Hfq Virulence Regulation in Enterohemorrhagic *Escherichia coli* O157:H7 Strain 86-24[▽]

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Enterohemorrhagic *Escherichia coli* O157:H7 (EHEC) causes bloody diarrhea and hemolytic-uremic syndrome. EHEC encodes the sRNA chaperone Hfq, which is important in posttranscriptional regulation. In EHEC strain EDL933, Hfq acts as a negative regulator of the locus of enterocyte effacement (LEE), which encodes most of the proteins involved in type III secretion and attaching and effacing (AE) lesions. Here, we deleted *hfq* in the EHEC strain 86-24 and compared global transcription profiles of the *hfq* mutant and wild-type (WT) strains in exponential growth phase. Deletion of *hfq* affected transcription of genes common to nonpathogenic and pathogenic strains of *E. coli* as well as pathogen-specific genes. Downregulated genes in the *hfq* mutant included *ler*, the transcriptional activator of all the LEE genes, as well as genes encoded in the *LEE2* to -5 operons. Decreased expression of the LEE genes in the *hfq* mutant occurred at middle, late, and stationary growth phases. We also confirmed decreased regulation of the LEE genes by examining the proteins secreted and AE lesion formation by the *hfq* mutant and WT strains. Deletion of *hfq* also caused decreased expression of the two-component system *qseBC*, which is involved in interkingdom signaling and virulence gene regulation in EHEC, as well as an increase in expression of *stx*_{2AB}, which encodes the deadly Shiga toxin. Altogether, these data indicate that Hfq plays a regulatory role in EHEC 86-24 that is different from what has been reported for EHEC strain EDL933 and that the role of Hfq in EHEC virulence regulation extends beyond the LEE.

Enterohemorrhagic *Escherichia coli* O157:H7 (EHEC) is a food-borne pathogen that causes severe bloody diarrhea and is often associated with complications, including hemolytic-uremic syndrome (HUS), seizures, cerebral edema, and/or coma (42). EHEC attaches intimately to intestinal epithelial cells and triggers extensive cytoskeletal rearrangements, resulting in attaching and effacing (AE) lesions and formation of a characteristic pedestal structure (40, 41, 46). Most of the genes involved in AE lesion formation are carried within a chromosomal pathogenicity island called the locus of enterocyte effacement (LEE) (53). The LEE contains five major operons (*LEE1* to -5) that encode a type III secretion (TTS) system and effector proteins. EHEC's arsenal of virulence factors also includes non-LEE-encoded effector proteins that increase adherence or mediate colonization of host epithelial cells (8, 16, 26, 27, 32, 84, 87, 88).

The mortality associated with EHEC infections is due to the production and release of a Shiga toxin (Stx) by these bacteria. EHEC expresses Stx, and this potent inhibitor of protein synthesis can be absorbed systemically, where it binds to receptors found in the kidneys and the central nervous system, thus causing the complications associated with EHEC disease (42).

Regulation of EHEC virulence genes is extremely complex and involves interkingdom signaling (12). EHEC senses the host-derived signals epinephrine and norepinephrine as well as the bacterial signal AI-3, which are present in the intestines, through a membrane-bound adrenergic sensor kinase, QseC. Upon detecting these signals, QseC autophosphorylates and subsequently phosphorylates the following three transcription factors: QseB, which regulates the flagellar operon (11, 12, 82); QseF, which plays a role in inducing the SOS response and Stx production as well as activating expression of *espFU* (66), which encodes an effector essential for AE lesion formation (8, 27); and KdpE, which activates *ler* (35).

In addition to QseBC, several other proteins play a role in LEE regulation. The regulator (Ler) that is encoded in *LEE1* is a master regulator of the LEE (33, 56, 69, 71, 79), and expression of Ler is tightly regulated by multiple proteins through direct or indirect interaction (3, 16, 25, 38, 39, 44, 69, 73, 74, 78, 92, 99). In enteropathogenic *E. coli*, the RNA binding protein CsrA and the nucleoid-associated protein Fis also regulate *ler* activity (4, 28). The global regulator H-NS is a negative regulator of *ler*, *LEE2*, and *LEE3* (7, 33, 56, 71, 79, 90), whereas the global regulator RpoS activates expression of *LEE3* (38, 80).

Recent studies have indicated that posttranscriptional regulation also plays a role in controlling expression of the LEE (4, 34, 49, 52, 72). Posttranscriptional gene regulation involves small RNAs (sRNAs) and allows bacteria to rapidly adapt to environmental signals (85). Although some sRNAs act by bind-

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or description	Reference or source
Strains		
86-24	Wild-type EHEC strain (serotype O157:H7)	30
MK08	86-24 <i>hfq</i> mutant	This study
EDL933	Wild-type EHEC strain (serotype O157:H7)	67
MK35	EDL933 <i>hfq</i> mutant	This study
MK10	EHEC strain MK08 with plasmid pCG52	This study
VS138	<i>qseC</i> mutant of 86-24	81
MC474	<i>qseB</i> mutant of 86-24	35
Plasmids		
pBADMycHis	C-terminal Myc-His tag vector	Invitrogen
pCG52	EHEC 86-24 <i>hfq</i> in pBADMycHis	This study

ing to and modulating protein activity, most characterized sRNAs act by base pairing with target mRNAs (83). These sRNAs are categorized as either *cis* or *trans* encoded. The *cis*-encoded sRNAs are transcribed from the DNA strand opposite to another gene on the bacterial chromosome and thus have perfect complementarity to its target gene (83). The *trans*-encoded sRNAs are encoded at chromosomal locations distinct from the mRNAs that they regulate, generally share little complementarity to their target genes, and often function in association with the chaperone protein Hfq (83).

Hfq was first identified as a host factor of RNA phage Q β in nonpathogenic *E. coli* (24), and subsequent studies have shown that Hfq is an important regulator of virulence traits in several bacterial species (10, 17, 55, 76, 77), including *E. coli* (34, 48, 72, 75). Hfq promotes interactions between an sRNA and its target mRNA to regulate gene expression (43, 51, 65); however, Hfq can also function independently by influencing polyadenylation or translation of mRNAs (91).

Two papers showing that the *hfq* deletion increased LEE gene expression in the EHEC strain EDL933 have been published (34, 72); however, our studies on the role of Hfq in EHEC strain 86-24 detected the opposite phenotype. EHEC strain EDL933 was isolated from a raw hamburger patty that was linked to the Oregon and Michigan outbreaks in 1982 (97) and carries both *stx*₁ and *stx*₂ (59, 89). EHEC strain 86-24 was isolated during the Washington state outbreak in 1986 from a patient experiencing hemorrhagic colitis and contains genes encoding solely Stx₂ (30). EHEC strain 86-24 is thought to be more virulent than EHEC strain EDL933, as strains that express *stx*₂ are more important to the development of HUS than strains that express *stx*₁ (29), and strains that produce only Stx₂ are more commonly associated with severe disease than are strains that produce both Stx₁ and Stx₂ (5, 23, 31). Additionally, strains that solely produced Stx₂ were more neurotropic for gnotobiotic piglets than strains producing only Stx₁ or both Stx₁ and Stx₂ (20). Differences in gene regulation among EHEC strains may lead to differences in pathogenicity, and thus the goal of this study was to examine the global role of Hfq in gene expression in EHEC strain 86-24 and, more specifically, Hfq regulation of genes important in EHEC viru-

TABLE 2. Primers used in this study

Primer	Sequence
tir_RT_F1	CCATGGAGAGCAGACGTAGCT
tir_RT_R1	CGGTGATCCTGGATTAAACCTT
escC_RT_F1	GCGTAAACTGGTCCGGTACGT
escC_RT_R1	TGCGGGTAGAGCTTTAAAGGCAAT
escV_RT_F1	TCGCCCCGTCCATTGA
escV_RT_R1	CGCTCCCGAGTGCAAAA
ler_RT_F1	CGACCAGGTCTGCCCTTCT
ler_RT_R1	GCGCGGAACATCGAAA
espA_RT_F1	TCAGAATCGCAGCCTGAAAA
espA_RT_R1	CGAAGGATGAGGTGGTTAAGCT
eae_RT_F1	GCTGGCCCTTGGTTTGATCA
eae_RT_R1	GCGGAGATGACTTCAGCACTT
MK064F	AGAATGGCTAAGGGGCAATCTTTACAAG ATCCGTTCTGAACGCACTGCGGTGTA GGCTGGAGCTGCTTC
MK065R	GCCCCAAACCTATTTCGGTTTCTTCGCTG TCCTGTTGCGCGGAAGTATTCCATATG AATATCCTCCTTAG
MK260F	CATGGTACCGCTAAGGGGCAATCTTTA CAAG
MK261R	GTAAAGCTTTTCGGTTTCTTCGCTGTCC
rpoA_F	GCGCTCATCTTCTTCCGAAT
rpoA_R	CGCGTCTGTGTTATGTG

lence. Here we report that in EHEC strain 86-24, Hfq is a positive regulator of the LEE and the QseBC two-component system and is a negative regulator of *stx*_{2AB}, which encodes Stx₂.

MATERIALS AND METHODS

Strains and growth media. All bacterial strains used in the study are listed in Table 1. Strains were grown at 37°C in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) or in LB (Invitrogen). Antibiotics were added at the following concentrations: ampicillin, 100 μ g/ml; streptomycin, 50 μ g/ml; and kanamycin, 50 μ g/ml. The signaling compounds AI-3 (30 μ M) and epinephrine (50 μ M) were added as indicated.

The *hfq* deletion strains were constructed as described by Datsenko and Wanner (14). Briefly, 86-24 or EDL933 cells containing pKD46 were electroporated with a *hfq* PCR product generated by primers MK64 and MK65 (Table 2) using pKD3 as a template. After electroporation, cells were incubated at 25°C overnight (OVN) in Super Optimal Broth with Catabolite repression (SOC) medium. Cells were then plated on medium containing chloramphenicol and incubated at 37°C. Resulting colonies were patched for chloramphenicol resistance and ampicillin sensitivity and PCR verified for the absence of *hfq*. The chloramphenicol cassette was then resolved using pCP20-mediated recombination, with additional patching for chloramphenicol and ampicillin sensitivity. The generated 86-24 *hfq* mutant MK08 was then complemented with pCG52. Plasmid pCG52 was constructed from the PCR product of primer set MK260F and MK261R using EHEC as a template, and this product was digested with KpnI and HindIII and inserted into pBADMycHis.

Recombinant DNA techniques. Standard methods were used to perform plasmid purification, PCR, ligation, restriction digests, transformations, and gel electrophoresis. All oligonucleotide primers are listed in Table 2.

RNA extraction. Cultures of strains 86-24, MK08, MK10, EDL933, MK35, VS138, and MC474 were grown aerobically in LB medium at 37°C overnight and then were diluted 1:100 in DMEM and grown at 37°C. RNA from three biological replicate cultures of each strain/condition was extracted at the early exponential growth phase (optical density at 600 nm [OD₆₀₀] of 0.2), mid-exponential growth phase (OD₆₀₀ of 0.5), late exponential growth phase (OD₆₀₀ of 1.0), or stationary growth phase (OD₆₀₀ of 1.5) using the RiboPure Bacteria RNA isolation kit (Ambion).

Microarray preparation and analyses. Affymetrix 2.0 *E. coli* gene arrays were used to compare gene expression in strain 86-24 to that in strain MK08 (*hfq* mutant), grown aerobically in DMEM at 37°C, at late exponential growth phase. The GeneChip *E. coli* Genome 2.0 array (Affymetrix, Santa Clara, CA) includes approximately 10,000 probe sets for all 20,366 genes present in the following four

strains of *E. coli*: K-12 lab strain MG1655, uropathogenic strain CFT073, O157:H7 enterohemorrhagic strain EDL933, and O157:H7 enterohemorrhagic strain Sakai. The RNA processing, labeling, hybridization, and slide-scanning procedures were performed as described in the Affymetrix Gene Expression technical manual.

The array data analyses were performed as described previously (45). The output from scanning a single replicate of the Affymetrix GeneChip *E. coli* Genome 2.0 array for each of the biological conditions was obtained using GCOS v 1.4 according to the manufacturer's instructions. Data were normalized using Robust Multiarray analysis (6, 36) at the RMAExpress website (<http://rmaexpress.bmbolstad.com/>), and the resulting data were compared to determine features whose expression was increased or decreased in response to the *hfq* deletion. Custom analysis scripts were written in Perl in order to sort the Affymetrix output data and complete the multiple-array analyses. The results of the array analyses were further confirmed using quantitative reverse transcription-PCR (qRT-PCR) as described below. We note that the isolate used in these studies has not been sequenced and thus is not fully contained on the array and that differences in genome content are evident.

Real-time qPCR. RNA was extracted as described above from three biological replicates each of strains 86-24, MK08, MK10, EDL933, MK35, VS138, and MC474. The primers used in the real-time qPCR assays were designed using Primer Express v1.5 (Applied Biosystems) (Table 2). The amplification efficiency and template specificity of each of the primer pairs were validated and reaction mixtures were prepared as described previously (96). Real-time reverse transcription-PCR was performed in a one-step reaction using an ABI 7500 sequence detection system (Applied Biosystems).

Data were collected using the ABI Sequence Detection 1.2 software (Applied Biosystems). All data were normalized to levels of *rpoA* and analyzed using the comparative cycle threshold (C_T) method (2). The expression levels of the target genes under the various conditions were compared using the relative quantification method (2). Real-time data are expressed as the changes in expression levels compared to the wild-type (WT) levels. Statistical significance was determined by Student's *t* test, and a *P* value of ≤ 0.05 was considered significant.

FAS assay. Fluorescein-actin staining (FAS) assays were performed as previously described by Knutton et al. (47). Briefly, OVN bacterial cultures grown aerobically in LB at 37°C were diluted 1:100 to infect confluent monolayers of HeLa cells. HeLa cells were grown on glass coverslips for 6 h at 37°C with 5% CO₂. The coverslips were then washed, permeabilized with 0.2% Triton X, and treated with fluorescein isothiocyanate (FITC)-labeled phalloidin to visualize actin accumulation, and finally propidium iodide was added to stain the bacteria.

Northern analysis. Northern blotting was completed as described previously (70). Samples were transferred OVN and then UV cross-linked to the membrane. Membranes were incubated in Ambion hybridization buffer for 30 min at 68°C. RNA probes were incubated in hybridization buffer for 5 min at 95°C and then incubated with the membrane OVN at 68°C. The following day, the membrane was washed two times for 5 min at 68°C with low-stringency buffer (2× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate] diluted from 20× stock [3.0 M NaCl, 0.3 M sodium citrate, pH 7.0] and 0.1% sodium dodecyl sulfate [SDS]) and then washed twice with high-stringency buffer (0.1× SSC, 1% SDS) for 15 min at 68°C. The membrane was exposed to a phosphorimager for 2 h to OVN.

Secreted protein and whole-cell lysate immunoblotting. Secreted proteins from strains 86-24 and MK08 were harvested as described by Jarvis et al. (40). Cultures were grown aerobically in DMEM at 37°C and collected at late exponential growth phase. Total secreted protein from culture supernatants was separated from bacterial cells using centrifugation and filtration. SDS-PAGE and immunoblotting were completed as previously described (70). The samples were then subjected to immunoblotting with rabbit polyclonal antiserum to EspA and visualized with enhanced chemiluminescence (Bio-Rad). Coomassie blue staining was used to visualize bovine serum albumin (BSA) loading controls.

Whole-cell lysates from WT EHEC strains 86-24 and EDL933 and the respective *hfq* mutant strains MK08 and MK35 were prepared from strains grown in DMEM to late exponential growth phase. Cells were collected by centrifugation and then lysed with urea lysis buffer (100 mM NaH₂PO₄, 10 mM Tris, 8 M urea, pH 6.3). Samples were probed using monoclonal antisera against Stx_{2A} (Santa Cruz Biotechnology) and RpoA (Neoclone).

Microarray data accession number. Expression data can be accessed using accession number GSE30893 at the NCBI GEO database.

TABLE 3. Comparison of K-12 and pathovar-specific genes with altered expression in the 86-24 *hfq* mutant strain compared to the WT

Strain	No. of genes with expression:		
	Increased	Decreased	Unchanged
MG1655	156	535	3,059
EDL933	47	368	1,266
Sakai	4	80	273
CFT073	11	339	2,082

RESULTS

Hfq is a global regulator in EHEC. In order to have a global snapshot of Hfq regulation in EHEC, we constructed an isogenic deletion of *hfq* in the EHEC O157:H7 strain 86-24. EHEC strain 86-24 was isolated in 1986 from a patient in Seattle, WA, experiencing hemorrhagic colitis (30). Moreover, strain 86-24 has been used extensively to study EHEC infection and disease in all EHEC animal models (15, 19, 54, 57, 58, 63, 94, 98). Transcriptome analyses were undertaken using Affymetrix gene arrays. The arrays contain approximately 10,000 probe sets for all 20,366 genes present in the genomes of the sequenced EHEC strains, EDL933 and Sakai, the uropathogenic *E. coli* strain CFT073, and K-12 strain MG1655 and 700 probes covering intergenic regions that can carry nonannotated small open reading frames (ORFs) or putative sRNAs.

Gene expression was compared between WT EHEC strain 86-24 and the 86-24 *hfq* mutant grown in DMEM culture medium to late logarithmic growth, conditions under which the LEE is optimally expressed (22, 96). The microarray data indicated that 249 probe sets were increased and 1,673 were decreased in the *hfq* mutant. Of these, approximately 700 altered probes were derived from the *E. coli* K-12 strain MG1655, which contains a common *E. coli* backbone conserved among all *E. coli* pathovars (64), and nearly 900 altered probe sets were pathovar-specific genes (Table 3), suggesting that Hfq plays an extensive role in EHEC gene regulation. Additionally, the arrays indicated that genes encoding iron acquisition, regulatory proteins, sRNAs, the LEE, and QseBC were differentially regulated in the *hfq* mutant compared to the WT. The regulation of these genes was explored in more detail.

In EHEC strain 86-24, Hfq is a positive regulator of the LEE. Because the microarray data indicated that LEE-carried genes were differentially regulated in the 86-24 *hfq* mutant strain compared to the 86-24 WT strain, we first examined expression of *ler*, the master regulator of the LEE. Expression levels of *ler* were measured from cells grown in DMEM to middle, late, and stationary growth phases. In all cases, *ler* expression was decreased in the *hfq* mutant compared to the WT (Fig. 1A to C; Table 4). Transcription of *ler* could be restored to WT levels upon complementation with a functional copy of *hfq* (86-24 *hfq*-*phfq*) (Fig. 1A). Because previous studies using the EHEC strain EDL933 analyzed *ler* expression in cells grown in LB medium, we also examined *ler* expression in the EHEC 86-24 WT and *hfq* mutant strains grown in LB to mid-exponential growth phase. The results were consistent

FIG. 1. (A) Transcriptional profile of *ler* gene expression for WT EHEC 86-24, the isogenic *hfq* mutant, and the *hfq* complemented strain grown in DMEM to late logarithmic growth phase as measured by qRT-PCR and expressed as fold differences normalized to WT strain 86-24. (B) qRT-PCR measuring gene expression of *ler* of WT 86-24 and the *hfq* mutant grown in DMEM to mid-logarithmic growth phase. (C) qRT-PCR measuring gene expression of *ler* of WT 86-24 and the *hfq* mutant grown in DMEM to stationary growth phase. (D) qRT-PCR measuring gene expression of *ler* of WT 86-24 and the *hfq* mutant grown in LB to mid-logarithmic growth phase. (E) qRT-PCR measuring gene expression of *ler* of WT EDL933 and the *hfq* mutant grown in DMEM to late logarithmic growth phase. (F) qRT-PCR measuring gene expression of *ler* of WT EDL933 and the *hfq* mutant grown in DMEM to mid-logarithmic growth phase. (G) qRT-PCR measuring gene expression of *ler* of WT EDL933 and the *hfq* mutant grown in LB to mid-logarithmic growth phase. For all of the qRT-PCR analyses, the levels of *rpoA* transcript were used to normalize the C_T values to account for variations in bacterial numbers. In all graphs, the error bars indicate the standard deviations of the $\Delta\Delta C_T$ values (2), and significance is indicated as follows: one asterisk, $P \leq 0.05$; two asterisks, $P \leq 0.005$; three asterisks, $P \leq 0.0005$.

TABLE 4. Relative change in expression in the *hfq* mutant strain MK08 compared with WT strain 86-24 as determined by qRT-PCR

Growth phase	Medium	Gene	Fold change in expression
Mid-log	LB	<i>ler</i>	−6,330
		<i>eae</i>	−520
		<i>espA</i>	−2
	DMEM	<i>ler</i>	−94
		<i>eae</i>	−320
		<i>tir</i>	−34,000
		<i>espA</i>	0
Late log	DMEM	<i>ler</i>	−1,000
		<i>escC</i>	−10,000
		<i>escV</i>	−4,200
		<i>tir</i>	−11,500
		<i>eae</i>	−6,650
		<i>espA</i>	−8.5
		<i>stx_{2A}</i>	4
		<i>qseB</i>	−4
		<i>qseC</i>	−3
Stationary	DMEM	<i>ler</i>	−950
		<i>tir</i>	−5,200
		<i>eae</i>	−410

with the DMEM data (Fig. 1D), in which *ler* expression is significantly decreased in the 86-24 *hfq* mutant.

Previous reports indicated that the *hfq* deletion in EHEC strain EDL933 increased expression of *ler* compared to that in the WT (34, 72). To confirm that our data were not due to growth media or other experimental variables, we constructed an *hfq* isogenic mutant of strain EDL933 and used qRT-PCR to compare *ler* expression in the EDL933 WT and *hfq* mutant strains at various growth phases and in LB and DMEM culture media. Under all conditions, our results agreed with previous reports in that the EDL933 *hfq* mutant displayed increased *ler* expression (Fig. 1E to G) compared to the WT strain. These results suggest that the variation in Hfq phenotypes is a result of differences in the genetic backgrounds of EHEC strains.

Ler regulates all of the LEE genes; thus, if *ler* expression is diminished, expression of other genes contained in the LEE pathogenicity island is also expected to be diminished. Indeed, the array analyses detected lower expression of genes contained in *LEE1* to *-5*. We used qRT-PCR data to confirm the array data, and the results were consistent, showing that expression of the LEE was decreased in the 86-24 *hfq* mutant strain compared to the WT when grown in DMEM or LB medium to the mid-exponential, late exponential, or stationary growth phase (Fig. 2A to D; Table 4). Negative regulation of TTS by the 86-24 *hfq* mutant was also observed at the trans-

FIG. 2. (A) qRT-PCR transcriptional profile of *LEE* gene expression for WT EHEC 86-24 and the isogenic *hfq* mutant grown in DMEM to mid-logarithmic growth phase. (B) qRT-PCR of *LEE* gene expression for WT EHEC 86-24 and the isogenic *hfq* mutant grown in DMEM to late logarithmic growth phase. (C) qRT-PCR of *LEE* gene expression for WT EHEC 86-24 and the isogenic *hfq* mutant grown in DMEM to stationary growth phase. (D) qRT-PCR of *LEE* gene expression for WT EHEC 86-24 and the isogenic *hfq* mutant grown in LB to mid-logarithmic growth phase. (E) Western blotting was performed on secreted protein preparations from WT 86-24 and *hfq* mutant strains grown in DMEM to late logarithmic growth phase.

lational level. The 86-24 *hfq* mutant strain secreted less effector protein EspA than the WT when the strains were compared by Western blot analysis (Fig. 2E).

The *hfq* mutant is deficient in forming AE lesions. Because the 86-24 *hfq* mutant diminishes transcription of *LEE* expression *in vitro*, we tested the ability of the *hfq* mutant to form AE lesions on epithelial cells using the fluorescein-actin staining (FAS) test (47). In this test, the epithelial cell actin is stained with FITC-labeled phalloidin, and the bacteria and HeLa cell nuclei are stained with propidium iodide. Pedestals are visualized as brilliant patches of green underneath a red bacterium via fluorescence microscopy. After 6 h, patches of pedestals could be visualized when HeLa cells were cultured with the 86-24 WT strain; however, when the HeLa cells were cultured with the 86-24 *hfq* mutant cells, no pedestals were observed, even though bacterial cells were present (Fig. 3). Altogether,

FIG. 3. Detection of AE lesion formation using the FAS test on HeLa cells, comparing the WT 86-24 and *hfq* mutant. Green shows the HeLa cell actin cytoskeleton, and red shows the bacteria and cell nuclei. WT EHEC forms pedestals (left panel), but the *hfq* mutant does not (right panel). Cells were viewed at a magnification of $\times 640$.

these results suggest that Hfq has a novel function of *LEE* regulation in strain 86-24, being a positive regulator of TTS and AE lesion formation in this strain.

The *hfq* mutant displays increased expression of *stx*_{2AB}. Expression of the *stx*_{2AB} genes, which encode Shiga toxin in EHEC, may lead to HUS and other fatal sequelae. Regulation of *stx*_{2AB} by Hfq in EHEC has not previously been investigated; therefore, we compared mRNA and protein expression in the 86-24 and EDL933 WT and *hfq* mutant strains. In both the 86-24 and EDL933 strains of EHEC, expression of the *stx*_{2A} genes is increased in the *hfq* mutant strain compared to the WT at both the transcriptional and translational levels in the cells (Fig. 4A to D; Table 4). WT expression levels of *stx*_{2AB} were restored upon complementation with *hfq* encoded on a plasmid (86-24 *hfq*-*phfq*) (Fig. 4A and B).

The QseBC two-component system is posttranscriptionally regulated. The microarray data indicated that expression of *qseC* and *qseB* was decreased in the EHEC strain 86-24 *hfq* mutant compared to the WT. We confirmed these data by qRT-PCR and also confirmed that levels of *qseC* and *qseB* could be restored upon complementation with *hfq* carried on a plasmid (86-24 *hfq*-*phfq*) (Fig. 5; Table 4).

Decreased expression of *qseBC* in the *hfq* mutant strain suggests that an sRNA(s) is involved in the posttranscriptional regulation of this two-component system. We have previously examined *qseC* autoregulation at the translational level using *lacZ* reporter fusions and found that *qseC* expression was downregulated in the *qseC* mutant compared to the WT (13). Here, we used qRT-PCR and Northern analyses to examine mRNA levels in the cell. In both of these experiments, tran-

FIG. 4. (A) qRT-PCR transcriptional profile of *stx2A* gene expression for WT EHEC 86-24, the isogenic *hfq* mutant, and the *hfq* complemented strain. (B) Western blotting of whole-cell lysates from WT strain 86-24, the *hfq* isogenic mutant, and the *hfq* complemented strain with an antibody specific to Stx_{2A}. (C) qRT-PCR transcriptional profile of *stx2A* gene expression for WT EHEC EDL933 and the isogenic *hfq* mutant. (D) Western blotting of whole-cell lysates from WT strain EDL933 and *hfq* isogenic mutant with an antibody specific to Stx_{2A}. In all experiments, cells were grown in DMEM to late logarithmic growth phase.

scription of *qseC* was increased in the *qseC* mutant compared to the WT (Fig. 6A and B), and addition of the signaling molecule epinephrine or AI-3 did not affect the *qseC* positive autoregulation (Fig. 6A).

DISCUSSION

Recent studies have identified an important role for Hfq and sRNAs in pathogenesis for both Gram-negative and Gram-positive bacteria (reviewed in references 9 and 85). EHEC integrates multiple environmental signals to coordinate timing of gene expression. sRNAs allow a rapid response to environmental signals, and thus it is not surprising that Hfq is part of the EHEC regulatory cascade. We examined the role of Hfq in strain 86-24. This strain was isolated in 1986 from a patient in Seattle, WA, who suffered from hemorrhagic colitis after eating undercooked meat from a fast food restaurant (30). The WT strain 86-24 expresses Stx and has been used in all EHEC

FIG. 5. Transcriptional profile of *qseB* and *qseC* gene expression for WT EHEC 86-24, the isogenic *hfq* mutant, and the *hfq* complemented strain grown in DMEM to late logarithmic growth phase as measured by qRT-PCR.

FIG. 6. (A) Transcriptional profile of *qseBC* expression for WT EHEC and the *qseC* mutant grown in DMEM, DMEM with added epinephrine, or DMEM with AI-3. (B) Northern analysis of *qseBC* expression for the WT 86-24, the *qseC* mutant, and the *qseB* mutant.

animal models to study EHEC infection and disease (15, 19, 54, 57, 58, 63, 94, 98).

Hfq is a positive regulator of TTS in *Salmonella enterica* serovar Typhimurium (76). Data presented here indicated that Hfq in EHEC 86-24 is a positive regulator of TTS and AE lesion formation (Fig. 1 to 3). This regulation is distinct from that in strain EDL933, in which Hfq is a negative regulator of *ler* and therefore of TTS and AE lesion formation (34, 72) (Fig. 1E to G).

Because other reports document the opposite phenotype in strain EDL933 grown in LB or to mid-exponential phase, we constructed an *hfq* mutant of EDL933 and tested both 86-24 and EDL933 under various growth conditions and with various growth times. In all cases, the 86-24 *hfq* mutant presented decreased expression of the *ler* and LEE-carried genes, whereas the EDL933 *hfq* mutant showed an increase in *ler* expression. In 86-24, the decrease in *ler* transcript levels was correlated with the decrease in transcription of genes carried in *LEE2* to -5 (Fig. 2A to D), the decrease of the TTS effector EspA into the supernatant (Fig. 2E), and the absence of formation of pedestals on HeLa epithelial cells by the *hfq* mutant strain (Fig. 3). These results present new findings on Hfq regulation of virulence genes in EHEC.

EHEC strains are rapidly evolving through the integration of laterally acquired virulence genes (62, 84) and vary in their ability to adhere to epithelial cells, cause disease in humans, colonize animals, and survive in the environment (1, 21, 68, 93). Differential regulation of the LEE among EHEC strains 86-24, EDL933, and Sakai has been observed in studies examining the roles of RpoS and GrlA in EHEC virulence (18, 37, 38, 49, 80, 86). Moreover, Islam et al. reported that the *ler* P1 promoter was the major promoter activating LEE expression in EHEC strains Sakai and EDL933 (37), whereas other studies have found that the *ler* P2 promoter was the major promoter in EHEC strain 86-24 (74, 78). Islam et al. suggested that the differences are likely due to the genetic backgrounds of the strains and/or the exact fusions and measurement conditions used in the experimental design (37). The differences in

FIG. 7. Model of Hfq regulation in EHEC strain 86-24.

hfq regulation between the 86-24 and EDL933 strains reported in this study most likely arises due to variations in the genetic backgrounds of strains 86-24 and EDL933, as we constructed *hfq* isogenic mutants of both strains in order to control for variations in experimental conditions.

In addition to regulating genes encoding AE lesion formation and TTS, Hfq also regulates genes encoding Stx. Stx is a major virulence factor of EHEC and is responsible for HUS and other sequelae associated with EHEC disease (42). The genes encoding Stx are located within the late genes of a λ -like bacteriophage and are transcribed when the phage enters its lytic cycle upon induction of an SOS response in the bacterial cell (60, 61, 95). The phage replicates, produces Stx, and lyses the bacteria, releasing Stx. Many bacterial sRNAs are involved in regulating stress pathways (50), and here we show that in both EHEC strains 86-24 and EDL933, Hfq decreases *stx*_{2A} expression (Fig. 4A to D). Therefore, it is possible that Hfq and sRNAs act to define a threshold for expression of Stx (50).

EHEC senses the bacterial signal AI-3 as well as the mammalian stress hormones epinephrine and norepinephrine through the membrane-bound sensor kinase QseC. QseC relays this information to the response regulators QseB, QseF, and KdpE to activate expression of virulence genes (12, 35, 81, 82). Hfq is a positive regulator of QseBC (Fig. 5). Expression of these virulence genes is energetically expensive and in the case of Stx is lethal to the bacterial cell; thus, EHEC employs posttranscriptional regulation to precisely coordinate timing of their expression.

The data presented here reveal that Hfq plays an important role in fine-tuning of EHEC virulence gene expression. Hfq synchronizes gene expression from the level of cell-to-cell signaling, to host attachment and colonization, to expression of Stx (Fig. 7). This study further unravels the complex regulatory networks involved in EHEC virulence regulation and highlights the importance of understanding gene regulation in the rapidly evolving pathogenic *E. coli* (62, 64, 84) as well as the possibility that EHEC strains EDL933 and 86-24 have evolved various means of achieving pathogenicity.

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