INFECTION AND IMMUNITY, July 2008, p. 3019–3026 Vol. 76, No. 7
0019-9567/08/$08.00+0 doi:10.1128/IAI.00022-08

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

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Received 7 January 2008/Returned for modification 7 March 2008/Accepted 28 April 2008

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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† Supplemental material for this article may be found at http://iai.asm.org/.

▽ Published ahead of print on 5 May 2008.

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 Silver-Snap 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

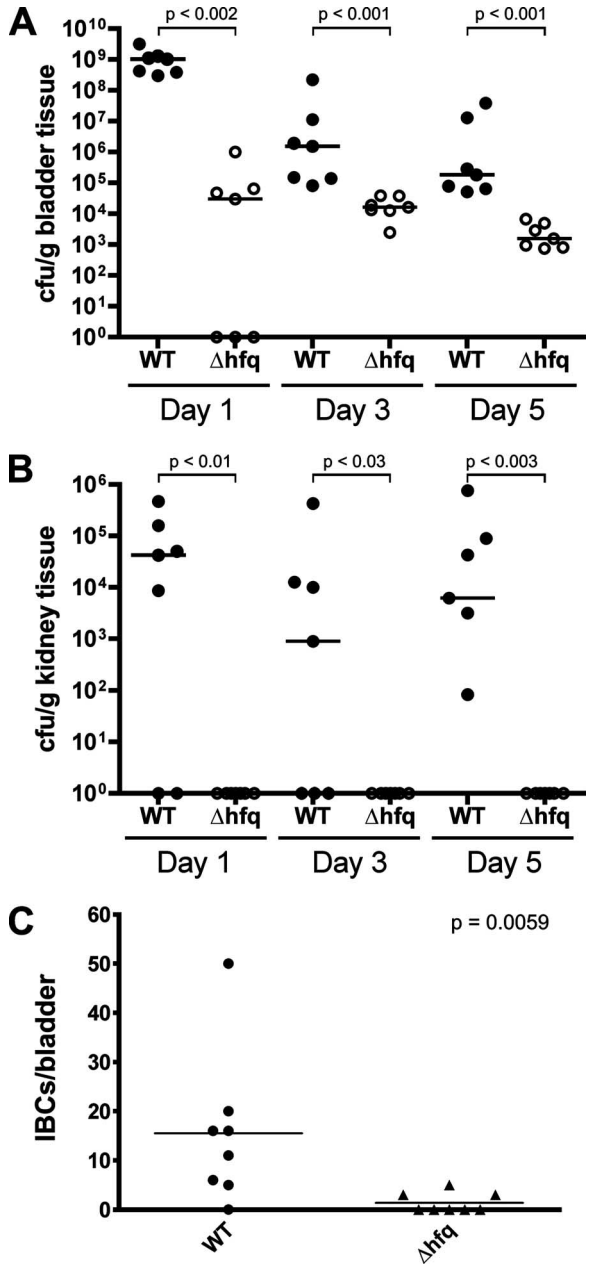

FIG. 1. Hfq is required for effective UPEC colonization of the urinary tract. (A and B) Adult female CBA/J mice were infected with 10^8 CFU of UTI89 or UTI89 Δ*hfq* via transurethral catheterization. Bacterial titers in bladder (A) and kidney (B) homogenates were determined at the indicated times postinoculation. 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

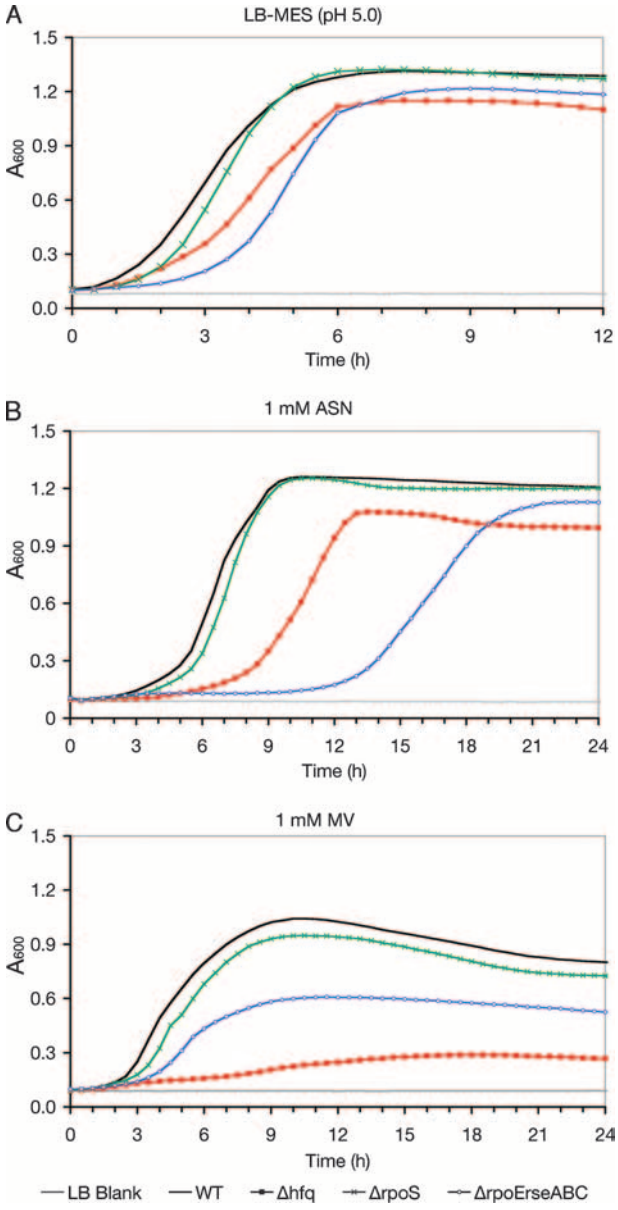

FIG. 2. UPEC resistance to low pH, RNS, and ROS is differentially affected by Hfq, RpoS, and RpoE. Overnight cultures of wild-type UTI89 (WT), UTI89 Δ*hfq*, UTI89 Δ*rpoS*, and UTI89 Δ(*rpoE-rseABC*) were diluted to an A_{600} of 1.0 and subcultured at 1:100 in LB-MES (pH 5.0) (A), LB-MES–1 mM ASN (B), 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.

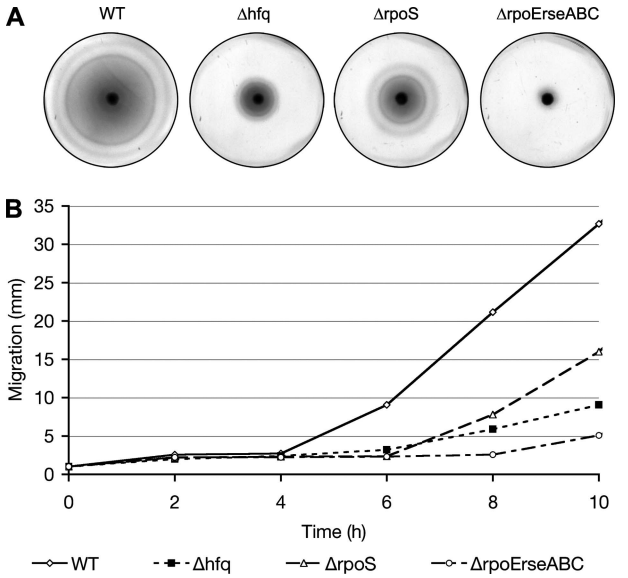

FIG. 3. Effects of *hfq*, *rpoS*, and *rpoE-rseABC* disruption on UPEC motility. (A) Images show the spread of UTI89 (the wild type [WT]) and its derivatives at 37°C 8 h after inoculation onto motility agar plates. (B) The change over time in the diameter (in millimeters) of the area covered by each 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.

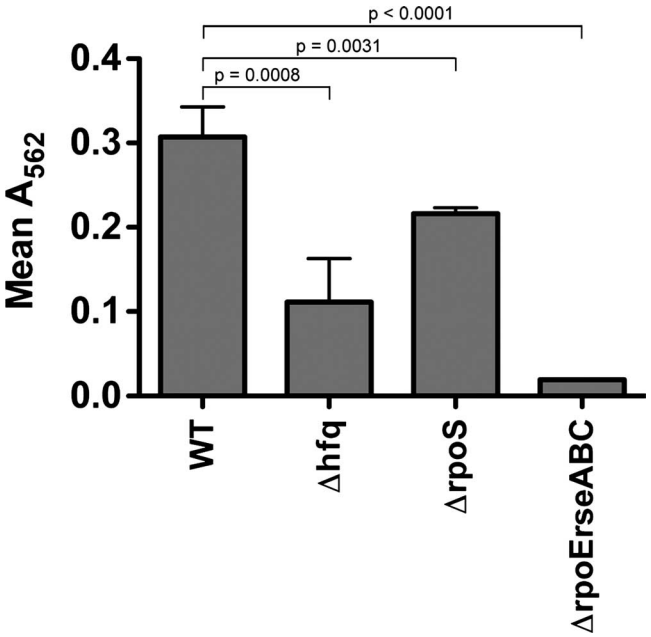

FIG. 4. Effects of *hfq*, *rpoS*, and *rpoE-rseABC* disruption on biofilm formation by UTI89. The wild-type (WT) UTI89 strain and its derivatives were grown in static M9 medium on flat-bottomed microtiter plates at 37°C for 48 h. Added crystal violet that was retained within wells by bacterial biofilms 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.

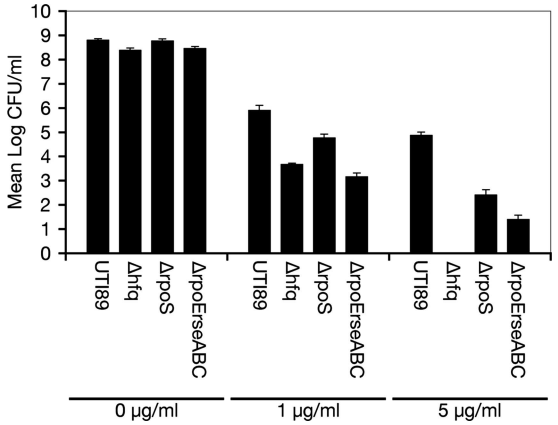

FIG. 5. UTI89 Δ*hfq* is hypersensitive to the cationic peptide polymyxin B. Equal numbers of CFU of UTI89 and its mutant derivatives were subcultured in LB broth with or without 1.0 or 5.0 μg of polymyxin B/ml, as indicated. After shaking at 37°C for 1.5 h, bacterial titers (in CFU per milliliter) in 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.

ACKNOWLEDGMENTS

This study was funded by NIH grants DK068585 and DK069526. R.R.K. and D.S.E. were supported by NIH microbial pathogenesis training grant T32 AI055434, and E.S.S. was supported by NIH postdoctoral fellowship DK070507. K.D.-P. contributed to this work as part of the BioURP Research Experience for Undergraduates Program funded by the National Science Foundation.

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Editor: F. C. Fang