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RESEARCH ARTICLE

Hfq and three Hfq-dependent small regulatory RNAs—MgrR, RyhB and McaS—coregulate the locus of enterocyte effacement in enteropathogenic *Escherichia coli*

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One sentence summary: This manuscript describes identification of the first regulatory small RNAs that control the virulence of enteropathogenic *Escherichia coli*.
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ABSTRACT

Enteropathogenic *Escherichia coli* (EPEC) is a significant cause of infantile diarrhea and death in developing countries. The pathogenicity island locus of enterocyte effacement (LEE) is essential for EPEC to cause diarrhea. Besides EPEC, the LEE is also present in other gastrointestinal pathogens, most notably enterohemorrhagic *E. coli* (EHEC). Whereas transcriptional control of the LEE has been meticulously examined, posttranscriptional regulation, including the role of Hfq-dependent small RNAs, remains undercharacterized. However, the past few years have witnessed a surge in the identification of riboregulators of the LEE in EHEC. Contrastingly, the posttranscriptional regulatory landscape of EPEC remains cryptic. Here we demonstrate that the RNA-chaperone Hfq represses the LEE of EPEC by targeting the 5′ untranslated leader region of *grlR* in the *grlRA* mRNA. Three conserved small regulatory RNAs (sRNAs)—MgrR, RyhB and McaS—are involved in the Hfq-dependent regulation of *grlRA*. MgrR and RyhB exert their effects by directly base-pairing to the 5′ region of *grlR*. Whereas MgrR selectively represses *grlR* but activates *grlA*, RyhB represses gene expression from the entire *grlRA* transcript. Meanwhile, McaS appears to target the *grlRA* mRNA indirectly. Thus, our results provide the first definitive evidence that implicates multiple sRNAs in regulating the LEE and the resulting virulence of EPEC.

Keywords: Hfq; RyhB; MgrR; McaS; LEE; EPEC

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INTRODUCTION

Enteropathogenic *Escherichia coli* (EPEC), enterohemorrhagic *E. coli* (EHEC) and *E. albertii* belong to the attaching and effacing (A/E) morphotype of diarrheic bacterial pathogens (Mellies *et al.* 1999; Mellies, Barron and Carmona 2007; Bhatt, Romeo and Kalman 2011; Franzin and Sircili 2015; Bhatt *et al.* 2016a,b; Egan *et al.* 2016). Members of this group are phenotypically distinguishable from other enteric pathogens by their propensity to form A/E lesions on infected intestinal cells—pathognomonic structures that facilitate bacterial colonization. A/E lesions are characterized by localized destruction of intestinal microvilli due to actin depolymerization (effacement) by the pathogen. This is followed by the recruitment and repolymerization of actin beneath the attached bacterium to form a signature membrane-enclosed evagination from the diseased cell called pedestal (attachment) (Mellies *et al.* 1999; Mellies, Barron and Carmona 2007; Bhatt, Romeo and Kalman 2011; Franzin and Sircili 2015). Destruction of intestinal microvilli reduces the ability of intestinal cells to absorb solutes, which is partly responsible for the observed diarrheal symptoms that accompany infections associated with A/E pathogens.

Biogenesis of A/E lesions requires the horizontally acquired pathogenicity island, locus of enterocyte effacement (LEE) (Donnenberg *et al.* 1993; McDaniel *et al.* 1995; McDaniel and Kaper 1997; Dziva *et al.* 2004; Ritchie and Waldor 2005; Mellies, Barron and Carmona 2007; Bhatt, Romeo and Kalman 2011). The LEE is a 35–40 kb AT-rich genomic island that houses the type III secretion system (T3SS) (Deng *et al.* 2001, 2004; Caron *et al.* 2006; Mellies, Barron and Carmona 2007; Bhatt, Romeo and Kalman 2011). Upon maturation, the T3SS directly connects the bacterial cytosol to that of the infected host (Ogino *et al.* 2006; Coburn, Sekirov and Finlay 2007; Mellies, Barron and Carmona 2007; Bhatt, Romeo and Kalman 2011). Subsequently, A/E pathogens traffic contraband effectors into the infected cell where they hijack host regulatory and structural factors to induce ultrastructural changes that remodel the host cytoskeleton to form A/E lesions (Croxen and Finlay 2010). The indispensable role of the LEE to EPEC and EHEC virulence has made this genetic locus the cynosure of regulatory, structural and mechanistic studies in A/E pathogens (Mellies, Barron and Carmona 2007; Bhatt, Romeo and Kalman 2011; Franzin and Sircili 2015).

The LEE is genetically organized into five polycistronic operons, *LEE1*-5, a bicistronic operon *grlRA*, and several monocistronic transcription units (Elliott *et al.* 1999; Mellies *et al.* 1999; Sanchez-SanMartin *et al.* 2001; Mellies, Barron and Carmona 2007; Bhatt, Romeo and Kalman 2011). Under nonpermissive conditions, the LEE is xenogeneically silenced by the nucleoid-associated protein H-NS (Umanski, Rosenshine and Friedberg 2002; Mellies, Barron and Carmona 2007; Bhatt, Romeo and Kalman 2011). Ler, an H-NS paralog, is encoded by the first gene in the *LEE1* operon and functions as an antisilencer that antagonizes the effect of H-NS and globally derepresses the other LEE operons (Friedberg *et al.* 1999; Mellies *et al.* 1999; Elliott *et al.* 2000; Mellies, Barron and Carmona 2007; Bhatt, Romeo and Kalman 2011). Recent data suggest that besides *ler*, the *grlRA* operon may also play a prominent role in synchronizing gene expression from the LEE (Deng *et al.* 2004; Yang *et al.* 2008, 2009). The *grlRA* operon encodes two transcription factors—Global Regulator of the LEE Repressor (GrlR) and Global Regulator of the LEE Activator (GrlA) (Deng *et al.* 2004). GrlA functions as a DNA-binding protein that recognizes sequences upstream of *LEE1* and stimulates transcription from this operon (Barba *et al.* 2005; Huang and Syu 2008; Jimenez *et al.* 2010). GrlR, on the other hand, antagonizes the effect of GrlA by binding to it and sequestering it, effectively preventing GrlA from binding to and activating transcription from *LEE1* (Jobichen *et al.* 2007; Padavannil *et al.* 2013). The importance of *ler* and *grlRA* in pedestal formation is highlighted by the observation that many environmental stimuli and their perceptive signal transduction pathways converge on the *ler*-encoding *LEE1* and *grlRA* operons to hone the transcriptional output from the LEE (Mellies, Barron and Carmona 2007; Bhatt, Romeo and Kalman 2011; Franzin and Sircili 2015).

Transcriptional regulation of the LEE, including the roles of *trans*-acting factors and *cis*-acting elements, has been systematically characterized in EPEC and EHEC (Mellies, Barron and Carmona 2007; Franzin and Sircili 2015). A range of structurally and mechanistically diverse transcription factors, responsive to an equally impressive array of stimuli, regulates the LEE in these pathogens (Kaper *et al.* 1997; Friedberg *et al.* 1999; Mellies *et al.* 1999; Elliott *et al.* 2000; Deng *et al.* 2001, 2004, 2005; Abe *et al.* 2002; Iyoda and Watanabe 2004; Porter *et al.* 2004, 2005; Sharma and Zuerner 2004; Barba *et al.* 2005; Mellies, Haack and Galligan 2007; Russell *et al.* 2007; Sharp and Sperandio 2007; Huang and Syu 2008; Jimenez *et al.* 2010; Kendall, Rasko and Sperandio 2010; Bustamante *et al.* 2011; Njoroge, Gruber and Sperandio 2013). These include global transcription factors, which are phylogenetically ubiquitous and affect diverse physiological processes, as well as local transcription factors, which are restricted to taxonomically related species and affect one or a few processes. By contrast, posttranscriptional regulation, particularly the role of small regulatory RNAs (sRNAs) in regulation of the LEE and/or virulence of A/E pathogens is only now beginning to be unraveled (Bhatt, Romeo and Kalman 2011; Bhatt *et al.* 2016a). More specifically, this has been limited to investigations in EHEC with very little knowledge of homologous or analogous mechanisms in EPEC (Bhatt, Romeo and Kalman 2011; Gruber and Sperandio 2014, 2015; Tobe *et al.* 2014; Bhatt *et al.* 2016a).

sRNAs comprise a structurally and mechanistically diverse group of molecules that are induced in response to different environmental cues, particularly stressors (Waters and Storz 2009; Papenfort and Vogel 2010). In bacteria, sRNAs typically range in size from 50 to 400 nucleotides in length and many of them are encoded within autonomous transcription units (Waters and Storz 2009; Papenfort and Vogel 2010; Storz, Vogel and Wassarman 2011). Whereas some sRNAs elicit their regulatory effect by binding to and modulating the activity of proteins, the vast majority base-pair to complementary mRNA targets and affect message stability and/or translation (Babitzke and Romeo 2007; Waters and Storz 2009; Papenfort and Vogel 2010; Storz, Vogel and Wassarman 2011). A subgroup of base-pairing sRNAs possesses short regions which are imperfectly complementary to target mRNAs. Base pairing between such sRNAs and their target mRNAs is often assisted by an RNA chaperone protein (Waters and Storz 2009; Papenfort and Vogel 2010; Storz, Vogel and Wassarman 2011). In Gram-negative bacteria, the principal RNA chaperone is Hfq (Valentin-Hansen, Eriksen and Udesen 2004; Link, Valentin-Hansen and Brennan 2009; Chao and Vogel 2010; Soper *et al.* 2010). Hfq and Hfq-dependent sRNAs together control many biological processes including motility, biofilm formation, adaptation to stressors, antibiotic resistance and virulence, among others (Gottesman *et al.* 2006; Hansen and Kaper 2009; Shakhnovich, Davis and Waldor 2009; Chao and Vogel 2010; Yamada *et al.* 2010; Kendall *et al.* 2011; Jorgensen *et al.* 2013). In particular, Hfq and Hfq-dependent sRNAs are instrumental in modulating virulence in phylogenetically distant bacterial pathogens including *Salmonella* Typhimurium, *Vibrio cholerae*, *Shigella* spp. and EHEC, amongst others (Sittka *et al.* 2007; Kulesus *et al.* 2008;

Mitobe *et al.* 2008, 2009; Hansen and Kaper 2009; Shakhnovich, Davis and Waldor 2009; Chao and Vogel 2010; Bhatt, Romeo and Kalman 2011; Kendall *et al.* 2011). In EHEC, Hfq functions as an activator or repressor of virulence depending on the bacterial strain. For instance, Hfq activates the LEE in the EHEC biotype 86-24 but represses the pathogenicity island in EDL933 (Hansen and Kaper 2009; Shakhnovich, Davis and Waldor 2009; Bhatt, Romeo and Kalman 2011; Kendall *et al.* 2011). Multiple conserved and pathogen-specific Hfq-dependent sRNAs that regulate the LEE have been identified in EHEC (Laaberki *et al.* 2006; Gruber and Sperandio 2014: e01025–13; Sudo *et al.* 2014; Tobe *et al.* 2014; Tree *et al.* 2014; Gruber and Sperandio 2015: e101582). For instance, the conserved sRNA DsrA activates *ler* in EHEC and this stimulatory effect occurs independently of GrlA (Laaberki *et al.* 2006). Recently, Gruber and Sperandio identified novel roles for the conserved Hfq-dependent sRNAs GlmY and GlmZ. Both GlmY and GlmZ antagonistically regulate the LEE and the non-LEE-encoded gene *espF*$_U$, despite the essentiality of the two genetic loci to pedestal biogenesis in EHEC (Gruber and Sperandio 2014: e01025–13). In a later study, the same authors further expanded on the posttranscriptional regulatory landscape of the LEE in EHEC by identifying seven more Hfq-dependent sRNAs (Gruber and Sperandio 2015). Besides sRNAs that regulate the LEE, other riboregulators have also been recognized in EHEC. Recently, a class of Hfq-dependent sRNAs, termed 'anti-sRNAs', has been identified in EHEC (Tree *et al.* 2014). These sRNAs mimic the cellular mRNA targets of sRNAs thereby competing with the mRNA for base-pairing to the sRNA. In doing so, the anti-sRNAs effectively prevent the sRNA from eliciting its regulatory effect on its mRNA substrates. Multiple anti-sRNAs, such as AgvB1, AgvB2 and AsxR, are expressed from genomic segments unique to EHEC and lacking in nonpathogenic and other pathogenic strains of *E. coli*. Many of these anti-sRNAs have been implicated in other facets of EHEC virulence (Tree *et al.* 2014).

In contrast to EHEC, to date, not a single Hfq-dependent sRNA has been implicated in the virulence of EPEC. Moreover, many of the Hfq-dependent sRNAs that control the LEE and/or virulence in EHEC may not function similarly in EPEC. For instance, DsrA activates the LEE of EHEC but has no effect on the LEE of EPEC (Laaberki *et al.* 2006). Additionally, GlmY and GlmZ exert their regulatory effects, in part, by stimulating translation of the adaptor protein EspF$_U$ in EHEC, which is absent in EPEC (Gruber and Sperandio 2014: e01025—13). Other Hfq-dependent sRNAs, such as AgvB1, AgvB2, AsxR, are unique to EHEC (Tree *et al.* 2014). These observations suggest that, unlike transcriptional regulatory circuits, posttranscriptional regulatory mechanisms of the LEE have diverged between EHEC and EPEC and cannot reliably be extrapolated in EPEC based upon results in EHEC and require empirical validation. Therefore, it is necessary to identify and characterize the posttranscriptional regulatory networks, in particular, the role of Hfq and Hfq-dependent sRNAs in the virulence of EPEC.

In the present study, we have corroborated previous findings that a loss-of-function mutation in *hfq* derepresses gene expression from the LEE of EPEC, specifically by targeting *grlRA* posttranscriptionally (Hansen and Kaper 2009; Shakhnovich, Davis and Waldor 2009). Moreover, we have extended these findings by showing that Hfq in conjunction with at least three conserved Hfq-dependent sRNAs—MgrR, RyhB and McaS—directly targets the 5′ leader region of the *grlRA* transcript. MgrR and RyhB base-pair to different regions within the 5′ untranslated leader region (UTR) of *grlR* and elicit different regulatory outcomes. Whereas MgrR selectively represses *grlR* but activates *grlA*, RyhB represses expression from the entire *grlRA* transcript. By contrast, McaS does not appear to base pair to *grlRA* and presumably exerts its effect indirectly. In summary, our results provide the first definitive evidence that implicates multiple Hfq-dependent sRNAs in controlling the LEE and the resulting virulence of EPEC.

MATERIALS AND METHODS

Bacterial strains, plasmids, primers and media

Bacteria were grown in Luria-Bertani (LB) broth or Dulbecco's Modified Eagle's Medium (DMEM) (pH ∼7.2 ± 0.2) lacking glutamine and phenol red and containing $NaHCO_3$ (44 mM) and NaH_2PO_4 (0.91 mM) as the buffering agents (Cellgro (Corning), Manassas, Virginia, USA). The media were supplemented with the appropriate antibiotics when necessary. Antibiotics used were streptomycin (100 μg/ml), chloramphenicol (12.5–25 μg/ml), kanamycin (50 μg/ml), tetracycline (15 μg/ml) and ampicillin (100 μg/ml). Strains, plasmids and oligonucleotides used are listed in Tables 1 and 2.

Chromosomal and plasmid manipulations

Chromosomal modifications, including allelic replacement and epitope tagging, were engineered in EPEC by using a modification of the lambda red-mediated recombineering technique as described previously (Murphy and Campellone 2003; Bhatt *et al.* 2009). All chromosomal modifications were verified by Polymerase Chain Reaction (PCR), and some of the mutations were further confirmed by sequencing.

Chromosomal modifications in *Escherichia coli* K-12 strains were constructed by lambda red recombineering as detailed previously by Court *et al.* with slight modifications (Court, Sawitzke and Thomason 2002; Court *et al.* 2003; Sharan *et al.* 2009; VanOrsdel *et al.* 2013). The reporter *E. coli* strain PM1205 was used for engineering chromosomal translational fusions with the *lacZ* gene (Mandin and Gottesman 2009). This transgenic strain harbors the defective and thermolabile mini-lambda prophage that expresses the lambda red recombinase genes under the control of the temperature-sensitive cI857 repressor. Furthermore, this strain contains a truncated '*lacZ* gene lacking its 5′ UTR and the first nine N-terminal codons. The P_{araBAD} promoter is located upstream of the '*lacZ* gene. However, transcription is interrupted by the presence of the *cat-sacB* cassette between the P_{araBAD} promoter and '*lacZ*. The relevant genotype of this reporter strain at the *lac* locus is P_{araBAD}-*cat-sacB*-'*lacZ* (Mandin and Gottesman 2009). As such, this strain is useful in constructing single copy translational fusions of a gene of interest with the *lacZ* gene so that the hybrid gene fusion is under the transcriptional control of the P_{araBAD} promoter but under the posttranscriptional regulatory elements of the gene of interest (P_{araBAD}-*yfg*'-'*lacZ*). Such translational fusions are informative about environmental stimuli and regulators that exert their effects posttranscriptionally on the cloned region of the gene of interest. An individual colony of PM1205 was inoculated into 5 ml of LB broth and incubated overnight at 30°C. The following day, after reaching stationary phase, bacteria were sub-cultured by diluting 100-fold in 30 ml of LB supplemented with tetracycline and allowed to grow at 30°C/250 rpm to an OD_{600} of ∼0.6–0.8. The lambda red recombinase genes *exo*, *bet* and *gam* were thermally induced by shifting the cultures to 42°C for 15–30 min in a water bath. The cultures were subsequently transferred to a pre-chilled 50 ml conical tube and cooled by swirling for ∼2 min. Cells were harvested by centrifugation at 4150 rpm/4°C/10 min. The supernatant was discarded and cells resuspended in 30 ml of chilled 10% glycerol (in distilled, deionized and autoclaved water) followed by one round of centrifugation at 4150 rpm/4°C/10 min. The supernatant was

Table 1. Bacterial strains and plasmids used in this study.

Strain	Relevant genotype	Reference or source
LS1148	EPEC 2348/69 Φ(*grlA*$^+$-3X FLAG), Kan^r Str^r	Bhatt *et al.* (2009)
LS1249	EPEC 2348/69 Φ(*grlA*$^+$-3X FLAG) Δ*hfq*::*cat*, Cm^r Kan^r Str^r	This study
LS5678	LS1249 (pUC18-*hfq*), Cm^r Kan^r Str^r Amp^r	This study
LS5360	EPEC 2348/69 Φ(*grlA*$^+$-3X FLAG) with pBR-plac, Kan^r Amp^r Str^r	This study
LS5365	EPEC 2348/69 Φ(*grlA*$^+$-3X FLAG) with pMgrR-EPEC, Kan^r Amp^r Str^r	This study
LS5392	EPEC 2348/69 Φ(*grlA*$^+$-3X FLAG) with pRyhB, Kan^r Amp^r Str^r	This study
LS4767 = PM1205	P_{araBAD}-*cat*-*sacB*-'*lacZ* mini-lambda, Cm^r Tet^r Suc^s	Mandin and Gottesman (2009)
LS4981	PM1205 P_{araBAD}-*grlR*'-'*lacZ*, Cm^s Tet^s, Suc^r	This study
LS4983	PM1205 P_{araBAD}-*grlR*'-'*lacZ* Δ*hfq*::*cat*-*sacB*, Cm^r Tet^s Suc^s	This study
LS5096	PM1205 P_{araBAD}-*grlR*'-'*lacZ* (pBR-plac), Amp^r Cm^s Tet^s, Suc^r	This study
LS5059	PM1205 P_{araBAD}-*grlR*'-'*lacZ* (pGadY), Amp^r Cm^s Tet^s, Suc^r	This study
LS5047	PM1205 P_{araBAD}-*grlR*'-'*lacZ* (pGlmZ), Amp^r Cm^s Tet^s, Suc^r	This study
LS5021	PM1205 P_{araBAD}-*grlR*'-'*lacZ* (pFnrS), Amp^r Cm^s Tet^s, Suc^r	This study
LS5084	PM1205 P_{araBAD}-*grlR*'-'*lacZ* (pRydC), Amp^r Cm^s Tet^s, Suc^r	This study
LS5082	PM1205 P_{araBAD}-*grlR*'-'*lacZ* (pMicF), Amp^r Cm^s Tet^s, Suc^r	This study
LS5035	PM1205 P_{araBAD}-*grlR*'-'*lacZ* (pMgrR), Amp^r Cm^s Tet^s, Suc^r	This study
LS5039	PM1205 P_{araBAD}-*grlR*'-'*lacZ* (pRybB), Amp^r Cm^s Tet^s, Suc^r	This study
LS5025	PM1205 P_{araBAD}-*grlR*'-'*lacZ* (pOmrA), Amp^r Cm^s Tet^s, Suc^r	This study
LS5029	PM1205 P_{araBAD}-*grlR*'-'*lacZ* (pSpot42), Amp^r Cm^s Tet^s, Suc^r	This study
LS5055	PM1205 P_{araBAD}-*grlR*'-'*lacZ* (pGlmY), Amp^r Cm^s Tet^s, Suc^r	This study
LS5045	PM1205 P_{araBAD}-*grlR*'-'*lacZ* (pOmrB), Amp^r Cm^s Tet^s, Suc^r	This study
LS5037	PM1205 P_{araBAD}-*grlR*'-'*lacZ* (pRseX), Amp^r Cm^s Tet^s, Suc^r	This study
LS5015	PM1205 P_{araBAD}-*grlR*'-'*lacZ* (pMcaS), Amp^r Cm^s Tet^s, Suc^r	This study
LS5013	PM1205 P_{araBAD}-*grlR*'-'*lacZ* (pRybD), Amp^r Cm^s Tet^s, Suc^r	This study
LS5080	PM1205 P_{araBAD}-*grlR*'-'*lacZ* (pCyaR), Amp^r Cm^s Tet^s, Suc^r	This study
LS5064	PM1205 P_{araBAD}-*grlR*'-'*lacZ* (pDsrA), Amp^r Cm^s Tet^s, Suc^r	This study
LS5007	PM1205 P_{araBAD}-*grlR*'-'*lacZ* (pMicC), Amp^r Cm^s Tet^s, Suc^r	This study
LS5017	PM1205 P_{araBAD}-*grlR*'-'*lacZ* (pArcZ), Amp^r Cm^s Tet^s, Suc^r	This study
LS5027	PM1205 P_{araBAD}-*grlR*'-'*lacZ* (pRyeB), Amp^r Cm^s Tet^s, Suc^r	This study
LS5032	PM1205 P_{araBAD}-*grlR*'-'*lacZ* (pMicA), Amp^r Cm^s Tet^s, Suc^r	This study
LS5011	PM1205 P_{araBAD}-*grlR*'-'*lacZ* (pGcvB), Amp^r Cm^s Tet^s, Suc^r	This study
LS5009	PM1205 P_{araBAD}-*grlR*'-'*lacZ* (pReg26-short), Amp^r Cm^s Tet^s, Suc^r	This study
LS5061	PM1205 P_{araBAD}-*grlR*'-'*lacZ* (pChiX), Amp^r Cm^s Tet^s, Suc^r	This study
LS5019	PM1205 P_{araBAD}-*grlR*'-'*lacZ* (pRprA), Amp^r Cm^s Tet^s, Suc^r	This study
LS5023	PM1205 P_{araBAD}-*grlR*'-'*lacZ* (pRyhB), Amp^r Cm^s Tet^s, Suc^r	This study
JR1008-2	PM1205 P_{araBAD}-*grlR*'-'*lacZ* (pRyhB-mut1), Amp^r Cm^s Tet^s, Suc^r	This study
LS5191	PM1205 P_{araBAD}-*grlR*(MgrR-mut1)'-'*lacZ*, Cm^s Tet^s, Suc^r	This study
LS5258	PM1205 P_{araBAD}-*grlR*'-'*lacZ* (pMgrR-EPEC), Amp^r Cm^s Tet^s, Suc^r	This study
LS5262	PM1205 P_{araBAD}-*grlR*'-'*lacZ* (pMgrR-mut1-EPEC), Amp^r Cm^s Tet^s, Suc^r	This study
DH5α	*supE44* Δ*lacU169* (Φ80 *lacZ*Δ*M15*) *hsdR17 recA1 endA1 gryA96 thi-1 relA1*	Bettina Bommarius
Plasmids		
pTP223	*gam, bet* and *exo* genes of the λ phage cloned under the control of the *lac* promoter, Tet^r	Kenan Murphy
pBR-plac	Cloning vector, Amp^r	Gisela Storz
pRyhB	*ryhB* wild type allele under an IPTG inducible promoter, Amp^r	Gisela Storz
pRyhB-mut1	*ryhB* mutant allele from EPEC under an IPTG inducible promoter, Amp^r	This study
pMgrR-EPEC	*mgrR* wild type allele from EPEC under an IPTG inducible promoter, Amp^r	This study
pMgrR-mut1-EPEC	*mgrR* mutant allele from EPEC under an IPTG inducible promoter, Amp^r	This study
pMcaS	*mcaS* wild type allele from *E. coli* under an IPTG inducible promoter, Amp^r	Gisela Storz
pUC18	Cloning vector, Amp^r	Gisela Storz
pUC18-*hfq*	Wild type *hfq* allele from *E. coli* K-12 under an IPTG-inducible promoter, Amp^r	Gisela Storz

Tet$^{r/s}$—tetracycline resistant/sensitive, Cm$^{r/s}$—chloramphenicol resistant/sensitive, Str$^{r/s}$—streptomycin resistant/sensitive, Amp$^{r/s}$—ampicillin resistant/sensitive, Suc$^{r/s}$—sucroses resistant/sensitive.

discarded and the cell pellet resuspended and washed in 1 ml of pre-chilled 10% glycerol, after which, it was transferred to a pre-chilled microcentrifuge tube and centrifuged at 10 000 rpm/4°C/2 min. The resuspension, washing and centrifugation step was repeated four times to engineer electrocompetency in the cells. Electrocompetent cells were electroporated (V = 1.8 kV, C = 25 μF, R = 200 Ω (low range) and 500 Ω (high range)) with ∼2 μg of a concentrated and ethanol-precipitated amplicon that contains two flanking homology arms of ∼40–45 nucleotides in length. The P_{araBAD}-*grlR*'-'*lacZ* amplicon was generated by regular PCR, whereas P_{araBAD}-*grlR*(MgrR-mut1)'-'*lacZ* was engineered by overlapping PCR. The upstream homology arm is identical to the P_{araBAD} core promoter, whereas the downstream homology arm is identical to the '*lacZ* gene. Electroporants were recovered in LB and subsequently plated on M63 or LB plates supplemented with 5% sucrose at 30°C to enrich for recombinants. Thereafter, ∼50 colonies were spot-streaked on LB, LB supplemented with chloramphenicol or LB supplemented with tetracycline plates

Table 2. Oligonucleotides used in this study.

Primers (purpose)	Sequence
SB2257 (5′ primer for deleting *hfq* in EPEC)	ATCGAAAGGTTCAAAGTACAAATAAGCATATAAGGAAAAGAGAGAcatatgaatatcctcctta
SB2258 (3′ primer for deleting *hfq* in EPEC)	AACGCAGGATCGCTGGCTCCCCGTGTAAAAAAACAGCCCGAAACCgtgtaggctggagctgcttc
SB2236 (5′ primer for generating P_{araBAD}-*grlR*'-'*lacZ* and P_{araBAD}-*grlR*(MgrR-mut1)'-'*lacZ* fusions)	ACCTGACGCTTTTTATCGCAACTCTCTACTGTTTCTCCATgcaatctggagaaaaagaaaggtct
SB2248 (3′ primer for P_{araBAD}-*grlR*'-'*lacZ* and P_{araBAD}-*grlR*(MgrR-mut1)'-'*lacZ* fusions)	TAACGCCAGGGTTTTCCCAGTCACGACGTTGTAAAACGACattgctaataaatataatgctatagatgcc
SB2314 (3′ primer used with SB2236 to generate the upstream fragment of *grlR*(MgrR-mut1)'-'*lacZ*)	ACCTCACTCCTTTCAATTTGTTCTA**agg**ATAAGCAATATCAAGAATAATGGAGAC
SB2315 (5′ primer used with SB2248 to generate the downstream fragment of *grlR*(MgrR-mut1)'-'*lacZ*)	GTCTCCATTATTCTTGATATTGCTTAT**cct** TAGAACAAATTGAAAGGAGTGAGGT
SB2181 (5′ primer for sequencing *lacZ* fusions	CGACGAATTCGCGCTTCAGCCATACTTTTCATAC
SB2180 (3′ primer for sequencing *lacZ* fusions	CGGGCCTCTTCGCTA
SB2311 (5′ primer to amplify *mgrR* or *mgrR-mut1* from EPEC)	tatactatGACGTCGATCCGTTATCAGTGCAGGAAAAT
SB2312 (3′ primer to amplify *mgrR-mut1* from EPEC)	gcgcgcAAGCTTAAAAAAAACCGCCAGTAAACCGGCGGTGAATGCTTGCAT**cct** TAGATTTGTGTTTTGCTTTTACGCT
SB2313 (3′ primer to amplify *mgrR* from EPEC)	gcgcgcAAGCTTAAAAAAAACCGCCAGTAAACCGG
SB2316 (5′ primer to amplify *mcaS* from EPEC)	tatactatGACGTCACCGGTCACCAGGACCCCAGG
SB2317 (3′ primer to amplify *mcaS* from EPEC)	gcgcgcAAGCTTAAAAAATAGAGCCTGTCGACATCC
SB2440 (3′ primer for site-directed mutagenesis of *ryhB* to *ryhB-mut1*)	GGGTCTTCCTGATCGC
SB2441 (5′ primer for site-directed mutagenesis of *ryhB* to *ryhB-mut1*)	TCGCGGAGAACCTGAAAGCACGAttcctctttaATTGCTTCCAGTATTACTTAGCCAG
5′ *grlR* (upstream primer for qRT-PCR)	TTAGCAATGAAGACTCCTGTGG
3′ *grlR* (downstream primer qRT-PCR)	AGAGAGAACCCCCTGATACAC
5′ *grlA* (upstream primer for qRT-PCR)	AGGCGGTTCCGATAGAAAGT
3′ *grlA* (downstream primer for qRT-PCR)	GCCTCAAGATCATTTCGTTCC
5′ *rrsB* (upstream primer for qRT-PCR)	CTTACGACCAGGGCTACACAC
3′ *rrsB* (downstream primer for qRT-PCR)	CGGACTACGACGCACTTTATG

Restriction sites are underlined. The boldface indicates the mutations that were introduced.

to identify potential recombinants that are phenotypically Cm^S Tet^S Suc^R. This specific phenotype suggests that the *cat-sacB* cassette has been replaced by the amplicon to generate a translational fusion (P_{araBAD}-*yfg*'-'*lacZ*) and the mini-lambda prophage has been successfully cured. The genotype of the recombinants was further verified by PCR and sequencing.

The *hfq* mutants of EPEC and *E. coli* were complemented with the wild-type allele of *hfq* from *E. coli* that was expressed from an Isopropyl β-D-1-thiogalactopyranoside (IPTG)-inducible promoter from the multicopy plasmid pUC18. The primary structure of the Hfq protein is identical between the prototypical EPEC strain 2348/69 and *E. coli* strain K-12 substrain MG1655.

β-Galactosidase assay

β-Galactosidase assays were performed as describe previously with slight modifications (Mandin and Gottesman 2009). An axenic bacterial colony was inoculated into 5 ml of LB or LB supplemented with the appropriate antibiotic and grown overnight at 37°C/250 rpm. Overnight cultures were diluted in replicates to a starting OD_{600} of ∼0.03 into 5 ml LB/(LB+Amp_{100}) + Arabinose (0.002%) + IPTG (1 mM) (if needed) in a borosilicate glass test tube and grown under shaking conditions to varying optical densities. All cultures, but RyhB overexpressors, were grown to an OD_{600} of ∼0.6–0.7. The RyhB overexpressors were grown to an OD_{600} of ∼1.2. β-Galactosidase assays were performed on 100 μl of permeabilized cell extracts. Each assay was performed on at least three separate occasions with replicates of two different isolates being used in each experiment.

RNA isolation and qRT-PCR

RNA isolation was performed by using Trizol reagent (Thermo Fisher Scientific, Grand Island, New York, USA) followed by isopropanol precipitation. Quantitative Real-time Reverse Transcription Polymerase Chain Reaction (qRT-PCR) was performed on 50 ng of DNase I-treated RNA essentially as described previously (Bhatt *et al.* 2009), with the exception that the RotorGene SYBR Green RT-PCR kit (QIAGEN, Germantown, Maryland, USA) was used.

Preparation of cell lysates, trichloroacetic acid precipitation and Western blotting

Bacterial cultures were grown under stationary conditions at 37°C in LB or DMEM lacking phenol red to an optical density of ∼0.5–0.7. Briefly, 1 ml of the culture was pelleted by centrifugation at 13 200 rpm for 2′. The supernatant was completely separated from the pellet and the two fractions were treated differently. The pellet was permeabilized by adding a volume of 1× SDS protein loading buffer, supplemented with ß-mercaptoethanol (5%) and Bromophenol blue (0.02%), that is 100× the optical density at the time of harvest of the culture (e.g. At OD_{600} of 0.5 and 0.6, 50 and 60 μl of the SDS protein loading buffer was added, respectively). The samples were heated at

95°C for 10′ and then centrifuged at 13 200 rpm for 2′. Approximately 30 μl of the cell extract was loaded onto pre-cast 4%–20% gradient or AnyKD sodium dodecyl sulfate-polyacrylamide gels (Bio-rad, Hercules, California, USA) and electrophoresed. Subsequently, the samples were electroblotted onto a nitrocellulose membrane. The membrane was then blocked in 5% nonfat dry milk dissolved in 1× phosphate-buffered saline supplemented with Tween 20 (0.1%) buffer and probed for the presence of the appropriate protein. The primary antibodies used were anti-FLAG (1:5000 dilution), anti-GroEL (1:2000 dilution), anti-Ler (1:2000 dilution), anti-EspB (1:10 000 dilution) and anti-Tir (1:5000 dilution). A secondary antibody conjugated to Horseradish peroxidase was used at 5000-fold dilution to detect each primary antibody except anti-FLAG, which is conjugated to alkaline phosphatase. The secretion of EspB and Tir in the culture supernatants was assayed by Western blotting essentially as described previously (Bhatt *et al.* 2009). Each experiment was repeated on at least two separate occasions with similar results in each experiment.

RESULTS

Inactivation of *hfq* leads to constitutive gene expression from the LEE of EPEC

The role of Hfq in the virulence of EHEC has been meticulously interrogated. Deletion of *hfq* results in constitutive expression of GrlR and GrlA, which appears to be mediated at the post-transcriptional level by affecting *grlRA* mRNA stability (Hansen and Kaper 2009). However, the detailed molecular mechanism, including the roles of Hfq-dependent sRNAs, remains to be determined. By contrast, the role of this RNA chaperone in EPEC pathogenicity is, at best, cryptic (Hansen and Kaper 2009). To elucidate the role of Hfq in the virulence of EPEC, we used lambda red recombineering to engineer an amorphic mutation in the *grlA*-3X-FLAG-tagged mutant derivative of EPEC. Thereafter, we compared the synthesis of GrlA and other LEE-encoded proteins in the mutant and its coisogenic unmutagenized parent. As seen previously, deletion of *hfq* led to increased steady-state levels of the GrlA-3X-FLAG-tagged protein in LB and DMEM (Fig. 1A). Consistent with the observed increase in GrlA levels in the *hfq* mutant, there was increased expression and ensuing secretion of the type three secretion (T3S) substrates, EspB and Tir, whose genes are positively regulated GrlA (Fig. 1B). Moreover, the phenotype of the *hfq* mutant was successfully rescued upon complementing it with the wild-type *hfq* allele, expressed from an IPTG-inducible promoter from the plasmid pUC18 (Fig. 1A). Overall, these results suggest that in EPEC Hfq represses gene expression from the LEE by targeting GrlA.

The 5′ region of *grlR* is sufficient for Hfq-dependent regulation

Whereas immunoblotting confirmed that Hfq negatively regulated GrlA protein levels, the assay did not reveal the hierarchical level at which Hfq exerts its effect and whether the observed effect is direct or mediated indirectly via an intermediate regulator. Hfq typically chaperones *trans*-encoded regulatory sRNAs to their target mRNAs to facilitate complementary base pairing (Link, Valentin-Hansen and Brennan 2009; Waters and Storz 2009; Papenfort and Vogel 2010). In many instances, sRNAs base-pair with the 5′ region of the first gene in a polycistronic mRNA. In EPEC, *grlA* is cotranscribed with the upstream gene *grlR* to generate the bicistronic *grlRA* transcript (Mellies

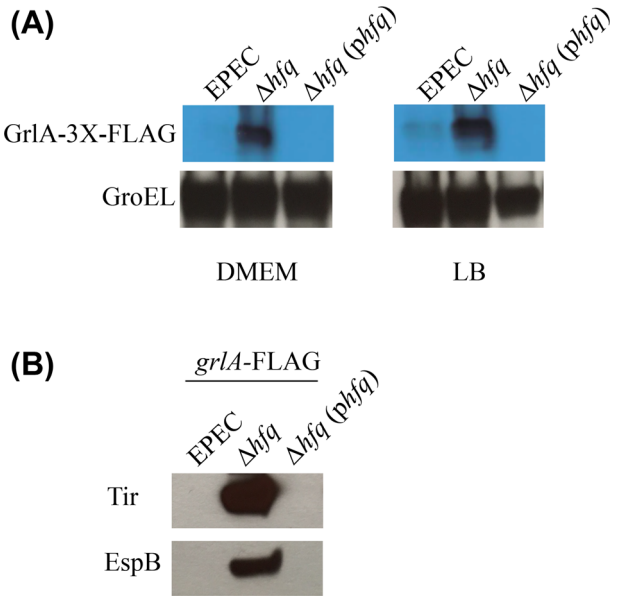

Figure 1. (**A** and **B**), Inactivation of *hfq* leads to increased expression of GrlA and GrlA-regulated genes. Overnight grown cultures of EPEC *grlA*-3X-FLAG, EPEC *grlA*-3X-FLAG Δ*hfq* and the *hfq* complemented strain were sub-cultured in DMEM and LB and grown to an OD_{600} of ∼0.5–0.6 as described in materials and methods. The steady-state levels of FLAG-tagged GrlA and native GroEL proteins in whole cell extracts were examined by Western blotting (A). Additionally, the secretion of Tir and EspB, that are transcriptionally activated by GrlA, was examined when bacteria were cultivated in DMEM, which represents LEE-inducing conditions (B). Each experiment was repeated on at least three separate occasions and similar results were obtained in each trial. The image shown is representative from one such experiment.

et al. 1999). A computational analysis of the 5′ region of *grlR* revealed the presence of a poly(A-R-N) motif (AGAAAAAGAAAG) 10 nucleotides downstream of the *grlRA* transcription start site (Fig. 2A), where A, R and N denote adenine, purine and any nucleotide, respectively. Such motifs are recognized by the distal face of Hfq (Link, Valentin-Hansen and Brennan 2009). This suggested that Hfq presumably targeted the entire *grlRA* mRNA by binding upstream of the *grlR* open reading frame (ORF). To test this hypothesis, we engineered a transgenic *Escherichia coli* reporter strain in which the entire 5′ UTR along with 45 nucleotides of the *grlR* ORF was fused in-frame with *lacZ* gene to generate a chromosomal *grlR*′-′*lacZ* translational fusion under the transcriptional control of the heterologous *araBAD* promoter (Fig. 2A). The P_{araBAD}-*grlR*′-′*lacZ* fusion is informative about environmental and regulatory controls that affect *grlR* posttranscriptionally. Deletion of *hfq* resulted in a reproducible ∼2–3-fold increase in β-galactosidase activity (mean ± SD of 14420 ± 3418) compared to the unaltered parent (5308 ± 547) (Fig. 2B), suggesting that the cloned 5′ region of *grlR* is sufficient for the observed Hfq-dependent repression of *grlRA*. Moreover, we also examined the steady state mRNA levels of the *grlRA* mRNA by qRT-PCR in the native EPEC background. The transcript levels of both *grlR* and *grlA* were strongly elevated in the *hfq* mutant grown in LB (Fig. 2C) or DMEM (Fig. 2D) in comparison to its coisogenic parent, with the effect being more pronounced in DMEM than in LB. For instance, *grlR* levels were elevated by ∼3-fold in LB (Fig. 2C) but ∼11-fold in DMEM (Fig. 2D). Likewise, *grlA* levels were derepressed by ∼8-fold in LB (Fig. 2C) but ∼59-fold in DMEM (Fig. 2D).

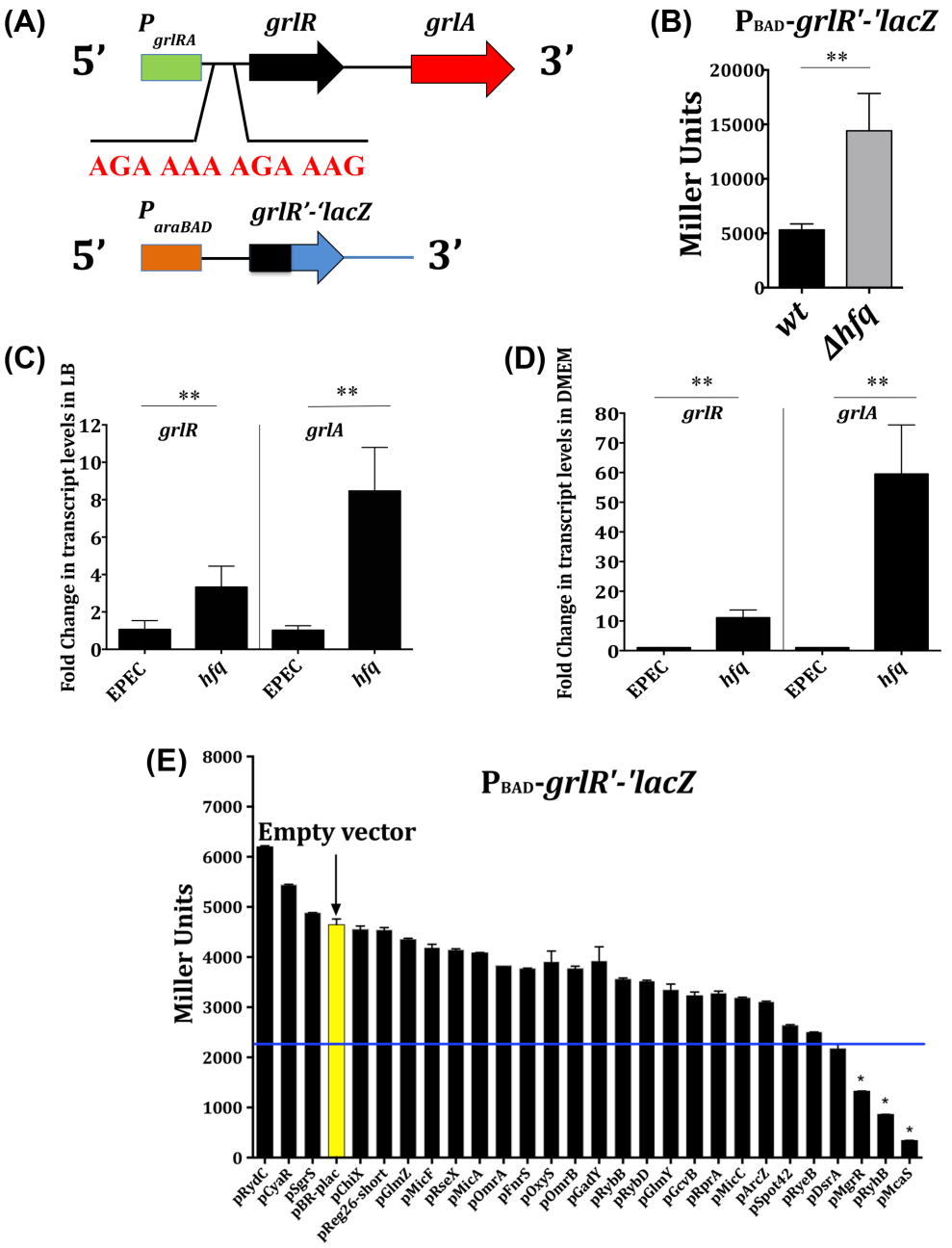

Figure 2. The 5′ leader region of *grlR* is sufficient for regulation by Hfq and the Hfq-dependent sRNAs MgrR, RyhB and McaS. A recognizable Hfq-binding site is located in the 5′ UTR of *grlR* within the bicistronic *grlRA* transcript. A reporter strain was engineered in which the 5′ UTR of *grlR* and 45 nucleotides into the *grlR* ORF were fused to a truncated *lacZ* gene, which lacks its native 5′ UTR and nine of the N-terminal codons, to generate a *grlR'-'lacZ* translational fusion. This fusion is transcriptionally driven by the heterologous P_{araBAD} promoter (**A**). Inactivation of *hfq* leads to increased β-galactosidase activity from the minimal P_{araBAD}–*grlR'-'lacZ* translational fusion (**B**). qRT-PCR was used to monitor the steady-state *grlRA* transcript levels in EPEC and its coisogenic *hfq* mutant grown in LB (**C**) and DMEM (**D**). The transcript levels for both *grlR* and *grlA* were profoundly derepressed in the *hfq* mutant with the effect being stronger in DMEM than LB. The *grlR'-'lacZ* reporter strain was transformed with individual members of plasmid library, each of which overproduces a conserved Hfq-dependent sRNA under the control of an IPTG-inducible promoter. MgrR, RyhB and McaS were the only Hfq-dependent sRNAs that reproducibly repressed the *grlR'-'lacZ* fusion in multiple experimental trials (**E**). The horizontal blue line indicates a 2-fold reduction relative to the empty vector (pBR-plac) containing strain. β-Galactosidase assay with each derivative was performed on at least three separate occasions, with duplicate samples being used for each derivative per experiment. Error bars depict standard deviation. Student's *t*-test was used to assay for statistical significance of the difference in the means between the wild type and the *hfq* mutant or the wild-type containing pBR-plac and the RyhB, MgrR or McaS overexpressors. A *P*-value of <0.02 was considered to be statistically significant. * denotes a *P*-value <0.02 and ** denotes a *P*-value <0.002.

The regulatory sRNAs MgrR, RyhB and McaS repress the *grlR'-'lacZ* fusion

The observation that Hfq represses translation of *grlR* in an *E. coli* genetic background suggested that ancestral Hfq-dependent sRNAs that are conserved between nonpathogenic *E. coli* and EPEC may be involved in coregulating *grlRA* transcript levels. A BLAST search revealed the conservation of many Hfq-dependent sRNAs of *E. coli* in EPEC (data not shown). To determine the involvement of any of these sRNAs in regulating *grlR*, the

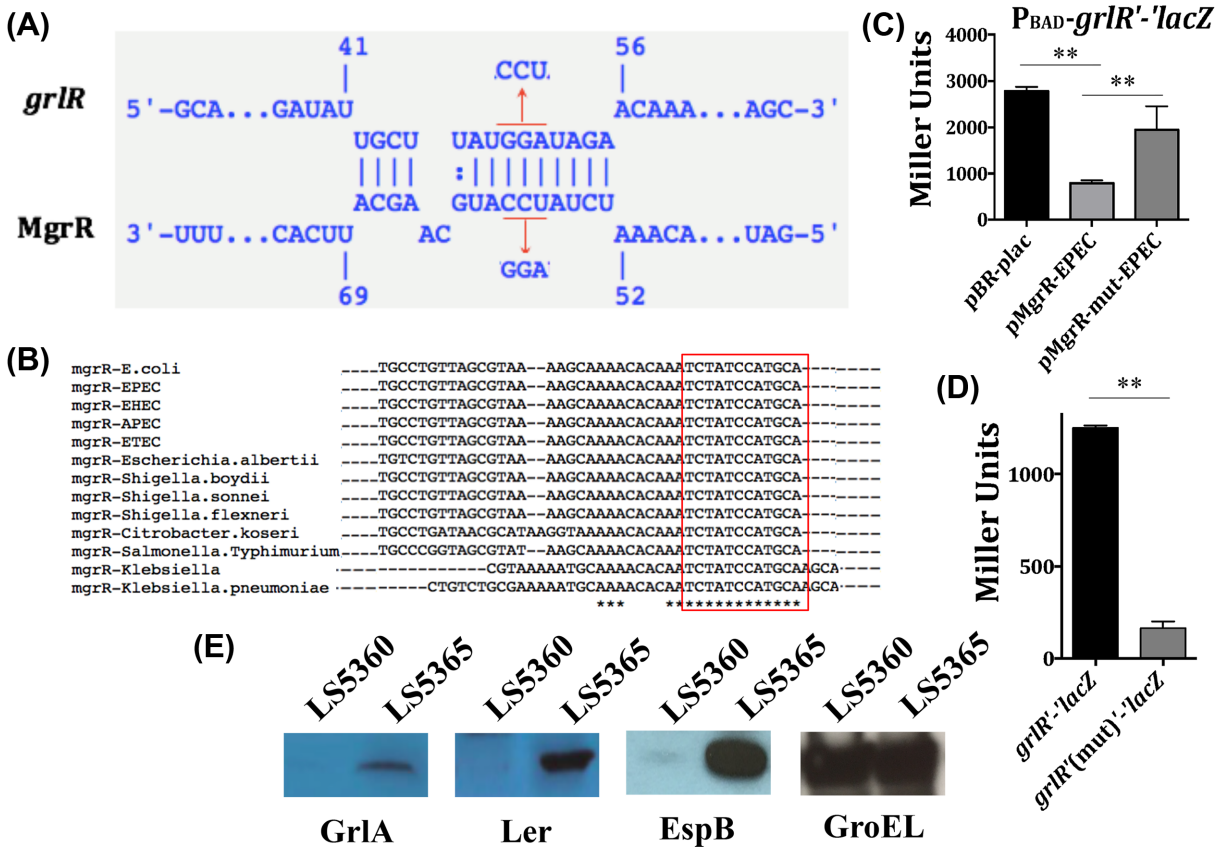

Figure 3. MgrR base-pairs to the 5′ UTR of *grlR* to repress *grlR* but activate *grlA* and the LEE. IntaRNA reveals a region of extensive complementarity between MgrR and the cloned region of *grlR* (**A**). The seed region of MgrR is the most conserved segment within the RNA-coding region (**B**). A trinucleotide mutation (UCC→AGG) within the predicted base-pairing region diminishes the ability of MgrR to repress the *grlR'-'lacZ* fusion (**C**). A compensatory trinucleotide mutation (GGA→CCU) in the predicted base-pairing region of the 5′ UTR of *grlR* diminishes the basal level β-galactosidase activity from the *grlR'-'lacZ* fusion even in the absence of *mgrR* overexpression (**D**). Overexpression of *mgrR* in EPEC results in increased steady state levels of GrlA, Ler and EspB in LB medium (**E**). β-Galactosidase assay with each derivative was performed on at least three separate occasions, with duplicate samples being assayed for each derivative per experiment. Error bars depict standard deviation. Student's *t*-test was used to assay for statistical significance of the difference in the means between the *grlR'-'lacZ* (pMgrR-EPEC) vs *grlR'-'lacZ*(pBR-plac) or *grlR'-'lacZ*(pMgrR-mut1-EPEC) strains. A *P*-value of <0.02 was considered to be statistically significant. ** denotes a *P*-value <0.002.

grlR'-'lacZ reporter strain was individually transformed with a plasmid overproducing one of the Hfq-dependent sRNAs of *E. coli* under the transcriptional control of an IPTG-inducible promoter (Mandin and Gottesman 2010). Three conserved sRNAs, McaS (mean β-gal activity of 343.2 $\pm$ 0.94), RyhB (mean β-gal activity of 862.8 $\pm$ 1.80) and MgrR (mean β-gal activity of 1328 $\pm$ 2.83) reproducibly repressed the *grlR'-'lacZ* by >2-fold in comparison to the empty vectors containing strain (mean β-gal activity of 4646 $\pm$ 115.15) (Fig. 2E). McaS, RyhB and MgrR are sRNAs that are induced in response to diverse stressors. McaS is expressed under nutrient deprivation conditions (Thomason *et al.* 2012; Jorgensen *et al.* 2013), whereas RyhB and MgrR are iron- and magnesium-responsive sRNAs, respectively (Masse and Gottesman 2002; Masse, Escorcia and Gottesman 2003; Masse, Vanderpool and Gottesman 2005; Moon and Gottesman 2009; Moon *et al.* 2013). In *E. coli*, these sRNAs enable the bacterium to rapidly adapt to fluctuating levels of their respective stressors.

MgrR directly represses *grlR* but activates *grlA*

McaS, MgrR and RyhB are antisense regulators that enact gene expression by base-pairing to their target mRNAs. Thus, we used the computational tool IntaRNA to identify regions of potential complementarity between the candidate sRNAs and the 5′ region of *grlR* that had been recombineered upstream of *'lacZ* (Busch, Richter and Backofen 2008). *In silico* analysis revealed that MgrR and RyhB could potentially duplex with the 5′ leader region of *grlR*, albeit at different sites (Figs 3 and 4). However, no obvious regions of complementarity were observed between McaS and *grlR*, suggesting that McaS presumably exerts its effect indirectly by means of transitional regulator. The remainder of the manuscript focuses on the molecular basis of regulation by MgrR and RyhB.

MgrR exhibited the most expansive and continuous region of complementarity with the 5′ leader region of *grlR* (Fig. 3A). MgrR was predicted to duplex between the 42 and 55th nucleotides (42-UGCUUAUGGAUAGA-55) downstream of the transcription start site of the *grlRA* mRNA. Importantly, the predicted base-pairing region of MgrR is the most conserved part within the RNA-coding region of MgrR (boxed region in Fig. 3B), suggesting that there is higher selective pressure to retain the nucleotide sequences, perhaps because of the important role of this domain in base-pairing interactions. Consistent with our observations, a previous report demonstrated that MgrR base-pairs to the *eptB* and *ygdQ* mRNAs in *E. coli* by means of the same oligonucleotide tract and represses gene expression (Moon and Gottesman 2009). Next, we introduced a trinucleotide substitution mutation (UCC→AGG) within the predicted base-pairing region of *mgrR* to generate the mutant allele, *mgrR-mut1* (Fig. 3A).

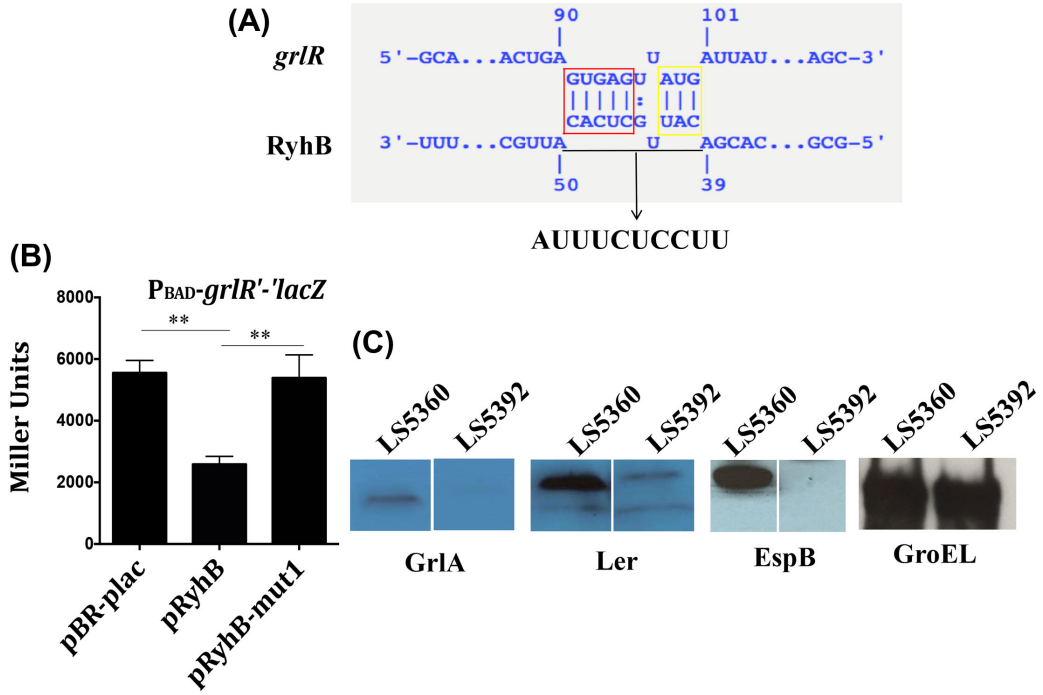

Figure 4. RyhB base-pairs with the *grlR* ribosome binding site and represses gene expression from the entire *grlRA* mRNA and the LEE. RyhB is predicted to duplex with the Shine-Dalgarno sequence and the translation initiation codon of *grlR* (**A**). A polynucleotide mutation within the predicted base-pairing region completely abolishes the ability of RyhB to repress the *grlR'-'lacZ* fusion (**B**). Overexpression of *ryhB* in EPEC represses the steady-state levels of GrlR, Ler and EspB in DMEM medium (**C**). Note that in (**C**), each paired sample (LS5360 and LS5392) is from the same experiment but they were loaded onto nonadjacent lanes in the gel. β-Galactosidase assay with each derivative was performed on at least three separate occasions, with duplicate samples being assayed for each derivative per experiment. Error bars depict standard deviation. Student's *t*-test was used to assay for statistical significance of the difference in the means between *grlR'-'lacZ* (pRyhB) vs *grlR'-'lacZ*(pBR-plac) or *grlR'-'lacZ*(pRyhB-mut1). A *P*-value of <0.02 was considered to be statistically significant. ** denotes a *P*-value <0.002.

This mutation is expected to abolish base pairing between MgrR and the *grlR'-'lacZ* fusion. Predictably, the *mgrR-mut1* allele no longer repressed the *grlR'-'lacZ* fusion (mean β-gal activity of 1941.4 ± 115.15) to the same degree as the wild-type allele (mean β-gal activity of 786.2 ± 64.4) (Fig. 3C), suggesting that the trinucleotide motif, UCC, is essential for MgrR to base-pair to and repress *grlR*. To further validate the direct base-pairing interaction, we generated compensatory mutations in the 5′ seed region of *grlR* by substituting the trinucleotide motif GGA (49–51st nucleotides) with CCU to generate a *grlR*(mgrR-mut1)′-'*lacZ* mutant allele (Fig. 3A), which is predicted to base-pair to the *mgrR-mut1*, but not *mgrR*, allele and specifically be repressed by it. However, we noted that the basal level of β-galactosidase activity from the *grlR*(mgrR-mut1)'-'*lacZ* fusion was inherently much lower (mean β-gal activity of 127.4 ± 1.26) than from the wild-type *grlR'-'lacZ* fusion (Fig. 3D), even without overproducing *mgrR-mut1*, implying that the trinucleotide motif GGA is an essential *cis*-regulatory element required for high levels of β-galactosidase activity from the *grlR'-'lacZ* fusion (Fig. 3D). We also examined the steady-state levels of FLAG-tagged GrlA when *mgrR* was overexpressed in EPEC grown in LB or DMEM. LB and DMEM represent LEE-repressing and LEE-inducing conditions, respectively. Surprisingly, we observed that GrlA levels, instead of being reduced, were profoundly elevated when the bacterium was cultured in LB (Fig. 3E). By contrast, the effect of *mgrR* on GrlA levels in DMEM was modest (data not shown). Consistent with the observed upregulation of GrlA in LB, the protein levels of Ler, which is transcriptionally activated by GrlA, and EspB, which is transcriptionally activated by Ler, were also elevated (Fig. 3E). Thus, these results suggest that MgrR directly base-pairs to the 5′ region of *grlR* on the *grlRA* mRNA and uncouples the expression of *grlR* and *grlA*. Whereas MgrR selectively downregulates the steady-state levels of GrlR, it upregulates the levels of GrlA to initiate gene expression from the LEE (Fig. 5).

RyhB base-pairs to the *grlRA* mRNA and represses expression from both *grlR* and *grlA*

In contrast to MgrR, RyhB was predicted to base-pair over a shorter tract within the *grlRA* leader segment that encompasses the Shine-Dalgarno sequence and the translation initiation codon (91-GUGAGUuAUG-100) of *grlR* ORF (Fig. 4A). Multiple Hfq-dependent sRNAs, including RyhB, repress translation and/or mRNA stability by antisense regulation at the ribosome-binding site (Vecerek *et al.* 2003; Moon and Gottesman 2009). This interaction sterically occludes the ribosome from the mRNA and inhibits translation, which is often accompanied by mRNA degradation (De Lay and Gottesman 2011). Initially, we introduced a mononucleotide (C→T at the 47th position) and a dinucleotide (CA→TC at 47–48th position) substitution in the *ryhB* RNA-coding region. However, these modest alterations did not impact the ability of RyhB to repress the *grlR'-'lacZ* fusion (data not shown). We reasoned that perhaps a more extensive base-pairing mutation was necessary to negate the base-pairing interaction between RyhB and *grlR*. To test this hypothesis, a novel *ryhB* mutant allele ($ryhB_{mut1}$) was engineered in which the entire seed region was substituted (CAUUGCUCAC→UUCCUCUUUA) (Fig. 4A) and the plasmid overexpressing the mutant allele was transformed into the *grlR'-'lacZ* reporter strain. The mean β-gal activity in the $ryhB_{mut1}$ overexpressor was restored to levels comparable to that of the empty vector containing strain (Fig. 4B). These results suggest that the

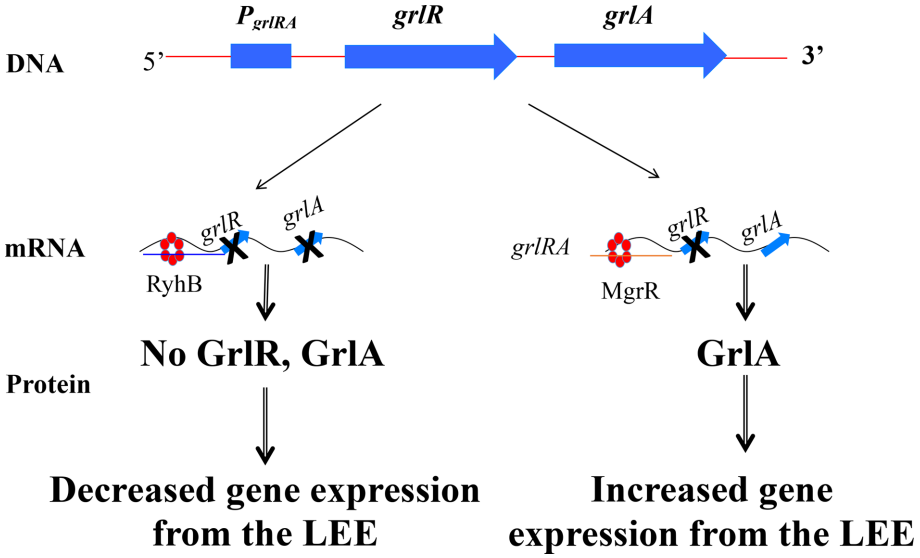

Figure 5. Model for the regulation of *grlRA* by the Hfq-dependent sRNAs RyhB, MgrR and McaS. Hfq represses the LEE by targeting the 5′ region of the *grlRA* mRNA. Three Hfq-dependent sRNAs—RyhB, MgrR and McaS—appear to target the 5′ UTR of *grlR* with varying regulatory outcomes. RyhB base-pairs to the ribosome-binding site and presumably destabilizes the entire *grlRA* mRNA thereby repressing the expression of both GrlR and GrlA. Because a reduction in GrlA would be epistatic to GrlR, thus gene expression from the entire LEE is repressed. MgrR base-pairs to a segment on the 5′ UTR of *grlR* that is located closer to the transcription start site. MgrR specifically represses the expression of GrlR while activating the expression of GrlA and initiating the LEE regulatory cascade. Unlike RyhB and MgrR, McaS does not appear to possess obvious regions of complementarity and presumably exerts its effect indirectly through an intermediate regulator, which is likely CsrA.

ryhB$_{mut1}$ allele no longer represses the *grlR*′-′*lacZ* fusion, arguing for an important role of this polynucleotide tract in antisense regulation by RyhB (Fig. 4B). We did not introduce complementary mutations in the *grlR*′-′*lacZ* region since these mutations would be expected to interfere with the recruitment of the ribosome and prevent translation, thereby making it difficult to uncouple ribosomal recruitment from RyhB-*grlR* base pairing. Moreover, overproduction of *ryhB* in EPEC also repressed the cotranscribed gene *grlA*, evident from reduced levels of the GrlA-3X-FLAG tagged protein (Fig. 4C). RyhB-dependent repression was particularly striking when the bacterium was cultivated in DMEM, which induces gene expression from the LEE (Fig. 4C). Consistent with the observed decrease in GrlA, the expression of Ler and EspB were also reduced in the *ryhB* overproducer (Fig. 4C). By contrast, overexpression of RyhB in LB did not significantly affect gene expression from the LEE (data not shown). Thus, these results suggest that RyhB base-pairs to the ribosome-binding site of *grlR* and, unlike MgrR, represses gene expression from the entire *grlRA* transcript to globally silence the LEE (Fig. 5).

DISCUSSION

In this report, we demonstrate that the RNA-chaperone Hfq and multiple Hfq-dependent sRNAs coregulate gene expression from the LEE of EPEC. Hfq targets the 5′ region of *grlR* posttranscriptionally and results in diminished levels of the *grlRA* transcript, likely by promoting mRNA decay. Additionally, three Hfq-dependent sRNAs MgrR, RyhB and McaS also regulate the *grlRA* mRNA. MgrR and RyhB base-pair to different segments in the 5′ leader region of the *grlRA* transcript to yield different regulatory outcomes. MgrR selectively represses *grlR* but activates *grlA*, thereby activating gene expression from the LEE (Fig. 5). On the other hand, RyhB represses gene expression from the entire *grlRA* mRNA to completely silence the LEE (Fig. 5). In contrast to MgrR and RyhB, McaS does not seem to base-pair to the *grlRA* mRNA and likely exerts its effect indirectly. Thus, our results expand the repertoire of sRNA regulators of the LEE and highlight the intricate nature of such networks in the virulence of A/E pathogens.

Hfq is a prominent regulator of pathogenetic pathways in virtually every conceivable Gram-negative eneteropathogen (Chao and Vogel 2010). This can be attributed to the global regulatory role of Hfq that stems from its ability to recognize short and/or recurring sequence motifs (Valentin-Hansen, Eriksen and Udesen 2004; Link, Valentin-Hansen and Brennan 2009; De Lay, Schu and Gottesman 2013). Such motifs are more likely to be present, or evolve, in horizontally acquired virulence loci. The proximal surface of Hfq recognizes U-rich sequences, whereas the distal surface binds to tandem A-R-N repeats, where R and N represent a purine and any nucleotide, respectively (Link, Valentin-Hansen and Brennan 2009; De Lay, Schu and Gottesman 2013). Hfq can simultaneously bind to discrete transcripts using both its proximal and distal surfaces bringing the two species in spatial proximity and facilitating antisense interactions (Link, Valentin-Hansen and Brennan 2009). The 5′ UTR of *grlR* mRNA possesses the oligonucleotide tract 5′-AGAAAAAGAAAG-3′, located 10 nucleotides downstream of the transcription initiation site, that conforms to the canonical poly A-R-N repeats. Thus, Hfq likely contacts the *grlRA* mRNA with its distal face. Complementary to this observation, RyhB and MgrR possess a Rho-independent transcription terminator, which encompasses a GC-rich inverted repeat sequence followed by a poly-U tract at the 3′ end (Moon and Gottesman 2009; Morita *et al.* 2015). Hfq presumably binds to these sRNAs with its proximal surface. Together, these observations provide a potential mechanism that enables Hfq to bring one of these sRNAs and *grlRA* in proximity and facilitate complementary base-pairing interactions to regulate the LEE.

It is intriguing that under our experimental conditions (i.e. LB and DMEM), Hfq, in concert with RyhB, represses GrlR and GrlA to silence the LEE. Yet, at least one Hfq-dependent sRNA, MgrR, activates GrlA and therefore the entire LEE. These results suggest that Hfq may function as a repressor or activator of the LEE in EPEC. For instance, when EPEC is cultivated under conditions that induce RyhB, such as iron-limitation (Masse and Gottesman 2002), Hfq would be expected to negatively regulate

the LEE. By contrast, when grown under Mg^{2+}-limiting conditions, which induce the transcription of MgrR (Moon and Gottesman 2009; Moon *et al.* 2013), Hfq would be expected to stimulate the LEE. In fact, there is precedence for such a bifunctional regulatory role of Hfq in EHEC. Kendall *et al.* reported that in the EHEC strain 86-24, Hfq functions as an activator of the LEE (Kendall *et al.* 2011). However, in another study, Shakhnovich *et al.* reported that under their experimental setup, Hfq represses the LEE in the same EHEC strain (Shakhnovich, Davis and Waldor 2009), thereby mimicking its observed role in the EHEC strain EDL933 (Hansen and Kaper 2009; Shakhnovich, Davis and Waldor 2009). It is important to note in the two reports EHEC 86-24, despite being cultivated in LB, was grown to different optical densities (Shakhnovich, Davis and Waldor 2009; Kendall *et al.* 2011). Collectively, these studies suggest that environmental conditions, developmental stages and/or strain genotype could dictate whether Hfq activates or represses the LEE in EPEC and EHEC.

The Hfq-dependent sRNAs, MgrR, RyhB and McaS, respond to different environmental cues and regulate different physiological processes in *Escherichia coli*. RyhB is an iron-responsive sRNA that is repressed by the Fur-Fe^{2+} holoprotein complex (Ernst, Bennett and Rothfield 1978; Masse and Gottesman 2002; Masse, Vanderpool and Gottesman 2005; Troxell and Hassan 2013;). Under iron-replete conditions activated Fur binds to the *ryhB* promoter to repress transcription, whereas under iron-deplete conditions Fur is rendered inactive and this results in derepression of *ryhB* (Ernst, Bennett and Rothfield 1978; Masse, Vanderpool and Gottesman 2005; Troxell and Hassan 2013). Derepression of RyhB reduces iron consumption by downregulating iron-containing proteins (Masse, Vanderpool and Gottesman 2005). Moreover, RyhB also stimulates the production of siderophores for scavenging extracellular iron and increasing its availability for the bacterium (Salvail *et al.* 2010). Fur and Fur-binding sites in the promoter of *ryhB* are conserved in EPEC (data not shown), suggesting that the Fur-Fe^{2+}-dependent transcriptional regulation of *ryhB* is intact in EPEC. Consistent with this prediction, inactivation of *fur* results in reduced β-galactosidase activity from the *grlR'-'lacZ* fusion, which is partly rescued by deletion of *ryhB* in the *fur* mutant (data not shown; manuscript in preparation). A previous report had shown that EPEC depletion of extracellular iron reduces the secretion of proteins via the T3S system (Kenny *et al.* 1997). However, the mechanism of action had not been identified. Our results suggest that the reduced gene expression and secretion of the LEE-encoded proteins under iron-deficient conditions may stem from derepression of RyhB. RyhB, in turn, would base-pair to the ribosome binding site located upstream of *grlR* in the *grlRA* mRNA and prevent the ribosome from docking and initiating translation. The exposed transcript is likely recognized by ribonucleases that effectively degrade the entire *grlRA* mRNA. Because expression of GrlA would be epistatic to that of GrlR, this would globally silence the LEE and lead to reduced protein synthesis, secretion and pedestal formation (Fig. 5).

MgrR is a Mg^{2+}-responsive sRNA that is induced under low Mg^{2+} concentrations (Moon and Gottesman 2009; Moon *et al.* 2013). Under such conditions, the membrane-bound sensor kinase PhoQ undergoes autophosphorylation and a phosphorelay ensues whereby PhoQ phosphorylates its cognate response regulator PhoP. Activated PhoP binds to a PhoP box located upstream of the promoter of its target genes, including *mgrR*, to activate transcription (Groisman 2001). The results presented above link Mg^{2+} sensing to the regulation of the LEE-encoded T3SS. In fact, in *Salmonella* Typhimurium, limiting Mg^{2+} concentrations induce the SPI-2 encoded T3SS in a PhoQP-dependent manner (Deiwick *et al.* 1999). Likewise, in EPEC, it would be expected that as Mg^{2+} concentration decreases, *mgrR* is transcribed. MgrR base-pairs to a site located distantly and upstream of the ribosome binding site of *grlR* to repress the expression of *grlR* and activate *grlA*. Collectively, these results suggest that it is unlikely that MgrR mechanistically operates by competing with the ribosome to occupy the ribosome binding site of *grlR* on the *grlRA* mRNA. A more plausible explanation is that base pairing of MgrR to the leader region of *grlR* recruits a ribonuclease that specifically cleaves the bicistronic transcript to generate an unstable *grlR* fragment and a stable *grlA* fragment. GrlA, in turn, would directly activate the transcription of *ler* to initiate the LEE signal transduction cascade, culminating in pedestal formation (Fig. 5).

In contrast to RyhB and MgrR, a complementary base-pairing region was not observed between McaS and the cloned leader region of *grlRA*. Perhaps, using IntaRNA with less stringent parameters may identify complementary regions of base pairing between McaS and *grlRA*. An alternative hypothesis is that McaS may not be exerting its effect by base-pairing to the *grlRA* mRNA but rather by binding to and sequestering the RNA-binding protein CsrA. McaS is an intriguing bifunctional sRNA that is not only capable of base-pairing to its target mRNAs but can also bind to and sequester the RNA-binding protein CsrA effectively reducing its free concentration and preventing CsrA from binding to its natural cellular substrates (Thomason *et al.* 2012; Jorgensen *et al.* 2013). Thus, McaS, like CsrB and CsrC, would function to counteract the effect of CsrA on its targets (Babitzke and Romeo 2007). In a previous study, we demonstrated that purified CsrA binds to the 5′ UTR of *grlR*. Perhaps, overexpression of *mcaS* exerts its effect by binding to and titrating out CsrA. Experiments in our lab are currently underway to test each of these hypotheses.

In summary, we have identified a novel role for multiple conserved Hfq-dependent sRNAs—RyhB, MgrR and McaS—in regulating the LEE-dependent virulence of EPEC. In this report, we only screened for Hfq-dependent sRNAs that regulate *grlR*. Preliminary computational studies in our lab suggest that some of the sRNAs from the library exhibit substantial complementarity with other LEE-encoded operons (data not shown). Future studies in our lab are aimed at screening the sRNA library against translational fusions constructed with each of the other LEE operons to identify a complement of Hfq-dependent sRNAs that regulate the LEE. A systematic investigation of the riboregulatory landscape of the LEE is essential towards understanding the plasticity of gene expression from the LEE and to develop any effective therapeutic measures to counteract EPEC infections. The latter is particularly relevant since RNA is a very attractive pharmacological target.

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