Enterohemorrhagic 
*Escherichia coli*

Virulence Gene Regulation

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ABSTRACT  Coordinated expression of enterohemorrhagic *Escherichia coli* virulence genes enables the bacterium to cause hemorrhagic colitis and the complication known as hemolytic-uremic syndrome. Horizontally acquired genes and those common to *E. coli* contribute to the disease process, and increased virulence gene expression is correlated with more severe disease in humans. Researchers have gained considerable knowledge about how the type III secretion system, secreted effectors, adhesin molecules, and the Shiga toxins are regulated by environmental signals and multiple genetic pathways. Also emergent from the data is an understanding of how enterohemorrhagic *E. coli* regulates response to acid stress, the role of flagellar motility, and how passage through the human host and bovine intestinal tract causes disease and supports carriage in the cattle reservoir, respectively. Particularly exciting areas of discovery include data suggesting how expression of the myriad effectors is coordinately regulated with their cognate type III secretion system and how virulence is correlated with bacterial metabolism and gut physiology.

As a species, *Escherichia coli* is highly successful, adapting to inhabit the lower intestine of warm-blooded animals. Commensal *E. coli*, part of the normal biota, resides harmlessly in the gut, producing vitamin K. However, *E. coli* also causes three types of disease in humans: urinary tract infections, sepsis in newborns, and diarrheal disease. Enterohemorrhagic *E. coli* (EHEC) plays a prominent role in the third type of illness. It has been estimated that, for the pan genome of *E. coli*, the nonpathogenic and pathogenic strains only contain a core set of genes comprising approximately 20% of any one genome (1, 2). Much of the horizontally acquired genetic information is clustered within genomic islands in pathogens. As for EHEC, this has allowed the organism to not only attach and colonize the large intestine of humans and other animals, to outcompete commensal *E. coli* and other bacteria at the site of infection, but also to cause serious disease.

Horizontally acquired genetic information in EHEC results in evolution into a specific pathotype—genotype dictates phenotype. How this genetic information is controlled is of equal importance for the success and virulence of the organism. Indeed, Abu-Ali et al. (3) investigated differences in virulence gene regulation in two distinct EHEC isolate lineages, clade 8 and clade 2. A clade is a group of EHEC isolates with one ancestor and all its descendants. Eight clades of *E. coli* O157 isolates were defined by single nucleotide polymorphism (SNP) analyses, where clade 8 was a group of hypervirulent bacteria compared to the other seven clades (4). By examining multiple strains per lineage, the investigators found increased expression of horizontally acquired virulence genes in clade 8 versus clade 2. Genes expressed to higher levels in clade 8, which is associated with a greater number of cases of *E. coli* hemorrhagic
disease compared to those in clade 2, included several virulence factors: rpoS, 29 of the 41 locus of enterocyte effacement (LEE)-encoded genes, gadX involved in acid tolerance, and the pO157 plasmid-encoded stcE adhesin and hlyA hemolysin. These data provide evidence that genetic regulation correlates with EHEC virulence.

However, our understanding of the regulatory network controlling EHEC pathogenesis remains incomplete. Some of the questions pursued by researchers include the following: What are the molecular signals perceived by EHEC strains in the human host and within cattle that allow the bacteria to properly express virulence traits? What are the signals and bacterial responses required to pass through the acid environment of the stomach, to ultimately reside in the large intestine, and to cause disease in humans but to attach to and colonize harmlessly the recto-anal junction (RAJ) of cattle (5)? How are expression of the type III secretion system (T3SS), attachment to host cell surfaces, the effector molecules destined for translocation into host cells, secretion itself, and the Shiga toxin leading to serious disease controlled? This article summarizes our current knowledge of EHEC virulence gene regulation, indicating that spatiotemporal control of pathogenesis in humans and carriage in cattle is coming to light.

**ACID TOLERANCE**

As for any intestinal pathogen that causes disease, EHEC must survive the acidic environment of the stomach. Three acid resistance systems have been described: a glucose-repressed, or oxidative, system and glutamate- and arginine-dependent systems (for review see reference 6). Evidence suggests that the glucose-repressed system is used for EHEC survival in acidic food items (7). For the glutamate-dependent system, glutamate decarboxylases GadA and GadB convert glutamate to γ-amino butyric acid, or GABA (8–10). The arginine decarboxylase AdiA converts arginine to agmatine (11). The Gad system is regulated by a number of environmental conditions and the global regulatory proteins H-NS and CRP and alternate sigma factors RpoS and RpoN (8–10, 12, 13) (Fig. 1). For both amino acid-dependent systems, pH homeostasis is maintained by displacing the α-carboxyl group of the amino acids with a proton that is transported from the environment to the cytoplasm. Cytoplasmic glutamate and arginine are restored by their respective antiporters, GadC and AdiC. The glutamate-dependent Gad system provides a high level of acid tolerance and is necessary for passage through the stomachs and for colonization of the RAJ in cattle (7).

![Control of acid tolerance by EHEC. Resident flora produce AHL signaling molecules that stimulate expression of the glutamate-dependent acid resistance system (14). The GAD system removes excess protons by exchanging the α-carboxyl groups of glutamate with a proton. The resulting GABA molecules are transported out of the cell in exchange for additional glutamate (143). doi:10.1128/microbiolspec.EHEC-0004-2013.f1](ASMscience.org/MicrobiolSpectrum)

In cattle, EHEC passes through the rumen to eventually colonize the RAJ, and the LuxR homolog SdiA is involved in this process. Although EHEC does not possess the LuxI N-acyl homoserine lactone (AHL) synthase, SdiA perceives the oxo-C6-homoserine lactone produced by other bacteria in the rumen (Fig. 1). In turn, SdiA increases gadX expression (14), which is a regulator of the gad genes, encoding glutamate-dependent acid resistance. Using a cattle model, investigators demonstrated that wild-type EHEC outcompeted the sdiA deletion strain, which was defective in colonization of the RAJ over the 6-day assay. The finding that an estimated 70 to 80% of cattle herds in the United States carry EHEC is a major step forward in our understanding of the infection process in the EHEC reservoir.

**MOTILITY**

Flagellar motility is important in the initial stages of infection for many bacterial pathogens. For EHEC, motility and taxis are stimulated through the two-component system, QseC and QseB, which perceives
the AI-3 signal and the interkingdom communication molecules epinephrine and norepinephrine (15–18) (Fig. 2). QseBC signaling activates expression of the flhDC master regulators and FlIA, an alternate sigma factor, ultimately turning on a number of operons necessary for flagellar biosynthesis and motility. Furthermore, motility and biofilm formation are enhanced in the presence of epinephrine and norepinephrine (19). It is important to note that the AI-3 quorum-sensing molecule is distinct from AI-2, which was initially thought to control motility and type III secretion (20–22). Unlike AI-2, AI-3 is not dependent on LuxS for synthesis. Deletion of the gene encoding the AI-3 and epinephrine- and norepinephrine-recognizing QseC sensor kinase attenuated EHEC virulence in a rabbit model of infection. Collectively, these data clearly demonstrate that flagellar motility, and the associated signaling, is necessary for virulence (15, 16).

Additional molecules are known to stimulate flagellar motility, and data indicate temporal control correlates with the infection process. Three classes of promoters are responsible for transcribing the flagellar operons (23). The flhDC operon is designated class I and is required for class II promoter activity, which includes the flIA operon. In turn, class II promoters are required for class III expression. Butyrate, a short-chain fatty acid found in the large intestine, increases expression of flhDC (24) (Fig. 2). Regulation of flhDC by butyrate is leucine-responsive regulatory protein (LRP)-dependent, whereas induction of flIA by this short-chain fatty acid is LRP-independent. This regulation does not occur in the presence of propionate or acetate. However, propionate and acetate do increase flIC (class III) expression but not through flhDC. Short-chain fatty acids are found in concentrations ranging from 20 to 140 mM in the large bowel, and recognition of this signal, along with the AI-3 and inter-kingdom signaling molecules, likely contributes to EHEC niche recognition and adaptation. Some evidence also suggests that the flagellum acts as an initial adhesin to epithelial cells from the bovine terminal rectum (25).

Once motility is no longer needed, or prior to its necessity for colonization, the flagella are downregulated, an important step toward avoiding immune recognition. One observed mechanism is that the LuxR homolog SdiA downregulates FlIC expression (26). In another mechanism, mucin, produced by epithelial tissues, diminishes expression of flagellar genes. Mucin on agar plates repressed motility, and transcriptome analysis and quantitative PCR confirmed these data (27). In addition, the GrlA regulator, expressed from the LEE, decreased flagella biosynthesis by downregulating flIC and flhDC (28) (Fig. 2). Consistent with flagella not being needed after surface adherence, Tobe et al. observed that after 5 h of attachment to epithelial cells, flagella were downregulated in a GrlA-dependent manner. Furthermore, in a neonatal meningitis-causing E. coli strain, the E. coli common pilus (ECP), found in pathogens and nonpathogens alike, is necessary for attachment and biofilm formation. The regulator of ECP, EcpR, also downregulates flagellar motility after adherence occurs (29). By downregulating the flagellar master regulators flhDC by GrlA in EHEC, spatiotemporal regulation controls pathogenesis in coordination with the transition from a planktonic to an adhesive lifestyle.

**CONTINUING THE INTESTINAL JOURNEY**

Carbohydrate recognition and metabolism are important for EHEC niche adaptation (30) and ensure that EHEC virulence proteins are expressed only at the appropriate site of infection. A glycolytic environment, at
0.4% glucose, inhibits ler and LEE expression, whereas 0.1% glucose mimicking gluconeogenesis conditions enhances their expression (31). The LEE pathogenicity island (PAI) encodes T3SS and is organized into five major polycistronic operons and a number of bicistronic and monocistronic genes (32, 33). In gluconeogenesis, instead of oxidizing glucose, glucose is produced from two to three carbon molecules, such as acetate, succinate, or pyruvate. Consistently, glucose and glycerol inhibit ler expression, while succinate stimulates ler, mimicking glycolysis and gluconeogenesis, respectively (Fig. 3). This regulation occurs through the proteins KdpE, a response regulator that also senses osmotic stress, and Cra, both of which bind to ler regulatory DNA. Deletion of kdpE and cra results in ablation of attaching and effacing (A/E) lesion formation by EHEC in vitro (31).

In the gut, EHEC also perceives and responds to the sugar fucose, which enhances EHEC colonization (34). In the mammalian intestine, fucose is abundant, reaching concentrations of ~100 μM (35), because it is cleaved from host mucin glycoproteins by fucosidases produced by the commensal bacterium Bacteroides thetaiotaomicron. EHEC perceives the fucose signal via the two-component regulatory pair FusK, the sensor kinase, and FusR, the response regulator (36) (Fig. 3). Transcriptional activity of the LEE1 operon-encoded ler is increased in ΔfusK and ΔfusR strains. Thus these data indicate that the fucose-sensing, two-component system represses Ler and ultimately T3SS expression. Regulation is direct, as purified FusR binds to ler regulatory sequences, and binding is enhanced when the protein is in the phosphorylated state. This carbohydrate signaling is independent of using fucose as a carbon source. The importance of fucose sensing in virulence is highlighted by the findings that in an infant rabbit model of infection, fusK and fusR deletion mutants are outcompeted by the wild-type strain (36). In sum, ler and the T3SS are repressed by high glucose concentrations, and the presence of mucus-derived fucose, while stimulated under gluconeogenesis conditions, is mimicked by the presence of molecules such as succinate, pyruvate, acetate, and propionate.

EHEC virulence gene regulation has been correlated further with intestinal physiology. Butyrate, found in the large intestine, increases expression of the T3SS (37) (Fig. 4). More recently, ethanolamine, a bacterial and animal cell membrane component that is released into the lumen of the gut upon normal epithelial cell turnover, has been implicated as a molecule important in EHEC virulence gene regulation. Ethanolamine is perceived by EHEC, leading to increased expression of the quorum-sensing regulators QseA, QseC, and QseE and Ler (38). In addition, the LEE operons 1 through 5 are thus stimulated in the presence of ethanolamine. Though there are 17 ethanolamine utilization genes, eut, necessary for the use of this compound as a nitrogen source, the observed signaling in EHEC is independent of ethanolamine metabolism, but partially dependent on the EutR transcriptional regulator that binds ethanolamine (38) (Fig. 4). Furthermore, there is evidence that ethanolamine confers a growth advantage to EHEC during the stationary phase of growth, when this compound is used as a nitrogen source in media mimicking bovine intestinal contents (39). Ethanolamine is a part of normal physiology of the human and bovine large intestine membranes, and for EHEC, it provides a growth advantage and signals for expression of the T3SS as part of ecological niche recognition and adaptation. This signaling most likely coincides with close proximity or attachment to the host epithelium.
Elaboration of the T3SS of EHEC allows for attachment to the intestinal epithelium in cattle, specifically to the epithelial cells of the RAJ (5, 40). Disruption or deletion of genes encoding components of this apparatus reduces colonization in the bovine host (41, 42). Furthermore, the translocated intimin receptor (Tir) and intimin proteins, facilitators of the tight attachment of the A/E intestinal lesions, play a critical role in colonization of a neonatal calf model of infection (43). In addition to binding to the bacterial-derived Tir molecule, intimin also binds to integrins and nucleolin on the host cell surface. The molecular syringe, the apparatus necessary for altering signaling events in the formation of actin-rich pedestals, ultimately injects ~50 distinct effector molecules into host cells (44). Assembly of the apparatus, expression of both LEE and non-LEE encoded effectors, and their translocation must be regulated in a spatiotemporally correct manner to establish infection and to avoid immune detection in both humans and cattle.

FIGURE 4 Stimulation of LEE and non-LEE effector molecules required for infection by EHEC. In the large intestine of humans and RAJ, the host-produced hormones norepinephrine and epinephrine, EHEC-derived signaling molecule AI-3, and sulfate and phosphate trigger the two-component sensors QseCF and/or QseEF to upregulate ler and espFu transcription (17, 85). In addition, ethanolamine, a cell membrane component, stimulates ler production through EutR and QseC (38). Gluconeogenic conditions of the large intestine activate the ler activators KdpE and Cra, and butyrate directly activates LEE transcriptional activity (37, 125). The RNA chaperone Hfq affects LEE expression through interactions with Ler mRNA but has negative or positive effects depending on the strain of EHEC, as indicated by a dashed arrow (71–73). Finally, nutrient deprivation associated with the infection site activates the stringent response leading to production of ppGpp, which promotes expression of the LEE transcriptional activators pchA and pchB and the non-LEE effector NleA (68, 117). The asterisk indicates that ler (LEE1) expression is upregulated by several other factors, including temperature, pH, iron, ammonium, calcium, bicarbonate, and the regulatory proteins IHF, Fis, BipA, PerC, and GadX (previously reviewed in reference 45). Blunted arrow indicates negative control. doi:10.1128/microbiolspec.EHEC-0004-2013.f4

T3SS EXPRESSION AND ATTACHMENT

LEE

Elaboration of the T3SS of EHEC allows for attachment to the intestinal epithelium in cattle, specifically to the epithelial cells of the RAJ (5, 40). Disruption or deletion of genes encoding components of this apparatus reduces colonization in the bovine host (41, 42). Furthermore, the translocated intimin receptor (Tir) and intimin proteins, facilitators of the tight attachment of the A/E intestinal lesions, play a critical role in colonization of a neonatal calf model of infection (43). In addition to binding to the bacterial-derived Tir molecule, intimin also binds to integrins and nucleolin on the host cell surface. The molecular syringe, the apparatus necessary for altering signaling events in the formation of actin-rich pedestals, ultimately injects ~50 distinct effector molecules into host cells (44). Assembly of the apparatus, expression of both LEE and non-LEE encoded effectors, and their translocation must be regulated in a spatiotemporally correct manner to establish infection and to avoid immune detection in both humans and cattle.
The LEE PAI, encoding the T3SS, is silenced by H-NS (for review see reference 45). Navarre et al. demonstrated that the function of H-NS, conserved across the Enterobacteriaceae, is to silence horizontally transferred genetic elements, indicated by a lower GC or higher AT content than the resident genome. Along with LEE1, multiple LEE operons are directly silenced by H-NS (46–48), and thus, much of the regulatory network described for the LEE directly involves relieving H-NS-mediated repression. The LEE1-encoded Ler protein, an H-NS homolog, is a key regulator of the LEE and acts as an antisilencer (32). As most H-NS-controlled genes are regulated in response to environmental adaptation (49), numerous environmental inputs and regulatory proteins control ler gene expression.

To demonstrate the many inputs controlling ler gene expression, researchers working with both EHEC and enteropathogenic E. coli (EPEC) found that Ler is stimulated in response to environmental signals such as temperature, pH, iron, ammonium, calcium, bicarbonate (50–54), and quorum-sensing signaling (55, 56), as well as the proteins IHF (57), Fis (58), BipA (59), PerC homologs, or Pch (60), GrlR (61, 62), and GadX (63) (Fig. 4). The GrlR regulators constitute a complicated feedback loop whereby GrlA directly activates ler expression, and GrlR acts posttranslationally to reduce the cellular quantity of the GrlA protein (28, 61, 64, 65). Once expressed, Ler, in turn, acts as an H-NS antisilencer to increase the expression of LEE2, LEE3, LEE4, and LEE5 operons (Fig. 4).

LEE gene expression is stimulated as EHEC transitions into stationary phase, controlled by quorum-sensing signaling, and also in response to nutrient deprivation, much through direct regulation of ler (21) (Fig. 4). Nutrient deprivation, the starvation for a number of nutritional requirements such as amino acids, carbon, nitrogen, or phosphorus, induces the stringent response (for review see reference 66). The stringent response produces the signaling molecule ppGpp, a process dependent on the synthase RelA and SpoT, a hydrolase and synthase (67). Expression of the EHEC LEE and adherence to Caco-2 cells in culture are increased in response to nutrient deprivation in a RelA-dependent manner, whereby increased pools of ppGpp are created (68). The protein DksA, which interacts with RNA polymerase in the transcription complex, is also involved in this regulation. Regulation of EspB and Tir, and thus the LEE4 and LEE5 operons, is controlled, in part, by ppGpp interaction with the ler, pchA, and pchB genes. Thus the stringent response rapidly activates expression of the T3SS components when nutrients are limiting and upon transitioning into stationary phase, conditions EHEC is predicted to encounter when entering the lower intestine.

A number of posttranscriptional control mechanisms add to the complexity and highlight the importance of coordinating expression of LEE genes within the host. RNaseE-dependent RNA processing occurs at the LEE4 operon, resulting in differential control of expression of the SepL protein. This RNA processing is thought to be involved in the contact regulation that controls the switch from translocator to effector type III secretion (62, 69) and the regulation of EspADB proteins necessary for forming the filament and pore that ultimately embed in the host cell membrane (70). In EHEC strain 86-24, serotype O157:H7, the RNA chaperone Hfq acts positively on the expression of ler and LEE operons 2 through 5 in all phases of growth (71) (Fig. 4). This study shows that Hfq increases the expression of the quorum-sensing, two-component system QseBC. QseC also acts to increase Ler expression and thus illustrates coordinated regulation. Other researchers, though working with strain EDL933 as opposed to 86-24, observed that the RNA chaperone Hfq negatively affects the expression of the LEE (72, 73). Shakhnovich et al. observed that Hfq negatively regulates expression of the LEE in a Ler-dependent manner and also controls expression of a number of non-LEE effector molecules. The ler, or LEE1 transcript, was a direct target of Hfq regulation. Congruently, Hansen and Kaper observed that Hfq affects Ler expression by negatively affecting the GrlR regulators through posttranscriptional control, the stability of the grlR transcript, while negatively affecting LEE expression in stationary phase in a manner independent of grlR. Consistently with this set of observations, researchers also demonstrated that Hfq negatively affects LEE expression in the related AE pathogen EPEC (73). Thus, LEE genes are apparently regulated by Hfq in an opposite manner in the O157:H7 strains 86-24 and EDL933. Collectively, these regulatory observations illustrate the intricate and variable nature of virulence gene control in related but distinct isolates associated with outbreaks of EHEC disease and the evolution of regulatory networks associated with niche adaptation.

Additionally, a number of O-island regulators contribute to the expression of the LEE. O-islands are horizontally acquired clusters of genes that reside in the EHEC genome. The regulators EtrA and EivF are encoded in a cryptic, second T3SS gene cluster in EHEC, and negatively regulate the LEE PAI and secretion (74). Encoded within O-island 51, the regulator RgdR is a
positive regulator of the LEE and secretion, through the Ler regulatory cascade (75). Thus expression of the T3SS is controlled not only by LEE-encoded regulators and regulatory elements that are part of the core E. coli genome but also by genes acquired by horizontal gene transfer.

PerC is an important regulator of the EPEC LEE, though a perC-like gene does not exist on the pO157 virulence plasmid of EHEC (33, 46, 76). However, in the O157:H7 Sakai stain, Iyoda and Watanabe (60) identified five chromosomally encoded PerC homologs (pch). Of those identified, pchA, pchB, and pchC, but not pchD or pchE, were confirmed to regulate the LEE when expressed in trans. Using double mutations, the authors demonstrated that, in combination, pchA and pchB, or pchA and pchC resulted in significantly decreased expression of the LEE, through the global regulator Ler, and reduction of adherence to HEp-2 epithelial cells in culture (Fig. 4). Interestingly, the pchABC genes of EHEC and perC of EPEC are interchangeable in their ability to activate expression of the LEE1 operons of both organisms (72). Thus the EHEC pch genes, with the exception of pchD and pchE, all encoded within phage-like elements, are involved in pathogenesis.

Intense investigation has been directed toward cell-to-cell communication or quorum-sensing signaling of the LEE and its cognate effector molecules. The best understood of the autoinducers, AHLs, are not produced by E. coli. Thus EHEC strains do not have the LuxI AHL synthase, though they do possess a LuxR-type transcriptional regulator SdiA (78, 79), and structural studies indicate that SdiA properly folds only in the presence of AHLs (80). Therefore, regulation through SdiA occurs by perceiving AHLs produced by other bacteria, leading to alterations in EHEC gene expression.

AHL molecules are produced by the resident flora in the rumen of the bovine intestine, indicated by an Agrobacterium reporter strain, but are not found in the terminal rectum, the site of EHEC colonization (14). Through SdiA sensing of the o xo-C6-AHL signal LEE genes, including ler located in LEE1 and LEE4 operons, are downregulated (Fig. 3). It has been proposed that quorum sensing facilitates EHEC escaping the rumen en route to the site of colonization, the RAJ in cattle, by downregulation and avoiding inappropriate expression of the LEE, while inducing the gad acid tolerances system, as described above (Fig. 1).

Elucidation of the interkingdom signaling systems of EHEC controlling virulence has revealed how these organisms communicate with members of their own species, resident bacteria, and the host organism. The QseA quorum-sensing regulator acts directly on the expression of the LEE1 operon, specifically at the P1 promoter (81, 82) (Fig. 4). Indeed, multiple two-component regulators perceive chemical signals and the EHEC-derived, aromatic AI-3 molecule to control expression of the T3SS. The sensor kinase QseE responds to sulfate, phosphate, and host-derived epinephrine to phosphorylate its cognate response regulator QseF that ultimately stimulates ler expression (83) (Fig. 4). The sensor kinase QseC perceives the catecholamines epinephrine and norepinephrine and the EHEC-produced AI-3 molecule to increase ler transcription through phosphorylation of KdpE (17), leading to the elaboration of the type III system. To add emphasis to the importance of quorum-sensing signaling controlling virulence, in an infant rabbit model of infection, deletion or inhibition of qseC in rabbit EPEC severely attenuated virulence (15, 84), and a qseF deletion mutant of EHEC does not form A/E lesions (85). Thus the ability to respond to host catecholamines and AI-3 is required for EHEC to stimulate T3SS expression and to establish infection.

Non-LEE

A number of non-T3SS adhesins, either demonstrated to be necessary for or hypothesized to be involved in attachment to host cells, have been described (Table 1). Upon sequencing the E. coli O157:H7 genome of strain EDL933, it was noted that this organism has at least 10 fibri lial gene clusters and 13 regions that encode nonfimbrial adhesins, some of which were not found in nonpathogenic E. coli (86, 87). Extracellular structures include the E. coli YcbQ laminin-binding fimbriae (ELF) (88), two long polar fimbriae (LPF) (89, 90), the F9 fimbriae (91), a type IV pilus called “hemorrhagic coli pilus” (HCP) (92), curli (93, 94), OmpA (95), the EHEC factor for adherence (Efa1) (96), the IgrA homolog adhesin (Iha) (97), the ECP, the autotransporter protein EhaG (98), and the pO157 virulence plasmid-encoded StcE (99, 100). Surely these molecules contribute to adherence to surfaces, both biotic and abiotic, but a comprehensive understanding of the role of these adhesins in human disease, carriage in cattle, and survival and propagation in food does not exist.

However, the long polar fimbria 1 (Lpf1) of E. coli O157:H7 is a well-characterized example of one such adhesion. Lpf1 facilitates binding of EHEC to not only epithelial cells but also extracellular matrix proteins, including fibronectin, laminin, and collagen IV (101). Lpf1 is tightly regulated, with maximal expression in
late exponential phase of growth in iron-deprived and slightly acidic environments (102) (Table 1). Two lpf loci exist in E. coli O157:H7, and when one or both are deleted, colonization in animal infection models is altered (103, 104) (Table 1). The Lpf1 fimbria is coordinately regulated with the LEE because the operon is silenced by H-NS, while Ler functions as its antisilencer (105).

ECP, common to both pathogens and nonpathogens, has also been described. Deletion of the ecp genes resulted in decreased adherence of both O157:H7 strain EDL933 and commensal E. coli to human epithelial cells in culture (106). EcpR, a LuxR-like regulator containing a helix-turn-helix DNA-binding motif, controls expression of the ECP (107). The EcpR protein was shown to bind to a TTCCT sequence upstream of the ecp operon, and deletion of ecpR resulted in decreased adherence by EHEC. H-NS silences ecp, and antisilencing occurs by EcpR, assisted by the protein IHF. Thus the ECP is most likely involved in colonization by pathogens and nonpathogens alike. Similarly, a trimeric autotransporter protein, EhaG, of EHEC has been described (98). As for EcpR, these conserved proteins are found in both pathogens and nonpathogens, and in EHEC EhaG is involved in autoaggregation, biofilm formation, and adherence to extracellular matrix proteins and colorectal epithelial cells. Not surprisingly, expression is, in part, controlled by H-NS (Table 1). Finally, the StcE zinc metalloprotease encoded on the pO157 virulence plasmid enhances pedestal formation and is predicted to facilitate adherence to epithelial cells in the host by cleavage of glycoproteins (99, 108). The stcE gene is coordinately regulated with the LEE through Ler and H-NS. Clearly there is much work to be done to understand the role of non-T3SS adhesins in niche recognition, pathogenesis in general, and how their expression is coordinated with other virulence factors.

### INJECTION OF EFFECTORS

Effector molecules slated for injection by the T3SS into host cells are encoded within the LEE, O-islands, phage, and other integrative elements located in the genome. Those located within the LEE, including Tir, EspF, Map, EspG, EspH, and EspZ, are of course coordinately regulated with the expression of the secretion apparatus itself. Evidence demonstrates that a number of non-LEE-encoded effectors, including EspJ, NleB, NleE, NleF, and NleH, are involved in EHEC colonization and survival, though experiments demonstrating these phenotypes were conducted in a number of different animal infection models (109–111). Thus further experimentation is necessary to determine the role of these non-LEE effectors in EHEC disease and carriage, particularly in colonization of the RAJ in cattle.

Assuming that the non-LEE effectors are important for disease in humans and carriage in cattle, they should be coordinately expressed with LEE-encoded effectors. Investigators have begun to unravel their regulation. In particular, several studies have focused on the NleA (also named EspI) effector because of evidence that it plays an important role in pathogenesis. After translocation into the host cell, NleA colocalizes with the Golgi apparatus, and has been associated with severe disease in the Citrobacter rodentium A/E mouse model (112). NleA is conserved in many A/E pathogens (113, 114), and evidence indicates that it affects protein trafficking and secretion through the rough endoplasmic reticulum by binding to the COPII coat protein (112, 113). NleA is secreted into the EHEC extracellular milieu (115) and is coordinately regulated with the LEE. As for the LEE, the regulatory proteins H-NS, Ler, and GrlA control expression of the phage-encoded nleA gene (115–117). Ler acts directly, binding to the regulatory region upstream of the nleA promoter. As with LEE1, nleA is regulated by quorum-sensing signaling, whereby QseC controls expression through inhibition of the positive-acting RcsB response regulator (118). Expression of NleA is induced by osmolarity, response to starvation signals, and RecA-dependent DNA-damage signaling, the latter also controlling LEE expression (117, 119) (Fig. 4).

#### TABLE 1  Regulation of non-LEE adhesins and factors involved in EHEC adherence

<table>
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<tr>
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<th>Type</th>
<th>Regulation</th>
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<tr>
<td>StcE</td>
<td>Zinc metalloprotease</td>
<td>H−NS, Ler</td>
<td>99, 100, 108</td>
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</table>
EspFu (also named TccP) is another non-LEE effector important for the EHEC A/E histopathology. Unlike EPEC where all genes required for A/E lesion formation are found within the LEE, EspFu is necessary for this phenotype in EHEC and is encoded within phage U (120–122). EspFu binds to the GTP-binding domain of N-WASP, mimicking the eukaryotic SH2/SH3 adapter protein and facilitates actin polymerization, and is subject to regulation by environmental signals (123). The genes espFu and espJ are influenced by changes in temperature, pH, osmolarity, and oxygen pressure (124). The regulators KdpE and Cra act to increase expression of espFu (125). The QseEF two-component, quorum-sensing system controls expression of EspFu through AI-3, epinephrine, and norepinephrine signaling (85) (Fig. 4). Thus the non-LEE effectors NleA and EspFu, important for virulence in EHEC, are coordinately controlled with the LEE by overlapping signaling: nleA expression is Ler-dependent and, in part, induced by the SOS response, while expression of espFu is controlled by the QseA and QseEF quorum-sensing pathways that directly stimulate the LEE (Fig. 4).

Data are emerging to show that there are at least two modes of regulation of the non-LEE effector molecules. The study by Garcia-Angulo et al. (116), addressing whether a common mechanism for the non-LEE effector molecules exists, is an important investigation because to date a common regulatory network linking their expression had not been established. Though performing much of their experimentation on the related A/E pathogen EPEC, investigators identified a 13-bp inverted repeat, with a 5-bp spacer upstream of many of the genes encoding the non-LEE effector molecules. They found in EPEC, by deletion of these sequences and mutagenesis, that the repeats were essential for transcription of the nleH1 and nleB2 genes. The authors termed these sequences NRIR, for nle regulatory inverted repeat. Transferring this information to EHEC virulence gene regulation, the authors found a number of nle genes in the EDL933 and Sakai EHEC strains that contain NRIR sequences in positions predicted to affect transcription by in silico analysis. These loci included, in both strains EDL933 and Sakai, nleH1-nleF1, nleB2-nleC, espX, a number of nleG genes, and espFu. The authors concluded that the NRIR sequences were involved in coordinated expression of the non-LEE effector molecules in a second mode of regulation, independent of the LEE regulators Ler and GrlA. Future work will undoubtedly involve identifying protein components, as well as additional genetic elements necessary for this regulatory network, and determining how these effector molecules are coordinately regulated with the type III system required for their translocation into host cells.

**TOXIN EXPRESSION AND PHAGE INDUCTION**

As with many of the non-LEE effector molecules and most major bacterial exotoxins, the EHEC stx1 and stx2 genes are encoded within prophages. The Shiga toxins Stx1 and Stx2 are responsible for bloody diarrhea associated with EHEC infection and the serious complication hemolytic-uremic syndrome. Toxin production occurs upon phage induction. The genes stx1 and stx2 are encoded in separate prophages, and both are expressed during the phage lytic cycle (126). Initial studies indicated that stx1 is also regulated by low levels of iron as a mechanism to acquire this micronutrient (127–129) (Fig. 5). As for induction of other lambdoid phage, Stx production is induced by DNA damage and regulated by RecA. Antibiotics that target DNA synthesis, such as quinolones and mitomycin C, stimulate Stx production.

**FIGURE 5** Stimulation of Shiga toxin expression. Low iron levels lead to upregulation of stx1 (127, 128). Hydrogen peroxide and antibiotics targeting DNA synthesis, such as mytomycin C and quinolones, lead to DNA damage and activate RecA to induce Stx1 production (130, 144, 145). Sulfate, phosphate, and epinephrine molecules signal through the two-component system QseCF to RecA, thus leading activation of Stx2 production. Ethanolamine also upregulates stx2 through the transcription factor EutR (38). doi:10.1128/microbiolspec.EHEC-0004-2013.f5
Similarly, hydrogen peroxide induces stx expression, most likely due to activation of the SOS response (131). In a seemingly contradictory study, the reactive oxygen species NO inhibited Stx phage induction and Stx production in the presence of the DNA-damaging agent mitomycin C (132). Here the authors claim that iNOS expressed in enterocytes, simulated by type I cytokines producing NO, leads to a bacterial NsrR-related stress response, sensitizing and protecting EHEC from DNA damage. Nonetheless, because the only known mechanism of Stx release is by lysing of the bacterium, combined with the now obvious reasons that DNA-damaging antibiotics lead to increased risk of hemolytic-uremic syndrome, there is a need for a more complete understanding of how stx genes are regulated.

More recent data correlate Stx production with signal molecules associated with specific regions of the human and bovine intestine. The two-component sensor kinase QseC regulates Stx2 expression through the SOS response, RecA cleavage of the lambda repressor cl (17) (Fig. 5). Consistently, recA expression was decreased in a qseC mutant. QseC responds to the interkingdom signaling molecules epinephrine and norepinephrine and the related AI-3 quorum-sensing molecule produced by E. coli. Several studies indicated that Stx production is controlled by AI-3. Another two-component pair, QseE, the sensor kinase, and QseF, the response regulator, affects Stx expression (85, 133). QseF can be phosphorylated by either QseE, responding to epinephrine, sulfate, and phosphate, or QseC to stimulate stx2. Ethanolamine, released from epithelium, also stimulates Stx2 production in EHEC (38). The genetic pathway necessary for the signaling involves the ethanolamine utilization regulator eutR, as deletion of the gene encoding this protein resulted in decreased expression of stx2 by microarray (Fig. 5). Thus we begin to see a clearer picture of Stx regulation in EHEC, correlating expression to bacteria-derived autoinducers, host-produced signal molecules, and stress-related signals, and ultimately the genetic pathways necessary for the bacterial response.

Because of the necessity of iron for colonization and pathogenesis within the animal host, the EhxA enterohemolysin is also an important EHEC virulence factor. EhxA is a member of the RTX family, and its function is to lyse red blood cells. The ehxCABD operon, found on the pO157 virulence plasmid (134), is positively regulated by DsrA and RpoS at host body temperature (135). DsrA is a small RNA that acts by allowing translation to proceed (136). The expression of ehx is also positively regulated by GrlA and Ler (64, 137), and thus the enterohemolysin is coordinately regulated with the T3SS.

A REGULATED INFECTION CONTINUUM

The work of many research groups leads to a model of EHEC pathogenesis correlating with gut physiology, bacterial metabolism along the route of infection, and host-microbe and microbe-microbe signaling. For both the human and bovine hosts, EHEC must pass through the acidic environment of the stomach and the rumen, respectively. Three acid tolerance systems assist the bacterium in this process. GadX is induced by AHL molecules produced by resident flora of the bovine rumen (14) and perceived by the LuxR homolog SdiA (Fig. 1). The LEE is downregulated by SdiA in this environment because it is not the normal site of attachment, allowing the bacterium to travel toward the RAJ in cattle (Fig. 3). Consistently, in a separate study, data indicated that flagellar motility is upregulated by acid stress while expression of Stx remains unchanged and the T3SS is downregulated by acute acid stress (138).

In the large intestines of humans, conditions mimicking gluconeogenic versus glycolytic metabolism are predicted to stimulate expression of the EHEC T3SS (31, 139) (Fig. 4). This observation is also understandable in colonization of the RAJ of cattle, where nutrients, particularly carbohydrates, are less abundant compared to the rumen. One might posit that EHEC avoids the small intestine in humans by downregulating expression of LEE genes in the presence of high-glucose, glycolytic conditions (Fig. 3). It is curious why EPEC, which infects the small intestine, also downregulates LEE genes under glycolytic conditions, whereas, identical to EHEC, LEE genes are stimulated under low-glucose conditions. This observation might be explained by the specificity of the infection process, that perception of multiple signals is necessary for proper niche recognition.

Carbohydrate metabolism and signaling are clearly linked to virulence, and several studies have indicated that fucose is important for EHEC colonization (34, 140). Resident Bacteroides spp. cleave fucose from the glycans found in the intestine, and suppression of Ler, T3SS function, and A/E lesions ensues from the fucose signal perceived by FusKR (36) (Fig. 3). EHEC can use a number of mucus-derived carbohydrates, in particular, manno-, N-acetylglucosamine, and N-acetylglucosamine, and galactose catabolism confers a competitive advantage against commensal bacteria, most likely assisting safe passage through the small intestine (39, 141). In both humans and cattle, EHEC expresses the T3SS in response to membrane-derived ethanolamine, clearly encountered at the sites of infection in both hosts. The catecholamine and AI-3 signals also contribute to elaboration of the secretion apparatus (Fig. 4). Why would epinephrine and
norepinephrine exist here when the adrenergic receptors are not located on the apical side of the intestinal lumen, but these molecules were detected in the lumen of the intestine of mice containing specific pathogen-free microbiota (142). It is also plausible that the epinephrine/norepinephrine signal becomes more available upon damage to the host epithelium, releasing these hormones into the lumen of the gut, enhancing expression of the T3SS and firmly establishing infection (Fig. 4). Nonetheless, researchers have established that three important signals, ethanolamine, epinephrine/norepinephrine, combined with the bacteria-derived AI-3, correlated with the sites of infection.

A number of signals, including the catecholamines, also control the expression of stx genes, mediated through two-component, quorum-sensing proteins QseC and QseE (Fig. 5). Membrane-derived ethanolamine and the well-studied RecA-dependent SOS response stimulate Stx production. EHEC is expected to encounter all of these conditions during niche recognition at the sites of attachment in humans and cattle. These observations are consistent with the presumption that substantive expression of Stx will occur when colonization has commenced.

CONCLUSIONS

Researchers have made significant progress toward understanding the genetic regulation controlling EHEC pathogenesis. How EHEC gains safe passage through the acidic environment of the stomach, how flagellar motility is turned on and turned off, and what signals and metabolites are necessary for outcompeting commensal flora prior to arriving at the sites of infection for humans and cattle are now recognized on a basic level. Researchers have discovered myriad regulatory networks and environmental signals that control type III secretion, and we are beginning to understand how effector molecules slated for translocation into host cells are coordinately regulated with the T3SS itself and how the dangerous Shiga toxin is expressed. Though the LEE-encoded effectors and non-LEE NleA and EspFU are controlled by Ler and quorum-sensing signaling, the majority of those proteins targeted for secretion are regulated by different mechanisms. The NRIR sequences found upstream of many of the phage- and O-island-encoded effectors most likely coordinate their expression, but this has yet to be established experimentally for EHEC. Similarly, with the exception of Lpf1 and StcE, we do not know if, or how, expression of most non-LEE adhesins is coordinated with that of the LEE (Table 1). Finally, EHEC tissue tropism is not well understood, but the regulatory networks described herein most likely play an important role in niche recognition in human and animal hosts.

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REFERENCES


Enterohemorrhagic Escherichia coli Virulence Gene Regulation


