

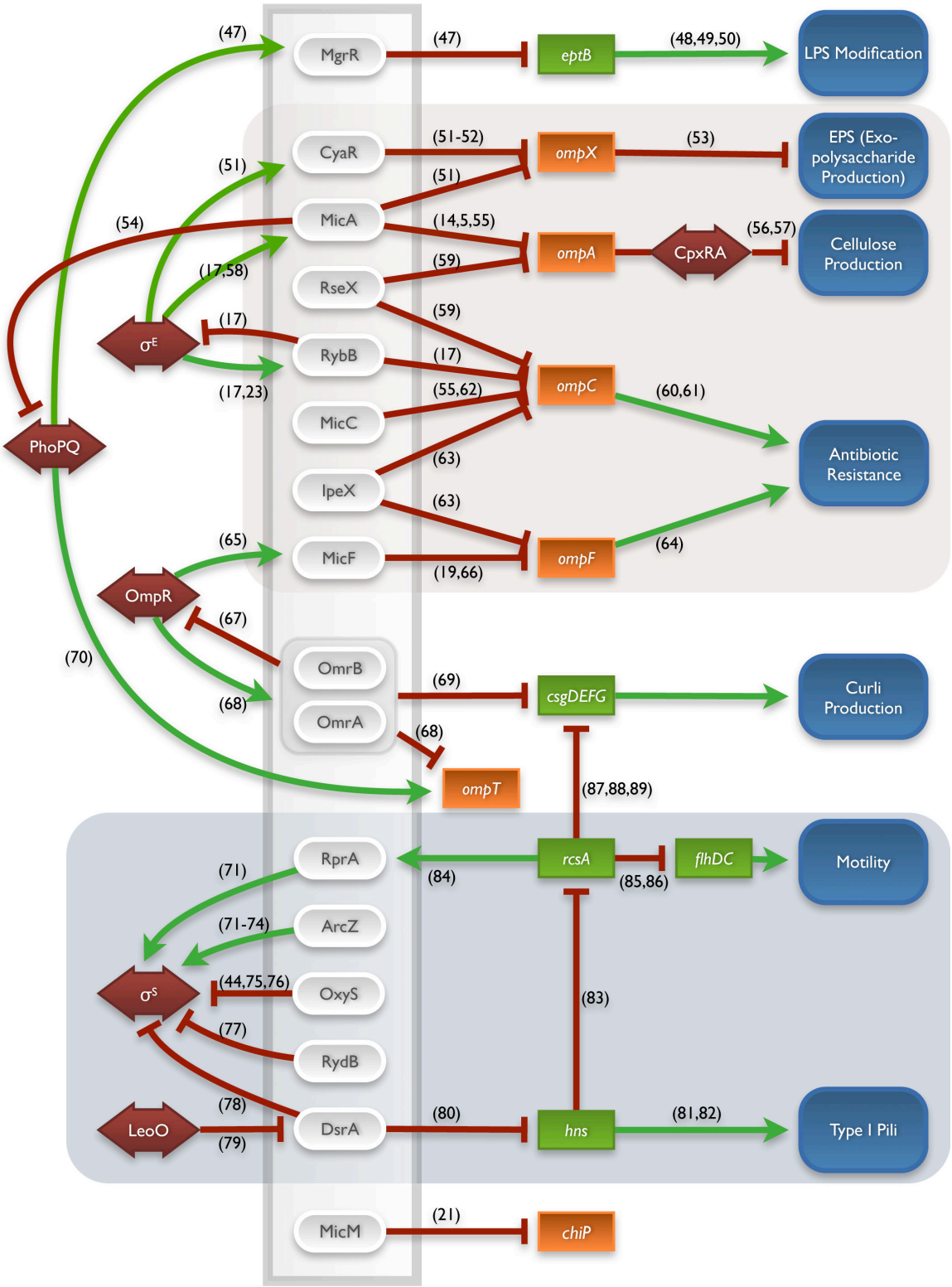


FIG. 4.3. sRNAs regulating LPS processes. Genes, shown in green, regulated by their respective Hfq-dependent sRNAs, shown in white. Affected downstream processes are shown in blue. Outer membrane protein (OMP) genes shown in orange. Upstream and downstream transcriptional regulators shown in red. Numbers correspond to references.

pili effects observed in *hfq* mutant UPEC could not be attributed singularly to alteration of OmpX levels. The possibility for synergistic effects with other misregulations resulting from inoperative sRNAs may explain these divergent phenotypes. Additionally, OmpX is required for full virulence in mouse and rat models of Yersinia pestis infection, and is reliant upon proper length of the LPS core (94). It is possible that derepression of OmpX expression in an *hfq* null mutant — and anticipated increase in virulence — is compensated for by the aberrant LPS profile of *hfq* mutant UPEC.

Besides regulating EPS through *ompX*, MicA sRNA also regulates production of cellulose, as can the RseX sRNA. Bacterial cellulose is another polymeric sugar which may be discerned on an LPS profile. The Hfq-dependent MicA and RseX sRNAs translationally repress expression of *ompA*, which encodes the outer membrane protein OmpA (51, 59) (see Fig. 4.3.). OmpA represses cellulose production via the CpxRA system (56, 57). Deletion of *ompA* produces sticky colonies due to enhanced cellulose production while overexpressed OmpA causes cell lysis (57). Loss of Hfq theoretically abrogates *ompA* repression by MicA and RseX, permitting OmpA translation and OmpA's repression of cellulose production, resulting in loss of cellulose signal on an LPS profile. Molecular weight for a polymeric carbohydrate can vary significantly, however, and may not appear as a discrete band on an LPS profile at all. Large polymers may be unresolvable as a high molecular weight smear or become trapped in the stacking gel. As yet, my existing LPS profiles have not differentiated cellulose, and this possibility remains to be addressed.

The role of the abnormal LPS profile of *hfq*-null UPEC is still an open question. Currently known sRNAs are likely not responsible for the aberrant *hfq*-null LPS profile, suggesting novel action by Hfq and other possible sRNAs. Is misregulated LPS responsible for the other envelope sensitivities observed, or merely symptomatic of greater aberrations? This could be tested by disrupting genes involved in LPS production, polymerization, transport, or turnover and performing LPS profiling to look for mutants phenocopying *hfq*-null UPEC's profile. A strain phenocopying *hfq*-null UPEC's LPS profile is an ideal candidate to explore LPS regulation by Hfq. These (and other LPS mutants) could be subjected to the same phenotypic assays used to characterize the UPEC *hfq* mutant observing for phenotypic overlap. If LPS mutants exhibited reduced motility, sensitivity to cationic peptides, ineffective murine colonization or IBC formation, this would signify the epistasis of the LPS alterations to the other observed phenotypes and highlight the paramount structural role of LPS in virulence. UPEC have already proven themselves formidable opponents against stresses that normally eliminate traditional lab strains of *E. coli* (95), however, and may be sufficiently resilient to withstand alterations to their abundant LPS. In this scenario, LPS mutants mimicking the *hfq*-null LPS profile do not recapitulate the other *hfq*-null phenotypes, signifying Hfq's control over other cellular processes as responsible for the deficient phenotypes. Many of the LPS biosynthesis and transport genes are known and were exploited to produce LPS cores of various lengths in Gram negative Yersinia (94). This paves the path for similar studies in UPEC to ascertain where Hfq intervenes in LPS biosynthesis or transport and what the direct consequences are.

Structural consequences of OMP misregulation

Alleviation of Hfq-mediated sRNA repression of OMPs may increase baseline envelope stress, sensitizing bacterial cells to further stresses. Many Hfq-dependent sRNAs translationally repress abundant outer membrane proteins, such as OmpA by MicA (14-16, 55) and RseX (59), OmpC by IpeX (63), MicC (55, 62), RybB 17, 51) and RseX (59), OmpF by IpeX (63) and MicF (18-20, 66), OmpX by CyaR (51, 52) and MicA (51), and ChiP by MicM (21). In the absence of Hfq, this repression is alleviated, resulting in OMP expression regardless of the regular translational inhibition mechanisms. This may result in activation of the σ^E pathway, which responds to overexpressed or misfolded OMPs by upregulating chaperones and proteases to manage these envelope stresses (96). Perhaps not surprisingly, the σ^E pathway also exerts translational control over OMPs via activation of the CyaR (51), MicA (17, 58), and RybB sRNAs (17, 23). The σ^E pathway thus adjusts OMP levels at the translational and posttranslational levels, demonstrating the necessity of OMP abundance control.

Regulation of OMPs by sRNA appears to be a common mechanistic motif (see Fig. 4.4.), which must be of evolutionary significance to the bacterium. If this were not the case PTR of OMPs would not be so universal nor common. It is reasonable the loss of repression of multiple OMPs simultaneously may impose an envelope stress of its own, which may be sufficient to occupy envelope stress response pathways. Additional stresses subsequently imposed upon this already stressed system then become insurmountable, producing the observed sensitivities to polymixin B, RNS, and ROS. This may explain the reduced motility of the *hfq* mutant as well, as transport or degradation of flagellin

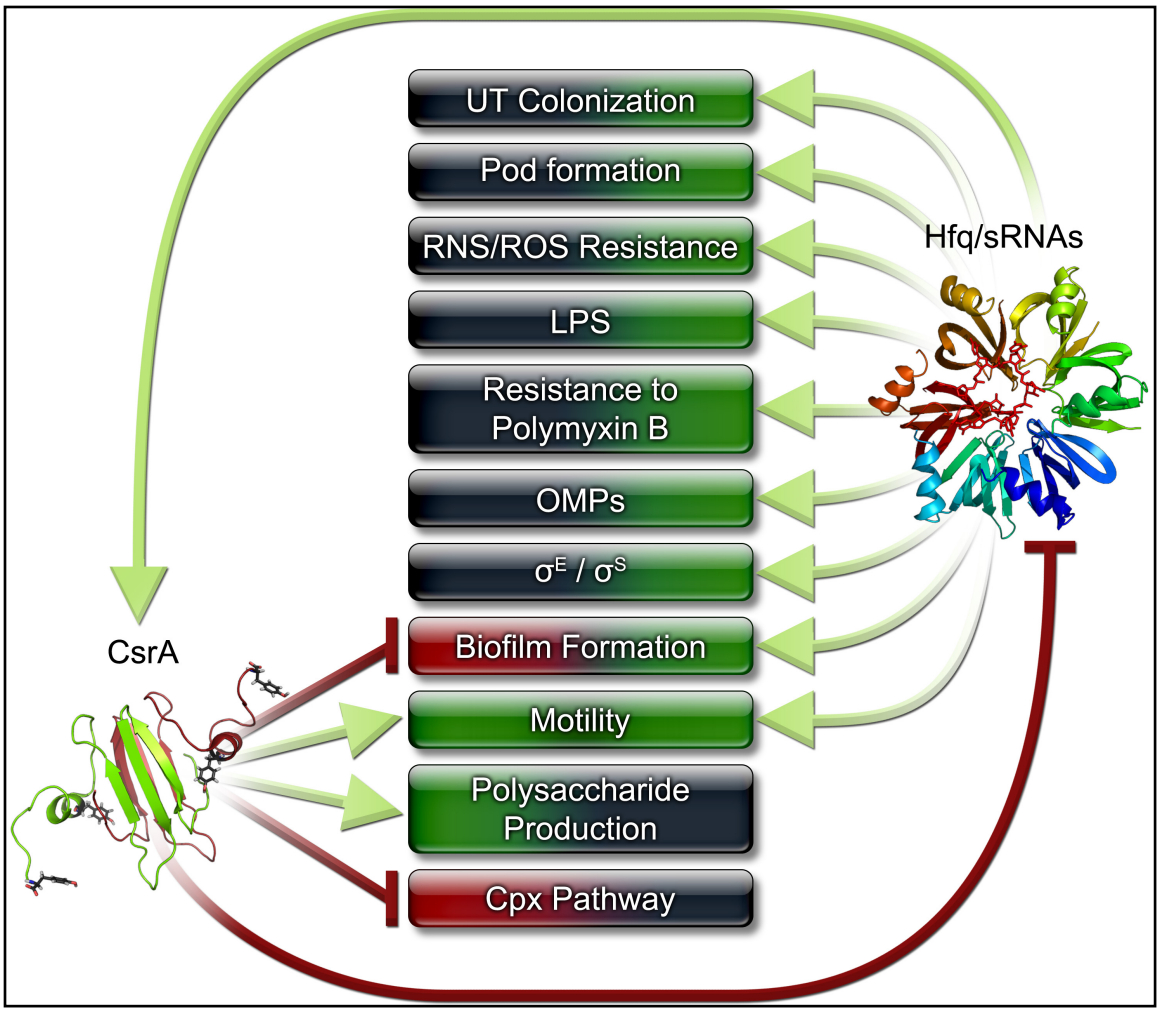


FIG. 4.4. Attributes regulated by PTR in UPEC. Green arrows indicate upregulation or activation while red arrows indicate inhibition.

subunits is partially compromised by a system barely able to handle a burden of beta-barrel OMPs.

Biofilm regulation by sRNAs

The *hfq*-null UPEC mutant exhibited notable deficiencies in microtiter-plate biofilm formation assays. As the body of biofilm literature and continued funding evidence, biofilm formation is a multifactorial process involving quorum-sensing,

production of exopolysaccharides, cellulose, and other extracellular matrix materials (97-104), the full extent of which is incompletely understood. Except for *E. coli* quorum sensing, all of these processes are regulated by Hfq-dependent sRNAs, possibly explaining reduced pathogenicity and biofilm formation in *hfq*-null UPEC.

Extracellular polysaccharides or exopolysaccharides (EPS), and cellulose are common bacterially-produced biofilm constituents (57, 103-105). Biosynthesis of EPS and cellulose are processes regulated by Hfq-dependent sRNAs (see Fig 4.4.). EPS biosynthesis is repressed by OmpX (53), and OmpX is repressed by the Hfq-dependent sRNAs CyaR (51, 52) and MicA (51). Cellulose production is inhibited by OmpA (56, 57) which is itself repressed by the Hfq-dependent sRNAs MicA (14, 15, 55), and RseX (59). Loss of Hfq should alleviate repression by these sRNAs and enhance expression of OmpX and OmpA, inhibiting EPS and cellulose production. This may answer how *hfq*-deleted UPEC produce reduced mitrotiter plate biofilms and may account for the reduced IBCs seen in vivo.

Also of note, MicA promotes both EPS and cellulose biosynthesis through repression of inhibitors to these pathways and is required for *Salmonella enterica serovar Typhimurium* biofilm formation (106). However, of all the sRNA mutants affecting OMP expression, LPS modification, motility, piliation, or curli production, only loss of MicA sRNA has shown any sensitivity to envelope stress (54). This strongly suggests MicA may be a key sRNA regulating pathogenic potential in UPEC, and is a current avenue of study.

Curli are extracellular amyloid-like fibrils which assist biofilm formation when expressed (101, 102, 107). DsrA sRNA regulates biofilm formation indirectly via the RcsAB pathway. When overexpressed, DsrA derepresses translation of *rcsA*, through an indirect mechanism involving the histone-like protein HNS (80). The *rcsA* gene encodes a component of the RcsAB transcriptional factor, which represses the curli biosynthesis operon *csgDEFG* (83), and as a side-note, the master regulator of motility, *flhDC* (86, 108). While DsrA expression indirectly represses transcription of the *csgDEFG* operon via the RcsAB transcription complex, two sRNAs directly inhibit translation of the operon. The highly homologous sRNAs, OmrA and OmrB, bind the 5' end of the *csgDEFG* transcript, inhibiting its effective translation (87) in an Hfq-dependent manner (67). The *csgDEFG* operon is thus regulated at both the transcriptional and translational levels by processes involving sRNAs. BLAST searches reveal the genomic presence of DsrA, OmrA, and OmrB in UPEC. Loss of Hfq, and concomitant function of the sRNAs DsrA, OmrA, and OmrB, would predictably result in upregulation of the curli biosynthesis operon. However, the *hfq* mutant UPEC mutant evidenced lower abundance of polysaccharides, with those present having decreased molecular weights. Thus the LPS profile seen cannot be attributed to the sRNAS DsrA, OmrA, or OmrB.

UPEC: The σ^E mutant that lived

In the course of studying Hfq effects on the virulence and fitness in UPEC, I generated a viable *rpoE* mutant as a control. The alternative sigma factor, σ^E, coded for by the *rpoE* gene, responds to heat-shock and misfolded outer membrane proteins (OMPs) by upregulation of periplasmic folding and degradation factors (109, 110). In

addition to these protein factors, sRNAs are upregulated that quickly inhibit translation of OMP transcripts (30, 31). OMPs are some of the most abundant bacterial proteins, and maintenance of appropriate OMP levels while avoiding their misfolding is a survival imperative. It comes as no surprise, therefore, that σ^E is considered an essential factor in K12 *E.coli* laboratory strains (111). However, second-site suppressor mutations have been isolated which permit deletion of *rpoE* in these strains (112).

So why then is an *rpoE* mutant in UPEC viable? The answer may lie in the accessory sequestration factors that are cotranscribed with *rpoE*. In UPEC, I generated a viable *rpoErseABC* deletion mutant, rather than deleting the *rpoE* gene individually (see Chapter 3). Although *rseABC* are co-transcribed with *rpoE,* there is an independent σ^E -type promoter downstream of *rpoE* (113, 114). The *rseABC* gene products, RseA and RseB, operate as anti-σ^E factors, sequestering σ^E at the inner membrane under non-inducing conditions (113). RseC assists reducing members of the SoxR reducing complex (115), and also promotes σ^E activity through an unknown mechanism (113).

I deleted *rseA*, *rseB*, and *rseC* simultaneously with *rpoE* for cloning convenience under the assumption that they would not be required due to loss of σ^E. The resulting mutant was viable, growing equivalent to the parental strain in shaking LB broth cultures, although it was significantly defective in resisting RNS and ROS. To address the possibility of secondary-site suppressor mutations permitting deletion of *rpoE,* I successfully trans-complemented the mutant with the entire *rpoErseABC* operon, restoring the wild type phenotypes. This implies that deletion of *rpoE* in UPEC is viable because 1) the expression of the *rseABC* genes is toxic in the absence of σ^E or 2) UPEC

possess some intrinsic difference relative to the K12 strains that permits disruption of *rpoE*.

Regarding the first possibility, deletion of *rseA* results in upregulation of σ^E activity (113, 116), while *rseB* deletion produces only marginal activation in non-inducing conditions and no change in σ^E-inducing conditions (116). Loss of *rseC* results in constitutive expression of oxidative stress responsive transcriptional activator *soxS* (115). SoxS then activates a network of genes involved in resistance to antibiotics (117-119), superoxide (120-122), organic solvents (123, 124), and heavy metals (125). Thus an *rseC* mutant might be anticipated to have a greater abundance of resistance proteins for antibiotics, superoxide, organic solvents and heavy metals. However, my work has demonstrated the lack of resistance to oxidative stress in the UPEC *rpoErseABC* mutant (126) despite anticipated constitutive activation of *soxS*. This seems to argue against the viability of Δ*rpoErseABC* UPEC hinging upon concomitant deletion of *rpoE* and *rseC*.

While envelope maintenance in K12 *E.coli* is dependent upon σ^E, it appears that UPEC may have redundant envelope maintenance capabilities, or perhaps not suffer the same degree of incipient envelope stress as laboratory strains. This latter possibility hints at additional UPEC factors enabling its survival. Interestingly, second-site mutation of the hypothetical gene *ydcQ*, of K12 *E. coli*, permits deletion of *rpoE* (112). BLAST alignments indicate this region contains a deletion in multiple UPEC strains, possibly making them amenable to *rpoE* deletion. Current lab experimentation is addressing this possibility.

The possibility also exists the original study characterizing σ^E as absolutely required was flawed. Prior studies determined the necessity of σ^E in K12 *E.coli* by non-targeted transposon mutagenesis (109-111, 127). Because *rpoE* lies upstream of *rseABC*, and can be contranscribed with them (113, 114, 128), it is possible the introduction of a transposon eliminates or otherwise alters transcription of the downstream *rseABC* genes. Forseeably, upregulation of the *rseABC* genes is possible. The possibility again becomes that expression of RseA, RseB, or RseC is toxic and follows the same analysis given several paragraphs prior. The overexpression scenario seems particularly unlikely, however, as SoxS upregulation should result in cells more resistant to oxidative and antibiotic stresses, not less, as observed in the UPEC *rpoErseABC* mutant.

Complicity of Hfq and CsrA

In a curious twist of regulatory fate, CsrA and Hfq regulate one another. Baker *et al*. have demonstrated CsrA binding to the *hfq* leader region inhibits *hfq* translation in *Escherichia coli* (129). Hfq also negatively regulates its own translation (130). Oppositely, the Hfq homolog in *Legionella pneumophila* upregulates *csrA* gene expression through an unknown mechanism (131). This suggests an interesting autoinhibitory loop whereby Hfq upregulates CsrA, eventually downregulating Hfq. This regulatory loop may imply a chronological cycle or hierarchy of PTR activity in which Hfq accomplishes its tasks and activates CsrA, which then reduces Hfq activity through translational inhibition.

In the context of UPEC infection, speculative scenarios come to mind in which colonizing bacteria immediately respond to environmental changes and host innate immune defenses through fast-acting sRNA-mediated methods, enabling the pathogens to survive. They later switch to CsrA-mediated PTR to activate glycolysis and motility to spread. This may involve temporal-spatial changes in CsrA and Hfq activity throughout the various phases of infection: attachment, invasion, IBC-formation, efflux and dissemination or ascension (see Fig. 4.1.). The fact that both varieties of PTR function rapidly may also suggest they both function at every step of infection as new environments and insults are encountered, with Hfq/sRNAs quickly readapting the bacterium to immediately cope with the stress and CsrA dictating lifestyle choices of whether to form biofilms or opt for motility. Lastly, it is possible that this regulatory loop represents a constant fine balance of background activities lacking major temporal-spatial fluctuations. Hfq and CsrA individually represent systems of incredible complexity, and their complicity in UPEC pathogenesis needs to be addressed.

Interplay between Hfq and CsrA is further suggested by their shared and unique phenotypes. Both PTR systems regulate motility and biofilm formation (see Fig. 4.4.). Both Hfq and CsrA positively regulate motility, as evidenced by the reduced motility of the *hfq* mutant UPEC and the known role of CsrA upregulating the FlhDC master motility regulator (26). Although the methods CsrA upregulates motility are well characterized, the pathway for Hfq control of motility is more speculative. The Hfq-dependent sRNA, DsrA, posttranscriptionally represses *hns*, coding for the nucleoid protein H-NS (80). H-NS exerts transcriptional activity, simultaneously promoting translation of the *flhDC*

master regulator of motility (132) and downregulating expression of *rcsAB* (83), which represses *flhDC* expression (85, 86) (see Fig. 4.3.). In short, Hfq promotes motility coordinately with DsrA, and loss of Hfq should result in inability of DsrA to repress *hns*, resulting in repression of motility. Although *hfq*-deleted UPEC were motile, they were significantly impaired in motility rate. So, both Hfq and CsrA function to promote motility.

While both Hfq and CsrA regulate biofilm production, they do so antagonistically. CsrA is known to inhibit biofilm elaboration in K12 laboratory strains of *E.coli*, and a Δ*csrA* mutant readily forms biofilms on glass (27, 133). Similarly, a UPEC Δ*csrA* mutant forms massive biofilms and seemingly grows slower and to a lower density than the parental wild type strain at 30º and 37ºC in LB broth and M9 minimal media. Growth of this mutant also yields thick mucus-like material at the air-fluid interface in glass tubes, but forms non-attached cellular aggregates in plastic tubes or microtiters plates. While presence of CsrA represses biofilm formation, Hfq promotes it, as *hfq* deletion resulted in significantly reduced microtiter-plate biofilms, IBCs, and no observable non-attached cellular aggregates (see Chapter 3). Thus, Hfq and CsrA differentially impact biofilm formation as evidenced by microtiter plate biofilm assays.

Considering possible cross-regulation of Hfq and CsrA, a hierarchical set of responses in which Hfq assists in biofilm formation and stress adaptation precede escape by motility, as CsrA becomes activated, is plausible. Given the specific regulation of *hfq* translation by CsrA (129), it is possible that some of the phenotypes observed in *csrA*-deleted UPEC result from enhanced Hfq production. This does not appear to be the case,

however, considering the apparent growth defects of the Δ*csrA*-deletion strain. The predicted upregulation of Hfq, and subsequent sRNA-mediated PTR in a *csrA*-null strain does not overcome the general decrease in fitness this mutant exhibits. Thus, while CsrA and Hfq are known to regulate each other in different organisms, these interactions and their functional consequences still need to be addressed in UPEC.

Another interesting connection implied by my work with Hfq and CsrA PTR systems is cross-regulation of σ^E. Hfq indirectly downregulates σ^E activity (22, 134, 135). This complex regulation involves multiple factors, such as DegS, the periplasmic protease responsible for degradation of the RseAB σ^E-sequestration complex, which is significantly increased in an *hfq*-deletion mutant (135). In addition, expression of the Hfq-dependent sRNA, RybB, is dependent upon, and downregulates, σ^E translation, providing a σ^E autoregulatory loop (17, 23). While sRNAs and Hfq downregulate σ^E, the Cpx pathway transcriptionally activates σ^E expression through the response regulator CpxR (136). Given the downregulatory effect of CsrA upon CpxP under nitrosative stress, CsrA may indirectly influence σ^E activity. Downregulation of CpxP by CsrA may result in activation of the Cpx pathway, which in turn upregulates σ^E. In this respect, the protein-mediated and sRNA-mediated classes of PTR may function antagonistically to one another with respect to σ^E. Downregulation of CpxP by CsrA under nitrosative stress is enigmatic, however, and it is not clear that RNS-mediated ablation of CpxP expression actually leads to increased Cpx pathway activation. Consequently, while it is possible that PTR by CsrA upon CpxP may affect σ^E, this remains to be tested.

Importance of PTR in UPEC

This study has demonstrated the unique roles of both CsrA-mediated and Hfq-mediated PTR regulating UPEC virulence by investigating chemical resistance and murine infection phenotypes in gene deletion mutants. Specifically, PTR regulation involving Hfq is dispensable for in vitro growth but is critical for infection in murine models of cystitis and pyelonephritis. My in vitro analyses clarify Hfq's requirement in resisting a variety of insults a host may present. Thus, Hfq is a key regulator of stress responses contributing to UPEC survival in adverse conditions, including life within a host.

CsrA, on the other hand, is required for normal in vitro growth of UPEC, although its activity may be detrimental under nitrosative stress by downregulating the periplasmic chaperone CpxP. In vitro CpxP overexpression proved beneficial under nitrosative stress, suggesting that downregulation of CpxP is an effect of host-supplied RNS limiting bacterial growth. However, I cannot rule out the possibility that this phenomenon serves another as-yet undefined purpose that may actually benefit the pathogens in the end. Downregulation of CpxP in the presence of RNS may be attributed to nitration of CsrA and may represent the first evidence of posttranscriptional regulation by nitration in *Escherichia coli*.

Besides demonstrating the importance of PTR in UPEC virulence, these discoveries raise additional questions. Regarding nitrated CsrA, the question remains if nitrated CsrA actually binds the 5' UTR of the *cpxP* transcript, or if it exerts control indirectly. Binding parameters for nCsrA still need to be ascertained. Does nCsrA still

bind RNA, and if so, are its binding parameters altered by nitration? If RNA immunoprecipitation or footprinting analyses do confirm nCsrA RNA-binding activity, affinity studies can address if nCsrA has a novel binding consensus region. These data could subsequently lead to predictions of other RNA targets of nCsrA.

The discovery that Hfq influences UPEC pathogenesis strongly suggests sRNAs are involved in these regulations. The foremost question is which specific sRNAs are responsible for the pathogenicity defects observed in the *hfq* mutant? Gene deletion mutants for specific sRNAs may mimic a subset of conditions present in the *hfq* knockout, permitting association of particular sRNAs with observed *hfq*-null phenotypes. Such a study could not account for novel or UPEC-specific sRNAs which may be responsible for these phenotypes, however. Addressing this possibility requires much more involved methods, such as differential expression analyses or custom genome-tiling microarrays of UPEC during in vivo murine infection versus UPEC grown in vitro. Any sRNA or expressed intragenic regions could be deleted or overexpressed and subjected to the panoply of phenotypic assays assembled to assess *hfq* mutant UPEC.

The *hfq*-null UPEC mutant's phenotypes may be broadly characterized as envelope deficiencies and previously discussed the possibility that gross alterations to OMP expression or LPS misregulation may either contribute to or arise from the perturbed envelope. The genes coding for OMPs, OMP transport and assembly proteins, and LPS biosynthesis are well characterized. Tunable overexpression of multiple OMPs simultaneously or simultaneous deletion of the CyaR, MicA, RseX, RybB, MicC, IpeX, and MicF sRNAs regulating OMPs (14, 15, 17, 19, 51, 52, 55, 59, 62, 63, 66) may yield

UPEC phenocopying the *hfq* mutant, indicating the envelope deficiencies result from overloaded envelope stress response pathways. Similarly, if LPS biosynthesis deletion UPEC mutants produce the same sensitivities as the *hfq* mutant, these observations would support a model of LPS as a critical structural component whose alteration produces envelope sensitivity. Bioinformatics predictions would then help align putative sRNAs with known LPS biosynthesis genes to find potential regulatory sRNAS.

Another remaining question is how important is the MicA sRNA in UPEC pathogenesis? MicA regulates both *ompX* (51) and *ompA* (14, 15, 55), and thus EPS elaboration (53) and cellulose production (56, 57). MicA is required for biofilm formation in bacterial "cousin" *Salmonella enterica serovar Typhimurium* (106), and of all the OMP-regulating sRNAs, only deletion of MicA produces any envelope sensitivity (54). MicA thus seems an ideal sRNA candidate for biofilm regulation in UPEC, although its role in IBC formation or in vivo infection has yet to be assessed.

Of the questions raised in this study perhaps the most intriguing is how and why Hfq and CsrA interregulation occurs. Consider the critical importance of both Hfq and CsrA in UPEC virulence phenotypes demonstrated in this work, the role of this reciprocal regulation in the context of UPEC virulence is a tantalizing lead. Is there, in fact, a cycle of regulation oscillating between Hfq- or CsrA-dominated modes? If so, does this cycle decide motile or cessile lifestyles for UPEC? Expression studies of CsrA, CsrB, and Hfq in UPEC during infection of tissue culture or mouse bladders performed at high temporal resolution would begin to reveal any such cycle, or perhaps demonstrate expression patterns for either as UPEC progresses through infection.

This work links PTR of both the protein-mediated and sRNA-mediate varieties in regulation of specific UPEC virulence determinants and demonstrates the significance of both during in vivo murine infections. Many of the phenotypes resulting in abrogation of UPEC's PTR mechanisms resulted in envelope sensitivities, and while Hfq is known to regulate the SigmaE envelope stress response pathway, this work has shown regulation of the Cpx envelope stress response pathway by CsrA. Significantly, this demonstrates regulation of the two principle envelope stress response pathways by PTR, suggesting rapid translation regulation of envelope stress pathways is of great benefit to UPEC, particularly during infection. Not only has this study demonstrated PTR is required for proper envelope responses in UPEC, but, specifically in the case of CsrA, may also be a target of host innate immune responses. If the host modulates UPEC virulence via PTR mechanisms, this may represent a novel and exploitable means of pathogen control via targeting envelope stress response pathways. This gateway research has opened numerous avenues and opportunities for future pathogenesis research specifically regarding translation control of host stress resistance and virulence determinants.

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APPENDIX A

MECHANISMS AND CONSEQUENCES OF BLADDER CELL INVASION BY UROPATHOGENIC *ESCHERICHIA COLI*

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REVIEW ARTICLE

Mechanisms and consequences of bladder cell invasion by uropathogenic *Escherichia coli*

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ABSTRACT

Strains of uropathogenic *Escherichia coli* (UPEC) are the major cause of urinary tract infections worldwide. Multiple studies over the past decade have called into question the dogmatic view that UPEC strains act as strictly extracellular pathogens. Rather, bacterial expression of filamentous adhesive organelles known as type 1 pili and Afa/Dr fibrils enable UPEC to invade host epithelial cells within the urinary tract. Entry into bladder epithelial cells provides UPEC with a protected niche where the bacteria can persist quiescently for long periods, unperturbed by host defences and protected from many antibiotic treatments. Alternately, internalized UPEC can rapidly multiply, forming large intracellular inclusions that can contain several thousand bacteria. Initial work aimed at defining the host and bacterial factors that modulate the entry, intracellular trafficking, and eventual resurgence of UPEC suggests a high degree of host-pathogen crosstalk. Targeted disruption of these processes may provide a novel means to prevent and treat recurrent, relapsing and chronic infections within the urinary tract.

Keywords Fimbriae, integrin, invasion, UPEC, UTI, uroplakin.

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Introduction

The urinary tract is normally a sterile environment, kept free of microbes by the cleansing flow of urine and by myriad antimicrobial molecules and effector immune cells. However, despite these formidable host defences and the increasing usage of antibiotics, urinary tract infections (UTIs) remain among the most common of infectious diseases worldwide. Women are particularly susceptible to UTI in part, it is believed, due to unique anatomical features of the female genitourinary tract, which include a shorter urethra and the more proximal location of the urethral meatus to the anus [1]. About 50% of women will experience at least one UTI in their lifetime, and of those about 25% will suffer from one or more recurrent (or relapsing) infections. These infections represent a serious economic and medical burden, accounting for more than 7 million outpatient hospital visits and 1 million emergency room visits in the United States alone, with a cost greater than $2 billion annually [2,3]. About 95% of all UTIs are uncomplicated bladder infections (cystitis), occurring in anatomically normal individuals and characterized by burning sensations, frequent micturition, incomplete voiding, and suprapubic pain [1]. Infection of the kidneys (pyelonephritis) is more severe and is indicated by symptoms that include chills, high fever, nausea, joint and muscle aches and flank pain. Left untreated, pyelonephritis can result in renal failure, bacteraemia and sepsis [4].

UTIs are caused by a number of bacterial pathogens including *Klebsiella* spp., *Proteus* spp., *Enterobacter* spp., *Citrobacter* spp., *Staphylococcus aureus*, coagulase-negative *Staphylococcus*, Enterococci, and strains of uropathogenic *Escherichia coli* (UPEC) [5]. Among these, UPEC are the principal causative agents of UTIs, accounting for most community (up to 95%) and hospital acquired (~50%) infections [3,6,7]. UPEC isolates are classified as extra intestinal pathogenic *E. coli* (ExPEC), a genetically diverse group of *E. coli* strains that can cause sepsis and neonatal meningitis, in addition to UTIs [8]. Although these pathogens are believed to originate within the gut, they do not appear to cause any notable disease within the gastrointestinal tract. UPEC strains, which are closely related to avian pathogenic *E. coli* (APEC) isolates [9,10], can potentially be transmitted between individuals via faecal-oral routes, contaminated food products, and even sexual contact [11–13].

To successfully colonize the harsh environment of the host urinary tract, UPEC strains express an array of diverse virulence factors. These include various fimbrial and afimbrial adhesins, siderophores, and secreted toxins [14–16]. Adhesive organelles like type 1, P, S, and F1C pili (or fimbriae) promote UPEC adherence to host cells and tissues while siderophores like enterobactin, aerobactin, and bacteriocin allow UPEC to better sequester much needed iron away from the host. Secreted toxins,

which include α-haemolysin, cytotoxic necrotizing factor-1 (CNF1), and the secreted autotransporter toxin Sat, can alter host cell signalling cascades, modulate inflammatory responses, and stimulate host cell death, thereby releasing needed nutrients and providing UPEC with greater access to deeper tissues within the urinary tract. Experimental and epidemiological data highlight the fact that no single virulence factor is sufficient for UPEC to cause disease. Rather, a timely and stepwise expression of multiple, potentially redundant factors working in concert contributes to the successful establishment of a UTI.

Over the past decade, several studies have shown that UPEC, long considered an extracellular pathogen, can invade epithelial cells of the urinary tract in both *in vitro* and *in vivo* assays (see [15]). Host cell invasion provides UPEC with a distinct and quantifiable survival advantage within the urinary tract, making it clear that the ability of UPEC to act as an opportunistic intracellular pathogen can contribute significantly to the pathogenesis of UTIs. Here, we review the consequences and mechanisms of host cell invasion by UPEC.

UPEC as an opportunistic intracellular pathogen

The distinction between an invasive pathogen and one that is merely phagocytosed as part of a host defence mechanism is not always immediately obvious. In some of the initial microscopic studies of rodents that had been infected with UPEC, it was noted that the pathogens could be found both within membrane-bound compartments and free within the cytosol of the superficial facet cells that line the lumenal surface of the bladder epithelium [17,18]. These bacteria were assumed to be doomed, phagocytosed by the facet cells and awaiting imminent destruction. However, subsequent studies using mouse cystitis model systems have shown that entry of UPEC into host bladder epithelial cells could benefit the microbes, providing them with a niche in which to multiply and persist [19–25]. In addition, intracellular UPEC appear to be better protected from a number of antibiotics, including mecillinam, cefuroxime, gentamicin, and trimethoprim-sulfamethoxazole [21,26,27]. In mouse models, administration of antibiotics can effectively sterilize the urine of infected mice, but intracellular bacteria sequestered within the bladder epithelium remain viable and can persist for many weeks.

Upon entering host bladder epithelial cells, UPEC becomes localized within acidic membrane-bound vacuoles that have many characteristics of late endosomes and lysosomes [22]. The fate of UPEC within these compartments can vary depending on the differentiation status of the host cells and probably other factors. Within immature bladder epithelial cells, which underlie the terminally differentiated superficial facet cells, the UPEC-containing vacuoles are often enmeshed within a network of actin fibres and bacterial replication is fairly limited. It is proposed that UPEC can persist quiescently for long periods within these immature cells without causing any of the overt clinical symptoms of a UTI [20,22]. The quiescent status of these bacteria likely makes them less detectable by host immunosurveillance mechanisms and also less susceptible to many antibiotic treatments, which often target only replicating microbes. The eventual resurgence of these quiescent reservoirs of intracellular bacteria may give rise to a significant percentage of recurrent or relapsing infections that are experienced by many individuals throughout their lives.

In vitro studies using bladder epithelial cells in culture have shown that intravacuolar replication and efflux of UPEC can be enhanced by disruption or rearrangement of the host actin cytoskeleton [28]. The release of UPEC into the host cytosol stimulates bacterial replication further, allowing the pathogens to rapidly form large intracellular inclusions containing up to several thousand bacteria. These biofilm-like inclusions develop in association with host intermediate filaments and were initially called bacterial factories, in reference to the term ‘viral factories’ used in the virology literature [20,22]. Subsequent studies refer to these bacterial inclusions as intracellular bacterial communities (IBCs) or pods [23,24]. Although UPEC can invade all cell layers of the bladder epithelium, IBCs typically develop only within the superficial facet cells, in which the actin cytoskeleton is sparse and generally restricted to sites along the basolateral surfaces [20,28]. IBC formation appears to be a general attribute of most UPEC isolates, occurring in all mouse strains so far examined as well as in human patients with UTI [29,30]. Time lapse video microscopy of infected mouse bladder explants indicates that IBC formation is marked by different developmental phases in which the bacterial growth rates, morphology, and motility vary along with the organization of the IBC [24]. The net effect of this process is amplification of bacterial numbers, increasing the likelihood that some microbes will manage to persist within the urinary tract or go on to infect new hosts.

Dissemination of UPEC within the bladder epithelium

The bladder epithelium, which acts as a formidable permeability barrier, has an exceptionally slow turnover rate under normal conditions [31,32]. However, in the face of large numbers of UPEC or other stressors, uroepithelial cells will detach, to be subsequently rinsed from the body with the flow of urine (Fig. 1) [19]. These infection-induced events stimulate the expression of numerous pro-proliferation and differentiation genes within the bladder epithelium, promoting the rapid repair and regeneration of the bladder epithelium [19,33].

The exfoliation of infected facet cells, along with the targeting of IBCs by infiltrating neutrophils, naturally limits the lifespan of IBC-containing host cells within the urinary tract [19,20,34]. Consequently, it was hypothesized that the release, or efflux, of

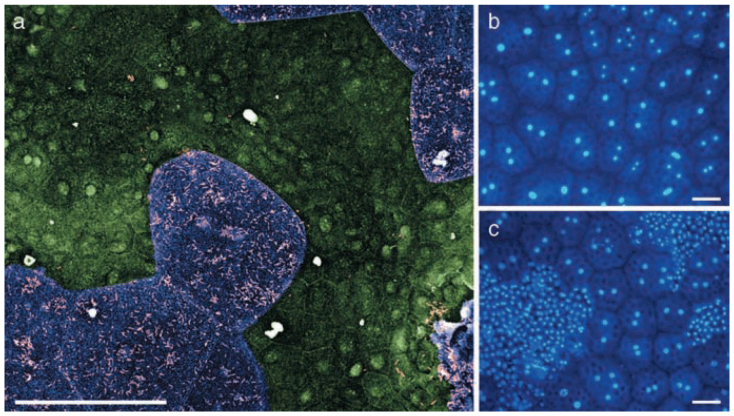

Figure 1 UPEC-induced exfoliation of bladder epithelial cells. (a) Scanning electron microscopy (SEM) image of an infected mouse bladder. Some of the large superficial facet cells (blue), which normally cover the entire luminal surface of the bladder, have exfoliated, exposing the much smaller immature cells (green) of the bladder epithelium. Bacteria are coloured red. (b and c) Images show the luminal surface of bladders taken from mice that were left uninfected (b) or infected with UPEC (c) for 6 h and subsequently stained using the nuclear dye Hoechst. The superficial facet cells are easily identified by their large size, hexagonal shape, and binucleate status, notably in contrast with the much smaller mononucleate immature cells of the bladder that are exposed following infection with UPEC. Scale bars = 100 μm.

UPEC from IBCs is needed to facilitate the colonization and long term persistence of UPEC within the urinary tract [20]. Both *in vitro* and *in vivo* studies have noted the ability of UPEC to escape from infected bladder cells [20,22,24,35]. Efflux is stimulated by the generation of the second messenger cyclic adenosine monophosphate (cAMP) in infected host cells, as well as by the breakdown of bladder cell integrity during the exfoliation process. Interestingly, bacterial efflux from IBCs often coincides with the transient appearance of long, filamentous microbes that can loop within and between adjacent superficial cells [20,24]. These filamentous bacteria arise due to activation of the SOS stress response in UPEC, and appear to enhance bacterial colonization of the urinary tract in the face of innate host defences [36].

While bladder cell exfoliation can be viewed as a host defence mechanism, providing a means for the host to clear out large numbers of bacteria (in IBCs, for example) with the flow of urine, it also leaves the underlying layers of the bladder epithelium temporarily exposed and more susceptible to infection. Tissue damage caused by the influx of neutrophils, as well as the generation of secreted toxins by UPEC, also likely facilitate bacterial dissemination throughout the multiple layers of bladder epithelium. For example, sublytic levels of the pore-forming toxin α-haemolysin (HlyA) produced by many UPEC isolates have been shown to trigger bladder cell apoptosis and exfoliation [37]. This process may be linked with HlyA-mediated inactivation of the host serine/threonine kinase Akt, which is a central regulator of several host cell survival, metabolic and inflammatory pathways [38]. Other factors associated with UPEC, including lipopolysaccharide and type 1 pili, may also stimulate bladder cell exfoliation independent of any secreted protein toxins [39–41].

Type 1 pili-mediated invasion

The major facilitators of host cell invasion by UPEC are filamentous adhesive organelles known as type 1 pili [42]. These hair-like fibres are expressed by more than 90% of all *E. coli* isolates, including both pathogenic and commensal strains, and are required for most UPEC isolates to effectively colonize the urinary tract [43–48]. Type 1 pili, which are distributed all around the bacterial surface, are assembled via a canonical chaperone-usher pathway and vary from a few fractions of a micron to greater than 5 μm in length [19,49–51]. The major portion of each pilus fibre consists of a 7-nm-wide helical rod comprised of thousands of repeating FimA pilin subunits. The distal part of the pilus tapers into a 3-nm-wide tip fibrillum structure formed by two adapter proteins, FimF and FimG, and the mannose-binding adhesin FimH [49,52]. FimH mediates bacterial adherence to a number of host glycoproteins and non-glycosylated peptide epitopes, including secretory IgA [53], the glycophosphatidylinositol (GPI)-anchored protein CD48 [54], carcinoembryonic antigen-related cell adhesion molecule (CEACAM) family members [55,56], Tamm-Horsfall protein (THP) [57], non-specific cross-reacting antigen (NCA)-50

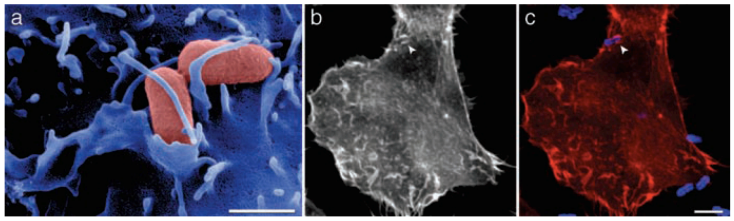

Figure 2 UPEC invasion of bladder epithelial cells. (a) SEM image of an infected mouse bladder showing the plasma membrane (blue) of a superficial facet cell zippering around and enveloping UPEC (red). (b and c) Actin localizes around invading type 1-piliated bacteria, as detected using confocal microscopy. In the merged image in (c) F-actin is red, while UPEC is blue. For clarity, F-actin is shown by itself in (b). The arrowhead indicates localized actin rearrangements taking place as UPEC enters the bladder epithelial cell. Scale bars = 1 μm (a) and 5 μm (b and c).

[56], leukocyte adhesion molecules CD11b and CD18 [58], α3 and β1 integrin subunits [28], uroplakin 1a (UP1a) [59], and the extracellular matrix-associated proteins type I and type IV collagens [60], laminin [61], fibronectin [62]. FimH interactions with these various receptors are competitively inhibited by D-mannose, which acts as a soluble receptor analogue.

In studies using cultured bladder epithelial cells, the FimH adhesin was shown to be both necessary and sufficient to initiate the invasion process [42]. Bacteria expressing type 1 pili lacking FimH fail to invade bladder cells, while latex beads coated with purified FimH (but not control proteins) are effectively internalized. FimH-mediated bacterial invasion of bladder cells requires localized rearrangements of the actin cytoskeleton, stimulating the host plasma membrane to zipper around and envelop the adherent bacteria (Fig. 2). Once UPEC is internalized, the continued expression of type 1 pili can also augment the development of IBCs, perhaps by stimulating bacterial aggregation and biofilm formation [63]. Intracellular biosynthesis of another adhesin, the autotransporter protein antigen 43 (Ag43), may contribute similarly to IBC development by promoting UPEC aggregation [22,23,64].

Recent work indicates that FimH-mediated bacterial invasion of host cells can be mediated by α3 and β1 integrin subunits [28]. Integrins are integral membrane proteins, made up of α and β subunits, which normally function as linkers between the extracellular matrix and the actin cytoskeleton [65]. FimH is able to bind α3 and β1 integrin subunits individually via N-linked high-mannose type glycan residues, independent of the canonical ligand-binding pocket formed by α3β1 heterodimers [28]. The α3 and β1 integrin receptors cluster around adherent bacteria, coincident with the accumulation of F-actin at sites of bacterial invasion. Phosphorylation sites within the cytoplasmic tail of β1 integrin modulate the conformation of β1 integrin as well as the recruitment and activation of various adaptor and signalling factors, several of which have been implicated in FimH-mediated bacterial invasion of host cells. These include stimulation of host Rho family GTPases like Rac1 and the activation of Src and focal adhesion kinase (FAK) [28,42,66,67]. Transient complex formation between FAK and PI 3-kinase, as observed during the invasion process, stimulates the generation of 3-phosphoinositide second messengers that can modulate actin dynamics, while complex formation between the adaptor proteins α-actinin and vinculin likely helps stabilize the induced cytoskeletal rearrangements [42,68–70]. Notably, mutations within the cytoplasmic tail of β1 integrin that change threonines 788 and 789 to alanines and tyrosines 783 and 795 to phenylalanine effectively inhibit FimH-mediated bacterial invasion [28]. In contrast, mutating serine 785 within the β1 integrin tail to alanine greatly enhances bacterial invasion frequencies for reasons not yet understood. None of these mutations interfere with bacterial adherence to the host cells. Interestingly, recent work indicates that target host cells may actively work to circumvent some of the signalling events that promote UPEC invasion. This is exemplified by results showing that elevated host cAMP levels linked with activation of the pattern recognition receptor Toll-like receptor 4 (TLR4) interfere with the invasion process by negatively affecting Rac1 activity [67]. Fig. 3 summarizes many of the known signalling events involved in FimH-mediated bacterial invasion of bladder epithelial cells along with some of the downstream consequences.

Both differentiated and immature epithelial cells at sites throughout the urinary tract, including the renal pelvis, ureter and bladder, express α3β1 integrins [28,71]. These integrin receptors are thus well situated to mediate UPEC entry into host cells throughout all strata of the urothelium at both early and late time points during the course of a UTI. However, it is probable that additional host receptors also have a role in FimH-mediated bacterial invasion [28]. High-resolution imaging of UPEC-infected mouse bladders showed that the FimH-containing tips of type 1 pili make intimate contact with the so-called asymmetric unit

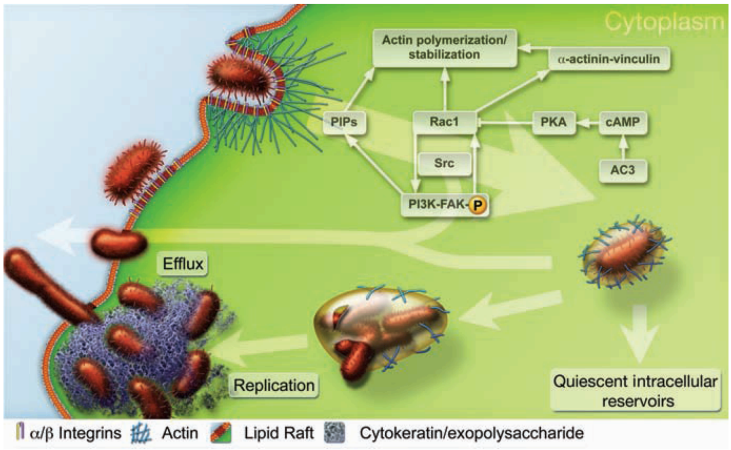

Figure 3 Overview of type 1 pili mediated invasion of bladder cells. FimH mediated binding of UPEC to host receptors, like α3β1 integrins, induces receptor clustering within cholesterol-rich lipid raft domains and subsequent activation of downstream signalling cascades. Invasion requires activation of focal adhesin kinase (FAK), which forms transient complexes with PI 3-kinase, leading to the generation of phosphotidylinositides (PIPs) that can directly and indirectly affect actin dynamics. Activation of Src and Rho family GTPases like Rac1, along with complex formation between α-actinin and vinculin, also contribute to localized actin and membrane rearrangements at sites of bacterial entry. Stimulation of protein kinase A (PKA) downstream of cAMP generation by adenylate cyclase 3 (AC3) and Toll-like receptors may inactivate Rac1 and thereby interfere with the invasion process. Once internalized, UPEC is either quickly trafficked back out of the host cell or is delivered into membrane-bound compartments having many features that are characteristic of late endosomes lysosomes. Within immature bladder epithelial cells, and along the basolateral edges of terminally differentiated facet cells, the UPEC-containing vacuoles are often enmeshed within a network of actin filaments, and bacterial replication is limited. These quiescent bacteria may persist for long periods, providing a source for recurrent UTIs. When actin fibres are redistributed or disrupted, intravacuolar growth of UPEC is stimulated. Continued growth may allow the microbes to eventually break out into the host cytosol where bacterial multiplication proceeds more rapidly, leading to the development of IBCs, often in association with host cytokeratin intermediate filaments and bacterial exopolysaccharide.

membrane (AUM) of facet cells within the bladder [19]. The AUM is embedded with a quasi-crystalline array of hexagonal complexes comprised of four or more integral membrane proteins collectively known as uroplakins [32]. One of these, the tetraspanin glycoprotein UP1a, interacts with FimH and is proposed to mediate FimH-dependent bacterial invasion of bladder cells [19,21,35,59]. The AUM has been observed to zipper around invading type 1-piliated bacteria via an apparent actin-regulated process that may involve conformational changes in the UP1a-containing hexagonal complexes and retraction of the type 1 pili [19,21,72]. However, signalling into host cells via UP1a has not yet been demonstrated, and no mechanism by which type 1 pili can be retracted is currently known.

Plasma membrane microdomains, which are enriched in cholesterol and glycosphingolipids and referred to as lipid rafts, are proposed sites of entry for type 1-piliated UPEC [73]. Specifically, a subset of lipid raft domains containing caveolin-1, and known as caveolae, were implicated in the invasion process. However, subsequent work has so far failed to substantiate a role for caveolae in FimH-mediated bacterial invasion of bladder cells [72]. More recently, it was suggested that UPEC invade host cells by high-jacking secretory lysosomes [35]. These vesicles store and secrete cellular products and participate in the repair of plasma membrane damage [74,75]. Secretory lysosomes are proposed to provide membrane needed to envelop and internalize adherent pathogens, although the protozoan parasite *Trypanosoma cruzi* is currently the only pathogen proven to utilize these vesicles [76,77]. Rab27b, a protein implicated in the transport of secretory lysosomes, also regulates the delivery of pre-assembled uroplakin arrays (plaques) to the AUM from the *trans* Golgi network within superficial facet cells [78]. UPEC was found to co-localize with Rab27b during the invasion process, raising the possibility that secretory lysosomes (or related secretory vesicles) are involved in the entry process [35]. However, while a functional role for Rab27b during UPEC invasion was validated using a gene silencing approach, further work is needed to verify a specific

requirement for secretory lysosomes in the infection process [79]. Considering the diversity of host proteins that FimH can bind, it is probable that type 1-piliated bacteria can enter host cells by more than one pathway.

Type 1 pili-independent entry

UPEC strains encode dozens of fimbrial and afimbrial adhesins with varying receptor-binding specificities distinct from type 1 pili. The functional significance of most of these mannose-insensitive adhesins is not yet well defined, either within or outside of the host. However, a few of these adhesive organelles, including P and S/F1C pili, as well as the Afa/Dr family of adhesins, can facilitate bacterial dissemination and persistence within the host [80–86]. In particular, Afa/Dr fimbrial adhesins have been shown to stimulate bacterial uptake by a number of different host cell types [14,87–89]. These adhesins are often encoded by so-called diffusely adhering *E. coli* (DAEC), and are epidemiologically associated with pathogenic strains that cause recurrent and chronic UTIs, cystitis in children, and pyelonephritis in pregnant women [14,90–94].

Typical Afa/Dr fimbrial adhesins bind the complement control protein repeats 2 and 3 (CCP2 and CCP3) domains of the Cromer blood group antigen Dr^a on human decay-accelerating factor (DAF, also known as glycophosphatidylinositol (GPI)-anchored complement regulatory molecule CD55) [95–97]. DAF acts as a complement regulatory molecule and is widely expressed by many host cells, including epithelial and endothelial cells within the urinary tract [98,99]. Several Afa/Dr fimbrial subunits, including AfaE-I, AfaE-III, AfaE-V, DraE, and DaaE, are associated with UTI isolates and bind members of the CEACAM family, as can the type 1 pilus adhesin FimH [55,100–102]. Interactions between Afa/Dr-associated subunits and host receptors like DAF, and possibly α5β1 integrins, stimulate bacterial entry into host cells [14,87–89,103]. DAF and α5β1 integrins cluster around the invading bacteria, which enter host cells via a zipper-like mechanism requiring cholesterol and, presumably, lipid rafts [88,103–105]. Afa/Dr-mediated invasion may also involve activation of the small Rho-family GTPase Cdc42 [102], but a functional actin cytoskeleton is surprisingly dispensable. Rather, microtubules are essential for Afa/Dr-mediated bacterial entry into host cells [89,103,105]. The specific signalling events involved in this microtubule-dependent entry process, and the precise roles of Afa/Dr adhesins in facilitating bacterial persistence within the urinary tract, remain to be defined.

In addition to promoting bacterial entry on their own, the many adhesins encoded by UPEC may also act synergistically with each other or with other virulence factors. For example, it has been hypothesized that P and S pili can work with UPEC-associated cytotoxic factors like α-haemolysin and CNF1 to facilitate bacterial invasion and dissemination within host tissues [85]. Both of these toxins, by disrupting tight junctions, modulating host signalling pathways, and/or inducing host cell death, can provide UPEC with access to deeper tissues where the bacteria may establish long-lived intracellular reservoirs [37,38,106–108]. In addition, CNF1 can indirectly promote UPEC entry into bladder cells by causing constitutive activation and subsequent degradation of Rho GTPases like Rac1 [109,110]. These events lead to enhanced membrane ruffling and uptake of bound bacteria along with increased migration of the intoxicated cells, phenomena that may facilitate the spread of UPEC within the bladder epithelium. While it is intuitively apparent that UPEC needs to coordinate the expression of CNF1 and its arsenal of other toxins and adhesins during the course of a UTI, the means by which this is accomplished is currently far from clear.

Conclusions

The recognition of UPEC as opportunistic intracellular pathogens, rather than strictly extracellular organisms, has significantly altered our view of how these bacteria can cause disease and persist within the urinary tract. Observations indicating that intracellular reservoirs of UPEC are unaffected by many of the antibiotics commonly used to treat UTIs argue for the development and/or use of more penetrant antimicrobials to treat or prevent chronic and recurrent UTIs. Extended treatments (beyond typical 1- or 3-day protocols) with antibiotics already in use to treat uncomplicated UTIs may also help ameliorate persistent/relapsing infections in some patients, although this approach may increase risks from emerging antibiotic-resistant strains. By defining more completely the repertoire of virulence factors that contribute to the establishment and persistence of UPEC within the urinary tract, it may be possible to develop more efficacious combinatorial vaccines or pharmacological therapies for the prevention and treatment of UTIs. At the same time, the identification and characterization of host receptors and signalling factors that facilitate UPEC adherence to and invasion of host cells and tissues may help explain the increased susceptibility of some individuals to UTIs, potentially highlighting additional targets for therapeutic intervention.

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APPENDIX B

ORIGINS AND VIRULENCE MECHANISMS OF UROPATHOGENIC *ESCHERICHIA COLI*

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Origins and virulence mechanisms of uropathogenic *Escherichia coli*

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ABSTRACT

Strains of uropathogenic *E. coli* (UPEC) are the primary cause of urinary tract infections, including both cystitis and pyelonephritis. These bacteria have evolved a multitude of virulence factors and strategies that facilitate bacterial growth and persistence within the adverse settings of the host urinary tract. Expression of adhesive organelles like type 1 and P pili allow UPEC to bind and invade host cells and tissues within the urinary tract while expression of iron-chelating factors (siderophores) enable UPEC to pilfer host iron stores. Deployment of an array of toxins, including hemolysin and cytotoxic necrotizing factor 1, provide UPEC with the means to inflict extensive tissue damage, facilitating bacterial dissemination as well as releasing host nutrients and disabling immune effector cells. These toxins also have the capacity to modulate, in more subtle ways, host signaling pathways affecting myriad processes, including inflammatory responses, host cell survival, and cytoskeletal dynamics. Here, we discuss the mechanisms by which these and other virulence factors promote UPEC survival and growth within the urinary tract. Comparisons are also made between UPEC and other strains of extraintestinal pathogenic *E. coli* that, although closely related to UPEC, are distinct in their abilities to colonize the host and cause disease.

Introduction

Escherichia coli is an incredibly diverse bacterial species with the ability to colonize and persist in numerous niches both in the environment and within animal hosts. *E. coli* and other commensal intestinal flora of mammals often form a beneficial symbiotic relationship with their host, providing nutrients, key signals for developmental and immune regulation, and protection against foreign pathogens (Yan and Polk, 2004). Some strains of *E. coli* can diverge from their commensal cohorts, taking on a more pathogenic nature and the ability to cause serious disease both within the intestinal tract and elsewhere within the host. These pathogenic strains are broadly categorized as either diarrheagenic *E. coli* or extraintestinal pathogenic *E. coli* (ExPEC) (Kaper et al., 2004; Russo and Johnson, 2000). Within each of these broad groups are sets of strains known as pathotypes that share common virulence factors and elicit similar pathogenic outcomes (Marrs et al., 2005). Several pathotypes of diarrheagenic *E. coli* give rise to gastroenteritis, but rarely cause disease outside of the intestinal tract. ExPEC, on the other hand, have maintained the ability to exist in the gut without consequence, but have the capacity to disseminate and colonize other host niches including the blood, central nervous system, and urinary tract, resulting in disease.

Among ExPEC, strains of uropathogenic *E. coli* (UPEC) are most commonly associated with human disease. These bacteria are the primary cause of community-acquired urinary tract infections (UTI) (70–95%) and a large portion of nosocomial UTIs (50%), accounting for substantial medical costs and morbidity worldwide (Foxman, 2003). Recurrent, or relapsing, UTIs are especially problematic in many individuals. UPEC strains act as opportunistic intracellular pathogens, taking advantage of host behavior and susceptibility by employing a diverse repertoire of virulence factors to colonize the urinary tract. It is believed that a primary reservoir of UPEC isolates is within the human intestinal tract, as the isolate responsible for a UTI in a given individual often matches rectal isolates from that same person (Russo et al., 1995). In some cases, dissemination of a single clonal group of UPEC isolates may occur within a community via contaminated food or other consumables (Manges et al., 2001). Additionally, UPEC strains isolated from sexually active patients often match fecal isolates from their partners, indicating that UTIs can be sexually transmitted (Foxman et al., 2002; Johnson and Delavari, 2002).

Once inside the urinary tract, UPEC preferentially colonizes the bladder and causes cystitis, but can also ascend through the ureters into the kidneys, causing pyelonephritis. In response to the breach by UPEC into the normally sterile urinary tract, host inflammatory responses are triggered leading to cytokine production, neutrophil influx, the exfoliation of infected bladder epithelial cells, and the generation of reactive nitrogen and oxygen species along with other antimicrobial compounds (Bower et al., 2005; Mulvey et al., 2000).

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UPEC have evolved a number of strategies to evade these innate immune responses, enabling the pathogens to more effectively colonize the urinary tract and persist. The ability of UPEC to bind host tissues is one of the paramount factors that facilitate UPEC colonization of the urinary tract, allowing the bacteria to withstand the bulk flow of urine and promoting UPEC invasion of urothelial cells. Within bladder epithelial cells, UPEC are trafficked into membrane-bound, acidic compartments with features similar to late endosome or lysosomes (Eto et al., 2007). In the large, terminally differentiated superficial umbrella cells that line the lumen of the bladder UPEC are able to break into the host cell cytosol and rapidly multiply, forming large intracellular biofilm-like communities that can contain several thousand bacteria. This phenomenon is observed in both mouse UTI model systems and in human patients with UTI (Anderson et al., 2003; Eto et al., 2007; Mulvey et al., 2001; Rosen et al., 2007). Bladder cells containing large numbers of UPEC are primed to exfoliate, providing a mechanism by which the host can rapidly clear many bacteria from the urinary tract with the flow of urine (Mulvey et al., 1998, 2001). This process, however, leaves the underlying layers of immature bladder epithelial cells exposed and more susceptible to infection. In mouse models, UPEC have been observed to invade the underlying immature urothelial cells, entering late endosome-like compartments that are often enmeshed within a network of actin filaments (Eto et al., 2006; Mulvey et al., 2001). Replication of these actin-bound bacteria is restricted, making them less susceptible to many antibiotics and perhaps less immunogenic. These quiescent bacteria may serve as reservoirs for recurrent (or relapsing) UTIs. The rearrangement of actin filaments during terminal differentiation of the infected immature cells may act as a trigger for increased intracellular multiplication of UPEC and the recrudescence of clinical symptoms. Fig. 1 provides an overview of many of the key events taking place during the course of a bladder infection. It is anticipated that a greater understanding of these events and other factors that promote UPEC colonization of the urinary tract, as well as the gut, will lead to the identification of novel vaccine targets and the development of more efficacious therapies.

In 2002 the genomic sequence of the pyelonephritis isolate CFT073 (O6:K2:H1) was published and more recently, in 2006, genomic sequencing of another pyelonephritis isolate, 536 (O6:K15:H31), and the cystitis isolate UTI89 (O18:K1:H7) were completed. Despite many similarities among UPEC isolates, genomic features that are specifically unique to UPEC have not yet been identified. Compared to K12 lab strains and commensal *E. coli* isolates, UPEC harbor more genes encoding virulent capsule antigens, iron acquisition systems, adhesins, and secreted toxins. These genes are often encoded within regions, referred to as pathogenicity islands (PAIs), with GC nucleotide content distinct from the rest of the genome (Gal-Mor and Finlay, 2006; Hacker and Kaper, 2000; Lloyd et al., 2007). Using recent epidemiological findings and genomic comparisons, this review describes the repertoire of key UPEC virulence factors and their prevalence among UPEC isolates. We also briefly discuss two other ExPEC pathotypes, avian pathogenic *E. coli* (APEC) and asymptomatic bacteriuria *E. coli* (ABU), and their usefulness in understanding UPEC virulence.

The armamentarium of UPEC

ExPEC genomes are generally larger than those of K12 or commensal *E. coli* isolates, presumably because they contain more genes required for survival outside the intestinal tract. The genomes of the UPEC isolates CFT073, 536, and UTI89 contain 8–22% more open reading frames and are 6–13% larger than the genome of the K-12 reference strain MG1655 (Brzuszkiewicz et al., 2006; Chen et al., 2006; Welch et al., 2002). PAIs acquired through horizontal gene transfer are partly responsible for the inflated size of UPEC genomes (Hacker et al., 1997). Diarrheagenic *E. coli* pathotypes also encode numerous virulence factors within PAIs, but the variety of virulence factors that they express can differ markedly from UPEC (Kaper et al., 2004). For example, UPEC typically express an array of adhesins and iron acquisition systems to facilitate extraintestinal survival, and generally lack the notorious type III secretion system used by many diarrheagenic *E.* coli isolates to inject virulence factors into target host cells. Even among UPEC strains there are considerable differences in the repertoire and expression levels of virulence factors that can affect bacterial growth and persistence within the urinary tract. Table 1 lists some of the major virulence factors associated with sequenced UPEC and related ExPEC strains.

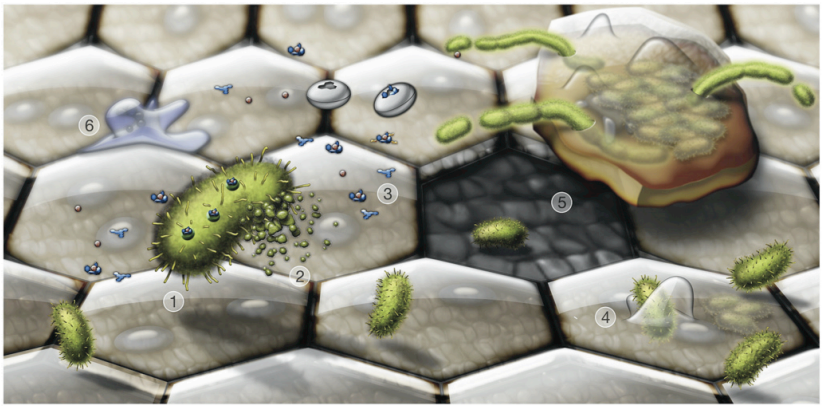

Fig. 1. Dynamic interplay between invading UPEC and the host during a UTI. Shown are key events taking place during bladder infection by UPEC. Type 1 pili-expressing UPEC (1, green) secrete toxins and other virulence factors, alone or in association with outer membrane vesicles (2). Siderophores like enterobactin and salmochelin (3, blue structures) released by UPEC scavenge iron, in competition with host iron-chelating molecules and lipocalin 2 (white discs). Type 1 pili mediate bacterial attachment to and invasion of the bladder epithelial cells (4). Large terminally differentiated superficial epithelial cells, which are often binucleate and have distinctive hexagonal or pentagonal shapes, line the lumenal surface of the bladder and are the primary targets of UPEC invasion. UPEC can rapidly multiply within the superficial cells, forming large biofilm-like communities. Exfoliation of infected bladder cells facilitates bacterial clearance from the host, but leaves the smaller underlying immature cells more susceptible to infection (5). The release, or efflux, of UPEC from infected host cells before they complete exfoliation likely promotes bacterial dissemination and persistence within the urinary tract. During efflux, UPEC often become filamentous, probably due in part to mounting stress arising from increased activation of host defenses. These include the influx of neutrophils (6), as well as the generation of reactive oxygen and nitrogen species and antimicrobial peptides.

Table 1
Key virulence factors in reference ExPEC strains

Category/gene(s)*	CFT073	536	UTI89	ABU 83972	APEC-01
Serotype	O6:K2:H1	O6:K15:H31	O18:K1:H7	OR:K5:H−	O1:K1:H7
Iron acquisition systems					
ent (enterobactin siderophore)	+	+	+	+	+
iro (salmochelin siderophore)	+	+	+	+	+
chu (hemin uptake system)	+	+	+	+	+
Sit (iron/manganese transport)	+	−	+	+	+
iutA (aerobactin siderophore)	+	−	−	+	+
fyuA (yersiniabactin siderophore)	+/nf	+	+	+	+
Pili					
fim (type 1)	+	+	+	+/nf	+
pap (P)	++	+	+	+/nf	+
sfa (S)	+	+	+	−	−
foc (F1C)	+	+	−	+/nf	−
Toxins					
hly (α-hemolysin)	+	++	+	+/nf	−
CNF1 (cytotoxic necrotizing factor 1)	-	-	+	+†	-
vat (vacuolating autotransporter toxin)	+	+	+	?	+
sat (secreted autotransporter toxin)	+	-	-	?	-

*Presence or absence of the indicated genes shown by + and - signs, respectively. nf, present but not functional; ?, presence not verified; †, functionality not known.

UPEC serotypes

Traditional classification of *E. coli* strains is based on the presence of certain O (somatic), K (capsular polysaccharide), and H (flagellar) antigens. An association between the expression of specific capsular antigens and *E. coli* pathotypes has been well documented, but the extent to which these antigens impact pathogenesis is not completely understood. The O antigen, which defines >176 serogroups, is a polysaccharide consisting of ~10–25 repeating sugar subunits anchored in the outer core of the lipopolysaccharide component of the bacterial membrane (Stenutz et al., 2006). There is a high frequency of the antigens O1, O2, O4, O6, O7, O8, O16, O18, O25, and O75 among UPEC, while specific K and H antigens have a less defined pattern (Bidet et al., 2007; Lloyd et al., 2007). This O antigen trend is reiterated with the sequenced UPEC isolates CFT073 (O6), 536 (O6), UTI89 (O18), as well as with two other often used UPEC strains, J96 (O4) and F11 (O6).

The K1 capsular antigen is typically associated with ExPEC strains that cause neonatal meningitis. Expression of the K1 capsule by so-called neonatal meningitis *E. coli* (NMEC) has been shown to protect these pathogens from both complement-mediated killing and bacteriophages, while also enhancing bacterial survival within brain microvascular endothelial cells and facilitating bacterial evasion of phagocytosis by professional phagocytes (Allen et al., 1987; Kim et al., 2003; Pluschke et al., 1983; Scholl et al., 2005; Van Dijk et al., 1979). Although there is no clear evidence for the involvement of specific K antigens in UPEC pathogenesis, it has been noted that UPEC isolates bearing the K1 or O18 antigens often encode more virulence-associated factors than other ExPEC isolates (Ewers et al., 2007). Interestingly, the well-studied human cystitis isolate UTI89 displays the prototypic NMEC O18:K1:H7 serotype, which in turn may contribute to the virulent nature of this particular strain.

Despite the higher prevalence of specific somatic antigens among certain ExPEC pathotypes like UPEC, expression of some O antigen types is much less biased. For example, the O45 antigen is similarly expressed in both UPEC and NMEC pathotypes, where it appears to provide a survival advantage for both types of pathogens (Bidet et al., 2007; Ewers et al., 2007). It has been suggested that ExPEC expressing the O45 antigen are evolutionarily young compared to O4-, O6-, or O18-expressing ExPEC isolates, and not yet settled into a particular niche requiring a more specific O antigen. While it is known that certain O and K antigens provide a survival advantage f some ExPEC strains, how these antigens specifically impact UPI virulence requires further investigation. On the other hand, flage (giving rise to H antigens) now have a more defined role in UPI pathogenesis, having recently been shown to facilitate the ascensi of UPEC from the bladder into the kidneys (Lane et al., 2007, 200 Wright et al., 2005).

Iron acquisition systems — virulence factors that pull their weight

A longstanding battle wages between pathogenic bacteria and the hosts for iron, an essential factor for many prokaryotic and eukaryo cellular processes (Andrews et al., 2003). In the mammalian host, fr iron concentrations are incredibly low, being approximately 10^{-25} M the blood and often lower at other sites (Barasch and Mori, 200 Fischbach et al., 2006a). For growth, bacteria require a cytoplasmic ir concentration of ~10^{-6} M (Andrews et al., 2003). Consequently, path genic bacteria, including ExPEC and more specifically UPEC, ha evolved multiple strategies for swiping iron from the host (Table These include the expression of iron acquisition systems that utili siderophores to scavenge iron from the environment and subsequent concentrate it in the bacterial cytosol. Siderophores are secreted lo molecular weight molecules that have a high affinity for ferric (Fe iron, which is insoluble as a free cation. Bacteria retrieve iron-bou siderophores through receptors that facilitate the transport of sid rophore–iron complexes through the bacterial membrane and into t cytosol where the iron is released.

Limiting iron availability is an important host defense agair invading bacterial pathogens. Just as bacteria use siderophores to bi and transport iron, eukaryotic organisms use iron-chelating proteins sequester and shuttle iron into and out of host cells. Transferrin, an ir carrier protein that is conserved among mammals, birds, fish, a amphibians, has a strong affinity for iron (K_d = ~10^{-20}) (Fischbach et a 2006a). However, a common siderophore known as enterobact which is expressed by both pathogenic and K12 *E. coli* strains, bin iron with an even lower K_d of ~10^{-49}, allowing enterobactin to ou compete transferrin for iron binding. Employment of enterobactin m afford bacteria like UPEC the ability to colonize iron-poor niches, su as the urinary tract. The host, however, is not defenseless agair siderophores like enterobactin. For example, the host protein lipocal 2 (also known as neutrophil gelatinase-associated lipocalin, sid rocalin, 24p3, or uterocalin) was recently identified as an effecti countermeasure for combating enterobactin-mediated iron scave ging by pathogens. Goetz et al. found that lipocalin 2 functions as bacteriostatic agent by specifically binding and sequestering ente obactin (Goetz et al., 2002). In addition, Flo et al. showed that bacte expressing only the enterobactin siderophore system were efficient cleared in wild type mice after intraperitoneal injection, where lipocalin 2 knockout mice were severely impaired in their ability eliminate the same bacteria (Flo et al., 2004). Lipocalin 2 is co stitutively expressed and released by activated neutrophils, the maj immune effector cells recruited to sites of infection within the urina tract. Expression of lipocalin 2 is also upregulated *in vivo* with urothelial cells harboring intracellular bacterial communities of UTI (Reigstad et al., 2007). These data indicate that lipocalin 2 is like employed by the host during a UTI, prompting the question: does UPI have the means to circumvent this host countermeasure?

In contrast to K12 *E. coli* strains, ExPEC pathotypes typically enco multiple iron acquisition systems aside from enterobactin. UPEC, particular, express a wealth of seemingly redundant iron acquisiti systems, including the siderophores salmochelin, yersiniabactin, a aerobactin. Interestingly, salmochelins are variants of enterobactin th have been modified by glucosylation via the action of a glucosyltransf ase encoded within the *iroA* gene cluster (Bister et al., 2004; Smit 2007). This modification to enterobactin prevents its recogniti and sequestration by lipocalin 2, giving *iroA*-positive bacteria a distir

advantage within the host (Fig. 2) (Fischbach et al., 2006b). Many UPEC isolates carry the *iroA* gene cluster within PAIs. Importantly, iron-bound salmochelin is also not recognized by the normal enterobactin receptor, FepA. However, the *iroA* gene cluster encodes another receptor, *iroN*, which does recognize iron-bound salmochelin and transports it into the bacterial cytosol (Hantke et al., 2003). These observations highlight the selective pressures on UPEC and other bacterial pathogens that drive the evolution of multiple iron acquisition systems and the challenges faced by the host in keeping its iron stores secure and out of reach.

Home is where your pili stick

UPEC colonization of the urinary tract hinges on its ability to bind host cells and tissues. Adherence also stimulates UPEC entry into host epithelial cells, a process that appears to promote UPEC survival within the urinary tract (Bower et al., 2005). The primary adherence factors encoded by UPEC, and many other microbes, are supramolecular, filamentous adhesive organelles known as pili or fimbriae. Common adhesive organelles elaborated by UPEC are type 1, P, S, and F1C pili encoded by the *fim*, *pap*, *sfa*, and *foc* operons, respectively. Individual UPEC genomes can carry >10 fimbrial gene clusters, the majority of which have been characterized in only cursory detail, making the contribution of each pilus type to UPEC virulence difficult to discern (Miyazaki et al., 2002; Snyder et al., 2005, 2006). Cross-talk among pilus operons within a bacterial cell, likely triggered by environmental cues, can result in a switch in expression from one pilus type to another, a process known as phase variation (Holden et al., 2007, 2006; Lindberg et al., 2007; Xia et al., 2000). Phase variable expression of pilus genes within a single strain of UPEC can give rise to subpopulations expressing functionally distinct pili, increasing the probability of adherence to or invasion of host tissues and possibly broadening host specificity (Holden and Gally, 2004).

Two of the most studied adhesive organelles are type 1 and P pili, which are encoded by many UPEC strains. The expression of P pili is often associated with pyelonephritic UPEC isolates (Lane and Mobley, 2007). The genomes of 536 and UTI89 each contain one copy of the *pap* (pyelonephritis-associated pili) operon encoding P pili, while the CFT073 genome has two copies within separate PAIs. A specific adhesin protein, called PapG, which is localized at the distal tip of the P pilus, mediates bacterial adhesion to host cells. Three types of the PapG adhesin have been identified (designated as PapG I, II, and III) that recognize globotriasylceramide variants on the surface of target cells, particularly in the kidney. The human pyelonephritis isolate CFT073 encodes two copies of the PapGII variant known to bind the globotetraosylceramide GbO4, a glycolipid that is abundantly expressed on the surface of human urothelium. PapGIII, on the other hand, is more common among cystitis isolates and is encoded by both 536 and UTI89. The PapGIII adhesin binds the globopentaosylceramide GbO5, which is predominantly found in the canine and not the human urinary tract, although alternate human receptors for PapGIII have been reported (Karr et al., 1990; Lindstedt et al., 1989; Stapleton et al., 1998; Stromberg et al., 1990). Despite its epidemiological association with UPEC strains that cause acute pyelonephritis, the exact role of P pili during the course of a UTI has remained elusive.

Type 1 pili are highly conserved and extremely common among both UPEC and commensal isolates and have come to be considered one of the most important virulence factors involved in the establishment of a UTI. Targeted knockout of the type 1 pilus adhesin FimH greatly diminishes the ability of UPEC to colonize the urinary tract in studies using both human volunteers and mice, in contrast to results from analogous studies with P pili (Bahrani-Mougeot et al., 2002; Connell et al., 1996; Langermann et al., 1997; Mulvey et al., 1998). FimH contains a binding pocket that recognizes mannose-containing host glycoprotein receptors (Hung et al., 2002). Interestingly, the FimH adhesin mediates both bacterial adherence to and invasion of host cells, and contributes to the formation of intracellular bacterial biofilms by UPEC (Martinez et al., 2000; Wright et al., 2007). The integral membrane glycoprotein uroplakin 1a, which is abundantly expressed on the apical surface of the bladder, appears to be a key receptor for the FimH adhesin, although FimH can also bind many other host proteins (Eto et al., 2007; Mulvey et al., 1998; Zhou et al., 2001). In particular, our laboratory recently showed that α3β1 integrin subunits expressed by many host cell types, including bladder epithelial cells, are bound by FimH and mediate uptake of type 1 piliated *E. coli* (Eto et al., 2007). Integrins are surface adhesion molecules that link extracellular matrix proteins with the actin cytoskeleton, providing signaling conduits between the intra- and extracellular environments. Manipulation of integrins and downstream signaling cascades is a common mechanism by which pathogens gain entry into host cells (Scibelli et al., 2007).

We found that the treatment of cultured bladder epithelial cells with blocking antibodies specific for either α3 or β1 integrin abrogated type 1 pilus-mediated bacterial invasion of host bladder cells without decreasing bacterial adherence (Eto et al., 2007). In addition, mutation of several phosphorylation sites within the cytoplasmic tail of β1 integrin (specifically, mutation of threonines 788 and 789 to alanines, and tyrosines 783 and 795 to phenylalanines) effectively attenuated FimH-mediated invasion. These cytosolic residues can affect the conformation of β1 integrin as well as the recruitment and activation of various adaptor and signaling factors. Intriguingly, mutation of serine 785 within the tail of β1 integrin to alanine enhanced bacterial invasion about 4-fold for reasons not yet fully understood. FimH-mediated bacterial invasion of host cells requires dynamic rearrangement of the host actin cytoskeleton, and a number of integrin-associated signaling factors and adaptor proteins known to regulate actin dynamics have been implicated in the invasion process (Fig. 3) (Eto et al., 2007; Martinez et al., 2000). These include Rho GTPases, focal adhesion kinase (FAK), Src kinase, and PI 3-kinase as well as the adaptor proteins α-actinin and vinculin. Recent work has indicated

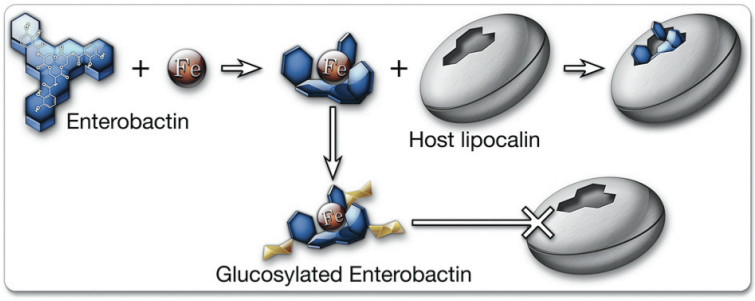

Fig. 2. Iron acquisition. Both host and pathogen compete for iron and have evolved multiple strategies to outdo the other. The bacterial siderophore enterobactin sequesters iron with high affinity, while host lipocalin 2 binds enterobactin and prevents its uptake by UPEC. Bacteria carrying the *iroA* gene cluster can modify enterobactin by glucosylation, creating salmochelin, which effectively binds iron but is not recognized by lipocalin 2.

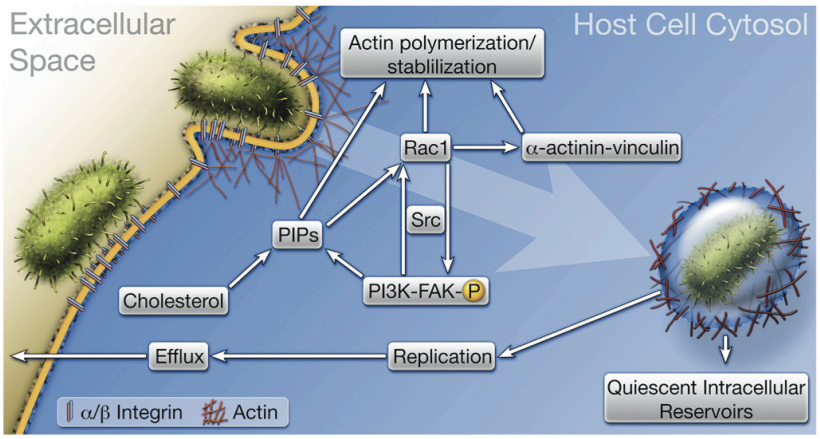

Fig. 3. Host cell invasion by UPEC. The FimH adhesin localized at the distal tips of type 1 pili engages α3β1 integrin receptors, and possibly other receptors, which likely cluster witl cholesterol-rich lipid rafts. Receptor binding triggers signaling cascades involving FAK, Src, PI 3-kinase, Rho GTPases like Rac1, phosphoinositides (PIPs), and transient comp formation between the cytoskeleton stabilizing and scaffolding proteins α-actinin and vinculin. These events stimulate actin rearrangements, causing the host plasma membrane zipper around and envelope bound bacteria. Once internalized, UPEC can be trafficked to late endosome-like compartments that are often localized within a meshwork of ac filaments. Bacteria exist quiescently within these actin-bound compartments and may serve as reservoirs for recurrent UTIs. Liberation of UPEC into the host cytosol stimulates ra bacterial growth and the formation of intracellular biofilm-like communities.

that elevated host cAMP levels linked with stimulation of the pattern recognition receptor TLR4 may also modulate the invasion process (Song et al., 2007). It is likely that other host receptors, in addition to α3 or β1 integrin, are also involved in FimH-mediated bacterial invasion. Identifying these putative receptors and defining their role(s) during UTI may aid the development of new treatment protocols. In addition, it is feasible that polymorphic variations in α3β1 integrins or other receptors may help explain why some individuals are more prone to recurrent and chronic UTIs.

UPEC ordnance — secreted toxins

The utilization of secreted toxins by pathogenic bacteria is well recognized. For the most part, UPEC lack the type III secretion system that some other pathogens use to inject effector molecules into host cells and instead often use so-called type I and type V secretion systems (see Henderson et al., 2004 for a recent review). Fig. 4 highlights some of the more thoroughly studied toxins commonly associated with UPEC. The prototypical type 1 secreted toxin, α-hemolysin (HlyA), is encoded by ~50% of UPEC isolates and its expression is associated with increased clinical severity in UTI patients (Johnson, 1991; Marrs et al., 2005). The UPEC isolates CFT073 and UTI89 each contain one copy of the hemolysin operon, *hlyCABD*, while the 536 strain encodes two copies. HlyA is a calcium-dependent toxin of 110 kDa that forms 2 nm-wide pores in host cells, leading to cell lysis when high levels of HlyA are reached (Bhakdi et al., 1988; Boehm et al., 1990a,b; Ostolaza and Goni, 1995). The host environments encountered by ExPEC are extremely nutrient poor and the function of HlyA has generally been thought to primarily be the destruction of host cells, thereby facilitating the release of nutrients and other factors, like iron, that are critical for bacterial growth. However, it is not clear how often HlyA reaches levels that are high enough to lyse target host cells during the course of an actual infection. Rather, sublytic concentrations of HlyA may be more physiologically relevant. Indeed, recent studies have demonstrated that sublytic concentrations of a number of pore-forming toxins can modulate a variety of host signaling pathways, including transient stimulation of calcium oscillations, the activation of MAP kinase signaling, and the alteration of histone phosphorylation and acetylation patterns (Hamon et al., 2007; Ratner et al., 2006). In addition, we have recently found that sublytic concentrations of HlyA potently stimulates inactivation of the serine/threonine kinase Akt, which plays a central role in host cell cycle progression, metabolis vesicular trafficking, survival, and inflammatory signaling pathwa (Wiles et al., 2008). These findings may help explain previously pu lished results implicating sublytic concentrations of HlyA in t inhibition of chemotaxis and bacterial killing by phagocytes, as well HlyA-mediated stimulation of host apoptotic and inflammatory pat ways (Cavalieri and Snyder, 1982; Koschinski et al., 2006; Mansson et a 2007; TranVan Nhieu et al., 2004; Uhlen et al., 2000).

ExPEC also encode several type V secreted toxins known colle tively as autotransporters (Henderson et al., 2004). Two of thes vacuolating autotransporter toxin (Vat) and secreted autotransport toxin (Sat), are often expressed by UPEC isolates (Ewers et al., 200 Restieri et al., 2007). Among the reference UPEC strains listed in Table CFT073 encodes both Vat and Sat, while 536 and UTI89 expresses or the former. Vat and Sat were initially characterized by their ability induce a variety of cytopathic effects in target host cells, includi vacuolation and swelling. Although the role of Vat in UTI pathogene has not been thoroughly studied, this toxin has been shown to enhan APEC virulence in respiratory and cellulitis infection models usi broiler chickens (Parreira and Gyles, 2003). Sat expression by CFT07 on the other hand, has been shown to induce dramatic kidney dama in a mouse UTI model system, causing dissolution of the glomerul membrane, loss of tubular epithelial cells, and vacuolation of kidn tissue (Guyer et al., 2002; Maroncle et al., 2006). Paradoxically, Sat d not appear to influence the ability of CFT073 to colonize the muri urinary tract. The role(s) of several other autotransporter toxi encoded by UPEC awaits further investigation.

The toxins expressed by UPEC are not necessarily secreted as nak proteins, but instead may be associated with outer membrane vesicl (OMVs) that bleb from the bacterial surface. OMVs are utilized by ma bacterial species to facilitate intra- and interspecies communication, t exchange of genetic material, bacterial adherence to and invasion of hc cells, and the delivery of toxins (Kuehn and Kesty, 2005; Mashbur Warren and Whiteley, 2006). The release of OMVs by pathogenic ba teria is thought to protect toxic cargos in transit to target host cells, wh also providing an effective way of delivering concentrated bursts effector molecules that can modulate host activities. HlyA and cytoto necrotizing factor 1 (CNF1) are two UPEC-associated toxins that appe to be delivered to target host cells primarily via OMVs (Balsalobre et a 2006; Davis et al., 2006; Kouokam et al., 2006).

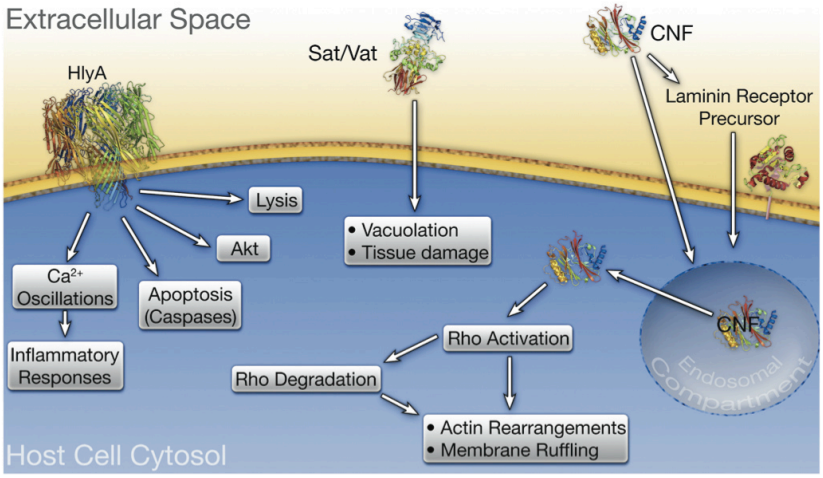

Fig. 4. UPEC-associated toxins. α-hemolysin (HlyA), Vat, Sat, and cytotoxic necrotizing factor 1 (CNF) are encoded by many UPEC isolates. (left) At high concentrations, HlyA inserts into the membrane of target host cells and promotes their lysis, whereas at sublytic concentrations HlyA can modulate signaling cascades affecting host cell survival and inflammatory responses. (middle) Intoxication of host cells by Vat or Sat induces vacuolation and other cytopathic effects, leading to host tissue damage. (right) Endocytosis of CNF occurs through the laminin receptor precursor, or via alternative delivery mechanisms in association with outer membrane vesicles. Conditions within the endosomal compartment stimulate translocation of the CNF catalytic domain into the host cytosol where it activates Rho GTPases, inducing their eventual degradation and subsequent membrane ruffling.

Approximately one third of UPEC isolates encode CNF1, including the reference cystitis isolate UTI89 (Table 1). The toxicity of this 113 kDa protein is attributed to its ability to constitutively activate the Rho family GTPases RhoA, Rac, and/or Cdc42 (Lemonnier et al., 2007). Activation of Rho GTPases affects numerous eukaryotic cellular functions, including the formation of actin stress fibers, lamellipodia, filopodia, the induction of membrane ruffling, and the modulation of inflammatory signaling pathways (Etienne-Manneville and Hall, 2002). To exert its effects, CNF1 must gain access to the host cytosol by binding to the laminin receptor precursor on the surface of target cells, triggering uptake and subsequent trafficking of the toxin into a late endosomal compartment. Acidic conditions within this compartment induce the translocation of the CNF1 catalytic domain across the vesicular membrane and into the host cytosol where it stimulates Rho family GTPases (Lemonnier et al., 2007). Prolonged activation of Rho GTPases leads to their ubiquitination and subsequent proteasomal degradation. CNF1-mediated Rho GTPase activation is therefore a temporary phenomenon, and the cytotoxicity associated with CNF1 is due to both aberrant Rho activation and subsequent Rho degradation. The mechanism by which CNF1 is incorporated into OMVs, and the specific role that OMVs have in CNF1 delivery to target host cells is currently not known.

Under some experimental conditions using mouse UTI model systems, CNF1 provides UPEC with a notable advantage within the bladder and kidneys (Bower et al., 2005; Rippere-Lampe et al., 2001). CNF1 expression by UPEC may impact UTI pathogenesis in multiple ways. Specifically, CNF1 can promote apoptosis of bladder epithelial cells, possibly stimulating their exfoliation and enhancing bacterial access to underlying tissue (Mills et al., 2000). In addition, proteasome-mediated degradation of the CNF1-activated Rho GTPase Rac was shown to stimulate plasma membrane ruffling and filopodia formation, resulting in increased host cell motility and bacterial uptake (Doye et al., 2002). Finally, Davis et al. have recently reported that the secretion of CNF1-containing OMVs by the UPEC isolate CP9 inhibits the phagocytic and chemotactic activities of neutrophils (Davis et al., 2006,2005). In total, these CNF1-mediated effects may facilitate the dissemination and persistence of UPEC within the urinary tract.

APEC, ABU and the rise and fall of UPEC

Despite the identification and characterization of numerous virulence factors and colonization strategies utilized by UPEC isolates, no single feature accurately defines an ExPEC isolate as UPEC. A better understanding of UPEC-associated virulence factors and the coordination of their activities during the course of a UTI might significantly enhance our ability to both predict and rationally redirect disease outcome. In recent years, work with the ExPEC pathotypes asymptomatic bacteriuria *E. coli* (ABU) and avian pathogenic *E. coli* (APEC) has provided greater insight into the evolution of UPEC strains, giving us a clearer picture of what makes UPEC pathogenic.

ABU, housebroken UPEC

ABU *E. coli* strains have evolved to persist for many months to years within the urinary tract without eliciting overt clinical symptoms. These bacteria exist in a commensal-like relationship with the host, and in some cases appear to protect the urinary tract from colonization by more pathogenic UPEC strains. The ABU strain 83972, in particular, can grow to very high titers within the bladder of human patients and can effectively outcompete prototypic UPEC isolates during growth in human urine as well as in a mouse UTI model (Roos et al., 2006c). For these reasons, the 83972 strain has been used prophylactically to reduce the occurrence of symptomatic UTIs in human volunteers and is currently under investigation in clinical trials in both the USA and Europe (Hull et al., 2000).

The genome of the 83972 ABU strain harbors many UPEC-associated genes encoding virulence-related determinants like type 1, P, S, and F1C pili, α-hemolysin, and multiple iron acquisition systems (see Table 1). Except for the latter, the genes encoding all of these putative virulence determinants were found to be nonfunctional and in various states of genomic decay (Hull et al., 1999; Klemm et al., 2006; Roos et al., 2006b). These observations suggest that ABU strains are descended from more toxic and inflammatory UPEC isolates (Klemm et al., 2007). After growth in the human or mouse urinary tract, ABU 83972 shows significant transcriptional upregulation of iron acquisition systems (enterobactin, aerobactin, salmochelin, *chu*, and *sit*) and notable downregulation of

fimbrial gene clusters, the α-hemolysin operon, and *rfaH*, a global regulator of UPEC virulence (Nagy et al., 2002; Roos and Klemm, 2006; Roos et al., 2006c). ABU strains are also more adept at biofilm formation relative to several reference UPEC isolates (Hancock et al., 2007). These genomic and behavioral modifications likely help 83972 and other ABU strains grow to high titers within iron-poor environments like urine without provoking significant immune responses (Roos et al., 2006a,c; Zdziarski et al., 2007). A better understanding of how ABU strains manage to proliferate and persist within the urinary tract without irritating the host may not only aid the development of ABU isolates as prophylactic agents, but may also help elucidate important virulence factors utilized by UPEC when causing disease.

APEC — how UPEC got its wings

APEC causes respiratory tract infections and septicemia in domesticated birds, and consequently can have a severe economic impact (Ewers et al., 2003). Sequencing of the prototypic APEC strain APEC-01, coupled with the genomic analysis of other APEC isolates, has revealed a significant amount of genomic overlap with UPEC. The majority of open reading frames identified in APEC-01 are highly similar to those within the reference UPEC strains UTI89 (93%), CFT073 (90%), and 536 (87%) (Johnson et al., 2007; Moulin-Schouleur et al., 2007). Genomic variations among UPEC and APEC isolates presumably account for their varying specificity for different niches and different animal hosts. Although a loose virulence gene profile exists for both UPEC and APEC, no single profile is unique to either pathotype, emphasizing the potential for both to be zoonotic risks (Kariyawasam et al., 2007; Moulin-Schouleur et al., 2007; Ron, 2006). Interestingly, the human UPEC isolate CFT073 was shown to be virulent in an avian respiratory infection model, but APEC isolates have not yet been shown to cause disease in humans (Moulin-Schouleur et al., 2007).

The primary reservoir for UPEC is believed to be within the human intestinal tract, but how this pathotype comes to inhabit the gut is not clear. It has been proposed that APEC ingested from contaminated retail poultry serves as a source of UPEC-like strains and virulence factors (Rodriguez-Siek et al., 2005a; Ron, 2006). APEC isolates express numerous UPEC-like virulence factors that are often encoded on plasmids, and although UPEC does not typically carry plasmids, some of the PAIs contained on the UPEC chromosome are remarkably similar to the PAIs on APEC plasmids (Rodriguez-Siek et al., 2005b). Further support for a link between APEC and UPEC virulence factors comes from a recent study showing that the introduction of APEC virulence plasmids into a nonpathogenic avian commensal isolate increased its virulence in both chick embryo lethality assays and in a mouse UTI model system (Skyberg et al., 2006). These observations suggest that APEC strains being maintained in domestic bird populations are the ultimate reservoir for UPEC-like strains. However, APEC isolates very rarely express archetypal UPEC-associated toxins, including Sat, α-hemolysin, and CNF1 (Ewers et al., 2007), indicating that additional reservoirs for UPEC-like strains and virulence factors likely exist.

Concluding remarks

The diversity of known and putative UPEC-associated virulence genes, coupled with high levels of genetic overlap seen among both pathogenic and nonpathogenic extraintestinal *E. coli* isolates, makes it difficult to attribute UPEC virulence to any one set of factors. Rather, it is likely that UPEC and other ExPEC strains have evolved multiple and redundant mechanisms to overcome the many challenges encountered within the host environment. Consequently, targeting multiple virulence factors simultaneously by use of combinatorial vaccines or pharmacological cocktails may be the best approach in combating UPEC and related pathogens. In addition, as we gain a clearer understanding of the ways in which ExPEC colonize and persist within the host, it may become possible to customize treatments based on the specific virulence traits of the infecting strain found within an individual patient.

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