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Hfq affects the expression of the LEE pathogenicity island in enterohaemorrhagic *Escherichia coli*

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Abstract

Colonization of the intestinal epithelium by enterohaemorrhagic *Escherichia coli* (EHEC) is characterized by an attaching and effacing (A/E) histopathology. The locus of enterocyte effacement (LEE) pathogenicity island encodes many genes required for the A/E phenotype including the global regulator of EHEC virulence gene expression, Ler. The LEE is subject to a complex regulatory network primarily targeting *ler* transcription. The RNA chaperone Hfq, implicated in post-transcriptional regulation, is an important virulence factor in many bacterial pathogens. Although post-transcriptional regulation of EHEC virulence genes is known to occur, a regulatory role of Hfq in EHEC virulence gene expression has yet to be defined. Here, we show that a *hfq* mutant expresses increased levels of LEE-encoded proteins prematurely, leading to earlier A/E lesion formation relative to wild type. Hfq indirectly affects LEE expression in exponential phase independent of Ler by negatively controlling levels of the regulators GrlA and GrlR through post-transcriptional regulation of the *grlRA* messenger. Moreover, Hfq negatively affects LEE expression in stationary phase independent of GrlA and GrlR. Altogether, Hfq plays an important role in coordinating the temporal expression of the LEE by controlling *grlRA* expression at the post-transcriptional level.

Keywords

enterohaemorrhagic *Escherichia coli*; Hfq; LEE; *grlRA*

Introduction

Enterohaemorrhagic *Escherichia coli* (EHEC) O157:H7 is a human pathogen that causes diarrhea, haemorrhagic colitis and haemolytic uremic syndrome (Nataro and Kaper, 1998). EHEC colonization of the intestinal epithelial surface is accompanied by a characteristic attaching and effacing (A/E) histopathology marked by localized destruction of brush border microvilli, intimate attachment to enterocytes and rearrangement of cytoskeletal components beneath adherent bacteria (Frankel *et al.*, 1998). The genes required for the A/E phenotype are primarily contained in the 35–45 kb locus of enterocyte effacement (LEE) pathogenicity island, which is conserved among EHEC strains and other A/E pathogens including enteropathogenic *E. coli* (EPEC), atypical EPEC, rabbit EPEC, *Escherichia albertii* and *Citrobacter rodentium* (McDaniel *et al.*, 1995; Nataro and Kaper, 1998; Huys *et al.*, 2003; Rasko *et al.*, 2008).

The LEE contains five major operons (*LEE1* to *LEE5*) that encode a type III secretion system (TTSS), the adhesion factor intimin, a translocated intimin receptor (Tir),

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translocator proteins, secreted effectors and regulators (McDaniel *et al.*, 1995; Elliott *et al.*, 1998; Perna *et al.*, 1998; Garmendia *et al.*, 2005). Proper coordination of virulence gene expression is crucial for the bacterial pathogen to successfully colonize the host. Under conditions suboptimal for infection, the global modulator H-NS silences LEE expression (Bustamante *et al.*, 2001; Umanski *et al.*, 2002; Haack *et al.*, 2003; Barba *et al.*, 2005). The *LEE1*-encoded regulator Ler is required for transcriptional activation of LEE genes and virulence genes located outside the LEE (Mellies *et al.*, 1999; Friedberg *et al.*, 1999; Elliott *et al.*, 2000; Sperandio *et al.*, 2000; Sanchez-SanMartin *et al.*, 2001; Haack *et al.*, 2003; Abe *et al.*, 2008) by relieving H-NS-mediated silencing (Sperandio *et al.*, 2000; Bustamante *et al.*, 2001; Umanski *et al.*, 2002; Haack *et al.*, 2003; Torres *et al.*, 2007; Mellies *et al.*, 2008). A complex regulatory network controlling *LEE1* expression reflects the importance of Ler in virulence gene expression. Factors including H-NS, IHF, Fis, Hha, GrlA, GrlR, BipA, Pch, EtrA, EivF, GrvA, QseA, and the alarmone ppGpp integrate environmental signals to regulate EHEC *LEE1* transcription in response to changing environmental and host conditions (for reviews see Kaper *et al.*, 2004; Mellies *et al.*, 2007). The LEE-encoded regulators GrlA and GrlR activate and repress LEE transcription, respectively (Barba *et al.*, 2005; Iyoda *et al.*, 2006). Specifically, GrlA binds to the EHEC *LEE1* promoter to activate *ler* transcription (Laaberki *et al.*, 2006; Russell *et al.*, 2007; Huang and Syu, 2008). Ler and GrlA then form a positive transcriptional regulatory loop in part by counteracting H-NS repression (Barba *et al.*, 2005). GrlR interacts with GrlA to prevent the formation of higher order complexes between GrlA and *LEE1* promoter DNA, thereby fine-tuning GrlA-mediated activation of *ler* transcription (Creasey *et al.*, 2003; Jobichen *et al.*, 2007; Huang and Syu, 2008). Consequently, GrlA and GrlR control activation of LEE transcription by optimizing Ler expression. Since GrlA is required for transcriptional activation of *LEE1* (Barba *et al.*, 2005), factors controlling GrlA expression are important for *ler* expression, and thereby for initiating expression of the Ler regulon.

Many transcriptional regulators are subject to post-transcriptional regulation mediated by small RNAs (sRNAs) in conjunction with Hfq, enabling the bacteria to rapidly respond to changes in the environment. Hfq is a conserved global post-transcriptional regulator, originally identified as an essential factor for phage Q β replication (Franze de Fernandez *et al.*, 1968), which functions in a hexameric ring-shaped structure as an RNA chaperone (Møller *et al.*, 2002; Schumacher *et al.*, 2002; Moll *et al.*, 2003; Geissmann and Touati, 2004; Brennan and Link, 2007) by mediating the binding of sRNAs preferentially to single-stranded AU-rich regions of messenger RNA (mRNA) targets (Zhang *et al.*, 2002; Brescia *et al.*, 2003; Kawamoto *et al.*, 2006). Small RNAs often bind to the 5'-untranslated leader region of cognate messengers to modulate their stability and/or translation (for reviews see Valentin-Hansen *et al.*, 2004; Majdalani *et al.*, 2005; Repoila and Darfeuille, 2009; Waters and Storz, 2009). A classic example is Hfq-mediated regulation of the global regulators RpoS and H-NS by sRNAs including DsrA. Upon binding to *rpoS* and *hns* mRNAs, DsrA stimulates translation and decreases messenger stability, respectively (for reviews see Lease and Belfort, 2000; Repoila and Gottesman, 2003; Majdalani *et al.*, 2005). Since the RpoS and H-NS regulons are important for the general stress response, a *hfq* mutant shows pleiotropic phenotypes (Tsui *et al.*, 1994). In recent years, Hfq has been established as an important virulence factor in bacterial pathogens including *Salmonella enterica* serovar Typhimurium (Sittka *et al.*, 2007; Wilson *et al.*, 2007), *Shigella sonnei* (Mitobe *et al.*, 2008), *Vibrio cholerae* (Ding *et al.*, 2004), *Pseudomonas aeruginosa* (Sonnleitner *et al.*, 2003), *Brucella abortus* (Robertson and Roop, Jr., 1999), *Yersinia enterocolitica* (Nakao *et al.*, 1995), *Legionella pneumophila* (McNealy *et al.*, 2005), *Listeria monocytogenes* (Christiansen *et al.*, 2006), *Neisseria meningitidis* (Fantappiè *et al.*, 2009), *Francisella tularensis* (Meibom *et al.*, 2009) and uropathogenic *E. coli* (Kulesus *et al.*, 2008). Although post-transcriptional regulation for EHEC virulence gene expression occurs (Roe *et al.*, 2003; 2004; 2007; Walters and Sperandio, 2006; Campellone *et al.*, 2007; Lodato and Kaper,

2008) a regulatory role of Hfq in EHEC virulence gene expression has yet to be defined. Here, we demonstrate that Hfq negatively affects LEE expression in exponential phase by regulating the expression of the LEE-encoded regulators GrlA and GrlR at the post-transcriptional level by destabilizing *grlRA* mRNA. In addition, we show that Hfq negatively affects LEE expression in stationary phase independent of *grlRA*. Our data suggest that Hfq plays an important role in controlling GrlA and GrlR levels to prevent induction of LEE expression during exponential growth, and thereby ensure proper temporal expression of the LEE.

Results

Hfq affects EHEC acid resistance, motility and protein secretion

To evaluate the effect of *hfq* on EHEC virulence, we constructed an in-frame deletion of *hfq* in the EHEC O157:H7 EDL933 *stx* mutant background, TUV93-0. Acid resistance is an essential virulence trait of EHEC O157:H7 as it enables survival of bacteria passing the acidic gastric barrier of the host (Price *et al.*, 2000). Since Hfq affects the expression of RpoS and H-NS regulons, Hfq is important for bacteria to respond to various environmental stresses such as low pH conditions they encounter during infection (Lin *et al.*, 1995; Atlung and Ingmer, 1997; Valentin-Hansen *et al.*, 2004). To determine the acid tolerance of an EHEC *hfq* mutant, we compared survival rates of stationary-phase cells of wild type and *hfq* mutant strains after one-hour of exposure to pH 2.5. The *hfq* mutant was significantly ($P < 0.004$) more sensitive to acid shock compared to the wild type and the complemented *hfq* mutant (Table 1), indicating an important role of *hfq* in acid tolerance of EHEC. The acid sensitive phenotype of the EHEC *hfq* mutant is likely due to strongly reduced RpoS levels (data not shown), which is consistent with previous findings for *hfq* mutants of *E. coli* K-12 and *Salmonella enterica* Typhimurium (Muffler *et al.*, 1996; Brown and Elliott, 1996).

The deletion of *hfq* impairs motility of the pathogens *Pseudomonas aeruginosa*, *Salmonella enterica* Typhimurium and uropathogenic *E. coli* (Sonnleitner *et al.*, 2003; Sittka *et al.*, 2007; Kulesus *et al.*, 2008). To test whether Hfq affects motility of EHEC, wild type and *hfq* mutant strains were grown on semi-solid agar plates. The *hfq* mutant showed significantly ($P < 0.02$) decreased motility compared to the wild type, as determined by the diameter of the swarming ring, whereas motility was restored in a complemented *hfq* mutant (Table 1).

Protein secretion and subsequent translocation of effectors into the host cell by the TTSS apparatus is crucial for A/E lesion formation to occur, and thereby for virulence of EHEC (Garmendia *et al.*, 2005). Indeed, deletion of *hfq* in *Salmonella enterica* Typhimurium abolishes secretion of effectors encoded by the pathogenicity island-1 (SPI-1), thereby reducing virulence (Sittka *et al.*, 2007). To assess the role of *hfq* in EHEC protein secretion, we compared the abundance of proteins in culture supernatants from TUV93-0 wild type and *hfq* mutant strains grown aerobically in DMEM. Proteins from equal volumes of culture supernatant were resolved by SDS-PAGE and visualized by silver staining. The *hfq* mutant exhibited increased levels of protein secretion in both exponential and transition phases compared to wild type (Fig. 1A, compare lanes 1 and 4 with 2 and 5), whereas the complemented *hfq* mutant had protein secretion restored to wild type levels (Fig. 1A, lanes 3 and 6). Comparison of the protein profiles revealed that the *hfq* mutant prematurely releases proteins in levels that exceeded those for wild type in the transition to stationary phase (Fig. 1A, compare lanes 2 and 5 with lane 4). The most abundant protein released to culture supernatants by the *hfq* mutant was identified as EspB by Western blot analyses (data not shown), suggesting a central role of Hfq in controlling LEE protein levels. Since Western blot analyses further revealed significantly increased levels of the cytoplasmic chaperonin GroEL in culture supernatants of the *hfq* mutant (data not shown), the increased abundance of proteins in these supernatants is most likely due to increased membrane perturbation,

which is consistent with the involvement of Hfq in the regulation of the σ^E -mediated envelope stress response (Guisbert *et al.*, 2007; Valentin-Hansen *et al.*, 2007).

LEE expression occurs prematurely at increased levels in a *hfq* mutant

To test whether Hfq affects LEE expression, we examined the temporal expression pattern of LEE-encoded proteins in TUV93-0 wild type, a *hfq* mutant and a complemented *hfq* mutant grown in both DMEM and LB using Western blot analyses (Fig. 1B and C, respectively). The LEE-encoded proteins EspA, EspB and Tir were expressed in a growth phase-dependent manner in the wild type with highest abundance in late exponential and stationary phases (Fig. 1B and C, lanes 1–4). Tir levels in the wild type were only detected by overexposing the immunoblot (data not shown). The *hfq* mutant expressed EspA, EspB and Tir prematurely in exponential phase and throughout growth at increased levels compared to wild type (Fig. 1B and C, lanes 5–8). Quantification of the immunoblots revealed that protein levels of EspA, EspB and Tir were increased by respectively 30, 14 and 16-fold in the *hfq* mutant grown in DMEM, and 19, 5 and 5-fold in the mutant grown in LB, compared to maximum levels detected for the wild type (Fig. 1B and C, lane 5 relative to 4). Consistent with our findings, while this paper was under review, an EHEC *hfq* mutant was reported to express elevated levels of EspA and EspB (Hirakawa *et al.*, 2009). The increased abundance of LEE-encoded proteins in the *hfq* mutant was abolished upon complementation with pAMH100 but not the empty vector pBR322 (Fig. 1B and C, lanes 9–12; data not shown). Moreover, the *hfq* mutant expressed the *LEE5*-encoded chaperone CesT and the LEE-encoded effector EspG prematurely at increased levels (data not shown). These results suggest that Hfq plays an important role in coordinating the temporal expression of both LEE- and non-LEE-encoded proteins. The increased expression of proteins in the *hfq* mutant was accompanied by a longer generation time (g) compared to wild type. Because the *hfq* mutant grew slower in DMEM ($g_{WT} \sim 25$ min and $g_{hfq} \sim 60$ min) than in LB ($g_{WT} \sim 25$ min and $g_{hfq} \sim 40$ min), LB medium was used for cell growth in the experiments below unless stated otherwise. The decreased growth phenotype of the *hfq* mutant, shared by other bacterial pathogens (Ding *et al.*, 2004; Sittka *et al.*, 2007; Attia *et al.*, 2008), might be due to drastically increased energy consumption caused by elevated LEE expression and subsequent protein secretion since a *hfq ler* double mutant, devoid of LEE expression, exhibited similar growth as an *E. coli* K-12 *hfq* mutant strain (data not shown).

To determine whether Hfq affects LEE expression on the transcriptional or post-transcriptional level, we examined LEE transcription in wild type and *hfq* mutant strains grown to exponential and stationary phases in LB. The relative expression of *ler* (*LEE1*), *sepZ* (*LEE2*), *escV* (*LEE3*), *espB* (*LEE4*), *tir* (*LEE5*) and the control *rpoB* was measured by quantitative real-time PCR (qRT-PCR) and normalized to 16S rRNA levels. Transcription of LEE in the *hfq* mutant grown to the exponential phase was significantly increased by 12 to 41-fold relative to wild type TUV93-0 ($P < 0.002$), whereas levels of the control transcript *rpoB* did not change significantly (Fig. 2A). Moreover, levels of LEE-encoded transcripts of the *hfq* mutant in exponential phase significantly exceeded those of wild type in stationary phase, which represent the maximum levels for the wild type, by 3 to 7-fold ($P < 0.002$). LEE transcription in stationary phase in the *hfq* mutant relative to wild type was also significantly increased but to a lesser degree than in exponential phase by 3 to 6-fold for *LEE2-5* ($P < 0.002$), while *LEE1* transcript levels did not change significantly (Fig. 2B). The finding that transcript levels of *LEE2-5* in the *hfq* mutant were the same in exponential and stationary phase (data not shown) correlates with the continuously increased levels of LEE proteins in the *hfq* mutant throughout growth (Fig. 1B and C, lanes 5–8). Thus, Hfq negatively affects LEE transcription especially during exponential growth. As Ler is an activator of LEE expression (Elliott *et al.*, 2000), increased abundance of the *ler* transcript in

exponential phase, and consequently increased Ler levels, most likely accounts for the increased LEE transcription in the *hfq* mutant.

Hfq negatively affects Ler levels

Since Hfq often affects gene expression by controlling the levels of global regulators, Hfq could potentially affect LEE transcription through post-transcriptional regulation of *ler*. The *LEE1* operon in EHEC is expressed from two promoters, the proximal and distal promoters, with transcriptional start sites located 32 nt and 163 nt upstream of the *ler* translation initiation codon, respectively (Sperandio *et al.*, 2002; Yerushalmi *et al.*, 2008). The *ler* messenger is primarily expressed from the distal promoter (Sperandio *et al.*, 2002), resulting in a transcript encoding a 163 nt-long untranslated 5'-untranslated leader region, which provides a potential target for Hfq-mediated regulation by small RNAs. The length of a leader region was shown to determine the efficiency of interaction between Hfq, a small RNA and target mRNA (Soper and Woodson, 2008; Updegrave *et al.*, 2008). To test whether the *ler* mRNA leader region is required for the Hfq-mediated regulation of *LEE1*, we deleted the region between the transcription initiation sites of the two promoters including the proximal promoter and ~130 nt of the leader region. The resulting construct, pAMH105, expressed a *ler* transcript encoding a truncated 5' leader from the distal promoter (Fig. 3A). Western blot analyses of His-tagged Ler, expressed from the two native *ler* promoters (pAMH103) and from the distal *ler* promoter in the presence of a truncated leader region (pAMH105), revealed a two to three-fold increase in Ler expression from both constructs in *ler* mutant backgrounds in the absence of *hfq* (Fig. 3B, compare lanes 1 and 4 with 2 and 5), whereas no Ler was detected in a LEE-deleted strain as expected (Fig. 3B, lanes 3 and 6). This result indicates that the *ler* messenger leader region is not required for Hfq-mediated regulation of Ler levels. The five-fold decrease in Ler expression observed from the construct encoding a truncated leader sequence (pAMH105) relative to the wild type construct (pAMH103) (Fig. 3B compare lanes 1 and 4) could be due to the absence of the proximal *ler* promoter in pAMH105 or alternatively due to a regulatory effect of the *ler* leader region on Ler expression.

To test whether Ler is required for a Hfq-mediated increase in expression from the *ler* promoter, we examined levels of Kan-His expressed from an in-frame kanamycin cassette under the control of a native *ler* promoter (pAMH113, fig. 3A) in *ler* and *ler hfq* mutant backgrounds. Western blot analyses revealed increased levels of Kan-His in the *ler hfq* double mutant in both exponential and stationary phase cells by four and twelve-fold, respectively, compared to the *ler* mutant (Fig. 3C, compare lanes 1–2 and 3–4). Furthermore, we measured transcript levels by qRT-PCR analyses using primers specific to the *ler* leader sequence of a corresponding chromosome-encoded in-frame kanamycin cassette, expressed from the native *ler* promoter during exponential growth of TUV93-0 $\Delta ler::kan$ and its *hfq* mutant derivative. Results revealed a four to five-fold (4.6 ± 0.2 , $P < 0.01$) increase in transcription from the *ler* promoter in the *ler hfq* mutant compared to the *ler* mutant. These findings indicate that Hfq negatively affects *LEE1* expression independent of Ler, possibly by controlling levels of a transcriptional regulator of *LEE1*. It should be noted that Hfq controls the transcriptional repressor of *LEE1*, H-NS (Porter *et al.*, 2005). However, as Hfq and DsrA act in concert to negatively regulate H-NS, levels of H-NS are increased in a *hfq* mutant (Lease *et al.*, 1998; Sledjeski *et al.*, 2001), and LEE expression would therefore be expected to decrease. Thus, the effect of Hfq on LEE expression is unlikely to be mediated by H-NS.

Hfq affects LEE expression in EPEC

Because transcriptional regulation of *LEE1* in EHEC and EPEC is similar (Mellies *et al.*, 2007), Hfq is likely to affect LEE expression in EPEC as well. To investigate whether Hfq

also negatively modulates LEE expression in EPEC, we deleted *hfq* in EPEC strain E2348/69 using lambda-red-mediated recombination as described in *Experimental procedures*. Western blot analyses revealed increased expression of EPEC Ler-His from pAMH104 in exponential and stationary phase cells of the *hfq* mutant compared to wild type (Fig. 4A, compare lanes 1 and 3 with 2 and 4). Hence, levels of EspB in the *hfq* mutant were also increased (Fig. 4B, lanes 2 and 4). Among the multiple transcriptional activators described for EPEC *LEE1* is PerC, which is encoded by the *perABC* operon located on the EPEC adherence factor plasmid pEAF (Mellies *et al.*, 1999; Porter *et al.*, 2004). To test whether Hfq affects LEE expression in EPEC through regulation of *per*, we deleted *hfq* in the E2348/69 *per* mutant derivative, JPN15, and examined the expression of EspB by Western blot analyses. Results revealed a similar increase in EspB levels in *hfq per* double and *hfq* single mutants (Fig. 4C, compare lanes 2 and 6 with 4 and 8), indicating that the abundant LEE expression in a *hfq* mutant mostly occurs independent of *per*. However, EspB levels in exponential phase cells of the *hfq per* double mutant were three-fold lower than in the *hfq* mutant (Fig. 4C, lanes 2 and 4), yet significantly increased relative to the *per* single mutant (Fig. 4C, lane 3). Thus, we cannot rule out the possibility that Hfq in part affects EPEC LEE expression by indirectly affecting *per* expression. Previous work from our lab demonstrated that transcription of *perC* is indirectly negatively regulated by the acid resistance regulator GadX (Shin *et al.*, 2001), which inhibits the expression of the *per* activator PerA and thereby PerC under low pH conditions (Shin *et al.*, 2001; Porter *et al.*, 2004). Since the Hfq-associated small RNA GadY positively regulates *gadX* mRNA stability (Opdyke *et al.*, 2004), Hfq most likely affects EPEC LEE expression at low pH in conjunction with GadY by indirectly affecting *perA*, and thereby *perC* expression, through post-transcriptional regulation of *gadX*. However, we did not address the effect of Hfq during growth at low pH as it was beyond the scope of this study.

Hfq negatively controls LEE expression through *grlRA*

Besides Ler, the LEE encodes at least three regulators (GrlA, GrlR and Mcp) that affect LEE expression (Deng *et al.*, 2004; Tsai *et al.*, 2006). Whereas Mcp affects Ler function, GrlA and GrlR regulate *LEE1* transcription. Since GrlA controls the spatiotemporal transcription of LEE-encoded genes by optimizing *LEE1* transcription, and thus Ler expression (Barba *et al.*, 2005), we raised the question of whether Hfq mediates the regulation of LEE expression through GrlA and GrlR. To address this issue, we deleted the *grlRA* operon in EHEC TUV93-0, and subsequently introduced an in-frame deletion of *hfq* creating a *grlRA hfq* double mutant. To determine the role of *grlRA* in the Hfq-mediated regulation of LEE, we examined protein levels of Tir, EspA and EspB in exponential (Fig. 5, lanes 1–6) and stationary phase cells (Fig. 5, lanes 7–12). Interestingly, the prematurely increased expression of LEE-encoded proteins in the *hfq* mutant (Fig. 5, lane 2) was absent in the *grlRA hfq* double mutant in exponential phase (Fig. 5, lane 4), indicating that *grlRA* is epistatic to *hfq* in regulating LEE expression. As expected, complementing the *grlRA hfq* mutant with *grlRA* from pAMH116 restored LEE protein levels nearly to that of the *hfq* mutant (Fig. 5, lane 5), whereas complementation with either *hfq* from pAMH100 or the empty vector did not affect protein levels (Fig. 5, lane 6; data not shown). Moreover, complementing the *grlRA hfq* mutant with *grlA* expressed *in trans* from an arabinose inducible promoter was sufficient to restore LEE expression to levels of a *hfq* mutant (data not shown), suggesting that the effect of Hfq on LEE expression in exponential phase is mediated by *grlA*. Interestingly, the effect of *grlA* on the Hfq-mediated regulation of LEE expression was abolished in stationary phase cells, as LEE protein levels in the *grlRA hfq* mutant were increased to the levels of a *hfq* mutant (Fig. 5, compare lanes 8 and 10). Complementation of stationary phase *grlRA hfq* cells with *grlRA* from pAMH116 did not affect levels of LEE-encoded proteins (Fig. 5, compare lanes 10 and 11), whereas complementation with *hfq* from pAMH100 decreased protein levels to that of the *grlRA*

mutant as anticipated (Fig. 5, compare lanes 9 and 12). Furthermore, qRT-PCR analyses revealed a 44-fold (44 ± 0.7 , $P < 0.0007$) increase in *ler* transcript levels in a *hfq* mutant relative to a *hfq grlRA* mutant in exponential phase, whereas *ler* transcription was unaffected in stationary phase (0.75 ± 0.17 , $P = 0.08$), indicating that *grlRA* affects *ler* transcript levels primarily in exponential phase. These results indicate that the Hfq-mediated regulation of LEE expression through *grlRA* occurs specifically in exponential phase, and that Hfq affects LEE expression in stationary phase independent of *grlRA*.

The increased levels of EspA, EspB and Tir in the *grlRA hfq* mutant in stationary phase could be due to either increased transcript abundance or protein stability. To determine whether the *grlRA*-independent effect of Hfq on LEE expression in stationary phase was due to increased transcription, we used qRT-PCR to compare *espB* transcript levels in exponential and stationary phase cells of the *hfq grlRA* mutant. Results revealed a seven-fold (7 ± 1.5 , $P < 0.01$) increase in *espB* transcript levels in stationary compared to exponential phase, indicating that increased EspB protein levels in stationary phase cells of the *hfq grlRA* mutant are due to increased *espB* transcript abundance. Furthermore, we measured levels of *ler* transcript in exponential and stationary phase cells of the *hfq grlRA* mutant using qRT-PCR, and found that *ler* transcript levels were five-fold (5 ± 0.7 , $P < 0.0001$) increased in the *hfq grlRA* mutant in stationary relative to exponential phase. Thus, increased levels of *ler* transcripts in stationary phase, followed by an increase in LEE transcription, could account for the observed increase in LEE-encoded proteins in stationary phase cells of the *hfq grlRA* mutant (Fig. 5, lane 10). To test whether RpoS is involved in the Hfq-mediated regulation of LEE expression in stationary phase, we expressed RpoS *in trans* from an arabinose inducible promoter in the *hfq* mutant to levels comparable with that of the wild type strain, and determined levels of EspB and Tir by Western blot analyses. Results revealed increased levels of EspB and Tir in the *hfq* mutant regardless of the presence of RpoS (data not shown), suggesting that RpoS is not implicated in the Hfq-mediated regulation of LEE expression in stationary phase.

Hfq regulates *grlRA* expression independent of Ler

The increased expression of LEE in exponential phase *hfq* mutant cells was absent in the *hfq grlRA* double mutant, raising the question of whether Hfq affects expression of *grlRA*. To investigate this, we used Western analyses to examine protein levels of His-tagged GrlA (pAMH117) and GrlR (pAMH118) in TUV93-0 wild type and *hfq* mutant backgrounds. Both exponential and stationary phase cells of the *hfq* mutant expressed significantly increased levels of GrlA-His (Fig. 6A, lanes 2 and 4) and GrlR-His (Fig. 6B, lanes 2 and 4) compared to wild type, indicating that Hfq negatively affects GrlA and GrlR protein levels. Likewise, Western analyses revealed increased levels of GrlA-His in a *hfq* mutant of EPEC strain E2348/69 grown to exponential and stationary phases (data not shown), suggesting a similar regulatory mechanism by Hfq in these EHEC and EPEC backgrounds.

Ler-independent expression of *grlRA* from its native promoter as reflected by transcriptional activation of EHEC LEE was observed in *E. coli* K-12 (Russell *et al.*, 2007). This along with our finding that Hfq negatively regulated expression of *LEE1* in the absence of *ler* (Fig. 3C) raised the question of whether Hfq regulates *grlRA* independent of Ler. To address this, we examined the expression of His-tagged GrlA and GrlR in *ler* and *ler hfq* mutant strains by Western blot analyses. Results revealed an increased expression of GrlA-His (Fig. 6C, lanes 2 and 4) and GrlR-His (Fig. 6D, lanes 2 and 4) in the *hfq* mutant background in the absence of *ler*. Thus, Hfq affects LEE expression in exponential phase through negative regulation of GrlA and GrlR levels independent of Ler.

Hfq controls *grlRA* transcript abundance post-transcriptionally by affecting messenger stability

To examine if increased protein levels of GrlA and GrlR in the *hfq* mutant was due to increased transcript levels, we used qRT-PCR to quantify the amount of *grlA* and *grlR* transcripts in total RNA isolated from wild type and *hfq* mutant strains grown to exponential phase in LB. Indeed, levels of *grlA* and *grlR* transcripts were increased in the *hfq* mutant compared to wild type by sixteen-fold (16 ± 1 , $P < 0.003$) and eleven-fold (11 ± 1 , $P < 0.001$), respectively. The increase in *grlA* transcript levels was less severe with a five-fold (5 ± 1 , $P < 0.001$) increase in the *hfq ler* double mutant compared to the *ler* mutant. This might be due to the absence of a Ler-GrlA positive regulatory loop in the *hfq ler* background. The abundance of *grlRA* transcript in the *hfq* mutant could be due to either increased transcription or increased messenger stability. First, we determined whether the increased *grlRA* transcript level in a *hfq* mutant was due to increased transcription from the *grlRA* operon promoter. To this end, we measured the level of transcription from the *grlRA* promoter in exponentially grown *grlRA* and *grlRA hfq* mutant strains, which have the coding region of *grlRA* replaced with a scar sequence that was introduced upon deletion, by qRT-PCR analyses using oligos specific to the *grlRA* leader region and the scar sequence, respectively, as described in *Experimental procedures*. Transcriptional activity from the *grlRA* promoter was similar in the *grlRA* and *grlRA hfq* mutant backgrounds, as reflected by a relative fold difference in transcript levels of about one (1.2 ± 0.1 , $P = 0.2$), indicating that Hfq more likely affects *grlRA* messenger stability. Second, to determine *grlRA* mRNA stability in wild type and *hfq* mutant strains, we grew the strains in LB to exponential phase, then treated cells with rifampicin to stop additional transcription, and extracted RNA at various times thereafter. The amount of *grlA* transcript remaining at 0, 4, 8 and 12 minutes upon transcription inhibition was measured by qRT-PCR using oligos specific to *grlA*. The *grlA* messenger was more stable in the *hfq* mutant, with 33% of transcripts remaining compared to 9% in the wild type, after four minutes of rifampicin exposure (Fig. 6E). The 16S rRNA levels remained unchanged in both strains at all time points tested (data not shown). The increased stability of the *grlA* messenger in the *hfq* mutant was reflected by a two-fold longer RNA half-life compared to the wild type. Since Northern blot analyses of total RNA using probes specific to *grlR*, *grlA* and *grlRA* revealed similar RNA banding patterns for RNA isolated from wild type and *hfq* mutant strains (data not shown), the stability of the *grlA* transcript in a *hfq* mutant likely also reflects that of the *grlR* transcript, which is apparent as *grlR* and *grlA* are in an operon (Barba *et al.*, 2005). Thus, Hfq controls *grlRA* expression post-transcriptionally by negatively modulating messenger stability.

Hfq-mediated regulation of *grlRA* requires a factor located outside of the LEE

Hfq often affects transcript stability in concerted action with sRNAs. A potential factor, such as a sRNA, mediating the Hfq-dependent regulation of *grlRA* is either located on a pathogenicity island or on the K-12 backbone of the EHEC chromosome. To test whether a potential sRNA is located outside of the LEE pathogenicity island, we constructed a *hfq* mutant derivative of a LEE-deleted EHEC EDL933 strain and measured the expression of GrlA and GrlR from pAMH125 and pAMH126, respectively, in Δ LEE and Δ LEE *hfq* backgrounds. Western analysis revealed increased levels of GrlA and GrlR in the Δ LEE *hfq* mutant relative to the Δ LEE strain especially in exponential phase (Fig. 7A and B, lanes 2 and 4), suggesting that a potential sRNA is located outside of the LEE either on another pathogenicity island or on the K-12 backbone of the chromosome. The *LEE* operons are expressed in *E. coli* K-12 (Mellies *et al.*, 1999), raising the question of whether *hfq* also influences *grlRA* expression in this background. To address this, we examined the expression of GrlA and GrlR from pAMH117 and pAMH118, respectively, in wild type and *hfq* mutant derivatives of the *E. coli* K-12 strain MG1655. Western analyses revealed increased expression of both GrlA and GrlR in the MG1655 *hfq* mutant strain compared to

wild type (Fig. 7C and D, lanes 2 and 4), indicating that a factor(s) such as a sRNA that potentially is required for the Hfq-mediated regulation of *grlRA* is located on the K-12 backbone of the chromosome rather than on an EHEC specific genetic island.

Hfq controls the timing of A/E lesion formation

The expression of the LEE-encoded factors is required for EHEC to trigger actin polymerization, and thus form A/E lesions on the eukaryotic epithelial cell. Since the *hfq* mutant strain prematurely expresses LEE-encoded proteins at increased levels in a *grlRA*-dependent manner, we predicted that Hfq would affect the kinetics of A/E lesion formation. To characterize the effect of Hfq on the ability of EHEC to form A/E lesions *in vitro*, we infected HeLa cells with wild type, *hfq*, *grlRA* and *hfq grlRA* mutant derivatives of strain TUV93-0 for 2, 3, 4 and 6 h. We then determined the presence of A/E lesions using the fluorescent actin staining (FAS) test, where the A/E lesions are visualized as condensed actin beneath the adherent bacteria on infected cells which actin filaments were FITC-phalloidin stained. Figure 8 shows phase-contrast and fluorescence actin images representing FAS assays at 3h and 6 h postinfection. The A/E lesion phenotype of wild type TUV 93-0 was diminished in exponential (3 h postinfection) compared to stationary phase (6 h postinfection) (Fig. 8, panels A and G), which is consistent with a previous study (Campellone *et al.*, 2007). The *hfq* mutant formed A/E lesions prematurely at 3 h postinfection compared to wild type and the complemented mutant (Fig. 8, panels A–C). The premature A/E lesion phenotype was diminished in a *hfq grlRA* double mutant and restored when complementing with *grlRA* from pAMH116 (Fig. 8, panels E–F), which is consistent with Hfq affecting LEE expression through *grlRA* (Fig. 5). Similar results were obtained for FAS assays carried out 2 h postinfection (data not shown). At 6 h postinfection both wild type and *hfq* mutant strains formed A/E lesions with the *hfq* mutant forming a greater number of A/E lesions (Fig. 8, panels G–H). The absence of A/E lesions upon infection with the complemented *hfq* mutant could be due to a two-fold increase in Hfq levels expressed from the mid-copy number plasmid pAMH100 (Fig. 8, panel I). Consistent with data from 3 h postinfection, the enhanced A/E lesion phenotype of the *hfq* mutant at 6 h postinfection was diminished in a *hfq grlRA* double mutant and partially restored in a *grlRA*-complemented *hfq grlRA* mutant (Fig. 8, panels, K–L). Interestingly, regardless of the increased LEE expression in stationary phase (Fig. 5, lane 10), the *hfq grlRA* mutant was only capable of forming A/E lesions at 6 h postinfection, when complemented with *grlRA*. It was previously reported that GrlA-dependent repression of the flagellar operon through *flhD* is crucial for bacterial adhesion to HeLa cells (Iyoda *et al.*, 2006). Thus, the inability of the *hfq grlRA* mutant to form A/E lesions at 6 h postinfection could be due to elevated *flhD* expression, leading to decreased cell adhesion of this mutant. Altogether, the regulatory effect of Hfq on LEE expression correlates with the A/E phenotype of a *hfq* mutant strain.

Discussion

Coordinating gene expression in response to environmental stimuli is important for the bacterial pathogen to survive in hostile environments in the host and to evade the host immune response, thereby successfully colonizing the host intestine. Consequently, virulence specific regulators often are subject to both transcriptional and post-transcriptional control through complex regulatory networks that integrate environmental signals. The global regulator Ler coordinates virulence gene expression to ensure optimal fitness of bacteria during infection by differentially regulating expression of the LEE-encoded TTSS and a group four capsule to ensure transient shielding of intimin and the TTSS early in infection (Shifrin *et al.*, 2008). Furthermore, the GrlR-GrlA regulatory system inversely controls the expression of the TTSS, enterohaemolysin and flagella (Iyoda *et al.*, 2006; Saitoh *et al.*, 2008). Whereas the complex regulatory network controlling the induction of

LEE1/ler expression in response to environmental signals has been extensively studied, the regulatory basis for *grlRA* expression is less defined. Our study on the effect of the RNA chaperone Hfq on EHEC virulence gene expression revealed an important role of Hfq in controlling LEE expression. We demonstrated that Hfq negatively affects LEE expression in exponential phase through post-transcriptional regulation of *grlRA*, and in stationary phase independent of the GrlR-GrlA regulatory pathway as depicted in our current model of Hfq-mediated regulation of the LEE (Fig. 9). We further showed that the regulatory effect of Hfq on LEE is reflected by a premature and enhanced A/E histopathological phenotype of a *hfq* mutant strain.

Many virulence genes acquired by horizontal transfer, including LEE island-encoded genes (Perna *et al.*, 1998), are silenced by H-NS (Deng *et al.*, 2001; Navarre *et al.*, 2006). H-NS levels change during growth with high levels present in exponential phase followed by decreased levels in stationary phase, consequently, the expression of the H-NS regulon including the LEE is growth phase-dependent (Ali *et al.*, 1999; Roe *et al.*, 2003; Hansen *et al.*, 2005). Counter silencing by DNA-binding H-NS paralogues such as Ler, is among the mechanisms by which H-NS-mediated repression is overcome (reviewed by Stoebel *et al.*, 2008). Thus, increased Ler levels are most likely required to antagonize H-NS-mediated repression for LEE expression to occur in exponential phase. We demonstrated that the requirement of *grlRA* for LEE expression to occur in a *hfq* mutant during exponential growth is diminished in stationary phase, suggesting that GrlA is primarily required for the induction of *ler* expression during conditions when H-NS levels are increased. Indeed, a recent study showed that GrlA positively controls *ler* expression in a H-NS-dependent pathway (Laaberki *et al.*, 2006). The Hfq-mediated control of *grlRA* expression occurs independent of *ler* (Fig. 6C and D), indicating that induction of *grlRA* expression occurs independent from the Ler-GrlA positive regulatory pathway. Thus, expression of GrlA is most likely crucial for initiating the Ler-GrlA positive regulatory loop.

In line with the finding that Hfq prevents the induction of LEE expression in exponential phase through *grlRA*, we showed that Hfq negatively regulates *grlRA* transcript abundance and consequently GrlR and GrlA protein levels (Fig. 6A and B). We demonstrated that the *grlA* transcript during exponential phase is more stable in a *hfq* mutant compared to wild type (Fig. 6E), indicating that Hfq affects *grlRA* expression on the post-transcriptional level by destabilizing *grlRA* mRNA. Hfq-mediated destabilization of mRNAs often involves the concerted action with sRNAs most of which promote target mRNA decay (Aiba, 2007; Repoila and Darfeuille, 2009). However, whether Hfq regulation of *grlRA* transcript stability involves a sRNA has yet to be determined. While Hfq acts as an adaptor between the sRNAs and RNaseE, the sRNAs recruit the degradosome to target mRNAs, thereby promoting rapid degradation of mRNA (Morita *et al.*, 2005). We showed that Hfq also negatively affects GrlA and GrlR levels in an *E. coli* K-12 background (Fig. 7), suggesting that a sRNA, which along with Hfq potentially mediates the decay of *grlRA*, is encoded by the K-12 backbone of the EHEC and EPEC chromosomes. Interestingly, a previous study identified a negative regulatory *cis*-acting element, located between positions +143 to +397 relative to the transcription initiation site of *grlR*, which in addition to H-NS negatively affects *grlRA* expression (Barba *et al.*, 2005). This element could provide a potential target for post-transcriptional regulation by a sRNA. Alternatively, Hfq also affects messenger stability by regulating polyadenylation-dependent messenger decay (Mohanty *et al.*, 2004). We are currently investigating whether the Hfq-mediated destabilization of *grlRA* mRNA involves a sRNA.

Regulation by Hfq in conjunction with sRNAs is an important means to control temporal expression of virulence-specific regulators by bacterial pathogens including *Vibrio cholerae* and *Salmonella enterica* Typhimurium, where Hfq and sRNAs affect levels of the virulence

gene master regulator HapR (Lenz *et al.*, 2004) and the major activator of the SPI-1-encoded TTSS and effector genes Hila (Sittka *et al.*, 2007), respectively. Here we show, to our knowledge for the first time, that Hfq controls the temporal expression of virulence genes in EHEC and EPEC through post-transcriptional control of the LEE-encoded regulators GrlA and GrlR. We showed that LEE expression in exponential phase is negatively regulated by the Hfq-mediated regulation of GrlA and GrlR (Fig. 5 and Fig. 6). This suggests that a signal inducing GrlA expression during exponential growth is required to relieve the Hfq-mediated repression of *grlRA*, for LEE expression to occur in a growth phase-independent manner. Interestingly, qRT-PCR analyses revealed similar levels of *grlRA* mRNA in exponential and stationary phases (1.5 ± 0.1 , $P = 0.04$), suggesting that a pool of *grlRA* mRNA, available throughout growth, enables cells to rapidly synthesize Grl proteins in response to environmental signals whenever needed. It has yet to be determined whether certain environmental conditions or the host induces such a signal, which is expected to negatively affect the abundance of a putative sRNA that could cause the destabilization of the *grlRA* messenger. LEE expression is activated by quorum sensing via the signaling molecules AI-3, epinephrine and norepinephrine, which signal through the QseEF two-component system to direct transcriptional activation of *LEE1* by QseA (Sperandio *et al.*, 1999; 2003; Walters and Sperandio, 2006; Sharp and Sperandio, 2007). Although Western blot analyses of EspB expression in a *hfq grlRA* mutant revealed that *qseA* is not epistatic to *hfq* in regulating LEE expression (data not shown), we cannot exclude the possibility that quorum sensing provides a signal to relieve the Hfq-mediated decay of *grlRA* mRNA. In addition to controlling *grlRA* mRNA stability in exponential phase (Fig. 6E), we showed that Hfq negatively regulates LEE expression in stationary phase by affecting *ler* transcript abundance and thereby Ler levels independent of *grlRA* (Fig. 5, data not shown). However, the mechanism behind Hfq-mediated regulation of *ler* in stationary phase has yet to be resolved. Moreover, Hfq might be involved in the post-transcriptional regulation of Tir and EspA, which is mediated by non-LEE-encoded factors and results in heterogeneous expression within an EHEC cell population (Roe *et al.*, 2003; 2004).

We showed that Hfq negatively controls levels of GrlA in exponential phase to ensure proper temporal expression of the LEE. By regulating GrlA, Hfq in turn affects expression of the two global regulators, Ler and FlhD, important in coordinating virulence and flagellar gene expression, respectively (Iyoda *et al.*, 2006; Abe *et al.*, 2008). The important impact of Hfq on the virulence potential of EHEC is reflected by the following phenotypes of the *hfq* mutant: (i) The *hfq* mutant of EHEC has decreased motility (Table 1), which most likely is caused by increased GrlA levels (Iyoda *et al.*, 2006) and might reduce the ability of the mutant to penetrate the intestinal mucus layer to colonize the host. (ii) The *hfq* mutant expresses strongly reduced RpoS levels leading to vulnerability to a variety of stress conditions including acid stress (Table 1), which diminishes its ability to survive passage through the acidic gastric barrier (Lin *et al.*, 1995; Price *et al.*, 2000). (iii) The *hfq* mutant expresses the virulence-associated regulators Ler, GrlR and GrlA prematurely at increased levels (Fig. 3 and Fig. 6), thereby not only disrupting proper temporal expression of virulence genes but also expending energy unnecessarily on protein synthesis (Fig. 1). Untimely expression of the TTSS during infection may expose the bacteria to the host immune system (Shifrin *et al.*, 2008). Thus, despite an increased ability to form A/E lesions on HeLa cells, the *hfq* mutant is expected to exhibit strongly reduced virulence due to the inability to appropriately coordinate the expression of stress response and virulence genes. In summary, our data establish a role of Hfq in virulence gene regulation of EHEC, where through negative post-transcriptional regulation of *grlRA* expression, Hfq ensures repression of LEE expression during exponential growth, and yet provides a means to induce *grlRA* and subsequently LEE expression if necessary. We are currently investigating the mechanism by which Hfq destabilizes the *grlRA* messenger with emphasis on identifying a potential Hfq-associated sRNA involved in the decay of *grlRA* mRNA.

Experimental procedures

Standard procedures

Standard DNA techniques, liquid media and agar plates were used as described (Sambrook and Russell, 2001). Restriction endonucleases, T4 DNA kinase and ligase were used as recommended by the manufacturer (New England Biolabs). DNA used for cloning and recombination purposes was PCR amplified using the high-fidelity DNA polymerases PfuUltra™ II Fusion HS or Easy-A™ (Stratagene). The University of Maryland Biopolymer Core Facility performed primer synthesis and DNA sequencing. Bacterial techniques were carried out as described (Miller, 1972). Bacteria were grown at 37°C in LB or DMEM (Invitrogen #11965) media supplemented with ampicillin (100 µg/ml), chloramphenicol (cm) (30 µg/ml), kanamycin (kan) (50 µg/ml) or tetracycline (20 µg/ml) as needed. HeLa cells (ATTC #CCL-2) were cultured in DMEM/F12 (Invitrogen #11330) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C in 5% CO₂.

Bacterial strain constructions

Bacterial strains used in this study are listed in Table 1. Gene deletions in *E. coli* K-12 MG1655, EHEC TUV93-0, EDL933 ΔLEE, EPEC E2348/69 and JPN15 were constructed using Lambda Red-mediated recombination using linear DNA fragments as described (Datsenko and Wanner, 2000; Murphy and Campellone, 2003). To generate deletions in *hfq* and *grlRA*, DNA fragments encoding a kanamycin resistance cassette flanked by Flipase Recognition Target (FRT) site-inverted repeats and sequences homologous to regions flanking the gene of interest, were PCR amplified from pKD13 using primer sets K4928/K5045 and K5663/K5664, respectively (oligonucleotides are listed in Table 3). The DNA fragments were electroporated into appropriate target strains, where the Red recombinase system was expressed from arabinose and IPTG inducible promoters on pKD46 and pKM201, respectively, to allow recombination into the chromosome. Recombinants were selected and purified on LB agar plates containing kanamycin at 37°C. The kanamycin resistance marker was eliminated from recombinant strains using a pCP20-encoded FLP flipase, which acts specifically on FRT sites when expressed at 42°C. The plasmids pKD46, pKM201 and pCP20 encode temperature-sensitive replicons and were cured by growing cells at 37°C or above. Strain EDL933 ΔLEE was deleted for *hfq* by recombining a DNA fragment, which was PCR amplified from TUV93-0 Δ*hfq::kan* genomic DNA (gDNA) using primers K5445/K5446, into EDL933 ΔLEE expressing the Red recombinase system from pTP223. The resulting strains deleted for *hfq* (AMH100, AMH109-112, and AMH115) or *grlRA* (AMH108) carried deletions of 303 and 828 nt starting from the translation initiation codon, respectively, which in place of the disrupted gene was proceeded by an 81 nt-long in-frame scar sequence that encodes a 27 amino-acid residue peptide. The kanamycin cassette was retained in *hfq* derivatives of EPEC and EDL933 ΔLEE (AMH110-111 and AMH115). Deletion mutants with *ler* replaced by an in-frame kanamycin cassette were generated by recombining a DNA fragment, amplified from SE1101 gDNA with primers K1372/1420, into TUV93-0 and AMH100, creating strains AMH101 and AMH102, respectively. To generate the double mutant strains AMH102 and AMH109, an *hfq* deletion was introduced into the strains as described above. Gene deletions of *hfq*, *ler* and *grlRA* were verified by PCR amplification analysis using the respective primer sets K4785/4786, K1372/K1420 and K5665/K5666. In addition, *hfq* deletions were confirmed by Western blot analysis using an Hfq-specific polyclonal antibody (Kindly provided by Ding J. Jin).

Plasmid constructions

Oligonucleotides used for cloning purposes are listed in Table 3.

pAMH100—A 1378 bp DNA fragment encoding *hfq* was PCR amplified from TUV93-0 gDNA using primers K4980/K4981, digested with *Bam*HI and *Hind*III and cloned into the corresponding sites of pBR322. pAMH100 encodes *hfq* expressed from its three native promoters P1–3 and was used to complement a *hfq* mutant.

pAMH103 and pAMH104—The 796 bp DNA fragments encoding His-tagged EHEC and EPEC Ler expressed from native *ler* promoters were PCR amplified from TUV93-0 and E2348/69 gDNA, respectively, using primers K1372/K4797 and cloned into the *Eco*RI/*Bam*HI sites of pBR322.

pAMH105—A plasmid encoding *ler-his* expressed from a modified *ler* promoter with ~120 nt of the *ler* leader sequence including the proximal *ler* promoter deleted, was obtained by combining two DNA fragments. The two fragments encode a 237 bp region upstream of the transcription initiation site of the distal *ler* promoter and a 425 bp region encoding the region downstream of the transcription initiation site of the proximal *ler* promoter comprising *ler-his*, respectively. The DNA fragments were PCR amplified from TUV93-0 gDNA using primer sets K1372/K5373 and K1420/K5374, phosphorylated using T4 polynucleotide kinase, respectively digested with *Eco*RI and *Bam*HI, and cloned into the corresponding sites of pBR322.

pAMH113—A 856 bp fragment encoding *kan-his* under the control of the *ler* promoter was PCR amplified from SE1101 gDNA using primers K1372/K5425 and cloned into the *Eco*RI/*Bam*HI sites of pBR322.

pAMH116—A 1601 bp DNA fragment encoding *grlRA* and the native promoter was PCR amplified from TUV93-0 gDNA using primers K5665/K5666 and cloned into the *Bam*HI/*Hind*III sites of pBR322.

pAMH117—A 1337 bp DNA fragment encoding *grlRA-his* and the *grlRA* promoter region was amplified from TUV93-0 gDNA with primers K5665/K5667 and cloned into the *Bam*HI/*Hind*III sites of pBR322.

pAMH118—A 869 bp DNA fragment encoding *grlR-his* preceded by the *grlRA* promoter region was amplified from TUV93-0 gDNA using primers K5665/K5822 and cloned into the *Bam*HI/*Hind*III sites of pBR322.

pAMH125—A 1032 bp DNA fragment encoding a cm resistance gene was amplified from pKD3 using primers K5377/K5378 and cloned into the *Msc*I site of pAMH117.

pAMH126—A 1032 bp DNA fragment encoding a cm resistance gene was amplified from pKD3 using primers K5377/K5378 and cloned into the *Msc*I site of pAMH118.

Assays for strain phenotype determinations

Motility assay—The motility of TUV93-0 pBR322, AMH100 pBR322 and AMH100 pAMH100 was determined as described (Bertin *et al.*, 1994). Semi-solid tryptone plates (1% bacto-tryptone, 0.5% NaCl, 0.3% bactoagar, pH 7.4) containing ampicillin were inoculated with a drop of 1×10^9 cells of each strain grown in LB medium at 37°C for 14 h. The motility of the strains was measured after incubation at 37°C for 10 h by measuring the diameter of the swarming ring.

Acid resistance assay—Stationary phase-induced acid resistance was determined for the TUV93-0 and *hfq* mutant strains carrying pBR322 or pAMH100 as described (Masuda and

Church, 2002). Stationary phase cultures (18 h) grown at 37°C in LB medium containing ampicillin were diluted 1:1000 in warm LB, adjusted to pH 2.5 with HCl and incubated for 1 h at 37°C. Serial dilutions of cells in 1X phosphate-buffered saline (PBS, pH 7.4) before and after acid exposure were plated onto LB agar plates and incubated overnight at 37°C. Cell survival was measured as the percentage of colony forming units remaining after exposure to low pH compared to before exposure. Each assay was carried out independently at least three times.

Protein secretion—Overnight cultures of TUV93-0, AMH100 and AMH100 pAMH100 were diluted 1:1000 in DMEM and grown aerobically at 37°C. Culture supernatants were collected at the optical cell densities at 600 nm (OD_{600}) values 0.2 and 1.0 by centrifugation followed by passage through a 0.22- μ m pore size Millex GP filter (Millipore). Secreted proteins were precipitated with 20% (vol/vol) trichloric acid and 5% (vol/vol) deoxycholic acid for 15 min on ice, collected by centrifugation (16,100 \times g for 10 min at 4°C), and resuspended in 2X SDS Blue loading buffer (New England Biolabs) with 0.5 M Tris base added. Protein samples corresponding to 300 μ l of culture supernatant were boiled for 5 min and analyzed by SDS-12% polyacrylamide gel electrophoresis (SDS-PAGE) along with a PAGERULER™ Prestained protein Ladder Plus molecular weight marker (Fermentas). Proteins were visualized using the SilverQuest™ silver staining kit (Invitrogen) as recommended by the manufacturer.

Western analysis

Total protein was prepared from cultures grown in LB or DMEM to the optical cell densities indicated in the figure legends. Overnight cultures were diluted 1:1000 in medium with appropriate antibiotics added to strains carrying plasmids and grown aerobically at 37°C. Total protein was precipitated from cell culture samples with 5% (vol/vol) trichloric acid for 15 min on ice, collected by centrifugation at 16,100 \times g for 10 min at 4°C, and washed with 80% acetone. Precipitated protein was resuspended in 1X SDS Blue loading buffer (New England Biolabs) in volumes normalized to the optical densities of cultures and boiled for 5 min. Protein sample amounts equivalent to 0.03 OD_{600} units of culture were resolved by SDS-PAGE along with a MagicMark™ XP western protein standard (Invitrogen) using 10% NextGel (Amresco) and Criterion precast 4–20% Tris-HCl protein gels (BioRad) according to the manufacturers instructions. Proteins were transferred onto Immobilon-FL polyvinylidene difluoride membranes (Millipore) using a Trans-Blot SD Semi-Dry Transfer Cell (BioRad). Membranes were blocked overnight at 4°C with 5% non-fat drymilk in 1X PBS containing 0.1% Tween-20 (PBST). Polyclonal antibodies produced in rabbits against EspA, EspB, Tir, GroEL (Sigma) and Hfq (Sledjeski *et al.*, 2001) were used diluted 1:6000 in 1X PBST. A monoclonal Tetra-His™ anti-mouse antibody (QIAGEN) was used in a 1:2000 dilution to detect His-tagged proteins. Alexa Fluor 680-conjugated goat anti-rabbit and Alexa Fluor 680-conjugated goat anti-mouse diluted 1:10000 in 1X PBST were used as secondary antibodies. Proteins were visualized and quantified using an Odyssey Infrared Imaging System with application software version 2.1 (Li-Cor) as recommended.

RNA isolation and qRT-PCR analysis

RNA isolation—Cultures of TUV93-0 and mutant derivatives were grown aerobically in LB at 37°C to OD_{600} values of 0.6 and 3.0. RNA used to determine *grlA* mRNA stability was isolated from cultures grown in LB at 37°C to an OD_{600} value of 0.6 (0 min) and 4, 8 and 12 min after rifampicin (250 μ g/ml) addition. Total RNA was isolated from culture samples corresponding to $\sim 2.3 \times 10^{10}$ cells using hot phenol extraction as described (Dam Mikkelsen and Gerdes, 1997). Contaminating DNA in the RNA preparations was removed by treating three times with TURBO™ DNase (Ambion). RNA samples were quantified based on absorption at 260 nm. The integrity of RNA samples was verified by determining

the ratio of absorption at 260 and 280 nm and by agarose gel electrophoresis. Removal of contaminating DNA in the RNA samples was confirmed by PCR analysis using oligos specific to *rrsB* (K5365/K5366, Table 3).

Quantitative Real-Time PCR—Quantitative Real-Time reverse transcription-PCR (qRT-PCR) was performed in a one-step reaction using the Superscript III™ Platinum SYBR Green One-Step qRT-PCR Kit (Invitrogen) and a Chromo 4™ real-time detector attached to a PTC-200 DNA engine base (MJ Research/BioRad). The qRT-PCR reactions contained 100 ng of total RNA in 25 μ l reactions, prepared as described by the manufacturer, and was carried out using the following conditions for cDNA synthesis and amplification: 1 cycle at 50°C for 3 min, 1 cycle at 95°C for 5 min, and 40 cycles at 95°C for 15 s and 60°C for 30 sec. Transcripts encoding *ler*, *sepZ*, *escV*, *espB*, *tir*, *grlA*, *grlR*, *rrsB*, *rpoB*, the *ler* leader and the *grlRA* leader were detected using the primer pairs K5359/K5360, K5833/K5834, K5831/K5832, K5361/K5362, K5930/K5931, K5653/K5656, K6046/K6047, K5365/K5366, K6088/K6089, K6021/K6022 and K6038/K6039, respectively (Table 2). Template specificity for each primer pair was verified by melting curve analysis, where products were heated from 50°C to 95°C during monitoring of fluorescence. The qRT-PCR reaction was carried out in duplicates on RNA isolated from at least three independent cultures.

qRT-PCR data quantification and statistical analysis—Data were collected using the MJ Opticon Monitor analysis software version 3.1 (BioRad), normalized to transcript levels of *rrsB* (16S rRNA), and analyzed using the comparative critical cycle threshold (C_T) method (Livak and Schmittgen, 2001). The expression levels of target genes in wild type and mutant derivatives were compared using the relative quantification method. Statistical significance between means was calculated using the unpaired *t*-test with a threshold *P* value of < 0.01. Relative expression levels are expressed as the means \pm the standard deviation of the $\Delta\Delta C_T$ value.

Fluorescent actin staining test

The ability of EHEC TUV93-0 and mutant derivatives to form A/E lesions on HeLa cell monolayers at 2, 3, 4 and 6 h post infection was evaluated using the fluorescent actin staining (FAS) test as described (Knutton *et al.*, 1989). Bacterial strains were inoculated from single colonies on LB agar plates into tryptic soy broth (TSB), containing antibiotics as needed, and grown without aeration for 16–18 h at 37°C. Prior to infection, strains were diluted 1:5 into the infection medium (DMEM supplemented with 2% FBS and 0.5% mannose) and primed for infection by incubation at 37°C in 5% CO₂ for 2 h. Semiconfluent HeLa cell monolayers were grown on glass coverslips, followed by addition of 2×10^7 bacteria (4 h and 6 h infections) or 4×10^7 bacteria (2 h and 3 h infections). Monolayers were fixed after 2, 3, 4 and 6 h of incubation with 4% formamide in PBS, washed three times with 1X PBS, permeabilized with 0.1% Triton X-100 in PBS, and stained with Alexa Fluor 488 phalloidin (Invitrogen) diluted 1:50. Coverslips were mounted on slides using Prolong Gold antifade reagent (Invitrogen) and the edges of the coverslip were sealed with cytooseal-60 (Richard-Allan Scientific). The FAS assay was carried out independently at least three times for each strain. The samples were visualized using an inverted Nikon TE-2000 microscope equipped with a 40X objective.

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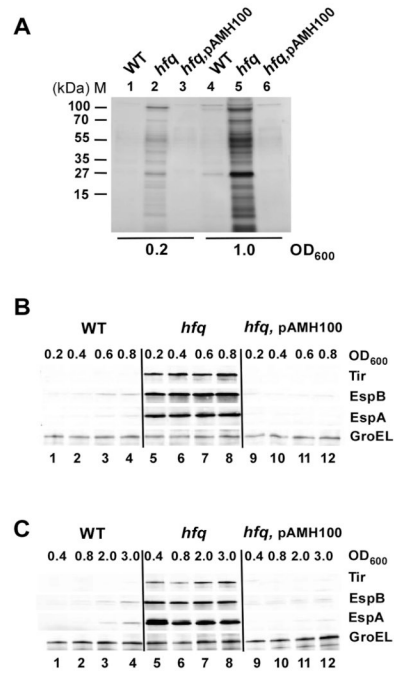


Figure 1.

Hfq affects the abundance of proteins in culture supernatants and LEE expression in EHEC. (A) Proteins present in culture supernatants of EHEC TUV93-0 wild type, *hfq* mutant and the mutant strain complemented with *hfq* from pAMH100 grown in DMEM at 37°C to an optical density at 600 nm (OD₆₀₀) ~ 0.2 (lanes 1–3) and 1.0 (lanes 4–6). Proteins corresponding to 300 μl of culture supernatant were prepared as described in *Experimental procedures*, resolved in a 12% SDS-PAGE and visualized by silver staining. (B and C) The temporal expression profiles of LEE-encoded proteins in wild type TUV93-0, a *hfq* mutant and a complemented *hfq* mutant were determined by Western blot analyses as described in *Experimental procedures*. Equal amounts of total protein including both whole cell and secreted proteins, was prepared from cultures grown at 37°C in DMEM (B) and LB (C) to the cell densities indicated, resolved by 4–20% SDS-PAGE and blotted onto nylon membranes. Levels of Tir, EspA, EspB and GroEL were detected by Western blot analyses using polyclonal antisera against the respective proteins. GroEL served as an internal control for the total protein amount.

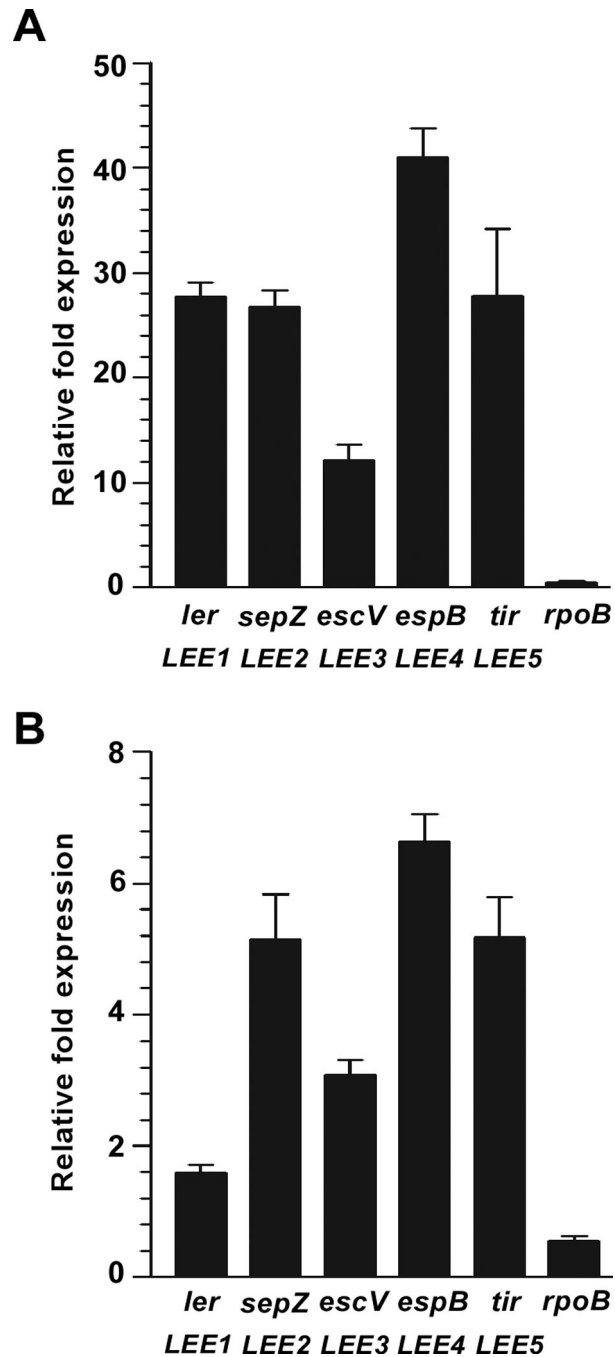


Figure 2.

LEE transcript levels are increased in a *hfq* mutant. Quantitative real-time PCR measurements of LEE transcripts in total RNA isolated from TUV93-0 wild type and *hfq* mutant strains grown at 37°C in LB to OD₆₀₀ ~ 0.6 and 3.0 were performed as described in *Experimental procedures*. The relative fold expression representing the change in transcript levels of *ler*, *sepZ*, *escV*, *espB* and *tir* in a *hfq* mutant relative to wild type grown to OD₆₀₀ ~ 0.6 (A) and 3.0 (B) was determined. Detection of the *rpoB* transcript served as a control for a gene which expression is not significantly stimulated in a *hfq* mutant. Results represent means and standard deviations from triplicate experiments. Error bars show the standard

deviations of $\Delta\Delta C_T$ values. Levels of 16S rRNA were used to normalize the C_T values of target genes to variations in bacterial numbers.

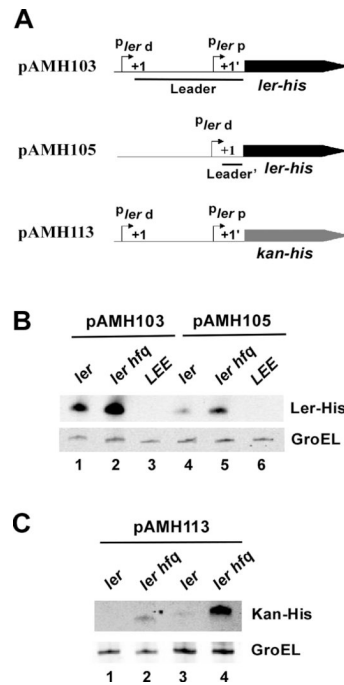


Figure 3.

Hfq indirectly affects Ler levels. (A) Schematic outline of constructs used to determine *ler-his* expression from *ler* distal and proximal promoters (pAMH103), a *ler* promoter with 120 nt of the 160 nt leader sequence including the proximal promoter region deleted (pAMH105) and *kan-his* expressed from a native *ler* promoter (pAMH113). $P_{ler\ d}$ and $P_{ler\ p}$ designate the distal and proximal *ler* promoter, respectively. (B) The *ler* leader region is not required for Hfq-mediated regulation of Ler levels. Western blot analysis of Ler-His expressed from pAMH103 (lanes 1–3) and pAMH105 (lanes 4–6) in TUV93-0 *ler*, TUV93-0 *ler hfq* and EDL933 Δ LEE strains grown in LB at 37°C to OD₆₀₀ ~ 0.4. (C) Expression of Kan-His from the *ler* promoter is negatively affected by *hfq*. Western blot analysis of Kan-His expressed from pAMH113 in TUV93-0 *ler* and *ler hfq* strains grown in LB at 37°C to OD₆₀₀ ~ 0.4 (lanes 1–2) and 3.0 (lanes 3–4). Ler-His, Kan-His and GroEL were detected using antibodies against the His-tag and GroEL, respectively. GroEL served as an internal loading control.

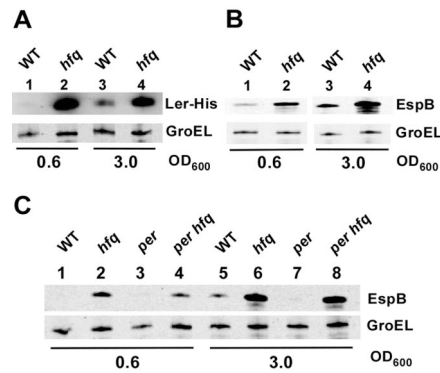


Figure 4.

Hfq affects LEE expression in EPEC E2348/69. Wild type and *hfq* mutant strains grown in LB at 37°C to OD₆₀₀ ~ 0.6 (lanes 1–2) and 3.0 (lanes 3–4) were assayed for Ler-His expressed from pAMH104 (A) and EspB (B) by Western blot analyses using His and EspB specific antibodies, respectively. (C) Hfq affects LEE expression independent of *per*. Western blot analyses to measure EspB levels in E2348/69, E2348/69 *hfq*, JPN15 (*per*) and JPN15 *hfq* strains, grown in LB at 37°C to OD₆₀₀ ~ 0.6 (lanes 1–4) and OD₆₀₀ ~ 3.0 (lanes 5–8), were performed using antibodies specific to EspB and GroEL.

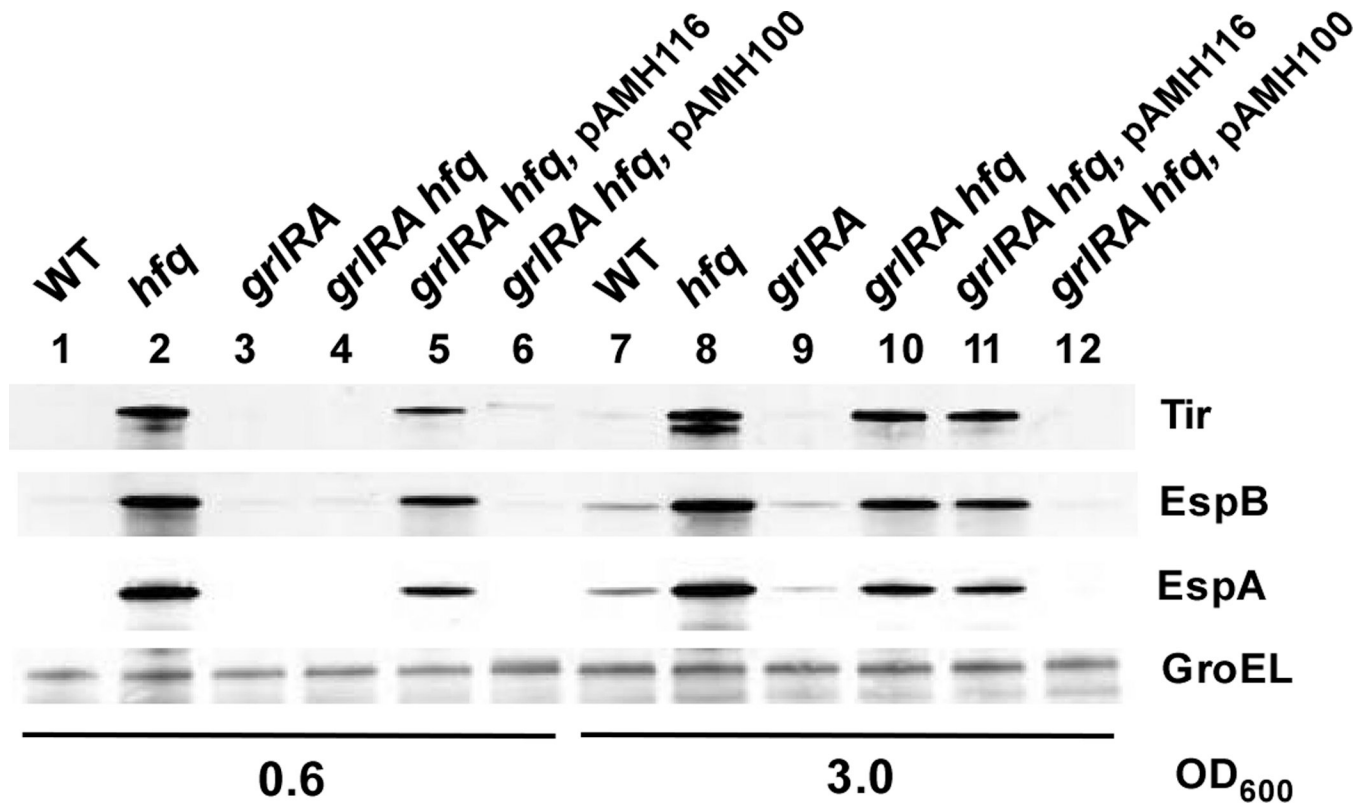


Figure 5.

Hfq negatively regulates levels of LEE-encoded proteins through *grlRA*. The effect of *grlRA* on LEE expression was measured by Western blot analyses of TUV93-0 wild type, a *hfq* mutant, a *grlRA* mutant, a *hfq grlRA* double mutant and a *hfq grlRA* double mutant strain complemented with *grlRA* (pAMH116) and *hfq* (pAMH100) grown in LB to exponential (OD₆₀₀ ~ 0.6, lanes 1–6) and stationary phase (OD₆₀₀ ~ 3.0, lanes 7–12). Levels of Tir, EspA, EspB and GroEL were detected using polyclonal antibodies against the respective proteins. GroEL served as an internal control for total protein levels.

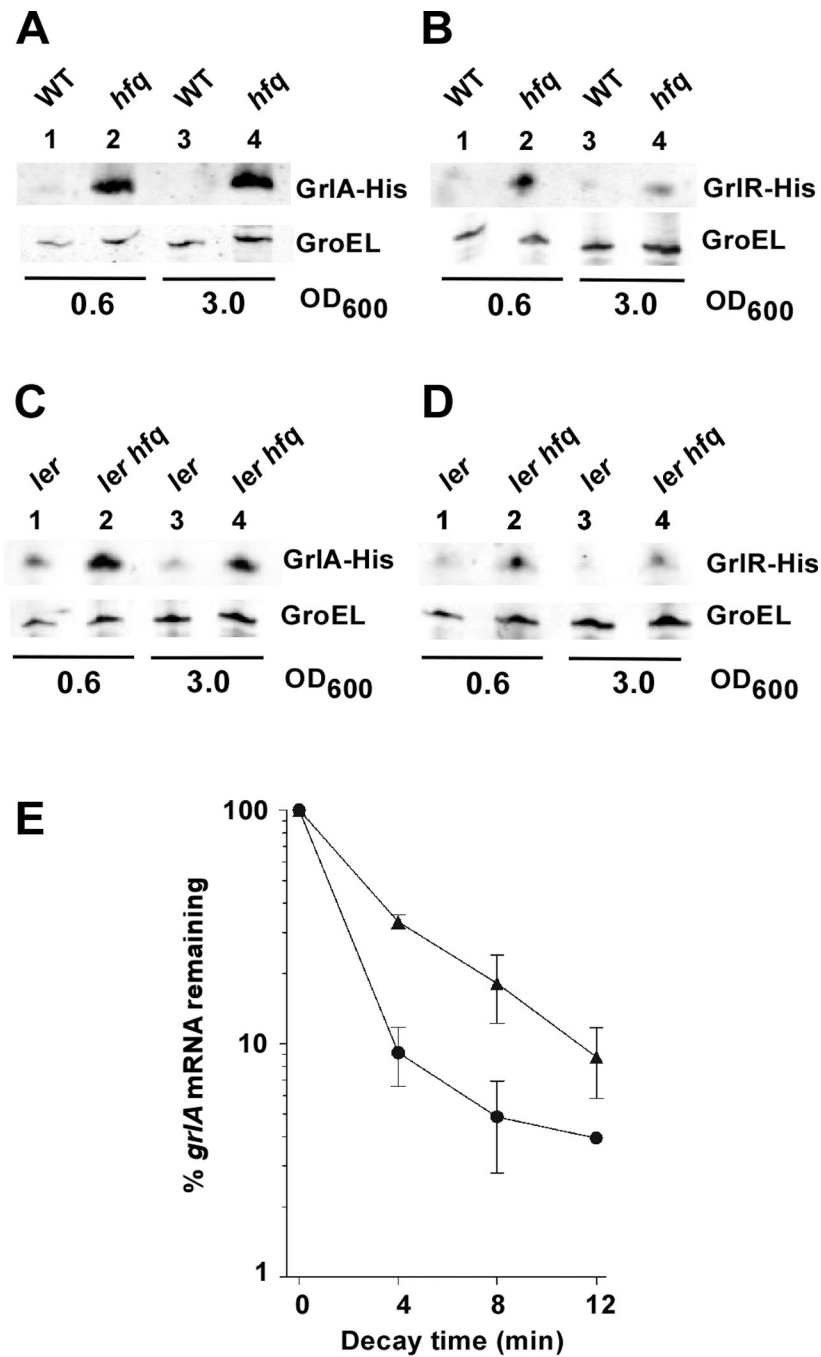


Figure 6. Hfq affects *grlA* expression independent of Ler. The expression of GrIA (A and C) and GrIR (B and D) was determined in TUV93-0 wild type and *hfq* backgrounds in the presence (A and B) and in the absence (C and D) of *ler* by Western analyses using a His specific antibody. TUV93-0 wild type, *hfq* and *ler* mutant strains, harboring plasmids encoding *grlA-his* (pAMH117) and *grlR-his* (pAMH118), were grown in LB at 37°C to exponential (A–D lanes 1–2) and stationary phases (A–D lanes 3–4). His-tagged GrIR and GrIA proteins were detected by Western blot analyses using a His specific antibody. Detection of GroEL served as an internal control. (E) Hfq affects *grlA* mRNA stability. Wild type TUV93-0 and *hfq* mutant strains were grown in LB at 37°C to exponential phase (OD₆₀₀ ~ 0.6),

transcription was blocked by rifampicin (0 min) and aliquots were collected at the time indicated, and total RNA was extracted as described in *Experimental procedures*. Analysis of *grlA* mRNA stability was carried out using qRT-PCR to measure the amount of *grlA* transcript normalized to levels of 16S rRNA. Data represent the mean \pm SD percentage of *grlA* transcript remaining in three independent RNA samples isolated from wild type (circles) and the *hfq* mutant (triangles) at the time indicated after rifampicin addition relative to time 0. The percentage of transcript remaining for each time point was significantly different in RNA isolated from wild type and *hfq* mutant strains as determined by the *t*-test ($P < 0.01$). Half-lives of *grlA* mRNA in wild type and *hfq* mutant strains were calculated from the lin-log graph to 1.5 min and 3 min, respectively.

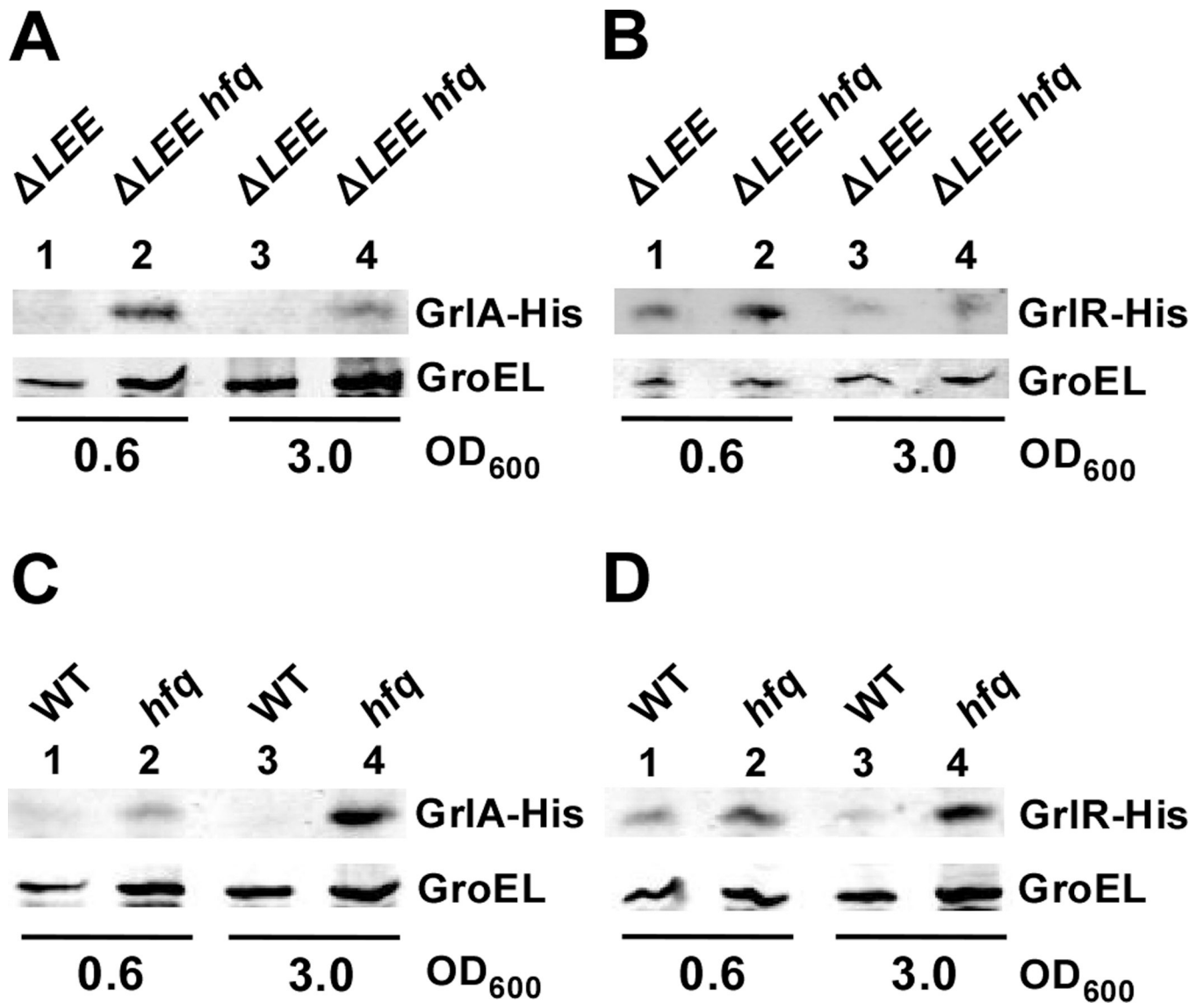


Figure 7.

The Hfq-mediated regulation of *grlRA* requires a factor that is located outside the LEE. Western blot analyses were used to detect levels of GrlA (A and C) and GrlR (B and D) in Δ LEE and Δ LEE *hfq* mutant derivatives of EHEC EDL933 (A and B) and wild type and *hfq* mutant backgrounds of *E. coli* K-12 MG1655 (C and D) grown in LB at 37°C to OD₆₀₀ ~ 0.6 (lanes 1–2) and 3.0 (lanes 3–4). His-tagged GrlR and GrlA were expressed from pAMH117/pAMH118 and pAMH125/pAMH126 in K-12 and EHEC backgrounds, respectively. His-tagged proteins were detected using a His specific antibody as described in *Experimental procedures*. GroEL served as an internal control for total protein levels.

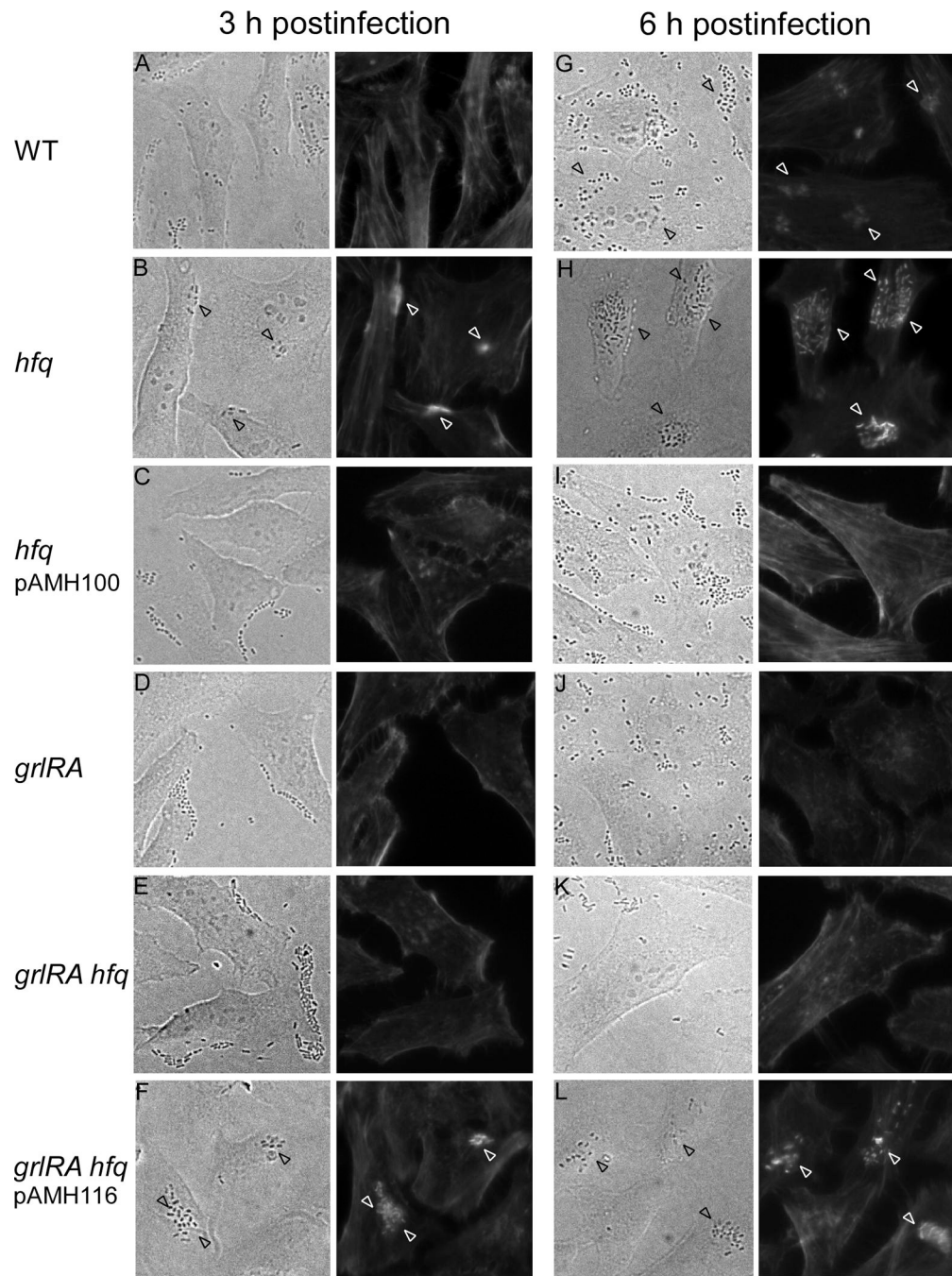


Figure 8.

Hfq affects the A/E phenotype through *grlRA*. FAS test using HeLa cells infected for 3 h (A–F) and 6 h (G–L) with TUV93-0, TUV93-0 *hfq*, TUV93-0 *hfq* pAMH100, TUV93-0 *grlRA*, TUV93-0 *grlRA hfq* and TUV93-0 *grlRA hfq* pAMH116. The actin cytoskeleton of infected HeLa cells was stained with FITC-phalloidin. Representative phase-contrast (left panels) and fluorescence actin (right panels) images are shown. Black and white arrowheads indicate bacteria and A/E lesions, respectively.

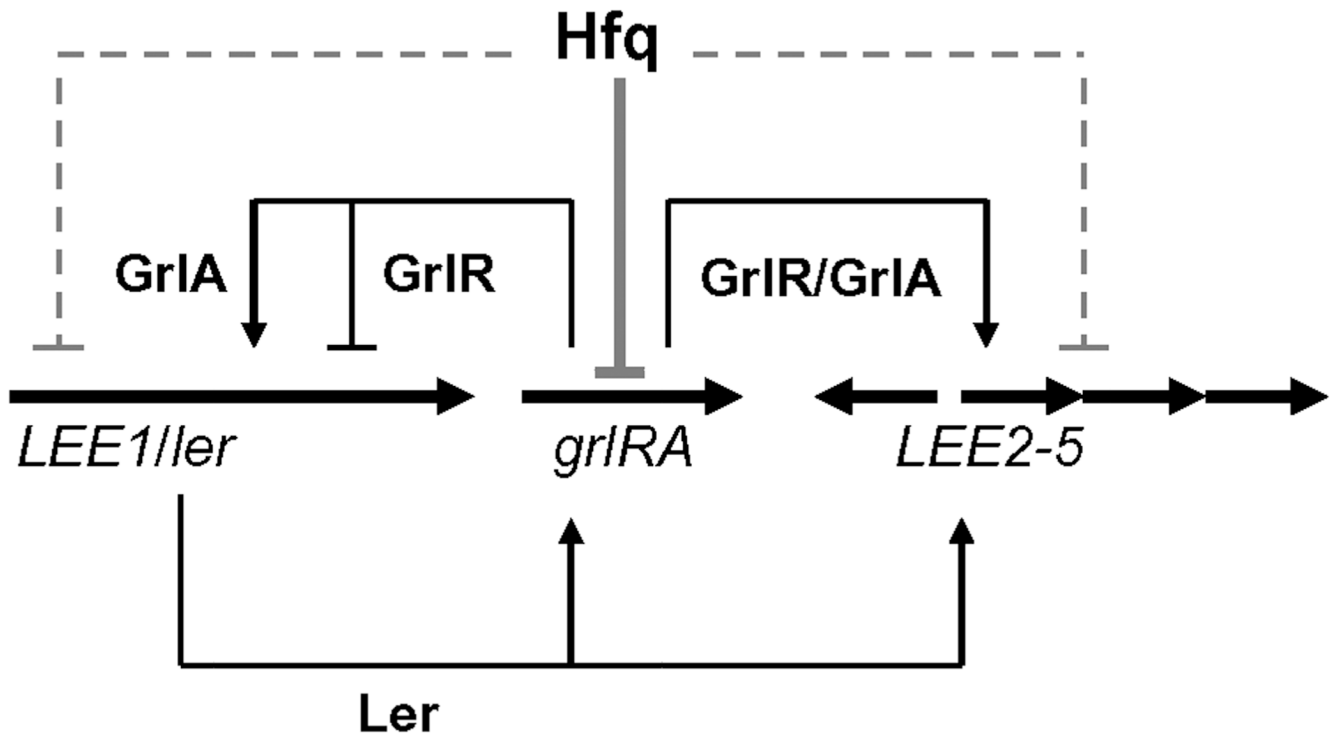


Figure 9.

Model depicting the regulation of LEE by Hfq, GrIA, GrIR and Ler. Black arrows and T-lines represent positive and negative regulation, respectively. Gray solid and broken T-lines indicate Hfq-mediated regulation in exponential and stationary phase, respectively. Ler is a positive regulator of *LEE2-5* and *grlRA* expression, whereas GrIA and GrIR activate and repress *LEE1* transcription, respectively, as described in the introduction. Moreover, GrIA and GrIR positively affect transcription of *LEE2* and *LEE4* independent of Ler (Russell *et al.*, 2007). Hfq negatively affects *grlRA* expression by modulating transcript stability to prevent expression of GrIA and GrIR, and thereby the initiation of the Ler-GrIA positive regulatory loop and subsequent LEE expression during exponential growth. In addition, Hfq negatively affects *LEE4* and *LEE5* expression independent of *grlRA* in stationary phase. To emphasize the impact of Hfq on LEE expression, the regulation of LEE by additional regulators is excluded from this model (for a detailed model of LEE regulation see Mellies *et al.*, 2007).

TABLE 1Acid resistance and motility of a *hfq* mutant.

	Acid resistance^{a,c}	Motility^{b,c}
TUV93-0, pBR322	5 ± 0.2	25 ± 5
TUV93-0 <i>hfq</i> , pBR322	< 0.001	10 ± 0
TUV93-0 <i>hfq</i> , pAMH100	9 ± 1	19 ± 3

^aPercent survival of cells after one hour of incubation in LB at pH 2.5.

^bCell motility in 0.3% agar expressed as the diameter of the bacterial swarming ring in mm.

^cValues represent the means ± SD of three independent experiments.

TABLE 2

Strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Reference or source
Strains		
TUV93-0	EHEC O157:H7 strain EDL933 Δ <i>stx1</i> Δ <i>stx2</i>	Donohue-Rolfe <i>et al.</i> , 2000
E2348/69	Wild-type EPEC O127:H6	Levine <i>et al.</i> , 1978
JPN15	E2348/69 spontaneously cured of pMAR2	Jerse <i>et al.</i> , 1990
MG1655	Wild-type <i>E. coli</i> K-12	Blattner <i>et al.</i> , 1997
EDL933 Δ LEE	EHEC O157:H7 EDL933 with LEE deleted	Posfai <i>et al.</i> , 1997
SE1101	EHEC O157:H7 84–86 Δ <i>ler::kan</i>	Elliott <i>et al.</i> , 2000
AMH100	TUV93-0 Δ <i>hfq</i>	This study
AMH101	TUV93-0 Δ <i>ler::kan</i>	This study
AMH102	TUV93-0 Δ <i>hfq</i> Δ <i>ler::kan</i>	This study
AMH108	TUV93-0 Δ <i>grlRA</i>	This study
AMH109	TUV93-0 Δ <i>grlRA</i> Δ <i>hfq</i>	This study
AMH110	E2348/69 Δ <i>hfq::kan</i>	This study
AMH111	JPN15 Δ <i>per</i> Δ <i>hfq::kan</i>	This study
AMH112	MG1655 Δ <i>hfq</i>	This study
AMH115	EDL933 Δ LEE Δ <i>hfq::kan</i>	This study
Plasmids		
pBR322	Medium-copy number cloning vector	Bolivar <i>et al.</i> , 1977
pCP20	FLP-flipase expressing plasmid; <i>repA101</i> (ts) $P_{\lambda R}$ - <i>FLP</i> λ cI857	Cherepanov and Wackernagel, 1995
pKD3	FRT-flanked <i>cm</i> resistance cassette	Datsenko and Wanner, 2000
pKD13	FRT-flanked <i>kan</i> resistance cassette	Datsenko and Wanner, 2000
pKD46	λ Red recombinase expressing plasmid; <i>repA101</i> (ts) P_{araB} - <i>gam</i> - <i>bet</i> - <i>exo</i>	Datsenko and Wanner, 2000
pKM201	λ Red recombinase expressing plasmid; <i>repA101</i> (ts) P_{lac} - <i>gam</i> - <i>bet</i> - <i>exo</i>	Murphy and Campellone, 2003
pTP223	λ Red recombinase expressing plasmid; P_{lac} - <i>gam</i> - <i>bet</i> - <i>exo</i>	Poteete and Fenton, 1984
pAMH100	pBR322:: <i>hfq</i> _{EHEC}	This study
pAMH103	pBR322:: <i>ler-his</i> _{EHEC}	This study
pAMH104	pBR322:: <i>ler-his</i> _{EPEC}	This study
pAMH105	pBR322:: $P_{ler\Delta Pprox}$ - <i>ler-his</i> _{EHEC}	This study
pAMH113	pBR322:: $P_{ler(EHEC)}$ - <i>kan-his</i>	This study
pAMH116	pBR322:: <i>grlRA</i> _{EHEC}	This study
pAMH117	pBR322:: <i>grlRA-his</i> _{EHEC}	This study
pAMH118	pBR322:: <i>grlR-his</i> _{EHEC}	This study
pAMH125	pAMH117:: <i>cm</i>	This study
pAMH126	pAMH118:: <i>cm</i>	This study

TABLE 3

Oligonucleotides used in this study.

Name	Oligo sequence (5' to 3')
K1372	CCGGAATTCCTGTAACCTGAATTAAGTAGAGT
K1420	CGCGGATCCAGCTCACGTTATCGTTATCATT
K4785	GAGACGTATCGTGCGCAAT
K4786	GAACGCAGGATCGCTGGCT
K4797	GCACCGGGATCCTCAGTGATGGTGATGGTGATGAATATTTTTCAGCGGTA TTATTTCTTCTT
K4928	ACGCAGGATCGCTGGCTCCCGTGTAACAAAAACAGCCCGAAACCATTG TGTAGGCTGGAGCTGCTTCG
K4980	GTCAGGATCCAGTAAGGCGAGCCTGCCTAAGG
K4981	GTCAGAAGCTTGAACGCAGGATCGCTGGCT
K5045	ATCGAAAGGTTCAAAGTACAAATAAGCATATAAGGA AAAGAGAGAATGTCCGGGGATCCGTCGACCT
K5377	TGTGTAGGCTGGAGCTGCT
K5378	CATATGAATATCCTCCTTAGTTC
K5373	CTTTTTTCAAATGTGTAATAACATTATCA
K5374	CTTAAATATTAAGCATGCGGAGAT
K5359	CACATAACAAGTCCATACATT
K5360	CGAGTCCATCATCAGGCACATTA
K5361	GCAACCGCTGCTTTGGTTGG
K5362	CGACATCAGCAACTTTCCG
K5365	TGCAAGTCGAACGGTAACAG
K5366	AGTTATCCCCCTCCATCAGG
K5425	GCACCGGGATCCTCAGTGATGGTGATGGTGATGAAACAATTCATCCAGT AAAATATAATATTTTATTTTC
K5445	GATCTCGAGCGAAACAGTAGGAACGTCGGCTTTG
K5446	CTAGAGCTCGCAGAGCAAGGTTGGGAGTCATT
K5653	GCGACTATGTAATTCCTGACTCAGT
K5656	CCATAACTAACATATCTGACACGAAATG
K5663	GAAAGGAGTGAGGTTAGTATGAAACTGAGTGAGTT ATGATTATGTCCGGGGATCCGTCGACCTGC
K5664	GCTTTATTTTTATTCTTCTATAAAAATACTCAAAAAATTACGTCTAGTGT AGGCTGGAGCTGCTTCGA
K5665	CACCGAAGCTTGCAACAGATATGTTTATACAGGCATCAT
K5666	GCACCGGGATCCACTGGTAGATTATCTACAGGTG
K5667	GCACCGGGATCCTAGTGATGGTGATGGTGATG ACTCTCCTTTTTCCGCCTCATGATC
K5822	GCACCGGGATCCTTAGTGATGGTGATGGTGATGTTTAAATAAACTTGTG GCATTCTGTGTTTT
K5831	AGTGCTCGTTTTCCCTTGA
K5832	AGCGAAGAAGCTTTGCCTCA
K5833	CTCAGTGGATGAGAAGACAGGAG
K5834	CCGCAGTAAGAGTACCTAGTACG
K5930	GATGTTCTGGACTTCTGTAA

Name	Oligo sequence (5' to 3')
K5931	CAACAGAGGTCTCAACACCATT
K6021	GGTGGTTGTTTGATGAAATAGATGTGTC
K6022	GCTATTAGCGACCTTATCAGGAAG
K6038	GGTCTCCATTATTCTTGATATTGCTTATG
K6039	GGAACTTCGAACTGCAGGTCTG
K6046	GCAATGAAGACTCCTGTGGGGA
K6047	CATGAAGTATGATGCCTCATCTTCA
K6088	GCATCATCCCTTACCGTGGTTC
K6089	GGATCTGCTCTGTGGTGTAGTTCA