Posttranscriptional Control of Microbe-Induced Rearrangement of Host Cell Actin

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ABSTRACT Remodeling of the host cytoskeleton is a common strategy employed by bacterial pathogens. Although there is vigorous investigation of the cell biology underlying these bacterial mediated cytoskeleton modifications, knowledge of the plasticity and dynamics of the bacterial signaling networks that regulate the expression of genes necessary for these phenotypes is lacking. Enterohemorrhagic Escherichia coli attaches to enterocytes, forming pedestal-like structures. Pedestal formation requires the expression of the locus-of-enterocyte-effacement (LEE) and espFu genes. The LEE encodes a molecular syringe, a type III secretion system (T3SS) used by pathogens to translocate effectors such as EspFu into the host cell. By using a combination of genetic, biochemical, and cell biology approaches, we show that pedestal formation relies on posttranscriptional regulation by two small RNAs (sRNAs), GlmY and GlmZ. The GlmY and GlmZ sRNAs are unique; they have extensive secondary structures and work in concert. Although these sRNAs may offer unique insights into RNA and posttranscriptional biology, thus far, only one target and one mechanism of action (exposure of the ribosome binding site from the glmS gene to promote its translation) has been described. Here we uncovered new targets and two different molecular mechanisms of action of these sRNAs. In the case of EspFu expression, they promote translation by cleavage of the transcript, while in regard to the LEE, they promote destabilization of the mRNA. Our findings reveal that two unique sRNAs act in concert through different molecular mechanisms to coordinate bacterial attachment to mammalian cells.

IMPORTANCE Pathogens evolve by horizontal acquisition of pathogenicity islands. We describe here how two sRNAs, GlmY and GlmZ, involved in cellular metabolism and cellular architecture, through the posttranscriptional control of GlmS (the previously only known target of GlmY and GlmZ), which controls amino sugar synthesis, have been coopted to modulate the expression of virulence. These sRNAs quickly allow for plasticity in gene expression in order for enterohemorrhagic Escherichia coli to fine-tune the expression of its complex type III secretion machinery and its effectors to promote bacterial attachment and subsequent actin rearrangement on host cells. Pedestal formation is a very dynamic process. Many of the genes necessary for pedestal formation are located within the same operon to evolutionarily guarantee that they are inherited together. However, it is worth noting that within these operons, several genes need to yield more proteins than others and that these differences cannot be efficiently regulated at the transcriptional level.
more abundant classes is that of trans-acting small RNAs (sRNAs). The majority of these sRNAs require the RNA chaperone Hfq and act by directly binding to mRNAs at the ribosome binding site (RBS) to repress translation, cause direct degradation of the mRNA by recruitment of nucleases, or activate translation by relieving a hairpin that blocks the RBS. While genes of the LEE are known to be posttranscriptionally regulated, no sRNAs responsible for this have been identified to date.

The AI-3/Epi/NE interkingdom signaling cascade activates the expression of virulence genes in EHEC (21, 31–33). The host hormones Epi and NE are specifically sensed by two membrane-bound histidine sensor kinases, QseC and QseE, which are the first bacterial adrenergic receptors identified (34, 35). QseE is downregulated histidine sensor kinases, QseC and QseE, which are the first genes Epi and NE are specifically sensed by two membrane-located a hairpin that blocks the RBS. While genes of the LEE are mRNA by recruitment of nucleases, or activate translation by repressing translation, cause direct degradation of the RNA polymerase (40). The almost complete lack of AE lesion formation on HeLa cells was confirmed by Northern blot analysis for the GlmY RNA (Fig. 1E). Neither RR had any effect on glmZ expression (Fig. 1F).

The almost complete ablation of glmY expression in the ΔqseF mutant is due to the α34 RNA polymerase acting as a repressor in the absence of QseF (40). The α34 RNA polymerase cannot promote the formation of the DNA open complex to initiate transcription by itself; it requires a σ34 RR, such as QseF, for this process (46). Because the σ34 promoter overlaps the σ70 promoter and in the absence of QseF, the α34 RNA polymerase occupying the σ70 promoter prevents access to the σ70 RNA polymerase (40).

Electrophoretic mobility shift assays (EMSAs) demonstrated that both QseB and QseF directly bind to the glmY regulatory region (Fig. 1G). Interestingly, while the QseB consensus sequence within the glmY regulatory region differs slightly among EHEC strain 86-24, E. coli K-12 strain MG1655, and enteropathogenic E. coli (EPEC) strain E2348/69, all are capable of binding to QseB (see Fig. S1 in the supplemental material).

**Insights into GlmY and GlmZ regulation of EspFu.** Because the QseF RR controls AE lesion formation by indirectly promoting the expression of the EspFu T3SS effector (36), next we investigated whether GlmY and/or GlmZ also play a role in EspFu expression. EspFu interacts with another effector, Tir, through IRTKS and acts as an Nck mimic to recruit N-WASP and Arp2/3 to the site of bacterial attachment, causing the formation of the characteristic actin-rich pedestal (Fig. 2A) (15, 16, 47). The expression of this effector is dependent on QseF, with the qseF mutant having the same phenotype as the ΔespFu mutant, which is the almost complete lack of AE lesion formation on HeLa cells (Fig. 2B and 3A) (15, 16, 36).

Since the regulation of espFu by QseF is known to be indirect (36), we tested the ability of its known target, GlmY, as well as its downstream target, GlmZ, to complement a qseF mutant. The glmY and glmZ genes were cloned under the control of an inducible promoter and transformed into the ΔqseF mutant strain. These strains were then used to infect HeLa cells to perform the fluorescein actin staining (FAS) test to visualize AE lesions. In the FAS assay, the HeLa cytoskeleton was stained green with fluorescein isothiocyanate (FITC)-labeled phalloidin, the bacteria and nuclei were stained red with propidium iodide (PI), and pedestals were visualized as brilliant patches of green underneath a red bacterium. Both sRNAs were able to rescue AE lesion formation in the qseF mutant, indicating that these sRNAs are the intermediaries between QseF and EspFu (Fig. 2B).

Since sRNAs act posttranscriptionally, we investigated espFu mRNA levels by using Northern blot assays. EspFu is located outside the LEE within a prophage. Upstream of the espFu gene is the espJ gene, which encodes another T3SS effector (Fig. 2E) (48). In the WT, there is a major band the size of the predicted espFu transcript (1,100 bp), as well as a much fainter upper band 2,100 nucleotides in length. In the qseF mutant, the lower band is still

**RESULTS**

**Transcriptional regulation of glmY and glmZ.** The QseC/QseE signaling system controls a plethora of virulence genes in EHEC that have to be coordinately expressed to ensure optimal AE lesion formation on epithelial cells, leading to host infection (37, 38, 43, 44). AE lesion formation is a dynamic process that requires plasticity and rapid adaptation of bacterial gene expression. Coupling of transcriptional and posttranscriptional regulation within a signaling transduction cascade in the bacterial cell is key to ensuring fine-tuning and rapid adaptation of gene expression toward the regulation of complex processes such as AE lesion formation. Upstream of the qseEFGlnB operon is glmY (Fig. 1B). The glmY gene is known to have two overlapping promoters, one that is driven by a σ70 RNA polymerase (the homeostatic form of this enzyme) and another that is driven by a σ34 RNA polymerase (Fig. 1B and C). Transcription of glmY is known to be regulated by the σ34-dependent transcriptional activator QseF (40). Additionally, a sequence matching the known consensus sequence of QseB (45), another RR involved in interkingdom signaling, was identified in silico in this promoter region (Fig. 1C).

Transcriptional β-galactosidase reporters of the promoters of both glmY and glmZ were constructed. As previously reported, in the qseF mutant, glmY expression is starkly decreased and almost ablated. Meanwhile, the qseB mutant, while still expressing glmY, expressed significantly less than the wild type (WT) (Fig. 1D). This result was confirmed by Northern blot analysis for the GlmY RNA (Fig. 1E). Neither RR had any effect on glmZ expression (Fig. 1F). The almost complete ablation of glmY expression in the ΔqseF mutant is due to the α34 RNA polymerase acting as a repressor in the absence of QseF (40). The α34 RNA polymerase cannot promote the formation of the DNA open complex to initiate transcription by itself; it requires a σ34 RR, such as QseF, for this process (46). Because the σ34 promoter overlaps the σ70 promoter and in the absence of QseF, the α34 RNA polymerase occupying the σ70 promoter prevents access to the σ70 RNA polymerase (40).

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FIG 1  QseF and QseB regulation of glmY. (A) Schematic representation of the QseC and QseE transduction signaling systems. QseC responds to AI-3, epinephrine, and norepinephrine (Nor), and QseE responds to epinephrine (Epi), sulfate (SO₄), and phosphate (PO₄). Upon sensing their signals, these histidine sensor kinases autophosphorylate and then transfer their phosphate to their response regulators. QseC phosphotransfers to KdpE (activates the LEE genes), QseB (regulates the flagellum regulon), and QseF (regulates espFu). QseE phosphotransfers only to QseF. (B) Schematic representation of glmY and the qseEGFglnB operon depicting the locations of the /H9268₅₄ and /H9268₇₀ promoters. (C) Diagram showing the layout of the glmY regulatory region. QseF binding sites are red, QseB binding sites are blue, and the /H9268₅₄ and /H9268₇₀ promoters are magenta and yellow, respectively. (D) β-Galactosidase assay of the glmY::lacZ transcriptional fusion in the WT strain and the /H9004 qseB and /H9004 qseF mutant strains. The plasmid contains bp −250 to +20 from the transcription start site. (E) Northern blot assay with a...
present; however, the upper band is much more pronounced. This upper band corresponds to the expected size of an espJ-espFu transcript (Fig. 2C and E). There are 320 bp between these two genes, and previous work suggested that they are not cotranscribed. However, since only RNA from WT bacteria was used in those experiments, it is possible that the less abundant larger transcript was not detected (36). To confirm that this larger transcript is espJ-espFu, reverse transcription (RT)-PCR was performed with primers spanning this region. While a very faint band corresponding to the espJ-espFu (2,100-bp) transcript was observed in WT EHEC, it was much more pronounced in the qseF transcript (Fig. 2D). Hence, the lack of EspFu expression in a qseF mutant is due not to the absence of its transcript but to the lack of a processing event of the espJ-espFu transcript necessary for EspFu expression.

Transcriptional and translational reporters of espFu were constructed. As expected, the qseF mutant had no defect in espFu transcription (Fig. 2F); however, it is required for the translation of EspFu (Fig. 2G). To determine the regions of espJ-espFu required for this regulation, various deletions in the intergenic region between these genes were constructed. These deletions were cloned into a vector with a FLAG tag at the C terminus of espFu. The following four deletion mutants were constructed by using the previously identified 5′ untranslated region (UTR) of the espFu transcript as a reference point (36): p1, which lacks the entire intergenic region; p2, which lacks the espJ 3′ UTR; p3, which lacks the espFu 5′ UTR; and p4, which does not have the espJ gene but still has the intergenic region (Fig. 2H). Western blot assays with anti-FLAG antigen were performed with whole-cell lysates of EHEC expressing the WT espJ-espFu-FLAG plasmid and each of these four deletion constructs. The p2 and p4 constructs expressed levels of EspFu:FLAG similar to those of the WT plasmid, while p1 expressed more protein and p3 did not express EspFu (Fig. 2I). These data indicate that the 3′ UTR of espJ acts negatively on the translation of espFu and that this QseF/GlmY/GlmZ-mediated processing event is required for the translation of espJ-espFu since the resulting transcript lacks the 3′ UTR of espJ. Additionally, the presence of EspFu:FLAG from the p4 plasmid indicates that in addition to being cotranscribed with espJ, espFu also has its own promoter (Fig. 2I).

The roles of GlmY and GlmZ in pedestal formation. Given that EspFu is involved in pedestal formation, we further investigated the roles of GlmY and GlmZ in AE lesion formation by constructing ΔglmY and ΔglmZ mutant strains and performing FAS assays with them. Since they were both capable of rescuing the qseF phenotype, we expected that both mutants would have a decreased ability to form pedestals, similarly to the ΔqseF and ΔespFu mutant strains. Surprisingly, both sRNA mutants attached to and formed pedestals on HeLa cells at levels far higher than that of the WT strain. Both the glmY and glmZ plasmids were capable of complementing the glmY mutant, which is an expected result (Fig. 3A and B). The GlmY sRNA is known to stabilize the GlmZ RNA, which is an expected result (Fig. 3A and B). The GlmY sRNA is known to stabilize the GlmZ RNA, which is also its own promoter (Fig. 3I).

To rule out the possibility that the AE lesion phenotype governed by GlmY and GlmZ is due to issues with cell wall synthesis in these sRNA mutants because of the decreased expression of GlmS, FAS

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\text{glm}^\text{Y} \text{ probe of RNAs from the WT strain and the } \Delta\text{qse}^\text{F} \text{ and } \Delta\text{qse}^\text{E} \text{ mutant strains (left) and Northern blot assay of a SS RNA probe of the same RNAs as a loading control (right). (F) } \beta\text{-Galactosidase assay of the glmZ::lacZ transcriptional fusion in the WT strain and the } \Delta\text{qse}^\text{F} \text{ and } \Delta\text{qse}^\text{E} \text{ mutant strains. (G) EMSAs of the glm}^\text{Y} \text{ promoter with increasing amounts of QseF or QseB protein in the presence of acetyl phosphate (left) and EMSAs of the kan promoter with QseF and QseB as a negative control (right). The plasmid contains bp −246 to −20 from the transcription start site. The unpaired Student } t \text{ test was used to determine statistical significance. A } P \text{ value of } \leq 0.05 \text{ was considered significant.} \]
FIG 2  Posttranscriptional regulation of EspFu. (A) Schematic representation of AE lesion formation. (B) FAS of HeLa cells infected with the WT strain, the ΔqseF mutant, or the ΔqseF mutant complemented with pGlmY or pGlmZ. (C) Northern blot assay with an espFu probe of RNA from the WT and ΔqseF and ΔespFu mutant strains (top) and Northern blot assay with a 5S rRNA probe with the same RNAs as a loading control (bottom). (D) RT-PCR of cDNA from the WT and the ΔqseF mutant with primer sets to the entire espJ-espFu region or just espFu. (E) Schematic representation of the espJ-espFu region and the 2,100-bp espJ-espFu and 1,100-bp espFu transcripts. (F) β-Galactosidase assay of the espFu::lacZ transcriptional fusion in the WT and ΔqseF mutant strains with the plasmid containing bp -461 to +48 from the translation start site. ns, no statistically significant difference. (G) β-Galactosidase assay of an EspFu::LacZ
assays were repeated in medium containing 1% GlcNAC. The AE lesion phenotypes of the WT and the glmY and glmZ mutants, higher AE lesion formation by both mutants than by the WT, was the same both in the absence and in the presence of GlcNAC (see Fig. S2B and C). Additionally, point mutations in glmZ that abolish the regulation of glmS by GlmZ were created as previously reported, and the mutant GlmZ sRNA was named GlmZ* (see Fig. S2D) (42). GlmZ* was still capable of complementing pedestal formation in the glmZ mutant, indicating that the pedestal formation phenotype is not mediated through downstream effects of diminished glmS expression or issues with cell wall biosynthesis (see Fig. S2E and F). To further assess the levels of glmS expression promoted by GlmY and GlmZ in EHEC, Northern blot assays of glmS were performed with RNA from WT EHEC and from WT EHEC expressing GlmY, GlmZ, and GlmZ* on a plasmid. It has been previously reported that in E. coli K-12 strain MC4100, expression of the glmS mRNA is increased by the expression of these sRNAs on plasmids because of the more efficient translation of glmS (42). In EHEC, however, the expression of both of these sRNAs on plasmids did not affect the levels of the glmS transcript under the conditions we assayed (see Fig. S3A). We also constructed a translational reporter of GlmS. Previous studies indicate that overexpression of either glmY or glmZ should lead to an increase in β-galactosidase activity with this reporter construct. However, in EHEC, overexpression of glmY or glmZ did not change GlmS::LacZ expression (see Fig. S3B). This same reporter plasmid was then assayed in MC4100, the E. coli K-12 strain used in previous studies of glmS (40, 42), and it behaved as previously reported (see Fig. S3C). Since the sequences of both glmS and glmZ that interact are invariant between these two strains, it is likely that there is another level of regulation that is masking the regulation of glmS by GlmZ in EHEC 86-24 that is not present in strain MC4100.

Posttranscriptional regulation of LEE5 and LEE4 by GlmY and GlmZ. The LEE5 operon in EHEC consists of three genes that encode the translocated intimin receptor (Tir), its chaperone (cesT), and the bacterial adhesin intimin (eae) with which Tir interacts (Fig. 2A and 5A) (5, 7). While this operon is transcribed by a single promoter upstream of tir (5, 51, 52), there is a processing event that results in the separation of cesT-eae from tir (Fig. 5A to D). Inasmuch as GlmY and GlmZ overexpression decreased eae transcript levels (Fig. 3A), we investigated the mRNA levels of each gene in this operon by Northern blot assay (Fig. 5B to D). Overexpression of both sRNAs decreased the levels of the cesT-eae transcript (3,300 bp) (Fig. 5C and D), while the tir transcript was largely unaffected (1,600 bp) (Fig. 5B). The levels of transcription of the entire LEE5 operon (4,900 bp) were also decreased by these sRNAs (Fig. 5B to D). Since the first gene of this operon is unaffected, GlmY and GlmZ must be acting posttranscriptionally. One of the primary ways in which sRNAs affect gene stability is blocking of translation by binding to the RBS (53). An mRNA being translated is largely protected from nucleases by the ribosomes, so blocking of translation can lead to degradation of the transcript. To test this possibility, translational LacZ reporters of all three genes of LEE5 were constructed and β-galactosidase assays were performed (Fig. 5E to G). Neither the knockout of glmY and glmZ nor their overexpression had any effect on the translation of any of the three reporter proteins, suggesting that they are not acting through this mechanism.

The LEE4 operon encodes the SepL regulator of effector translocation into host cells; the EspA protein, which forms a filament that creates a sheath around the T3SS needle; EspBD, which create a pore through the eukaryotic cell membrane; a chaperone, CesD2; the EscF structural protein of the needle; an uncharacterized protein, Orf29; and the effector EspF (6, 7, 18, 54–56). The LEE4 operon has only one promoter upstream of the sepL gene and no internal promoters (6, 51, 57–59). The first gene of this operon (sepL) is processed from this operon in an RNase E-dependent manner (24), and there is a terminator in the cesD2 gene that leads to lower expression of the last three genes (6) (Fig. 6A). Similarly to LEE5, the overexpression of both sRNAs led to lower levels of the espA-cesD2 transcript, while the sepL transcript was mostly unaffected. The level of the espA-cesD2 transcript was noticeably higher in the sRNA mutants (Fig. 6B). The transcript of the last three genes of the LEE4 operon could not be detected by Northern blot assay because of their much lower expression due to the transcription terminator in cesD2, so a quantitative PCR (qPCR) was performed, and the results demonstrated that they were also downregulated by the overexpression of glmY and glmZ (Fig. 6C). Translational fusions of SepL, EspA, EspD, and EspB were constructed and assayed, and again the expression of these reporters was unaffected in the ΔglmY and ΔglmZ mutant strains and under the overexpression of the sRNAs (Fig. 6D to G). In a scenario similar to the posttranscriptional regulation of LEE5 (Fig. 5), Northern blot assays were performed for the LEE4 genes in the WT, the WT overexpressing glmY or glmZ, and the glmY and glmZ mutants (Fig. 6B). Similarly to LEE5, the overexpression of both sRNAs led to lower levels of the espA-cesD2 transcript, while the sepL transcript was mostly unaffected. The level of the espA-cesD2 transcript was noticeably higher in the sRNA mutants (Fig. 6B). The transcript of the last three genes of the LEE4 operon could not be detected by Northern blot assay because of their much lower expression due to the transcription terminator in cesD2, so a quantitative PCR (qPCR) was performed, and the results demonstrated that they were also downregulated by the overexpression of glmY and glmZ. In silico target transcript and cause its degradation through the recruitment of an RNase (53). The IntaRNA software (60) was used to predict potential sites of GlmY and GlmZ binding to the coding regions of LEE5 and LEE4, and then a point mutation that would affect binding was created for each prediction. The resulting mutants were then assayed for the ability to complement the glmY and glmZ mutants in FAS tests and quantitative RT-PCR (qRT-PCR) assays of target genes. While we have been unable to find any site (either in silico or empirically) of direct GlmY or GlmZ binding to the LEE5 transcript, one prediction of GlmZ binding to the orf29 region of the LEE4 operon was promising (Fig. 6H and I). We

Figure Legend Continued

translation reporter plasmid in WT, the ΔespF mutant, and the complemented ΔespF mutant. The translational fusion contains the region from –170 bp downstream of the translation start site to +48 bp upstream of it. (H) Diagram of the espJ-espFu WT deletion constructs. To create p1, the region stretching from 20 bp downstream of the espJ stop codon to 20 bp upstream of the espFu start codon was deleted. To create p2, the region stretching from 20 bp downstream of the espJ stop codon to 170 bp upstream of the espFu start codon was deleted. To create p3, the region stretching from 170 bp upstream of the espFu start codon to 20 bp upstream of the espFu start codon was deleted. p4 contains the region stretching from 262 bp within the espJ gene to the end of espFu. (I) Western blot assays of WT EHEC expressing the espJ-espFu WT and FLAG-tagged deletion constructs probed with an anti-FLAG antibody. Western blot assays with anti-RpoA antibody were used as loading controls. The unpaired Student t test was used to determine statistical significance. A P value of ≤0.05 was considered significant.
generated a GlmZ mutant by changing two C residues to G to prevent base pairing with the LEE4 operon (Fig. 6I). This GlmZ mutant, named GlmZ-CG, was unable to complement the pedestal formation of the glmZ knockout (Fig. 6K) or to complement the expression of the LEE4 gene espA (Fig. 6J). To confirm this result, we made the corresponding compensatory mutations in a plasmid containing the entire LEE4 operon, so that in the presence of both GlmZ-CG and LEE4-GC, the interaction would be restored (Fig. 6J). This was tested by qPCR for espA. The glmZ-GC plasmid was unable to complement the increased levels of espA in the glmZ mutant; however, LEE4-GC restored this regulation (Fig. 6J). This indicates that GlmZ is interacting with LEE4 at this predicted site and that this interaction is responsible for the regulation of LEE4 by GlmZ.

Since GlmY- and GlmZ-mediated regulation of LEE4 and LEE5 does not act through repression of translation, it is likely that they recruit a nuclease that then degrades these transcripts. The major E. coli enzyme RNase E is known to be recruited to many sRNA-mRNA complexes, and it has been previously shown to mediate the processing of the LEE4 transcript (24). While rre is an essential gene, there is a well-characterized temperature-sensitive E. coli K-12 mutant that we used to test whether RNase E is involved in the GlmZ-dependent posttranscriptional regulation of LEE4 and LEE5. Since this is a K-12 strain, plasmids containing the entire LEE region could be expressed, and qPCR could be used to determine whether the glmZ-K12 mutant complemented the regulatory function of GlmZ on LEE4 and LEE5.

**Quantification of FAS**

The number of bacteria per cell was quantified using qPCR for espA, eae, and stxA, with RNA extracted from the WT, the WT overexpressing pGlmY, and the WT overexpressing pGlmZ. The unpaired Student t test was used to determine statistical significance. A P value of \( p \leq 0.05 \) was considered significant.
LEE4 or LEE5 operon along with the glmZ overexpression plasmid were transformed into the rnc<sup>WT</sup> (WT) and rnc<sup>ts</sup> (temperature-sensitive mutant) strains. The bacteria were heat shocked for 15 min before RNA was extracted. This was sufficient to stop the RNase E-mediated processing of sepL from espA as previously reported (24); however, it had no effect on the GlmZ downregulation of the espA-cesD2 transcript (see Fig. S4 in the supplemental material). The processing of tir from cesT-eae is also RNase E dependent, but GlmZ again is able to function in the absence of RNase E (see Fig. S4). These data show that, in both cases, LEE4 and LEE5 are processed by RNase E; however, GlmZ does not act by recruiting this nuclease. Another potential nuclease known to be recruited by sRNAs is RNase III. While the rnc gene is not essential, its knockout produces severe growth defects in EHEC, preventing its characterization.

**DISCUSSION**

By asking the question of how an extracellular bacterial pathogen rapidly and precisely coordinates the expression of its molecular circuitry to engage the expression of an array of genes necessary to encode the molecular structures and effectors that rearrange host actin dynamics, we uncovered three new targets and two different molecular mechanisms of action of the GlmY and GlmZ sRNAs. Our data establish that both the LEE and espFu genes that are necessary for pedestal formation on epithelial cells are posttranscriptionally regulated by the GlmY/GlmZ sRNAs through two different mechanisms. Several previous reports recognized that the LEE region is highly posttranscriptionally regulated (19, 23, 24, 29, 61, 62). However, no sRNA has yet been shown to be responsible for this regulation. One system known to be involved is the RNA binding protein and global regulator CsrA, which directly binds to the LEE4 operon and regulates a wide array of virulence factors through indirect means (19). Additionally, the RNA chaperone Hfq is required for the proper expression of many virulence genes (22, 23, 63), which suggested that trans-acting sRNAs are involved at some level of regulation. Here we describe the first sRNAs known to regulate the LEE, GlmY and GlmZ. Previous to this work, GlmY and GlmZ had only one target, the glmS mRNA, and one known molecular mechanism of action. GlmY was described as a molecular mimic of GlmZ, protecting GlmZ from degradation and allowing GlmZ to base pair with the glmS mRNA to expose the RBS and promote the translation of this gene (41, 42). Our data unraveled two new mechanisms of action for these sRNAs. GlmY and GlmZ promote cleavage of the intergenic region between espJ and espFu to allow the translation of EspFu. We do not know whether this is a direct effect of these sRNAs in the espJ-espFu transcript or an indirect effect through QseF regulation of other regulatory elements controlling EspFu expression (Fig. 2). Moreover, through destabilization of the LEE4 and LEE5 transcripts, these sRNAs fine-tune LEE expression (Fig. 7). One of the key advantages of posttranscriptional regulation of the LEE by GlmY and GlmZ is that it also allows for the differential regulation of gene expression within the LEE4 and LEE5 operons. This decoupling of the regulation of the genes of a...
polycistronic mRNA from each other enables a much more varied pattern of gene expression. GlmZ specifically downregulates the downstream portion of the LEE4 operon, including the filament EspA, the pore EspDB, and the needle EscF, while leaving SepL unaffected (Fig. 6). SepL is an important regulator of the translocation of effectors (64) and is likely to be required for effector delivery to host cells even when many of the structural proteins of the T3SS translocon are not. The posttranscriptional regulation of LEE4 mediated by GlmZ enables EHEC to tightly regulate the process of AE lesion formation (Fig. 2 to 7). The role of GlmY and GlmZ in the regulation of AE lesion formation by promoting EspFu translation and destabilizing LEE transcripts, seems to be initially confounding. However, AE lesion formation is a dynamic process, and the precise modulation of the levels of LEE and EspFu expression are important for the efficiency of this phenotype. Coupling of transcriptional and posttranscriptional regulation within a signaling transduction cascade in the bacterial cell is key to ensuring fine-tuning and rapid adaptation of gene expression toward the regulation of complex processes such as AE lesion formation.

Core chromosome-encoded sRNAs that regulate metabolic functions in bacteria have been shown to be coopted to regulate virulence genes that are horizontally acquired by bacterial pathogens (65, 66). Pathogens evolve through the integration of horizontally acquired genetic material that is known to be integrated within existing transcriptional regulatory networks in the recipient cell (67). EHEC integrates the transcription of horizontally acquired virulence genes through the core QseC/QseE signaling system, which controls a plethora of virulence genes in EHEC that have to be coordinately expressed to ensure optimal AE lesion formation on epithelial cells, leading to host infection (37, 38, 43, 44). GlmZ is well characterized as the activator of glmS translation. This core metabolism-regulating sRNA was coopted to regulate the LEE and espFu, both horizontally acquired islands, at some point in evolutionary history. Horizontal acquisition of pathogenicity islands contributes to the virulence of an organism, allowing exploitation of other niches and hosts for colonization (68). Our results suggest that the interplay between ancient and recent evolutionary acquisitions has shaped EHEC pathogenicity. An inverse example of this phenomenon comes from the InvR sRNA from Salmonella enterica (69), where a coopted sRNA that is adjacent to the SPI-1 pathogenicity island regulates many core chromosomal genes.

While we did not directly observe the regulation of glmS by GlmZ under the conditions we assayed in EHEC, we have evidence from the ΔqseF mutant transcriptome that suggests that...
FIG 6  GmY and GmZ regulation of the LEE4 operon. (A) Schematic depiction of the LEE4 operon and its transcripts. (B) Northern blot assays with probes for sepL and espA with RNA from WT, WT/pGmY, WT/pGmZ, ΔglmY mutant, and ΔglmZ mutant cells, with the 5S rRNA serving as a loading control. (C) qPCR of escF and espF with cDNA from the WT or the WT overexpressing glmY or glmZ. (D) β-Galactosidase assays of the SepL::LacZ translational fusion in WT, WT/pGmY, WT/pGmZ, ΔglmY mutant, and ΔglmZ mutant cells. The plasmid contained the region stretching from bp -82 to +90 from the translation start site. (E) β-Galactosidase assays of the EspA::LacZ translational fusion in WT, WT/pGmY, WT/pGmZ, ΔglmY mutant, and ΔglmZ mutant cells. The plasmid contained the region stretching from bp -107 to +90 from the translation start site. (F) β-Galactosidase assays of the EspD::LacZ translational fusion in WT, WT/pGmY, WT/pGmZ, ΔglmY mutant, and ΔglmZ mutant cells. The plasmid contained the region stretching from bp -61 to +90 from the translation start site.
plasmid pBAD33, and the cloned. All plasmids were confirmed through sequencing. For the primers contained the region stretching from bp start codon to 20 bp upstream of the region starting 262 bp within the was cloned into plasmid pRS551. For pCG48 (CYC177, disrupting the ampicillin gene. Site-directed mutagenesis was performed with the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). For transcriptional reporters, the promoter region was cloned into plasmid pRS551. For pCG48 (glmY::lacZ), bp -250 to +20 from the transcription start site were used. For pCG49 (glmY::lacZ), bp -246 to -20 were used. For pCG50 (espFu::lacZ), bp -461 to +48 from the translation start site were used. The translation reporter system was constructed by cloning lacZ from MG1655 into the vector pBAD24 and then cloning the gene of interest from the transcription start site or processing site to a spot within the coding region, creating an in-frame reporter protein under the control of the arabinose promoter. pCG57 (EspFu::lacZ) contains bp -170 from the translation start site to +48. pCG59 (GlmS::lacZ) contains bp -160 to +117. pCG60 (Tir::lacZ) contains bp -137 to +90. pCG61 (Ces::lacZ) contains bp -71 to +90. pCG62 (Eac::lacZ) contains bp -62 to +90. pCG63 (Sep::lacZ) contains bp -82 to +90. pCG64 (Esp::lacZ) contains bp -107 to +90. pCG65 (EspD::lacZ) contains bp -61 to +90. pCG66 (EspB::lacZ) contains bp -68 to +90. The esp-espFu constructs were created by first cloning the region from the esp promoter to espFu with a FLAG tag added via a primer and inserting it into the Zero Blunt TOPO vector (Invitrogen). The deletion mutants were then created through sewing PCR. In p1, the region from 20 bp downstream of the esp stop codon to 20 bp upstream of the espFu start codon was deleted. In p2, the region from 20 bp downstream of the esp stop codon to 170 bp upstream of the espFu start codon was deleted. In p3, the region from 170 bp upstream of the espFu start codon to 20 bp upstream of the espFu start codon was deleted. In p4, the region starting 262 bp within the esp gene to the end of espFu was cloned. All plasmids were confirmed through sequencing. For the primers used, see Table S3 in the supplemental material.

Isogenic mutant construction. Nonpolar glmY and glmZ mutants were constructed by amplifying the transposable system (71). Briefly, PCR products (obtained with the primers listed in Table S3) were amplified from plasmid pKD3 with flanking regions matching glmY or glmZ and were transformed into EHEC expressing the Red genes from plasmid pKD46. After selection and confirmation, the resistance cassette was resolved with flippase from temperature-sensitive plasmid pCP20, which was then cured through growth at 37°C. This generated nonpolar mutants, which were confirmed by sequencing.

Reporters assays. Transcriptional and translational β-galactosidase assays were performed by using the same protocol. Bacteria containing the reporter plasmids were grown overnight in LB with the appropriate antibiotic and then diluted 1:100 in clear DMEM supplemented with 0.2% arabinose and grown to an optical density at 600 nm (OD600) of 0.8. These were then assayed for β-galactosidase activity with o-nitrophenyl-β-D-galactopyranoside as previously described (72). For the experiments with the GmY::LacZ reporter plasmid, bacteria were grown overnight in LB with 0.2% arabinose and specific activity (nM/min/mg protein) was measured by determining protein concentrations by the assay of Lowry et al. (73). The unpaired Student t test was used to determine statistical significance. A P value of ≤0.05 was considered significant.

EMSA. QseB and QseF proteins were His tagged and purified from BL21(DE3) and TOP10 cells, respectively, as previously described (36, 45). The glmY promoter and the kanamycin resistance-encoding gene were amplified by PCR (for the primers used, see Table S3 in the supplemental material) and radioabeled with [γ-32P]ATP and T4 polynucleotide kinase (NEB). The labeled DNA was then incubated with increasing amounts of protein and run on a 6% polyacrylamide Tris-Bis gel, dried, and exposed to film overnight.

Operon analysis by RT-PCR. WT and ΔqseF mutant bacteria were grown to an OD600 of 1.0 in low-glucose DMEM, and RNA was extracted with the RiboPure kit (Ambion). RNA was reverse transcribed and amplified by PCR with different sets of primers (see Table S3 in the supplemental material).

Western blot assays. Bacteria were grown overnight in LB and then diluted 1:100 in low-glucose DMEM and grown to an OD600 of 1.0. The
pellets were then lysed under denaturing conditions, subjected to 12% SDS-PAGE, and transferred to a polyvinylidine difluoride membrane. The membrane was probed with an anti-FLAG (Sigma) or anti-RpoA (Santa Cruz Biotechnology) antibody as the endogenous control with a horseradish peroxidase-conjugated secondary antibody that was visualized by ECL (GE Biosciences) and exposed to film.

**Cell culture and FAS.** 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% CO₂. 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 statically at 37°C at a 1:100 dilution. FAS assays were performed as described by Knutton et al. (36, 74) to examine pedes-
tal formation. After 6 h of infection at 37°C in 5% CO₂, the coverslips were washed, fixed, and permeabilized. The samples were then treated with FITC-labeled phalloidin and PI to visualize actin accumulation and bac-
teria, respectively. PI also stained HeLa nuclei red. The coverslips were then mounted on slides and visualized with a Zeiss Axiovert microscope. Pedestal formation was quantified by randomly imaging different fields of view and counting the first 100 cells while recording the number of bac-
teria attached to each one. Replicate coverslips from multiple experiments were quantified, and statistical analysis was performed with the Student t test. Serially diluted samples of the original bacterial cultures were also plated to confirm that similar CFU ratios were used for infection.

**Live-cell imaging.** The Lifeact::GFP-expressing cell line was created with the Flip-In System (Invitrogen). HeLa cells were transfected with pLacZ::zeocin by using Fugene 6 (Promega) to create flippase recognition with the Flip-In System (Invitrogen). HeLa cells were transfected with the RiboPure kit (Ambion), run on a 1% formaldehyde agarose gel, and transferred overnight to a Zeta-Probe membrane (Bio-
extracts were purified with the RiboPure kit (Ambion), run on a 1% formaldehyde agarose gel, and transferred overnight to a Zeta-Probe membrane (Bio-

**SUPPLEMENTAL MATERIAL**

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

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**TABLE S3, DOCX file, 0.1 MB.**

**TABLE S2, DOCX file, 0.1 MB.**

**TABLE S1, DOCX file, 0.1 MB.**

**MOVIE S2, AVI file, 1.5 MB.**

**MOVIE S3, AVI file, 2.7 MB.**

**Figure S4, PDF file, 0.7 MB.**

**Figure S2, PDF file, 1.9 MB.**

**Figure S3, PDF file, 0.2 MB.**

**Figure S4, PDF file, 0.7 MB.**

**Figure S5, AVI file, 2.8 MB.**

**Figure S6, AVI file, 1.5 MB.**

**Figure S7, AVI file, 2.7 MB.**

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