Regulation of the Locus of Enterocyte Effacement in Attaching and Effacing Pathogens

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ABSTRACT Attaching and effacing (AE) pathogens colonize the gut mucosa using a type three secretion system (T3SS) and a suite of effector proteins. The locus of enterocyte effacement (LEE) is the defining genetic feature of the AE pathogens, encoding the T3SS and the core effector proteins necessary for pathogenesis. Extensive research has revealed a complex regulatory network that senses and responds to a myriad of host- and microbiota-derived signals in the infected gut to control transcription of the LEE. These signals include microbiota-liberated sugars and metabolites in the gut lumen, molecular oxygen at the gut epithelium, and host hormones. Recent research has revealed that AE pathogens also recognize physical signals, such as attachment to the epithelium, and that the act of effector translocation remodels gene expression in infecting bacteria. In this review, we summarize our knowledge to date and present an integrated view of how chemical, geographical, and physical cues regulate the virulence program of AE pathogens during infection.

KEYWORDS EHEC, EPEC, locus of enterocyte effacement, type 3 secretion system, mechanosensing, metabolism, pathogenesis, quorum sensing, regulation, two-component system

Initially described by the German physician Theodor Escherich in 1885 as the “common colon bacillus” (1), *Escherichia coli* is a Gram-negative facultative anaerobe of the *Enterobacteriaceae* family and a major component of the normal human intestinal flora (2). Pathogenic *E. coli* strains have arisen through the acquisition of large groups of virulence factors encoded on plasmids, prophages, and pathogenicity islands (PAIs) and are capable of causing both diarrheal and extraintestinal diseases, including urinary tract infections (UTIs), wound infections, meningitis, and sepsis (2, 3). The attaching and effacing (AE) pathogens enteropathogenic *E. coli* (EPEC) and enterohemorrhagic *E. coli* (EHEC) represent an important subset of pathogenic *E. coli* and are responsible for a significant disease burden worldwide (4). EPEC and EHEC infections are characterized by the formation of ultrastructural lesions on the surface of the gut epithelium known as AE lesions (2, 5). The formation of these lesions, and the infectious process of the AE pathogens more broadly, depends on a type three secretion system (T3SS), encoded by the locus of enterocyte effacement (LEE) PAI (5), and a suite of locally and distally encoded effector proteins (6). The LEE PAI is also found in the AE pathogens *Citrobacter rodentium* and *Escherichia albertii* (previously known as *Citrobacter freundii* and *Hafnia alvei*, respectively) (7, 8) and some *Edwardsiella* spp. (9, 10). The regulation of the LEE is central to the pathogenesis of AE pathogens and has been the subject of extensive research. Our review focuses on the regulation of the EPEC and EHEC LEEs, as little is currently known regarding the regulation of the LEEs in other organisms. However, it is likely that many similarities exist between the regulation of these canonical LEEs and those found in other pathogens.

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THE LOCUS OF ENTEROCYTE EFFACEMENT

The 35-kb LEE PAI consists of 41 open reading frames (ORFs) and is the defining genetic feature of the AE pathogens. The LEE carries the genes for a T3SS, a number of bacterial effector proteins, the chaperones for these effectors, the adhesin intimin, and regulatory elements (7). As with many of the virulence-associated elements found in the pathogenic *E. coli*, the lower G+C content of the LEE (38.3%), in comparison to that of the *E. coli* chromosome as a whole (50.8%), suggests that it was originally acquired by horizontal gene transfer (11, 12). The core LEEs of EPEC and EHEC are 93.9% identical at the nucleotide level, although the EHEC LEE has an additional 13 ORFs at the 5' end due to the insertion of a cryptic prophage (13). However, the sequences of individual genes can be highly variable and range from complete invariance between the two organisms in the case of structural components of the T3SS to 33.52% identity, as is seen for the effector *tir* (13).

ORGANIZATION OF THE LEE

The LEE is arranged as five polycistronic operons, namely, *LEE1*, *LEE2*, *LEE3*, *LEE4*, and *LEE5*, a bicistronic operon (*grlAB*), and multiple monocistronic transcription units (5, 14). *LEE1* contains the genes encoding the master virulence regulator Ler, the chaperones CesAB, and structural components of the T3SS, namely, EscR, EscS, EscT, EscU, EscK (15), and EscL (16). *LEE2* encodes the chaperone CesD, three components of the T3SS machinery, namely, EscC, EscJ, and SepD, the effector EspZ, and one ORF of unknown function. *LEE3* encodes three components of the T3SS machinery, namely, EscV, EscN, and SepQ, the effector EspH, and two ORFs of unknown function. *LEE4* contains the genes encoding the translocator proteins, which form the tip of the T3SS (EspB and EspD) (17), the needle filament EspA (18), the needle protein EscF (19), the chaperone CesD2, the gatekeeper protein SepL (20), and the effector EspF. Finally, *LEE5* contains the effectors Tir and intimin and the chaperone CesT. The effector Map is located 5' to *LEE5* (21), and the effector EspG is located 5' to *LEE1* (22). The addition of the 933L cryptic prophage 3' to the core LEE genes in EHEC makes the EHEC LEE larger than the EPEC LEE (13).

The expression of the five LEE operons is controlled through the action of the LEE-encoded transcriptional regulator Ler, the expression of which is regulated by numerous transcription factors in response to an array of intra- and extracellular stimuli (summarized in Fig. 1), which are discussed further below.

LEE-ENCODED REGULATORS Ler, GrlA, AND GrlR

AE pathogens sense and integrate multiple host-, environment-, and microbiota-derived signals to control the expression of the LEE genes, with the integration of these diverse signals occurring at the transcriptional, translational, and posttranslational levels (23–28). Below 37°C, the global regulator histone-like nucleoid structuring protein (H-NS) (29) represses the transcription of the master positive regulator of the LEE, *ler* (encoded in *LEE1*) as well as *LEE2*, *LEE3*, *LEE4*, and *LEE5* (30–33). However, at 37°C, *ler* is transcribed and, through the coordination of many cues, relieves H-NS-mediated repression (30, 34) to allow expression of the LEE genes (23, 35, 36). More than 40 regulators of LEE transcription have been described to date, including both proteins and regulatory RNAs (23, 24, 31, 35, 37–49), not all of which can be discussed here. Many of these systems converge on *ler* (23, 37) (Fig. 1).

During Ler-mediated expression, the LEE-encoded regulators GrlA (positive) and GrlR (negative) (38) also influence expression of the LEE. GrlA functions in a manner similar to that of some MerR family transcriptional activators, despite sharing no significant sequence similarity with these proteins (50), and belongs to a novel family of transcriptional activators that also contains a GrlA homologue from *Salmonella enterica* and CaiF from *E. coli* and *Shigella flexneri* (38, 51). Upon expression, GrlA drives the expression of virulence genes, likely through direct binding to the suboptimal 18-bp spacer between the –10 and –35 elements of the distal *LEE1* promoter P1 (Fig. 1B) via a helix-turn-helix (HTH) DNA-binding motif (50, 52). This drives expression of *ler*

FIG 1 Regulation of the LEE. (A) The master LEE regulator, Ler (23, 37), integrates external signals from TCSs (FusR, QseBC, QseEF, and CpxAR), LysR-type transcription factors (QseA, QseD, and LeuO), and the regulators Cra, KdpE, and NagC, which act in concert to regulate the transcription of *ler*. Cra also controls the expression of the LEE-encoded effector protein EspG. Ler-mediated expression of the LEE is fine-tuned by the LEE-encoded regulators GrIA and GrIR (38). The sRNA Arl directly antagonizes the translation of the *ler* mRNA, whilst GlmY silences *LEE4* and *LEE5* upon induction by QseB/QseF. (B) Binding sites within the *LEE1* regulatory region for regulators that act directly on *ler* transcription. GrIA counteracts H-NS-mediated negative regulation of the *LEE1* promoter and directly binds the P1 promoter of *LEE1* (50, 52). QseA binds directly upstream of the -35 elements of both the P1 promoter and the EHEC-specific P2 *LEE1* promoter (57, 58). KdpE binds to AT-rich regions of DNA, and as such, its binding site upstream of *LEE1* is poorly defined but is likely between 173 and 42 bp upstream of the *LEE1* transcription start site. Cra binds at the 5' end of the *LEE1* regulatory region and acts synergistically with KdpE to promote DNA bending upstream of the P1 and P2 promoters, facilitating *LEE1* expression (83). LeuO binds AT-rich regions of the *LEE1* promoter and collaborates with PchA to remove H-NS-mediated repression at these same AT-rich regions, thus enhancing transcription

(Continued on next page)

and subsequently the entire LEE. Conversely, GrIR, the cellular levels of which are regulated in a growth phase-dependent manner (39), antagonizes the system by directly binding to GrIA and preventing its interaction with DNA (53). As demonstrated by Alsharif et al. (26), GrIR is not the only antagonistic mechanism acting upon GrIA. GrIA activity is regulated by the small RNA (sRNA) Arl (54) (discussed further below), while under some conditions, GrIA also requires external cues, such as attachment to host cells and the shear forces which act upon attached bacteria in the gut, to become fully activated and drive *ler* expression (discussed further below) (26). Thus, these autoregulatory mechanisms allow the LEE to act dynamically by fine-tuning its own expression.

REGULATION OF THE LEE BY LysR AND Fur FAMILY TRANSCRIPTIONAL REGULATORS

The LysR-type transcriptional regulators are perhaps the primary family of DNA-binding regulatory proteins in bacteria and interact with DNA via HTH domains at their amino termini (55). Two major quorum sensing responsive LysR-type regulators act upon the LEE, QseA, and QseD (Fig. 1B). QseA is part of the LuxS quorum sensing system and responds to microbiota-derived signaling molecules, thereby binding the *LEE1* regulatory region, inducing LEE gene expression through the increased transcription of *ler* (40, 56), and directing the transcription of *LEE1* (57), as well as inducing virulence genes in other areas of the chromosome (58). QseD responds indirectly to autoinducer 3 (AI-3) and is present across the AE pathogens but exists almost exclusively in EHEC O157:H7 as a truncated short isoform (sQseD) that lacks the classical HTH domains of the LysR-type regulators (59). While both long QseD (lQseD) and sQseD repress LEE transcription in AE pathogens, each isoform achieves this in a different way. lQseD binds directly to the *LEE1* regulatory region to repress LEE transcription, while sQseD is thought to interact with a second as-yet-unidentified LysR-type transcriptional regulator in order to bind DNA and affect LEE expression (59).

In addition to the two quorum sensing responsive regulators described above, additional cues such as the microbiota-derived short-chain fatty acid (SCFA) butyrate and the essential cofactor biotin are involved in the regulation of AE pathogen virulence. Butyrate is produced by the fermentation of dietary fiber by the gut microbiota and has wide-ranging effects on the health and physiology of the gut (60). The detection of intestinal butyrate, via the regulatory protein Lrp, induces the expression of the LEE genes through the activation of the LysR-type regulator LeuO. In turn, LeuO cooperates with the plasmid-borne virulence regulator PchA to establish a positive feedback loop and activate *LEE1* transcription (61, 62) and flagellar biosynthesis (63). Thus, this accessory regulatory pathway appears to couple virulence and motility and may aid in the movement toward the epithelium and the preparation of the T3SS machinery for the initiation of infection.

Biotin is an essential metabolic cofactor in all organisms and can be both absorbed and synthesized *de novo* by many microorganisms (64). Humans cannot synthesize biotin and instead acquire it from external sources, with the majority of dietary and microbiota-derived biotin being absorbed in the small intestine (65). This creates a gradient within the gut, with high luminal biotin concentrations in the small intestine and low concentrations in the large intestine and colon (65, 66). During infection, EHEC colonizes the large intestine. This is in contrast to EPEC, which preferentially infects the small intestine. During infection, EHEC senses the biotin gradient in the gut via the biotin ligase BirA (66). In the presence of biotin, BirA directly binds to the promoter of

FIG 1 Legend (Continued)

(62). The repressors FusR, NagC, and QseD also bind directly to the *LEE1* regulatory region. FusR binds to a region between the P1 and P2 promoters (69), NagC binds upstream of FusR, and the HTH-containing isoform of QseD, lQseD, binds to the same site as QseA. The binding site of sQseD remains to be determined (59). Sequence information for binding sites and regulatory elements is shown where it is known; -10 and -35 element sequences are shown in bold. Regulators are color-coded to indicate the type of signal they are responsive to: yellow, microbiota-derived quorum sensing molecules and/or host-derived hormones; gray, butyrate; blue, mucus-derived sugars; pink, bacterial metabolic status; gold, *Ler* and external mechanical cues; orange, growth phase.

the global transcriptional regulator Fur, acting as a negative regulator (66, 67). The precise mechanism by which Fur then regulates the LEE is still unknown. However, as Fur does not appear to affect known LEE regulators (66), it is possible that an as-yet-undiscovered LEE regulator allows the indirect control of LEE expression by Fur. Therefore, BirA serves to indirectly repress LEE expression in the presence of high biotin concentrations (i.e., in the small intestine) and induce LEE expression in low-biotin environments, limiting T3SS activation and AE lesion formation to the large intestine during EHEC infection.

REGULATION OF THE LEE BY BACTERIAL TWO-COMPONENT SYSTEMS

A number of bacterial two-component systems (TCSs) control the expression of the LEE. In their basic form, TCSs consist of a membrane-implanted histidine kinase and a cognate response regulator (RR), but in some cases, a histidine kinase can activate multiple RRs (68). The TCSs that control LEE expression can be broadly divided into two classes: those that repress transcription of the LEE and those that promote it.

Foremost among the TCS that repress the LEE genes is the FusKR system, which responds to the host sugar fucose (69). Fucose is found in the lumen of the intestine due to the activity of *Bacteroides thetaiotaomicron*, a major component of the intestinal microbiota, which cleaves fucose from the mucin glycoproteins that coat the gut epithelium (70). The FusR RR directly represses *ler* transcription, thus preventing LEE expression in the gut lumen (69).

The sensing of fucose by the FusKR TCS also has a secondary function, which is to promote the use of mannose and galactose as carbon sources by intestinal pathogenic *E. coli* via the repression of the *fuc* operon (69). As fucose is the primary carbon source for commensal *E. coli* and these organisms are unable to metabolize mannose and galactose (71), this prevents unnecessary competition between the infecting strain and the resident commensal *E. coli* population.

Like the fucose sensing FusKR system, the CpxAR two-component system serves to repress the transcription of the LEE genes (25). The Cpx envelope stress response is present across Gram-negative bacteria, responds to a variety of stimuli in the bacterial inner membrane and periplasmic space (72), and has been linked to virulence in a number of pathogens, including *S. enterica* serovar Typhimurium (73) and *Legionella pneumophila* (74). In AE pathogens, CpxR has been shown to repress the production of the T3SS (75) by interfering with the activity of Ler and GrlA (25). To achieve this, CpxR, activated by CpxA, indirectly induces the Lon protease, resulting in the degradation of both Ler and GrlA (25).

In contrast, the cooperating TCSs QseCB and QseEF sense and respond to the microbiota-derived quorum sensing molecule AI-3 and the host hormones adrenaline and noradrenaline to activate the transcription of the LEE genes (68, 76, 77) and allow colonization (78). Following autophosphorylation, QseC activates three RRs, namely, QseB (QseC's cognate RR), QseF, and KdpE (68), via a conserved 8-amino-acid motif in its periplasmic sensing domain (79). QseF is also phosphorylated by its cognate membrane-bound histidine kinase QseE (77). QseB indirectly represses *LEE4* and *LEE5* via the sRNA GlmY while activating flagellar and motility genes (24, 68, 80). QseF promotes the expression of the Shiga toxin (68, 79) and the T3SS effector TccP/EspF_U, which is necessary for AE lesion formation in EHEC (81). Importantly, QseB (directly) and QseF (indirectly) also repress the FusKR TCS, alleviating fucose-mediated repression and thus shifting the regulatory balance toward the expression of the entire LEE (69). Phosphorylated KdpE promotes *ler* transcription and thus LEE expression and is discussed further below.

SENSING METABOLIC CUES AND OXYGEN AVAILABILITY

In addition to the TCSs described above, AE pathogens integrate their own metabolic status into the regulatory network controlling LEE expression. The phosphorylation-independent transcription factor Cra directly senses metabolic intermediates in the glycolysis pathway, such as fructose-1-phosphate and fructose-1,6-

bisphosphate. Both of these metabolic intermediates antagonize Cra's ability to bind to target promoters through the interactions with its inducer-binding domain (82). This results in the suppression of *LEE1* expression during glucose-based metabolism and the promotion of *ler* transcription when mucin-derived sugars are the most abundant carbon source (83, 84).

In the context of the intestine, this suggests that Cra acts in opposition to fucose sensing by FusR to promote *LEE1* transcription in response to the gluconeogenic environment encountered as the pathogen moves through the mucus layer. Cra also responds to the presence of oxygen, further enhancing *LEE1* transcription as the bacterium approaches the epithelial barrier, where oxygen concentrations are higher (85). This further promotes LEE transcription, T3SS expression, and AE lesion formation as the bacterium moves from the anaerobic environment of the gut lumen to the microaerophilic environment directly adjacent to the epithelium (84).

Cra achieves efficient LEE expression in concert with the RR KdpE. As discussed above, KdpE is activated in response to AI-3, adrenaline, and noradrenaline by QseC (68) and binds to the *LEE1* promoter, as Cra does. However, KdpE binds the *LEE1* promoter at a site distinct from that of Cra, promoting DNA bending and thus efficient initiation of *ler* transcription (83). Cra and KdpE also cooperate to control the transcription of a number of other genes, including the TccP/EspF_U gene, and additional, uncharacterized putative virulence factors in EHEC (86). Interestingly, the genes regulated by Cra and KdpE do not overlap entirely, and transcription factor-specific targets have been described. These include the LEE-encoded effector *espG*, which is adjacent to *ler* and appears to be regulated by Cra but not by KdpE (86).

Recent work has also suggested that the regulator NagC directly influences *LEE1* transcription and sugar catabolism in EHEC in response to the mucin-derived sugars *N*-acetylglucosamine (NAG) and *N*-acetylneuraminic acid (NANA). NagC directly binds the *LEE1* regulatory region at a position that overlaps the -10 element of the P1 promoter (87) (Fig. 1B). This suggests that mucin-derived sugars in the intestine can both promote and repress LEE gene expression. When taken together with the well-established role of Cra in LEE expression, this suggests that metabolic status, oxygen availability, and virulence are intimately linked in the AE pathogens.

REGULATION OF THE LEE BY SMALL RNAS

In addition to the plethora of regulatory proteins that have been shown to modulate the expression of the LEE genes, the influence of posttranscriptional sRNA-mediated regulation on the deployment of the virulence arsenal of the AE pathogens is becoming increasingly apparent.

Usually between 100 and 200 nucleotides in length, sRNAs are a heterogeneous population of molecules that modulate the translation of mRNAs to proteins by base pairing with their specific target mRNAs. Bacterial sRNAs are grouped as *cis*- and *trans*-encoded sRNAs, where *cis*-sRNAs are encoded on the complementary strands of the genes that they target and *trans*-sRNAs are encoded distal to their target genes. Due to this, the majority of *trans*-sRNAs require an RNA chaperone to facilitate base pairing with their target genes, the most common of which is the Hfq gene (88–90).

The Arl sRNA, encoded within the LEE, is the only *cis*-encoded sRNA shown to influence LEE expression to date (Fig. 1A). Arl exhibits significant complementarity to the *ler* mRNA, preventing the translation of Ler and thus LEE expression in response to changes in cytosolic iron homeostasis (54). Hfq has also been shown to act as a negative regulator of the LEE genes in both EPEC and EHEC (27) in two distinct ways: through antagonism of the *grlRA* mRNA (47), which prevents any LEE-mediated amplification of *ler* transcription, and through direct translational repression of *ler* (27). However, in some EHEC strains, large numbers of Hfq-dependent *trans*-sRNAs are present, many of which have been shown to act as activators of LEE gene expression (49, 91). Consistent with the observation that horizontally acquired PAIs harbor an increased number of sRNAs (92), the diversity of PAIs in AE pathogens (93), and the significant strain-to-strain variation seen in the roles of Hfq on the expression of the

EHEC LEE (94), it is likely that differences in the roles of Hfq are due to the different PAIs carried in different strains. Indeed, the description of 63 novel EHEC sRNAs, 55 of which are encoded in bacteriophage-derived sections of the EHEC genome (49), suggests that the sRNA-mediated regulatory circuits that influence LEE expression may be unique to each AE pathogen strain. The exceptions to this situation are the ancestral Hfq-dependent sRNAs GlmY and GlmZ, which are found in both pathogenic and nonpathogenic *E. coli* (88). These molecules act to dampen the expression of *LEE4* and *LEE5* and thus the assembly of new T3SS injectosomes, while simultaneously promoting TccP/EspF_U expression (24) and thus AE lesion formation (81). Interestingly, the transcription of GlmY but not of GlmZ is induced in response to signals from the host that are detected by the infecting pathogen (24) (as discussed above and shown in Fig. 1B).

Finally, the description of bacteriophage-derived “anti-sRNAs,” which silence other *trans*-sRNAs, in AE pathogens (49) exemplifies the complexity, diversity, and potential for variation of sRNA-mediated LEE regulation.

REGULATION OF THE LEE BY HOST ATTACHMENT AND EFFECTOR TRANSLOCATION

It is becoming increasingly apparent that aside from responding to external cues and sensing their own internal metabolic state, pathogens respond to physical forces to regulate virulence gene expression (95). AE pathogens sense and respond to physical attachment to the epithelial surface using a number of mechanisms (26, 96) (summarized in Fig. 2). Notably, attachment to host cells, mediated by both the T3SS and non-LEE-encoded adhesins, activates the *LEE1* promoter by increasing GrlA activity, which is amplified further in response to laterally acting fluid shear forces (26). However, the mechanism by which GrlA responds to host cell attachment and mechanical forces has yet to be determined.

LEE gene expression is also controlled by the translocation of effector proteins by the T3SS (97). This occurs through a novel pathway regulated by the LEE-encoded effector chaperone CesT, which modulates the global translation inhibitor CsrA, after effector translocation (97). The release of CesT following the translocation of effectors blocks CsrA activity, allowing the expression of the important non-LEE-encoded (Nle) effector NleA and the suppression of *LEE4*. As CsrA regulates gene expression throughout the *E. coli* genome (97, 98), it is likely that effector translocation leads to widespread alterations of the translational program in actively injecting AE pathogens (97).

PERSPECTIVES AND FUTURE DIRECTIONS

The regulatory mechanisms described above establish a situation in which LEE gene expression is controlled through the sensing of both chemical and physical cues along the length of the intestine (summarized in Fig. 3). As the bacterium enters the host, the switch to 37°C alleviates the negative repression by H-NS (30). Once in the gut, microbiota-derived quorum sensing molecules (56, 76) (positive regulators of *ler*) provide an initial biogeographic signal as to where in the host the bacterium is located. However, virulence gene expression is repressed overall while in the lumen by the sensing of the microbiota-liberated sugars fucose (69), NAG, and NANA (87). This repression of the LEE is relieved as the bacterium moves laterally along the intestine in response to microbiota-derived butyrate and/or sensing of the host hormones adrenaline and noradrenaline (61, 76). LEE expression is further enhanced as the bacterium moves through the mucus layer, closer to the epithelium, in response to mucus-derived sugars (such as galacturonic acid) and an increasing oxygen concentration (84). The detection of these signals occurs through the cooperation of the QseBC and QseEF TCSs, QseA, and Cra/KdpE, which together remove the negative regulatory pressure provided by FusKR and stimulate the transcription of the T3SS machinery, allowing infection (78). Once in contact with the host epithelium, the attachment to host cells and the associated shear stress produced by fluid flow over the epithelial surface (26, 96), in conjunction with the act of effector translocation (97), remodel gene expression in the actively infecting bacteria to enable colonization of the mucosal surface. Both the

FIG 2 Regulation of the LEE by physical forces and effector translocation. AE pathogens integrate physical cues to regulate the expression of the LEE. Attachment to host cells, sensed by the bacterial outer membrane (OM) protein NlpE, induces the CpxAR two-component system (103). The RR CpxR indirectly promotes *ler* transcription via the LysR-type regulator LhrA (96). Attachment to host cells also induces GrlA-mediated *ler* transcription, which is further enhanced by the sensing of fluid flow at the epithelial surface (26). Finally, effector translocation by the LEE-encoded chaperone CesT generates free CesT, which causes indirect repression of *LEE4* via antagonism of the global gene regulator CsrA, as well as allowing expression of the important non-LEE-encoded effector protein NleA by relieving CsrA-mediated repression (97). PM, plasma membrane; IM, inner membrane.

synergistic and sequential effects of multiple protein regulators at the *LEE1* promoter region facilitate this exquisite level of control. Additionally, throughout the process described above, it is likely that sRNA-mediated regulation further fine-tunes the virulence program of AE pathogens in response to the changing host environment.

FIG 3 Cues influencing LEE expression in the gut. Upon ingestion and the accompanying shift to 37°C, H-NS-mediated repression of *ler* is relieved (30), priming the infecting bacteria for expression of the LEE. Sensing of microbiota-derived molecules (e.g., autoinducer 2, autoinducer 3, and butyrate) in the gut lumen further induces LEE expression, but the strong negative signal provided by fucose, via the FusKR TCS, and NAG/NANA via NagC, prevents the expression of the T3SS. As further positive stimuli, such as the abiotic cues SO_4^{2-} and PO_4^{3-} (77), the host hormones adrenaline and noradrenaline (the sensing of which is necessary for colonization [78]), and biotin (66), are detected, LEE expression is gradually induced. As the infecting bacterium moves through the mucus layer and closer to the epithelium, the presence of mucin-derived sugars and the increasing oxygen concentration (left panel, blue gradient indicates increasing O_2 concentration) as the bacterium approaches the epithelial surface cooperate to activate full LEE expression and the deployment of the T3SS (84). Attachment to the epithelium (26, 96) and the sensing of shear forces produced by fluid flow in the gut (26) further promote LEE expression. Finally, the act of effector translocation represses the T3SS machinery genes (*LEE4*) and causes global alterations in the expression of virulence-associated and metabolic genes (97) (right panel, feed-forward regulatory mechanisms triggered by effector translocation are indicated by circular arrows).

Unsurprisingly, given the numerous regulatory mechanisms acting upon it, correct regulation of the LEE is central to virulence. Mutants lacking the sensor kinases encoded by *fusK* (69), *qseC* (76, 78), *qseE* (78), or *cpxA* (25) are attenuated in *in vivo* models, while strains lacking the transcription factor encoded by *cra* exhibit reduced pedestal formation on cultured epithelial cells (83) and those lacking *cesT* exhibit reduced effector translocation (97, 99, 100). These observations provide support for the notion that targeting LEE regulation may provide novel opportunities for therapeutic intervention. Indeed, small-molecule inhibition of QseC has already been shown to be a viable antivirulence approach for multiple Gram-negative pathogens (101, 102). Future work to further understand the influence of biotin sensing, the molecular mechanisms coupling LEE expression and shear stress, the extent of the genetic reprogramming which occurs upon effector translocation, and the full role of sRNAs in virulence regulation will undoubtedly prove useful in this area. Irrespective, it is clear that the complex regulatory network employed by AE pathogens provides an exquisite level of control over LEE expression, ensuring efficient coordinated deployment of virulence factors. This undoubtedly contributes to the contin-

ued success of these important human pathogens and provides potential targets for the development of therapeutics targeting virulence rather than viability.

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