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Global Analysis of Posttranscriptional Regulation by GlmY and GlmZ in Enterohemorrhagic *Escherichia coli* O157:H7

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Enterohemorrhagic *Escherichia coli* (EHEC) is a significant human pathogen and is the cause of bloody diarrhea and hemolytic-uremic syndrome. The virulence repertoire of EHEC includes the genes within the locus of enterocyte effacement (LEE) that are largely organized in five operons, *LEE1* to *LEE5*, which encode a type III secretion system, several effectors, chaperones, and regulatory proteins. In addition, EHEC also encodes several non-LEE-encoded effectors and fimbrial operons. The virulence genes of this pathogen are under a large amount of posttranscriptional regulation. The small RNAs (sRNAs) GlmY and GlmZ activate the translation of glucosamine synthase (GlmS) in *E. coli* K-12, and in EHEC they destabilize the 3′ fragments of the *LEE4* and *LEE5* operons and promote translation of the non-LEE-encoded effector EspFu. We investigated the global changes of EHEC gene expression governed by GlmY and GlmZ using RNA sequencing and gene arrays. This study extends the known effects of GlmY and GlmZ regulation to show that they promote expression of the curli adhesin, repress the expression of tryptophan metabolism genes, and promote the expression of acid resistance genes and the non-LEE-encoded effector NleA. In addition, seven novel EHEC-specific sRNAs were identified using RNA sequencing, and three of them—sRNA56, sRNA103, and sRNA350—were shown to regulate urease, fimbria, and the LEE, respectively. These findings expand the knowledge of posttranscriptional regulation in EHEC.

Enterohemorrhagic *Escherichia coli* (EHEC) is a major cause of hemorrhagic colitis and hemolytic-uremic syndrome. A defining characteristic of EHEC during infection is its ability to form attaching and effacing (AE) lesions on epithelial cells of the intestine, where the bacterium effaces the microvilli and forms an actin-rich pedestal structure that cups the bacterium (1). This process requires a type III secretion system encoded within the locus of enterocyte effacement (LEE) pathogenicity island (2, 3), as well as a non-LEE-encoded effector, EspFu/TccP (4, 5). The LEE contains 41 genes, the majority of which are organized in five major operons named *LEE1* to *LEE5*. The first gene of the *LEE1* operon encodes the master regulator Ler that activates transcription of all LEE genes (6). The *LEE2* and *LEE3* operons encode the major structural components of the type III secretion system, and *LEE4* encodes the needle complex and the EspA filament that sheaths the needle and is part of the type III secretion system's translocon (7). The *LEE5* operon encodes the adhesin intimin and its receptor Tir (8). In addition to the LEE, the EHEC genome contains many regions that are not present in the *E. coli* K-12 genome and are referred to as O-islands. Many of these regions contain genes encoding other virulence factors such as adhesins, toxins, and additional type III secreted effectors (9).

To successfully infect the host, EHEC must tightly control expression of a complex array of virulence factors in response to several environmental signals (10). Bacteria use posttranscriptional regulation to fine-tune gene expression and respond more rapidly to changing environmental conditions (11). The most common forms of posttranscriptional regulation in Gram-negative bacteria are *trans*-encoded small RNAs (sRNAs). These sRNAs generally require the RNA chaperone Hfq and act by binding to the ribosome-binding site (RBS) and blocking translation, by binding to an anti-RBS hairpin and activating translation, or by the recruitment of RNases destabilizing transcripts (12). While much is known about posttranscriptional regulation in *E. coli* K-12, less is known about this process in EHEC. The sRNAs GlmY and GlmZ act in concert in *E. coli* K-12 to promote translation of *glmS*, which encodes the enzyme glutamine synthase necessary for the synthesis of *N*-acetylglucosamine-6-P, which is used for cell wall biosynthesis (13). It has been recently reported that in EHEC, GlmY and GlmZ selectively destabilize parts of the *LEE4* and *LEE5* transcripts and also promote the translation of the effector EspFu, playing an important role in the posttranscriptional regulation of AE lesion formation (14).

In addition to GlmY and GlmZ, EHEC-specific sRNAs encoded in O-islands have recently been discovered. The sRNA Esr41 regulates expression of flagella and motility (15). AsxR and AgvB act as anti-sRNAs and regulate heme oxygenase and amino acid metabolism, respectively. These O-islands are likely a source of many as of as-yet-unidentified sRNAs (16).

Here, we report a global investigation of the role of GlmY and GlmZ regulation in EHEC. Microarrays show these sRNAs are involved in the regulation of many systems, including *gad* acid resistance, curli adhesin, tryptophan metabolism, and a non-LEE-encoded effector. In addition, we identified new EHEC specific sRNAs using RNA sequencing and have identified a few potential targets.

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TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Genotype or content	Source or reference
Strains		
86-24	Wild-type EHEC O157:H7	52
CG06	Δ*glmY* (86-24)	14
CG07	Δ*glmZ* (86-24)	14
MK08	Δ*hfq* (86-24)	46
CG16	Δ*glmY* Δ*rapZ*	This study
Plasmids		
pBad33	Cloning vector	53
pCG30	GlmY in pBAD33	14
pCG31	GlmZ in pBAD33	14
pCG56	LacZ in pBAD24	14
pCG104	CsgD:LacZ in pCG56	This study
pCG105	NleA:LacZ in pCG56	This study
pCG106	sRNA56 in pBAD33	This study
pCG107	sRNA103 in pBAD33	This study
pCG108	sRNA350 in pBAD33	This study

MATERIALS AND METHODS

Strains and plasmids. All bacterial strains and plasmids used in the present study are listed in Table 1. All of the oligonucleotides used here are listed in Table 2. All *E. coli* strains were grown aerobically in Luria-Bertani (LB) medium or Dulbecco modified Eagle medium (DMEM) at 37°C unless otherwise specified. Antibiotics were added at the following concentrations: 100 μg/ml for ampicillin, 30 μg/ml for chloramphenicol, and 50 μg/ml for kanamycin.

Construction of isogenic mutants. The Δ*glmY* Δ*rapZ* (CG16) double mutant was created using the lambda red mutagenesis system as previously described (17). Briefly, the Δ*glmY* (CG06) mutant was transformed with the helper plasmid pKD46. The resulting strain was then grown in the presence of 25 mM arabinose to an optical density at 600 nm (OD_{600}) of 0.6 and then electroporated with a PCR product that was created by amplifying the chloramphenicol-resistant (Cm^r) cassette from pKD3 with the primers rapZREDF and rapZREDR. This PCR product has 70-bp flanking regions that overlap the gene encoding RapZ. The electroporated bacteria were then plated on chloramphenicol-containing media. Cm^r colonies were then analyzed by PCR to confirm insertion of the cassette. Positive insertions were transformed with the resolvase-containing plasmid pCP20. Bacteria were grown at 37°C to induce expression of the resolvase and then plated on LB medium. Colonies were patched to identify chloramphenicol sensitivity, and the resulting colonies were checked by PCR and sequenced to confirm the generation of the mutant.

Plasmid construction. Standard methods were used to perform restriction digestions, plasmid purification, PCR, ligation, transformation, and gel electrophoresis (18). Primers were designed using the IDT Oligoanalyzer 3.1. Coding regions from the EHEC strain 86-24 were amplified using Phusion polymerase (NEB).

The reporter plasmids pCG104 and pCG105 were created by amplifying the 5′ untranslated region (UTR) of *csgD* and *nleA* using the primer pairs csgDtslF/csgDtslR and nleAtslF/nleAtslR, respectively. These PCR products were cloned into the vector pCG56 using KpnI and PstI restriction enzymes. The sRNA overexpression plasmids were constructed by amplifying the approximate sRNA-containing regions using the primers 56F and 56R, 103F and 103R, and 350F and 350R. These PCR products were then cloned into pBAD33 using the restriction enzymes SacI and XbaI. The resulting plasmids were all sequenced to confirm proper insertion.

Fluorescent actin staining. HeLa cells were maintained in high-glucose DMEM supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin glutamine and grown at 37°C and 5% CO_2. Cells were split into a 12-well plate, grown to confluence, washed, given low-glucose DMEM supplemented with 10% FBS, and then infected with bacteria grown overnight in LB medium statically at 37°C at a 1:100 dilution. After 3 h of infection, the medium was removed and replaced, and the infection continued for three more hours. The bacteria were then washed, fixed in formaldehyde, permeabilized with 0.2% Triton X-100, and stained with fluorescein isothiocyanate (FITC)-phalloidin and propidium iodide as previously described (19). The cells were then visualized by fluorescence microscopy. Statistical analysis was then performed by randomly imaging different fields and counting the first 50 cells, while recording the number of bacteria attached to each one. The Student *t* test was used to determine significance.

Reporter assays. Translational reporter assays were performed by first transforming the appropriate strains and reporter plasmids: wild type (wt), wt/pCG30, and wt/pCG31 with pCG105, as well as wt, CG06, and CG07 with pCG104. The bacteria were then grown overnight in LB medium at 37°C with the appropriate antibiotics. Cultures were then diluted 1 to 100 in clear DMEM to an OD_{600} of 0.8 in the presence of 0.2% arabinose for pCG105 and 0.1% arabinose for pCG104. These cultures were then assayed for beta-galactosidase activity using *o*-nitrophenyl-β-D-galactopyranoside in a Miller assay (20).

RNA purification. Cultures grown overnight aerobically at 37°C in LB medium were diluted 1:100 into DMEM and grown in triplicate to an OD_{600} of 1.0 or grown for 6 h at 37°C and 5% CO_2, which is the optimal *in vitro* condition for virulence gene expression in EHEC, and then pelleted and suspended in TRIzol (Invitrogen). A RiboPure bacterial isolation kit (Ambion) was then used to extract RNA from these biological replicates according to the manufacturer's protocols except for two modifications: TRIzol was used instead of RNAwiz, and the cells were not disrupted by vortexing with beads. Samples were DNase I treated to the manufacturer's specifications, and the concentration of RNA was determined with a NanoDrop apparatus.

Quantitative real-time PCR (qRT-PCR). Quantification of RNA transcription was performed as described previously (21). Extracted RNA was diluted to a concentration of 50 ng/μl and mixed with SYBR green, validated primers (Table 2), RNase inhibitor and reverse transcriptase (AB). The mix was used in a one-step reaction utilizing an ABI 7500 Fast sequence detection system. The data were normalized to endogenous *rpoA* levels and analyzed using the comparative critical threshold cycle (C_T) method. The data are presented as the fold changes over the wt levels. The error bars in the figures represent the standard deviations of the $\Delta\Delta C_T$ value. A Student unpaired *t* test was used to determine statistical significance, with a *P* value of ≤0.05 being considered significant.

Microarrays. Microarray global analysis of the transcriptome of wt, Δ*glmY*, and Δ*glmZ* strains of EHEC were performed on extracted RNA according to the manufacturer's instructions outlined in the Affymetrix gene expression technical manual. RNA was extracted as previously described and used as a template for reverse transcription to cDNA. This DNA was then fragmented with DNase I, labeled, and hybridized to the *Escherichia coli* Genome GeneChip 2.0. The gene chips contain over 10,000 probe sets directed toward 20,366 genes from four different strains of *E. coli*: the K-12 laboratory strain MG1655, the O157:H7 EHEC strain EDL933, the O157:H7 EHEC strain Sakai, and the uropathogenic strain CFT073.

The results were gathered from scanning the chips, and the data were normalized using the MAS5 method. Expression between chips was compared using the using the Affymetrix GeneChip operating software v1.4 (22) (GEO database number GSE63336).

sRNA identification. RNA sequencing was performed on RNA extracted from wt EHEC grown statically at 37°C and 5% CO_2 for 6 h. Bacterial mRNA was selectively amplified with the Ovation RNA-Seq System (Nugene) and then processed with the Encore RNA complete RNA-seq library system (Nugene). A paired-end sequencing run was then performed on an Illumina sequencing machine.

All alignments were performed using Bowtie2 (23). To enrich for EHEC specific reads, the raw reads were aligned against the MG1655

TABLE 2 Oligonucleotides

Oligonucleotide	Sequence (5′-3′)
Primers for plasmid construction	
csgDtslF	CAT GGTACC CAG ATG TAA TCC ATT AGT TTT ATA TTT TAC CC
csgDtslR	GTA CTGCAG TGA TCA ACA ATA ATG TAT GAC CAT GAA TAC
nleAtslF	CAT GGTACC TGT CCA CAT CGG ATA TGT GAC AC
nleAtslR	GTA CTGCAG TCC AGA TTG TAT GGT CGG TTG AAT G
p56F	CAT GAGCTC GGC TTA GTT CTG G
p56R	GTA TCTAGA CAT GAA CCT TTT ATG CAG GC
p103F	CAT GAGCTC TAA CCT GAT TCG TGG TAT G
p103R	GTA TCTAGA GGC CCC TGC TAT GAG
p350F	CAT GAGCTC CTT GAT AAT TTC TGC GCT GG
p350R	GTA TCTAGA CCT GAC TTA ACT CCA GAA TAG
Lambda red primers	
rapZREDF	GTTCAGGTTCAGGTAAATCTGTCGCCCTGCGTGCGCTGGAAGATATGGGTGTGTAGGCTGGAGCTGCTTC
rapZREDR	CGATGGCGTGACTGGACGTTTTTACCGCGCGAGCGGAAGTAGTCTGCCAGCATATGAATATCCTCCTTAG
Primers for Northern probes	
56F	GGCTTAGTTCTGGACGC
56R	TAATACGACTCACTATAGGG CAG TAT TCC GTG TCT GAT ATC AAC
82F	CAT GGC GAA TCC CCC
82R	TAATACGACTCACTATAGGG GGG ATA ACG ATG ATA GTT TGA G
103F	CGC ATG GTG AAT CCC CC
103R	TAATACGACTCACTATAGGG CTG TAT ACT GCA TGG TGC C
108F	CTT TCG GCT GAT GGC TGG
108R	TAATACGACTCACTATAGGG CTA GAT TTT TGC GAC ATA GCG CTT G
110F	GAA AAA GTT GCG CAA ATG GC
110R	TAATACGACTCACTATAGGG GCG TAT ATT TGG ATA TAA GAA AGC CAT C
264F	GCA AAA CTG CGT CTA AAG TTA AAC
264R	TAATACGACTCACTATAGGG GTG TCA ATT CCT GAT ATT GTT TAT GGG
350F	CTT GAT AAT TTC TGC GCT GGA TGG
350R	TAATACGACTCACTATAGGG GAA TAG TTA TAA TTC TGG AGT TTT TCC GC
qPCR primers	
rpoA_F	GCGCTCATCTTCTTCCGAAT
rpoA_R	CGCGGTCGTGGTTATGTG
nlpD_F	CAGCAAAGGACAGGCAATTA
nlpD_R	ATTGTGTCGTTATGGGCGTA
osmY_F	CGTGGTTCAGCTCTCTGGTA
osmY_R	CTTTGGCGATACTTTCAGCA
sepZ_F	GAACAAATCGCACCGTTAGA
sepZ_R	CCCAGGGCTAAGTATTCTGTG
amiB_F	TTTAGCCATCAAAGCAAACG
amiB_R	CGTAGCGTTTGTGCATCTTT
gadA_F	TCGTCGCGGCTTCGAA
gadA_R	TGAGATATTTCAGGGAGGCTTTG
tnaA_F	GTACCGTGCGTAACGTCTATATC
tnaA_R	TCGGACCAACTTCTTCAATACC
cspH_F	GCGGCAAAGGATTCATTATC
cspH_R	GAGTGAATGCGGAAATATGG
csgD_F	GCCTGAAGATTACCCGTACC
csgD_R	TTGATCCTCCATGGCATAAA
csgA_F	GCCACTCTTGATCAGTGGAA
csgA_R	TGTTACCAAAGCCAACCTGA
csgB_F	CCGCAGCAGGTTATGATTTA
csgB_R	CCTGCCGTAACTGAGCACTA
csgE_F	CCGTTGAAAAGAGACTTCGAAAA
csgE_R	GCGACGATTTAGTGCTTCTTCAG
csgF_F	CGCATGGTGACCAACGATTA
csgF_R	TCTGTCACGTTCAACTGCAATTG
nleA_F	AGCCACTACTTCGACGGTAACC
nleA_R	ACGAACCACTTGAGCTGTTAATCC
ureG_F	AAATCCGACTTCCTCGTGAT

(Continued on following page)

TABLE 2 (Continued)

Oligonucleotide	Sequence (5′-3′)
ureg_R	TTGCAGACCTTCACCACTTT
fimZ_F	TCAAACAAATCCAGAGCACAG
fimZ_R	ATCATCTGAACGGCATGAAA
ler_F	CGACCAGGTCTGCCC
ler_R	GCGCGGAACTCATC
sepL_F	GCCTGGGATTCGCAAAGGT
sepL_R	CTCTTGCATATCATTGAGCAGCTT
espA_F	TCAGAATCGCAGCCTGAAAA
espA_R	CGAAGGATGAGGTGGTTAAGCT
tir_F	CCATGGAGAGCAGACGTAGCT
tir_R	CGGTGATCCTGGATTTAACCTT
eae_F	GCTGGCCTTGGTTTGATCA
eae_R	GCGGAGATGACTTCAGCACTT
escV_F	TCGCCCCGTCCATTGA
escV_R	CGCTCCCGAGTGCAAAA

genome, with those that were unaligned then being aligned to the EDL933 genome. Further processing of the data was done using S-MART in a manner similar to, and using tools from, the DETR'PROK Galaxy workflow (24–28). Reads that correspond to a known open reading frame (ORF) were removed, with the remaining reads clustered. Clusters that were less than 30 nucleotides and had fewer than 30 reads were discarded. The resulting 386 predicted sRNAs were visualized using the Integrated Genome Browser (29), and any that corresponded to a discrete region were then confirmed using Northern blots. Binding predictions were done using TargetRNA2 (30). Folding predictions were done using Mfold (31).

Northern blots. wt and Δ*hfq* strains were grown aerobically in low-glucose DMEM supplemented with 0.2% arabinose at 37°C to an OD_{600} of 1.0 from a 1:100 dilution of an overnight grown in LB medium. RNA was extracted, and 5 μg of each sample was run on a 1% formaldehyde agarose gel and then transferred overnight to a Zeta-Probe membrane (Bio-Rad). RNA probes were created by amplifying a segment of the gene of interest with the T7 promoter and *in vitro* transcription using a MAXIscript T7 kit (Ambion) with radiolabeled α-UTP. A probe to sRNA56 was created using 56F and 56R, sRNA82 using 82F and 82R, sRNA103 using 103F and 103R, sRNA108 using 108F and 108R, sRNA110 using 110F and 110R, sRNA264 using 264F and 264R, and sRNA350 using 350F and 350R. The membranes were then hybridized overnight using Ultrahyb (Ambion) at 68°C for the RNA probes and 37°C for the oligonucleotide probes. The membranes were washed and exposed to a phosphorimager screen overnight and then visualized with a Storm scanner (GE Healthcare).

Microarray data accession number. Complete transcriptome data were deposited in the GEO database under accession number GSE63336.

RESULTS

RapZ deletion complements the virulence phenotype of the *glmY* knockout. The GlmY and GlmZ sRNAs have very similar secondary structures. Expression of the *glmZ* gene is constitutive, while expression of *glmY* is tightly regulated by the QseEF two-component system (14, 32). The GlmY sRNA is known to stabilize the GlmZ sRNA (13); GlmZ then base-pairs with the *glmS* mRNA to expose the RBS of *glmS* to promote its translation. The effect of GlmY in *glmS* is indirect and solely attributed to its stabilization of GlmZ, which acts as the effector sRNA (13). The protein RapZ binds GlmZ and recruits RNase E, leading to its degradation in the absence of GlmY. GlmY is capable of binding to RapZ, sequestering it from GlmZ, thereby preventing its degradation (33) (Fig. 1A). A *glmY rapZ* double mutant has been reported to express GlmS at near wt levels (34).

GlmY and GlmZ have been shown to posttranscriptionally regulate virulence genes in EHEC, but the role of RapZ in this regulation has not been addressed. In EHEC GlmY and GlmZ promote degradation of the *LEE4* and *LEE5* transcripts, which encode proteins that play key roles in AE lesion formation. Consequently, the EHEC *glmY* and *glmZ* mutants show enhanced AE lesion formation compared to the wt (14). To investigate the role of RapZ in this strain, a nonpolar *rapZ glmY* double mutant was constructed. The ability of EHEC to form AE lesions on epithelial cells was determined using the fluorescein actin staining (FAS) test (35). HeLa cells were infected with EHEC, the host cell nuclei and bacteria were stained with propidium iodide in red, and F-actin was stained with fluorescein isothiocyanate-phalloidin in green. Pedestals characteristic of AE lesion formation were identified by concentrated actin beneath a bacterium. Wild-type (wt) bacteria formed few pedestals on HeLa cells, while the Δ*glmY* strain formed 16-fold more pedestals ($P < 0.001$) (Fig. 1B and C). This mutant could be complemented by expressing *glmY in trans* on a plasmid (*P* value of <0.01). The Δ*glmY* Δ*rapZ* strain formed pedestals at the same levels as the complemented *glmY* mutant. This indicates that the mutation in *rapZ* is complementing the GlmYZ cascade in EHEC, as reported in other strains of *E. coli*. In the absence of GlmY, GlmZ would bind to RapZ, which recruits RNase E to direct its degradation, leading to more *LEE4* and *LEE5* transcripts and enhanced AE lesion formation. In a double *glmY rapZ* mutant, even in the absence of GlmY, there is no RapZ to bind to GlmZ, and hence there is no GlmZ degradation.

Global regulation of GlmY and GlmZ in EHEC. GlmZ has been previously shown to activate the translation of *glmS* and destabilize the transcripts of the *LEE4* and *LEE5* operons (14, 36). However, the regulon of these small RNAs remain undefined. To identify other potential targets of GlmY and GlmZ, microarrays were performed with RNA extracted from wt, Δ*glmY*, and Δ*glmZ* strains using Affymetrix GeneChip *E. coli* 2.0 arrays. This array contains approximately 10,000 probe sets for 20,366 genes and several intergenic regions present in four strains of *E. coli*: MG1655 (K-12 laboratory strain), CFT073 (uropathogenic *E. coli* strain), and two different EHEC O157:H7 strains (EDL933 and

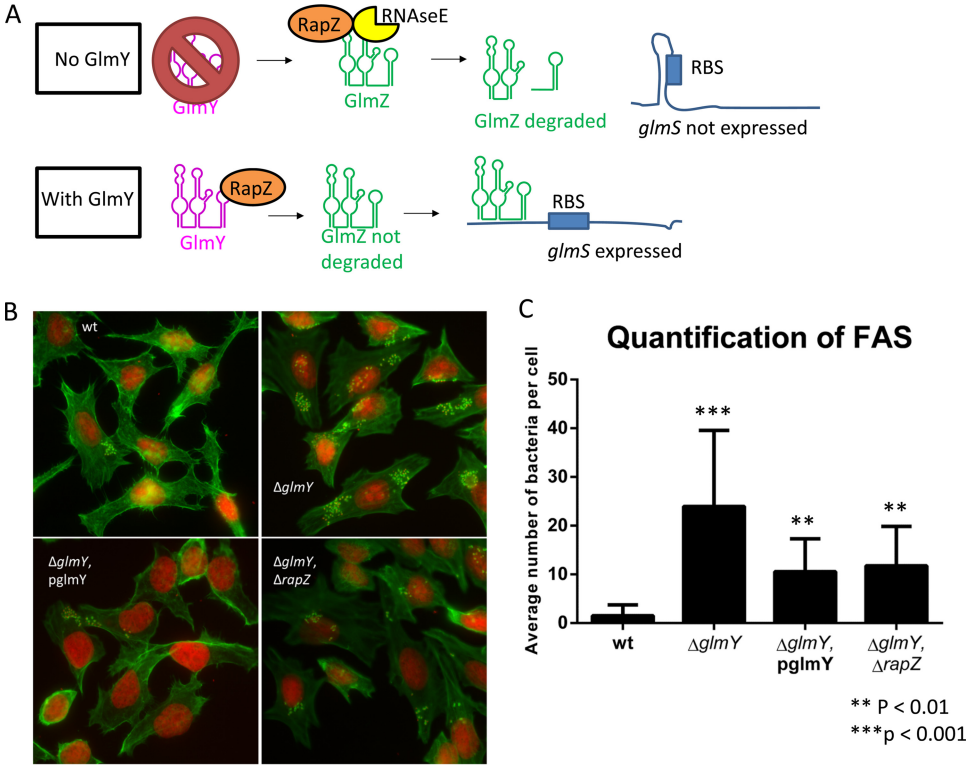

FIG 1 Coordination of LEE expression between GlmY, GlmZ, and RapZ. (A) Diagram of the RapZ-GlmYZ pathway. (B) FAS of wt, Δ*glmY*, Δ*glmY* pglmY (overexpressing GlmY on a plasmid), and Δ*glmY* Δ*rapZ* strains. The Δ*glmY* strain forms more pedestals on HeLa cells, and this can be complemented by adding GlmY expressed from a plasmid. The deletion of *rapZ* also complements the *glmY* mutant as expected. (C) Quantification of FAS.

Sakai). Analysis of these arrays showed significant differences between the two mutants and wt, with increased expression of 551 probe sets and decreased expression of 1,192 probe sets in the Δ*glmZ* strain and with 1,007 increased and 1,627 decreased in the Δ*glmY* strain. The *glmY* and *glmZ* mutants presented more-similar gene expression profiles; however, it is noteworthy that there were also significant differences between them, with 717 probe sets increased and 1,092 decreased in the Δ*glmZ* mutant compared to the Δ*glmY* mutant. Genes within the GlmY and GlmZ regulon include stress-related genes, adhesins, virulence factors (including the LEE and non-LEE-encoded effectors), acid resistance genes, sugar utilization genes, and genes involved in osmoregulation and tryptophan synthesis.

A subset of transcripts that were regulated in the array was confirmed using qRT-PCR (Fig. 2). As expected, the LEE is differentially regulated in the Δ*glmY* and Δ*glmZ* strains, with the LEE genes (*sepZ* and *eae*) being upregulated in these mutants. Expression of the *amiB* gene, which encodes an amidase involved in cell wall recycling, was decreased, likely due to the effect of GlmY and GlmZ on *glmS*. Expression of several stress genes, including the

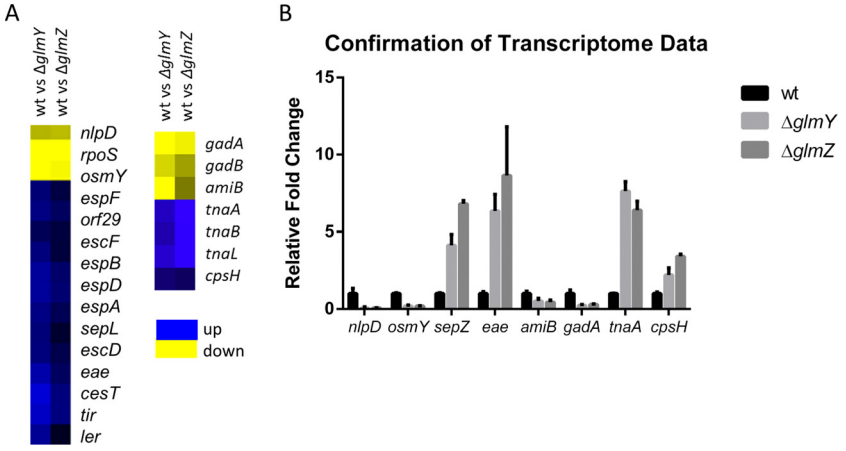

FIG 2 Regulation of *rpoS*-related genes by GlmY and GlmZ. (A) Heat map showing various genes regulated in the Δ*glmY* and Δ*glmZ* mutant microarrays. The LEE, acid resistance, and *rpoS*-related stress genes are regulated. (B) qRT-PCR confirming the microarray data.

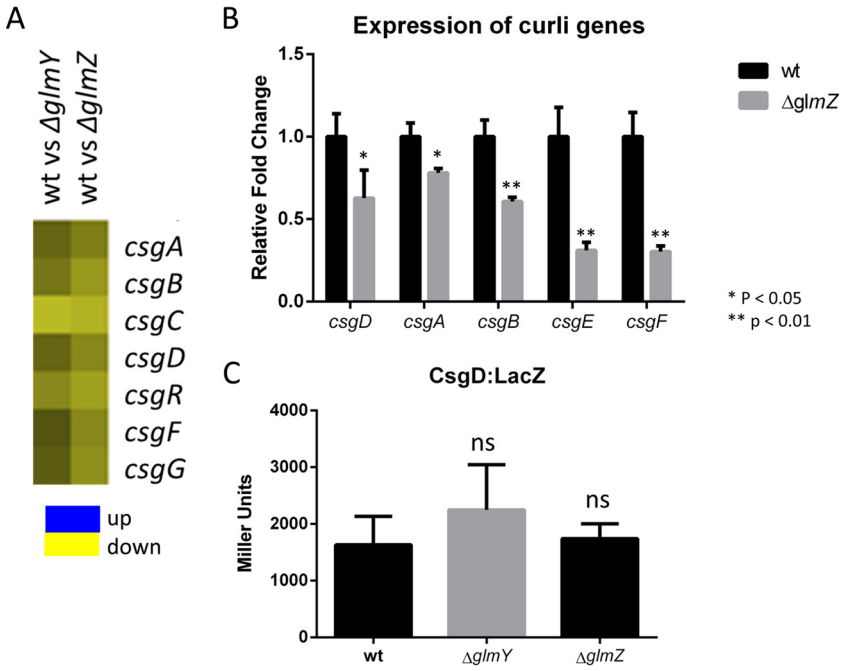

FIG 3 Curli regulation of GlmY and GlmZ. (A) Heat map showing the regulation of the curli genes by *glmY* and *glmZ*. In both mutants, the expression of curli is reduced. (B) Confirmation of the array data showing that the expression of curli genes is decreased in the Δ*glmZ* strain. (C) Expression of a translation reporter of the master regulator of curli CsgD in Δ*glmY* and Δ*glmZ* mutants. CsgD is not being regulated by GlmY and GlmZ at the translational level.

glutamate-dependent acid resistance genes (*gad*) and the osmoregulatory protein Y gene (*osmY*), was decreased in these mutants, whereas the transcript of the gene encoding the cold shock protein H (*cspH*) was increased. Expression of the genes required for tryptophan utilization and indole production (*tnaABL*) were also upregulated in the mutants. In addition to the previously known targets of GlmY and GlmZ, these sRNAs appear to have an effect on various stress pathways that are regulated in stationary phase (37).

Besides regulating stress pathways, the transcriptome data also indicated that GlmY and GlmZ play a role in the regulation of adhesins. Expression of the genes encoding the bacterial adhesin curli was decreased in Δ*glmY* and Δ*glmZ* mutants (Fig. 3A). This was then confirmed using qRT-PCR of several of the curli genes (Fig. 3B). Curli are a major class of adhesin molecules that are involved in bacterial attachment to abiotic surfaces, biofilm formation, and attachment to host cells (38, 39). The curli genes are regulated by the transcription factor CsgD that activates the transcription of the other *csg* operons. The other components are exported to the periplasm through the Sec secretion system. CsgE and CsgF stabilize the other subunits and transport them to CsgG, which exports them out of the cell. Then the major subunit, CsgA, is assembled by the homologous protein CsgB. A translational reporter of the master regulator of the curli operons, CsgD, was constructed. Beta-galactosidase assays of this reporter in the wt, Δ*glmY*, and Δ*glmZ* strains showed no GlmY- or GlmZ-dependent regulation of *csgD* at the translation level (Fig. 3C). Attempts were made to construct a transcriptional reporter of *csgD*; however, the resulting plasmid was lethal in EHEC (data not shown). These data indicate that GlmY and GlmZ may promote stabilization of the *csg* transcripts; however, the specific mechanism of this regulation remains unknown.

NleA is a non-LEE-encoded type III secreted effector protein that disrupts the tight junctions of epithelial cells, which has been shown to be important for virulence in animal models (40). Deletion of either *glmY* or *glmZ* led to a decrease in the transcript levels of *nleA* as determined by qRT-PCR, suggesting that these sRNAs stabilize the *nleA* transcript (Fig. 4A). To assess whether this regulation occurred at the level of translation, a translational LacZ reporter fusion of NleA was constructed. Beta-galactosidase assays of the NleA:LacZ translational reporter in the wt and in wt expressing *glmY* or *glmZ* on a plasmid (of note *glmY* and *glmZ* were cloned in the pBAD33 plasmid, which is compatible with the plasmid containing the NleA:LacZ fusion) showed that neither *glmY* nor *glmZ* had an effect on the translation of this reporter, suggesting that these sRNAs are not acting at the level of translation. No direct binding site for GlmZ was found within the *nleA* transcript, suggesting that the GlmZ-dependent regulation of *nleA* is indirect.

Discovery of novel EHEC specific sRNAs. The small RNAs of *E. coli* K-12 have been thoroughly investigated (41), while those encoded in EHEC specific genomic islands have only recently been identified (15, 16). To identify these EHEC sRNAs, RNA sequencing of RNA extracted from wt EHEC was performed. The resulting EHEC specific reads were clustered and then filtered by length and number of reads, resulting in 386 clusters. These were then analyzed, resulting in 35 potential sRNAs. This same method was performed on the genomic regions conserved with K-12 and successfully identified 22 of 55 known K-12 sRNAs. The existence of these 35 transcripts was then investigated using Northern blotting. In addition, they were also investigated for Hfq dependence, since the RNA chaperone Hfq is required for the stability of most *trans*-encoded sRNAs (42). Of the 35 predicted sRNAs, the existence of seven of these transcripts was confirmed, with all but one,

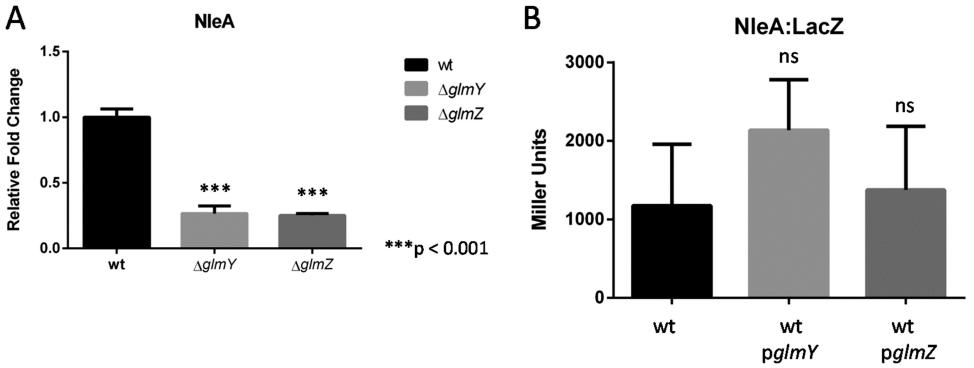

FIG 4 NleA regulation by GlmY and GlmZ. (A) qRT-PCR of *nleA* in wt, Δ*glmY*, and Δ*glmZ* strains. Deletion of the sRNAs leads to a decrease in *nleA* transcript levels. (B) Beta-galactosidase assay of a NleA translation reporter in wt, wt(p*glmY*), and wt(p*glmZ*) strains. No change was detected with overexpression of GlmY and GlmZ, indicating that the regulation of *nleA* by these sRNAs is not at the translational level.

sRNA108, being dependent on Hfq (Fig. 5). sRNA56, sRNA264, and sRNA350 require Hfq, given that we could not detect them on the *hfq* knockout. sRNA82, sRNA103, and sRNA110 seem to be dependent on Hfq, with decreased amounts of these sRNAs present in the *hfq* knockout, but did not require Hfq, given that we could still detect some sRNA in the knockout (Fig. 5). Three of them—sRNA83, sRNA110, and sRNA264—could potentially encode ORFs, but they were included due to the Hfq dependence. Further investigation focused on the remaining three. The regions in which they were encoded were cloned into a vector allowing for their overexpression, and potential targets were predicted using TargetRNA2 (30). Regulation of their predicted targets was then tested by qRT-PCR.

sRNA56 is located between the tellurite resistance genes *terE* and *terF* in O-island 43. In the EHEC strain EDL933 this O-island is duplicated, with the second copy being O48, while in the Sakai strain it is present in a single copy. This sRNA is predicted to bind to the urease accessory protein G (*ureG*) mRNA upstream of its RBS. qRT-PCR performed on RNA from bacteria overexpressing this sRNA revealed an almost 2-fold increase in levels of the *ureG* transcript ($P < 0.01$) (Fig. 6A). The *ureG* mRNA is also predicted to form a weak hairpin, blocking its RBS and interfering with translation initiation (Fig. 6C). Although sRNA56 is not predicted to bind to the anti-RBS sequence, it binds nearby, which could be sufficient to disrupt the weak hairpin. In addition, levels of the transcript encoding the filament protein EspA were also increased 1.5-fold ($P < 0.01$) (Fig. 6A), although this is likely indirect through an unknown mechanism because we could not find any binding regions for sRNA56 within the *espA* transcript. Interestingly, *ureG* is located within the same O-island, which suggests the regulator and its target would be acquired simultaneously.

The sRNA103 is located directly downstream of stx_{2b}, the gene encoding Shiga toxin, one of the major virulence factors in EHEC. Another sRNA, AsxR, was recently reported on the inverse strand directly of sRNA103 (16). This sRNA was predicted to bind within the coding region of *fimZ*, a transcription factor that regulates type 1 fimbriae. qRT-PCR showed that overexpression of sRN103 led to a 3.4-fold increase in *fimZ* transcripts ($P < 0.001$) (Fig. 6B).

A

Name	Start	Stop	Location	Strand
sRNA56	1105323	1105609	O43, between *terE* and *terF*	-
sRNA82	1267510	1267825	O44, CP933M, between Z1348 and Z1349	+
sRNA103	1353498	1353956	O45, BP933W, between *stx2b* and *asxR*	+
sRNA108	1376223	1376653	O45, BP933W, between *lomW* and Z1490	+
sRNA110	1388241	1388792	O45, BP933W, betwee Z1495 and Z1498	+
sRNA264	2966049	2966348	O93, upstream Z3305	+
sRNA350	4672252	4672450	LEE, cesF 3'UTR	+

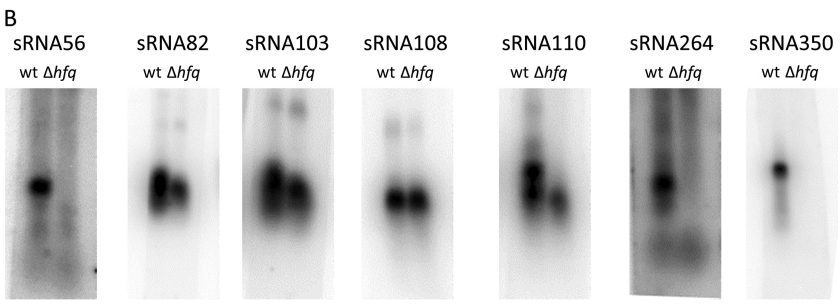

FIG 5 Prediction of EHEC specific sRNAs. (A) Seven predicted sRNAs, their locations, neighboring genes, and strand. (B) Northern blots confirming the existence of the seven predicted transcripts. Both wt and Δ*hfq* RNAs show that all but sRNA108 present in high levels in the presence of Hfq, suggesting they are Hfq-dependent sRNAs.

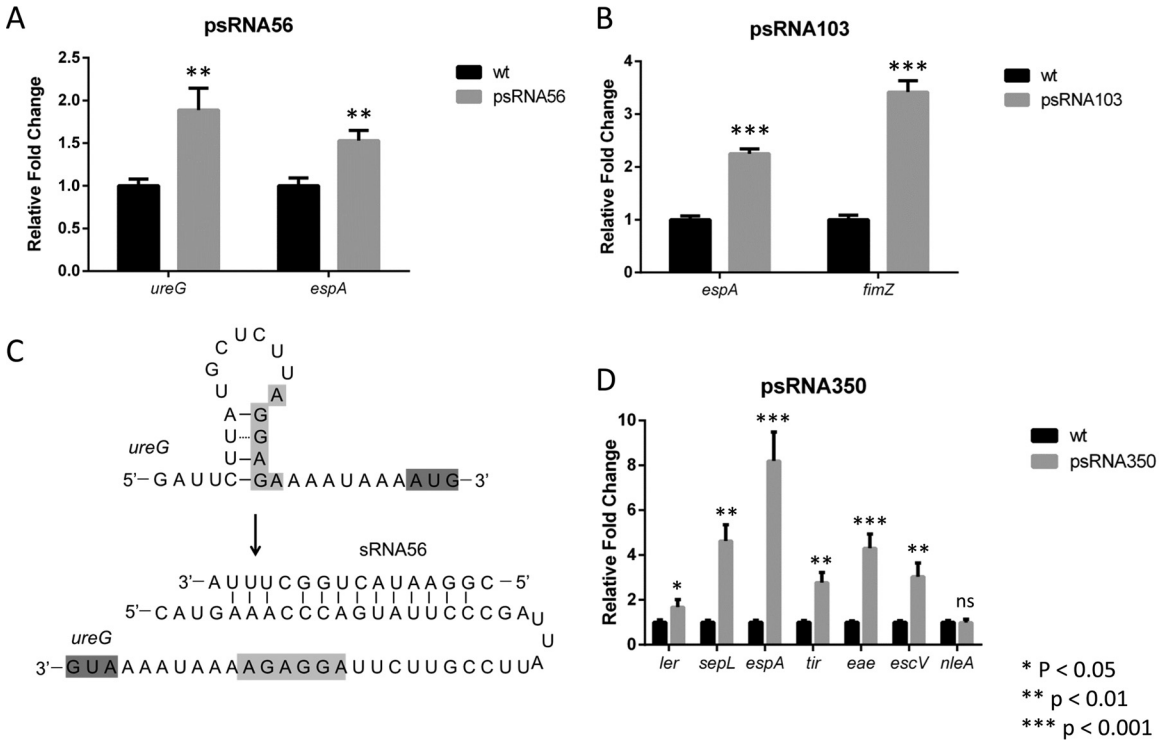

FIG 6 Function of EHEC specific sRNAs. (A) qRT-PCR of RNA from wt and wt psRNA56. Overexpression of sRNA56 leads to upregulation of *ureG* and *espA*. (B) qRT-PCR of RNA from wt and wt/psRNA103. Overexpression of sRNA103 leads to an increase in *fimZ* and *espA* transcripts. (C) Diagram showing the *ureG* mRNA and its anti-RBS hairpin, as well as the predicted binding side of sRNA56 to this mRNA. (D) qRT-PCR of RNA from wt and wt/psRNA350. Several genes of the LEE, including *ler*, *sepL*, *espA*, *tir*, *eae*, and *escV*, are upregulated, while transcripts of *nleA* are unaffected.

Binding within the coding region of a gene typically results in the downregulation of the gene transcript, so if the binding prediction is accurate, this may be a novel mechanism of posttranscriptional regulation in which binding of a sRNA within the coding region is enhancing the levels of transcripts. In addition, levels of *espA* were also increased 2-fold under overexpression of sRNA103 ($P < 0.001$) (Fig. 6B). We could not find any predicted binding sequences for sRNA103 in the *espA* transcript, again suggesting that sRNA103 regulates the levels of the *espA* transcript through an indirect mechanism of posttranscription regulation.

The sRNA350 is not a traditional sRNA. It was predicted to be within the LEE directly downstream of the *cesF* gene, which encodes the chaperone for the type III secretion effector EspF (43). Northern blots showed that the band corresponding to sRNA350 is actually larger than its predicted size, with the sRNA350 probe hybridizing with a band that corresponds to the size of the *cesF* transcript plus sRNA350, suggesting that this sRNA is the 3′ UTR of *cesF* (Fig. 5B). The *cesF* ORF is 384 nucleotides long, while the 3′ UTR is approximately 180 nucleotides. The 3′ UTR of genes have been identified as another source of regulatory RNAs (44); however, no predicted targets were found. Overexpression of this UTR in wt EHEC resulted in the upregulation of various genes within the LEE as measured by qRT-PCR, including *ler*, *sepL*, *espA*, *tir*, *eae*, and *escV*. However, the transcript levels of *nleA*, a non-LEE-encoded effector, were unaffected (Fig. 6D). This regulatory 3′ UTR may act as a global regulator of the LEE island.

DISCUSSION

The virulence genes of EHEC have long been known to be heavily posttranscriptionally regulated. The RNA-binding protein CsrA binds to the *LEE4* operon, and indirectly regulates many other targets (45). A large number of virulence genes are also known to be differentially regulated in the absence of the RNA chaperone Hfq (46–48), suggesting that posttranscriptional regulation by *trans*-encoded sRNAs governs expression of large portions of the EHEC genome. The GlmZ regulation of $espF_U$, *LEE4*, and *LEE5* is only a small part of this regulation. Recently, several EHEC specific sRNAs have been identified, but thus far all have been found to regulate conserved genes that are present in *E. coli* K-12. Regulation of core genes by horizontally acquired sRNAs is becoming a common theme in the evolution of pathogens (15, 16, 49). However, there are still many gaps in our knowledge of EHEC RNA regulation that need to be filled.

In the present study, we used microarrays to determine the regulon of the sRNAs GlmY and GlmZ in EHEC. These sRNAs were initially described as promoting the translation of *glmS* in *E. coli* K-12. The *glmS* gene encodes the enzyme glutamine synthase necessary for the synthesis of *N*-acetylglucosamine-6-P that is important for cell wall biosynthesis (13). We have previously reported that these two sRNAs also posttranscriptionally regulate the *LEE4* and *LEE5* operons and $espF_U$, all of which are necessary for the formation of AE lesions that are key to EHEC pathogenesis (14). We report here that in addition to these previously known targets, these sRNAs also regulate other genes. GlmY and GlmZ regulation of *amiB*, which encodes an amidase involved in cell wall recycling, is likely due to their regulation of *glmS*. Many stress-related genes, such as the *gad* system, *osmY*, and *cspH*, were also differentially regulated. Expression of *glmZ* has been reported to increase in response to exposure to hydroxyurea (50). This report,

coupled with our microarray data, suggests that GlmY and GlmZ may play an important role in response to various stressors. GlmY and GlmZ also positively regulate curli, an adhesin that has been shown to be important for the attachment of EHEC to abiotic surfaces and host cells (51). In addition, GlmY and GlmZ regulate the non-LEE-encoded effector, NleA. In both cases, no direct binding site was found, and it is possible that GlmY and GlmZ are working indirectly. All of these combined results suggest that a complex posttranscriptional regulatory system is likely tied to the timing of infection. Since sRNAs are capable of acting faster than transcriptional regulators, they may be key mediators of the quick responses to stimuli required during an infection.

Pathogens evolve by horizontal acquisition of pathogenicity islands. We describe here how two sRNAs involved in cellular metabolism, stress, and architecture have been co-opted to modulate virulence expression. Most importantly, they modulate expression of the type III secretion system and its effectors, which makes sense given that assembly of the type III secretion machinery requires cell wall rearrangement, because these syringe-like structures breach the peptidoglycan cell wall.

We also performed RNA sequencing on RNA from the EHEC strain 86-24 and identified putative EHEC-specific sRNAs. The seven we have discovered in addition to the three previously described bring the total of EHEC-specific sRNAs to ten. Of these ten, we focused our further investigation on the three that were the most promising. The first of these, sRNA56, regulates *ureG*, a gene within its O-island, likely by disrupting a hairpin that otherwise blocks the RBS and translation. The second, sRNA103, is located within the BP-933W bacteriophage that encodes Shiga toxin 2, a major virulence factor. Two other of the sRNAs we identified, as well as *asxR*, are located within this lysogenic phage. This phage may have such a high concentration of regulatory RNAs, since unlike the various cryptic phages present throughout the EHEC genome, it is still functional. Both sRNA103 and *asxR* are located directly adjacent to the stx_{2b} gene in a region not present in any sequenced lambda phage that does not contain the stx_2 genes. Overexpression of sRNA103 led to the upregulation of *fimZ* transcripts. The predicted binding site for this sRNA was found within the coding region of this gene. sRNA binding within the coding region normally leads to the recruitment of RNases, and the degradation of the transcripts. However, sRNA103 enhances stabilization of this transcript. Assuming the prediction is correct, this could be a new mechanism of sRNA regulation. The most interesting predicted sRNA is sRNA350, the 3′ UTR of the LEE *cesF* gene that encodes the CesF chaperone. The 3′ UTRs of transcripts have been considered a reservoir of regulatory RNAs (44); however, unlike some known examples, the *cesF* UTR is not processed off, and under the conditions we tested, it seems to stay attached to the open reading frame. This UTR is a significant fraction of the size of the *cesF* transcript and certainly serves some purpose. Overexpression of this fragment led to an increase in the transcripts of every gene of the LEE that we investigated, but not of the non-LEE-encoded effector NleA. This sRNA and UTR seem to be acting on the entirety of the LEE through an as-yet-unknown mechanism.

Altogether, we have added extensively to the knowledge of posttranscriptional regulation in EHEC. Understanding the role these newly identified sRNAs play will greatly expand our knowledge of virulence regulation in this pathogen, as well as providing clues to the integration of horizontally acquired genes into the whole-cell regulon.

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