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Molecular Mechanisms That Mediate Colonization of Shiga Toxin-Producing *Escherichia coli* Strains

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Shiga toxin-producing *Escherichia coli* (STEC) is a group of pathogens which cause gastrointestinal disease in humans and have been associated with numerous food-borne outbreaks worldwide. The intimin adhesin has been considered for many years to be the only colonization factor in these strains. However, the rapid progress in whole-genome sequencing of different STEC serotypes has accelerated the discovery of other adhesins (fimbrial and afimbrial), which have emerged as important contributors to the intestinal colonization occurring during STEC infection. This review summarizes recent progress to identify and characterize, at the molecular level, novel adhesion and colonization factors in STEC strains, with an emphasis on their contribution to virulence traits, their host-pathogen interactions, the regulatory mechanisms controlling their expression, and their role as targets eliciting immune responses in the host.

STEC O157:H7 AND NON-O157 STEC

Shiga-toxigenic *Escherichia coli* (STEC) O157:H7 is an important cause of human gastrointestinal disease and the best-studied STEC associated with large outbreaks worldwide (43, 74). STEC O157:H7 strains cause diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome (HUS) (59). Disease-associated human isolates of *E. coli* O157:H7 are characterized for the presence of specific sets of virulence genes, including those encoding Shiga-toxins (stx_1, stx_2), intimin (*eae*), hemolysin (*hlyA*), and long polar fimbriae (*lpf1* and *lpf2*) (45, 97). The production of Stx is the main virulence feature of STEC associated with the development of HUS (82) but cannot be solely responsible for full pathogenicity. STEC associated with severe human disease is usually capable of colonizing the intestinal mucosa and possesses mobile genetic elements carrying virulence genes, such as plasmids, transposons, phages, and pathogenicity islands (79, 92, 97). Several important colonization properties are carried by the locus of enterocyte effacement (LEE) pathogenicity island (92), which governs the ability of STEC to colonize the intestinal mucosa of the host and produces a peculiar pathogenic process known as the attaching-and-effacing (A/E) lesion (for a review, see reference 59).

Most virulence/colonization factors have been described for STEC O157:H7 isolates; however, it is important to identify and determine the virulence traits of other STECs important for disease in humans, the so-called "non-O157 STEC" strains. In addition to diarrhea, these isolates are also associated with severe disease. Several serogroups of non-O157 STEC have been described; however, the serogroups O26, O45, O103, O111, and O145 have been identified as the "big six" non-O157 STEC O serogroups, because they have been associated with increasing frequency in patients with bloody diarrhea and HUS (12, 29, 41, 55). Because the U.S. Food and Drug Administration recognizes that non-O157 STEC serogroups are emerging as an important cause of food-borne disease, these isolates impact both the imported and domestic food supply. Therefore, they represent significant scientific and risk management challenges. For this reason, it is important to identify and characterize the repertoire of specific virulence factors associated with their ability to colonize and cause disease. This review summarizes the current progress in the characterization of novel colonization factors in STEC O157:H7 and those adhesion factors described for non-O157 STEC strains.

INTIMIN AS THE KEY COLONIZATION FACTOR OF STEC STRAINS

Besides the *stx* gene(s), human pathogenic STEC strains often carry the *eae* gene, encoding the outer membrane adherence protein intimin (64). The *eae* gene is carried by the chromosomally located LEE pathogenicity island, which is required for intimate attachment to the host intestinal mucosa (43, 59). The interaction of intimin with the bacterial "translocated intimin receptor" (Tir) results in the formation of A/E lesions, which are critical for the pathogenesis of STEC, since mutants that cannot form lesions do not colonize their hosts or cause disease (59). Further, it has been shown that intimin has an affinity for the eukaryotic proteins nucleolin and β1 integrin, and they serve as potential receptors for intimin during STEC O157:H7 infection (76, 77). The intimin-Tir interaction has been extensively reviewed elsewhere (see reviews in references 15 and 16); therefore, the mechanism for A/E lesion formation will not be discussed here. However, it is well accepted that LEE-positive STEC strains deliver the Tir proteins into host cells (20), with subsequent insertion of Tir into the plasma membrane, thereby exposing its extracellular portion to bind intimin. The Tir and intimin proteins form multimers, and intimin binding results in a higher-order clustering of Tir, culminating in a signal that triggers the actin assembly that drives pedestal formation (Fig. 1A) (15, 16).

Although the essential role of intimin and Tir is the formation

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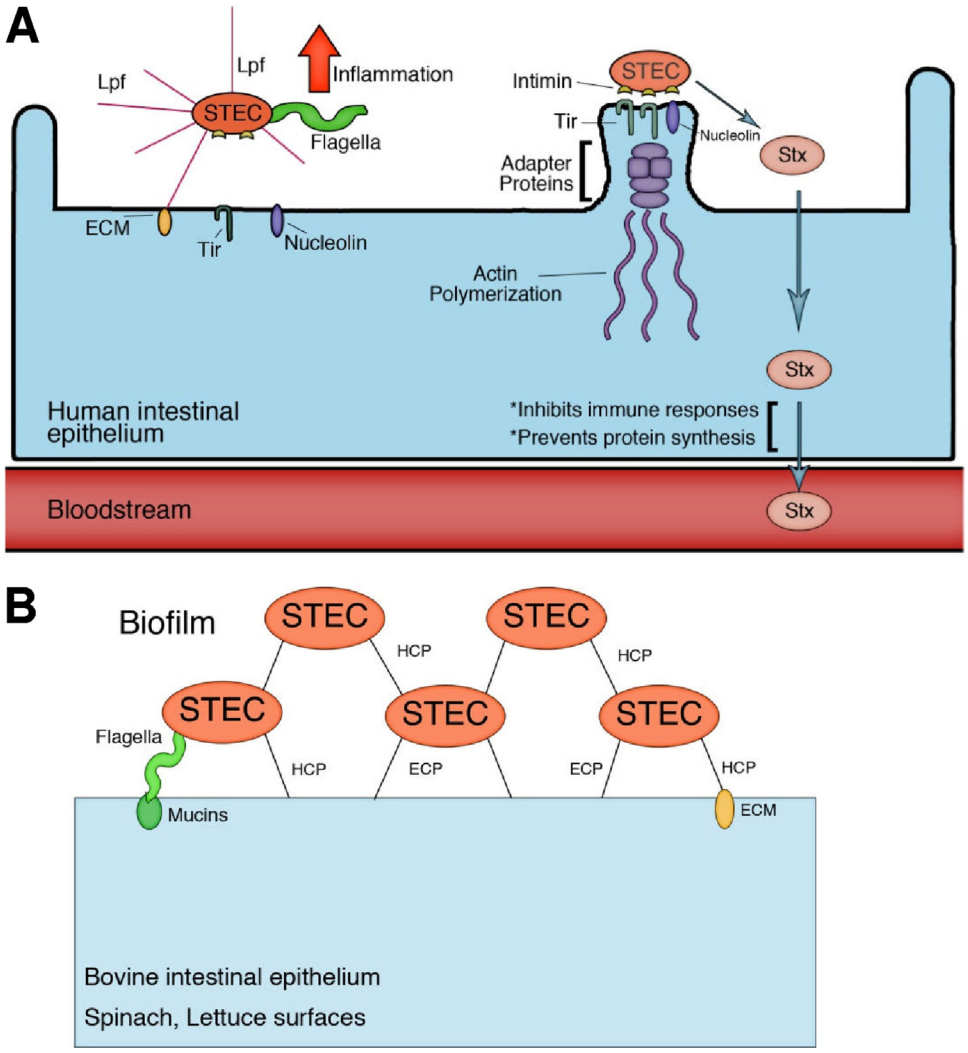

FIG 1 Working colonization model for STEC infecting humans and persisting in ruminants. (A) The diagram shows a human intestinal epithelial cell displaying the first stages of colonization by STEC in the lumen. STEC O157 or non-O157 strains interact with the intestine via the Lpf fimbriae; this is followed by the formation of A/E lesions. Stx production occurs in the intestinal tract, and translocation across the intestinal lumen to the bloodstream results in its distribution to target organs, such as the kidneys. (B) STEC O157 and non-O157 strains are also capable of adhering and colonizing other surfaces, such as the bovine intestinal epithelium and the surfaces of different vegetables. Several adhesins, such as HCP, ECP, and flagella, have been associated with persistence in the bovine intestine and the formation of biofilms.

of intimate cell attachment, it has been reported that STEC O157:H7 mutant strains lacking intimin or Tir do not colonize the intestine in the infant rabbit model of infection (69). However, the presence of the LEE island does not seem to be essential for full virulence, as a wide number of STEC LEE-negative strains have been associated with sporadic cases and small outbreaks of hemorrhagic colitis and the HUS (9). The increasing number of LEE-negative STEC strains associated with human infection (31, 32), and the identification of novel adhesins in STEC O157:H7 (91, 97), which are also present in other STEC strains, lead to further characterization of other non-LEE-encoded adhesion factors (Table 1). The following sections describe recent progress in the investigation of those novel adhesion/colonization factors in different STEC strains.

STEC O157:H7 FIMBRIAE OR PILUS FACTORS

Long polar fimbriae. When the sequence of the genomes of prototype STEC O157:H7 strains (36, 65) became available, DNA analysis revealed a total of 14 gene loci that could potentially encode fimbriae. Ten of those loci are conserved or partially conserved in *E. coli* K-12, and four were reported as unique to O157. Of those O157 specifically identified loci, two contained genes closely related to the long polar fimbriae (*lpf*) operon of *Salmonella enterica* serovar Typhimurium (6). The first locus is located in an O157-specific island (O-island 141 or OI141) of approximately 5.9 kb, inserted in the *yhjX-yhjW* intergenic region (in relation to the *E. coli* K-12 chromosome). The so-called *lpf1* operon contains 6 genes (*lpfABCC′DE*) similar in sequence and gene order to the *Salmonella lpfABCDE* genes (88). Expression of the STEC O157:H7 *lpf1* operon in a nonfimbriated *E. coli* ORN172 strain was reported to increase adherence to HeLa and MDCK cells, and peritrichous short fimbriae were observed (88). It was further demonstrated that *stx*-positive and *stx*-negative STEC O157:H7 mutated in the *lpfA1* gene (encoding the major fimbrial subunit), exhibited a reduction in adherence to epithelial cells,

TABLE 1 Properties and characteristics of the STEC adhesion factors[a]

Name	Type	Host(s) and/or cell adherence	Receptor	Other characteristics
Lpf	Fimbriae	Sheep, pigs, lambs, rabbits Intestinal ECs Human IVOC	Fibronectin, laminin, and type IV collagen	IL-8 release induction on intestinal ECs (unpublished observation)
HCP	Fimbriae	Human and bovine ECs	Fibronectin and laminin	IL-8 and TNF-α release induction on intestinal ECs
ECP (Mat)	Fimbriae	Human ECs	Not identified	Putative synergic role with BFP during EPEC microcolony formation
F9	Fimbriae	Bovine ECs	Fibronectin	
ELF	Fimbriae	Human intestinal ECs Cow and pig intestinal explants	Laminin	
Sfp	Fimbriae	Human intestinal ECs	Not identified	
EhaA-B-J	Autotransporter	Primary ECs, bovine rectum (EhaA)	Fibronectin, laminin, and type IV collagen (EhaB-J)	
EspP ropes	Autotransporter	Intestinal ECs, bovine Primary ECs, bovine rectum	Not identified	Bacterial protection from antimicrobial compounds
Sab	Autotransporter	ECs	Not identified	
Cah	Autotransporter	Not identified	Not identified	Bacterium-bacterium interaction
Flagella	Flagella	Primary ECs, bovine rectum Chicks, human colon xenografts	Bovine mucus, mucin protein, and extracellular matrix proteins	Induction of proinflammatory chemokines
EibG	Adhesin	Human and bovine intestinal ECs	Not identified	Binding to IgG and IgA
Efa-1	Adhesin	CHO cells, intestinal ECs	Not identified	Lymphostatin, induction of production and/or secretion

[a] ECs, epithelial cells; IVOC, *in vitro* organ cultures.

and displayed a diffuse adherence pattern (88, 90). A recent study has shown that STEC O157:H7 adhered more abundantly to surfaces coated with fibronectin, laminin, and collagen IV and that a reduced binding of the bacteria to these extracellular matrix (ECM) proteins was observed for an *lpf* mutant (*lpf1*) strain (27). This study demonstrated that Lpf1 and ECM proteins interact, and their interaction may contribute to STEC O157:H7 colonization of the gastrointestinal tract.

The second *lpf* operon, referred to as the *lpf2* locus, is approximately 6.8 kb and located in the O-island 154; it is inserted in the *glmS-pstS* intergenic region (90). The *lpf2* locus contains 5 genes (*lpfABCDD′*), but lacks a *lpfE* homolog and, instead, the *lpfD* gene is duplicated in O157 strains. The *in vitro* adherence phenotype conferred by this locus is not well understood. When a nonfimbriated *E. coli* K-12 strain was used to express the *lpf2* locus, a less adherent phenotype to Caco-2 intestinal cells was observed (90). However, a previous study utilizing a random transposon mutagenesis showed that an insertion in the *lpfD2* gene caused increased bacterial adherence to HeLa cells (91). Further, disruption of the gene encoding the major fimbrial subunit, the *lpfA2* gene, resulted in a reduction in initial adherence to Caco-2 cells, although adherence to HeLa cells was unaffected (90). Finally, expression of the *lpf2* gene in a nonfimbriated *E. coli* strain resulted in the appearance of thin, fibrilla-like structures on the bacterial surface that were structurally different from those observed for a strain expressing the cloned *lpf1* genes (90).

Homologs of *lpf* genes have also been identified for non-O157 STEC strains, and their role in adherence has also been explored. Doughty et al. found that a Tn*5phoA* mutant of the LEE-negative STEC O113:H21 strain exhibited reduced adherence to Chinese hamster ovary-K1 (CHO-K1) cells, and further analysis mapped the mutation to a gene with homology to the *lpfD2* gene (21). Sequencing analysis demonstrated that the STEC O113:H21 strain possesses an *lpf* operon (also referred as lpf_{O113}) containing 4 genes (*lpfABCD* genes) found at the same chromosomal location as the STEC O157:H7 *lpf2* locus (O-island 154) (21). Inactivation of the $lpfA_{O113}$ gene in STEC O113:H21 resulted in a significant reduction in microcolony formation on CHO-K1 cells, and when the lpf_{O113} genes were introduced into the nonfimbriated *E. coli* K-12 strain ORN103, the bacteria adhered in a localized pattern, as opposed to a diffuse adherence, indicating that the Lpf2 fimbria homologues may promote interbacterial interactions (21).

Regulation of the *lpf* loci. In comparison to the extensively studied regulatory mechanisms controlling the expression of STEC LEE-encoded gene products (for a review, see reference 79), very little is known about the mechanisms regulating other STEC O157:H7 colonization factors. In the case of the *lpf1* operon, its expression has been determined to be regulated in response to growth phase, temperature, and pH (94). Further characterization of the *lpf1* promoter region has clarified the connection between regulatory proteins and expression of the *lpf1* loci in response to environmental cues (93). It has been demonstrated that the silencing/antisilencing mechanisms mediated by the H-NS and Ler proteins that control several virulence-associated genes in STEC O157:H7 (reviewed in reference 80), including the LEE gene products, also influence expression of the *lpf1* loci (93, 96). The H-NS protein functions as a transcriptional silencer, binding to the regulatory sequence upstream of *lpfA1* and silencing its transcription, while the LEE-encoded Ler protein functions as an antisilencer of Lpf expression, by interacting directly with the *lpf* promoter region and outcompeting the repression exerted by H-NS (93, 96). Recently, it was investigated whether the regulation of the STEC O157:H7 *lpf1* operon requires binding of Ler and H-NS to specific sequences in the *lpf1* promoter or within the structural *lpfA1* gene (70). A mapping of the minimal regulatory region of the *lpf1* gene required for H-NS- and Ler-mediated regulation identified three

sites for H-NS binding. Two of them, named silencer regulatory sequence 1 (SRS1) and SRS2, were located on a region that covers the *lpf1* promoter, and the third one was located within the *lpfA1* gene. Ler interacts with Ler binding site 1 (LBS1) and LBS2, which were located upstream of the two promoters (70). The current model of regulatory control supports the hypothesis that H-NS silences *lpf1* expression by binding to both SRSs on the promoter region, forming an SRS-H-NS complex preventing RNA polymerase-mediated transcription. Under optimal conditions for expression of Ler, this regulator outcompetes the H-NS repression over the LEE-encoded gene products as well as the *lpf1* operon (70).

In the case of the *lpf2* operon, very little is known about the regulatory control mediating its expression; however, transcriptional fusion analysis indicates that the *lpf2* operon is induced *in vitro* at the late exponential growth phase and at 37°C. Further, expression of the *lpf2* operon was stimulated in minimal media depleted of iron, which may mean expression of this operon could be controlled by the ferric uptake regulator (Fur) protein in *E. coli* O157:H7 (94). Interestingly, an avian pathogenic *Escherichia coli* O78:K80 strain, chi7122, was reported to possess a fimbrial operon homologous to the *lpf2* operon, termed the *stg* operon, that contributes to adherence to avian lung sections and human epithelial cells *in vitro* (52). The Stg fimbrial locus, similar to the *lpf2* operon, is also located in the *glmS-pstS* intergenic region, and its expression has been shown to be influenced by carbohydrate source and iron depletion (52). Although further studies are required, it is plausible to suggest that the Fur protein plays a role in regulation of the *lpf2* fimbriae.

Colonization attributes of the long polar fimbriae. Diverse studies using a variety of animal models have supported the role of Lpf in intestinal colonization and persistence. For example, a significantly lower recovery rate for bacteria in the feces of experimentally inoculated sheep and conventional pigs was reported after an initial study with STEC O157:H7 mutants lacking both *lpfA1* and *lpfA2* genes (42). The STEC O157:H7 *lpfA1*/*lpfA2* double-mutant strain also induced fewer A/E lesions in neonatal gnotobiotic piglets than did the wild-type strain (42). In contrast, the single *lpfA1* mutant performed as well as the parent strain in colonizing the intestines of sheep and forming A/E lesions in gnotobiotic piglets (42).

Subsequent studies explored the contribution of Lpf to the persistence in the intestines of infected 6-week-old cross-bred lambs (94). A collection of *lpf* mutants constructed for the *stx*-negative STEC O157:H7 strain NCTC12900 was used to determine the influence of Lpf on colonization and persistence in experimentally infected lambs. Although the reduction in the number of bacteria recovered from the feces of the individual *lpf* mutants was significant, particularly in the case of the *lpfA1* mutant, the effect of the *lpfA1 lpfA2* double mutation had more pronounced effects (94). Further, the results of studies in which *in vitro* organ cultures were infected with the parent and *lpf* mutants of lamb revealed that the *lpf* double mutant adhered as well as the wild-type preferentially to the midrectum but also to the duodenum, jejunum, ileum, and colon. These findings indicated that the reduction in persistence was not due to the *lpf* double mutant's inability to colonize the lamb gastrointestinal tract (94).

Because homologues of the *lpf* operon have been identified for other A/E-producing pathogenic *E. coli*, their role in pathogenesis has also been investigated. For example, the rabbit enteropathogenic *E. coli* (EPEC) O15:H$^-$ strain 83/39 possesses two *lpf* loci. The first one encodes the *lpfABCDE* gene products (referred to as the lpf_{R141} locus) and is highly homologous to the STEC O157:H7 *lpf1* genes (60). Disruption of the $lpfB_{R141}$ (*lpfB1*) gene caused a modest reduction in adherence to CHO-K1 or HeLa cells but significantly reduced the severity of diarrhea in rabbits and impaired intestinal colonization in the early stages of infection without affecting the induction or distribution of A/E lesions (60). The second rabbit EPEC *lpf* locus (referred to as lpf_{R154}) is highly homologous to the STEC O157:H7 *lpf2* genes and is identical to the STEC O113:H21 lpf_{O113} loci. The mutation of the lpf_{R154} operon caused a modest reduction in adherence *in vitro* but did not show attenuation in rabbits (60). Recently, the role of the STEC O157:H7 *lpf* loci has also been further tested using an infant rabbit model, which mimics the diarrhea and gut pathology, including A/E lesions, seen in patients with STEC infection (69). Findings from competition experiments between the wild-type STEC O157:H7 and its isogenic *lpfA1/lpfA2* double mutant were that the mutant was outcompeted in the ileum, cecum, midcolon, and stool, thereby further supporting the role of Lpf in colonization (48a).

Finally, the role of Lpf in colonization and human intestinal tissue tropism was evaluated with the *in vitro* organ culture model (28). Based on previous observations (7) indicating that the *S.* Typhimurium *lpf* operon mediated adhesion to murine Peyer's patches, the authors of the study hypothesized that mutations in the STEC O157:H7 *lpf* operons may cause a reduction in the Peyer's patch follicle-associated epithelium (FAE) adhesion. However, rather than reducing the tropism of STEC O157:H7 for FAE overlying Peyer's patches of the terminal ileum, the *lpf* mutations extended adhesion to include both FAE and the small intestine (28). Therefore, the study demonstrated that the *lpf* fimbrial loci were the first genes to be identified outside the LEE pathogenicity island that influence STEC O157:H7 human intestinal tissue tropism.

Classification and distribution of *lpf* variants in different STEC strains. The *lpfA1* and *lpfA2* genes are highly prevalent among LEE-positive STEC O157:H7 strains associated with severe and/or epidemic disease (81, 86, 90). Further, cumulative evidence indicates that homologues to the *lpf* genes are found in other pathogenic *E. coli* and *Salmonella* strains (17, 21, 63, 81, 85, 90). However, the nomenclature to describe the *lpf* operons has become quite complex because several research groups have used different acronyms to define the same or highly homologous *lpf* loci. For example, the *lpf1* locus has been also described as $lpf_{O157/OI-141}$ for other STEC strains (85), $lpfA_{O26}$ for STEC O26:H11 (5), and lpf_{R141} for rabbit EPEC (60). Similarly, the *lpf2* locus has been described as $lpfA_{O157/OI-154}$ for other STEC strains (85), lpf_{O113} for STEC O113:H21 (21), lpf_{R154} for rabbit EPEC (60), and *stg* for avian pathogenic *E. coli* (52). In an effort to standardize the nomenclature, a recent study analyzed the DNA sequence of different *lpfA1* and *lpfA2* genes from a large number of A/E-producing *E. coli* strains and identified several polymorphisms, which allowed classification of the major *lpf* fimbrial subunits by distinct variants (87). The *lpfA1* genes can be grouped as five different types (the alleles were named *lpfA1-1*, *lpfA1-2*, *lpfA1-3*, *lpfA1-4*, and *lpfA1-5*), and the *lpfA2* genes as three distinct types (namely, *lpfA2-1*, *lpfA2-2*, and *lpfA2-3*). The different types of *lpfA1* and *lpfA2* genes were associated with specific intimin gene variants and some general trends were established. (i) The *lpfA1-1* variant was commonly present in those *E. coli* strains possessing the intimin gene types $\alpha1$, δ/κ, $\eta1$, $\eta2$, λ, μ, and π. (ii) In the case

of *lpfA1-2*, this variant is associated with *E. coli* strains carrying intimin types β1, γ2/θ, ε1, and ε2. (iii) The *lpfA1-3* gene was found only in *E. coli* strains belonging to the serotypes O157:H7 and O55:H7, both possessing the intimin γ1 variant. (iv) The *lpfA2-1* gene was associated with *E. coli* strains carrying intimin types β1, γ2/θ, ε2, ζ, and ι1. (v) Finally, the *lpfA2-2* variant is associated with the *E. coli* strains carrying intimin types γ1 (EPEC O55:H7 and STEC O157:H7) (87). In addition to defining the specific *lpf* variants in a large collection of EPEC and STEC strains isolated around the world, this study established that the combination of the *lpfA1-3* and *lpfA2-2* types is found only in serotypes O55:H7 and O157:H7, and because O55:H7 clinical isolates are rarely found, the detection of *lpfA* variants could be used to specifically identify STEC O157:H7 strains (87). A subsequent study determined the distribution of the *lpfA* gene types in a collection of LEE-negative (intimin-negative) STEC strains from a wide variety of serotypes (other than O157:H7) and found no apparent association between the presence of specific *lpfA1* or *lpfA2* gene variants and the severity of human disease (32). However, the *lpfA2-1* gene was the most prevalent variant identified, present in 95.8% of the isolates, and the *lpfA1-3* and *lpfA2-2* variants, specific biomarkers of STEC O157:H7, were not found in any of the serotypes studied (32).

The cumulative evidence indicates that the detection of *lpfA* gene variants in combination with intimin types could be included in a specific diagnostic test to identify STEC strains and differentiate them from closely related EPEC or other pathogenic *E. coli* strains. A recent study incorporated the detection of *lpf* variants with well-defined virulence marker genes of STEC and EPEC strains in a multiplex PCR approach and resulted in an assay successful in predicting the correct pathotype for 59 of 78 isolates (76%) tested from a diarrheagenic *E. coli* collection (DEC). Moreover, the assay was also specific and highly sensitive for screening *E. coli* pathotypes in clinical samples from HUS and diarrheal patients (D. J. Botkin, L. Galli, V. Sankarapani, M. Soler, M. Rivas, and A. G. Torres, unpublished data).

HEMORRHAGIC COLI PILUS

Type 4 pili (T4P) are long bundles of flexible, filamentous polymers described for several Gram-negative pathogenic bacteria, such as EPEC, enterotoxigenic *E. coli* (ETEC), STEC, *Moraxella catarrhalis*, some species of *Neisseria*, *Pseudomonas aeruginosa*, and *Vibrio cholerae*. Moreover, T4P have been associated with numerous pathogenic processes, such as host cell adherence, biofilm formation, bacterial aggregation, phage receptors, immune evasion, twitching motility, DNA uptake, and cell signaling (for a review, see reference 18). Under normal laboratory growth conditions, such as those using DMEM or LB media, T4P cannot be visualized on the STEC bacterial surface, and no expression of T4P-encoding genes is observed. However, when STEC O157:H7 is grown at 37°C on minimal casein (Minca) medium, bundles of fibers greater than 10 μm in length are observed by electron microscopy (102, 103). Since these structures are morphologically reminiscent of the ETEC Longus T4P, these T4P were named hemorrhagic coli pili (HCP). Further characterization showed that HCP are composed of a 19-kDa pilin subunit, called HcpA, encoded by the *hcpA* chromosomal gene (called the prepilin, peptidase-dependent or *ppdD* gene on *E. coli* K-12 genome). Interestingly, the purified HcpA protein is recognized by the sera of HUS patients but not by those of healthy individuals, possibly meaning that HCP are produced *in vivo* (102). Inactivation of the *hcpA* gene in STEC O157:H7 reduced adherence to human and bovine epithelial cells, supporting the function of HCP as an adhesion factor in STEC. Other features of HCP are its ability to hemagglutinate rabbit red blood cells, bind to the ECM proteins fibronectin and laminin, contribute to STEC biofilm formation, and mediate twitching motility (103). More recently, HCP have been shown to be associated with the release of proinflammatory cytokines, particularly interleukin-8 (IL-8) and tumor necrosis factor alpha (TNF-α), from intestinal epithelial cells infected with STEC by modulating the NF-κB and AP-1 pathways (47). Overall, HCP appears to be an important virulence factor in STEC O157:H7 that might participate not only in the adherence of the bacteria to the host cell but also in the inflammation caused by STEC. The elucidation of the *in vivo* conditions that allow the expression of this pilus will be helpful in correlating them with findings observed in *in vitro* studies.

E. COLI COMMON PILUS

The ability of bacterial pathogens to bind to the host mucosa is a critical step in the pathogenesis of many bacterial infections, but this characteristic is also present in commensal bacteria, since they have to adhere and colonize specific niches. Both commensal and pathogenic bacteria have several putative adhesins that might participate in the adherence process. The best-studied example is the type 1 fimbriae that mediate binding to the intestinal cell surface (25). Scanning electron microscopy visualization of STEC strains at the surface of epithelial cells has revealed the presence of peritrichous thin (4-nm-wide) flexible fibers that promote bacterium-to-bacterium interaction (68). Further characterization showed that the major component of these fibers was a 21-kDa protein encoded by the *yagZ* gene, widely present among pathogenic and commensal *E. coli*, a situation leading to the designation of these pili as *E. coli* common pili or ECP (4, 11, 68). It is worth noticing that this operon was originally described as meningitis-associated and temperature-regulated (Mat) fimbriae of extraintestinal *E. coli* isolates (66). Expression analyses of ECP have indicated that these pili are regulated by temperature, oxygen tension, and growth media; host cell products and contact with the host cell are not required to induce ECP expression (68). Even though the adherence of the *ecpA* mutant of STEC and commensal *E. coli* to epithelial cells is reduced compared to that in the parental strain (68), no contribution to adherence to human or bovine intestinal cells has been reported for ECP (23, 84). In addition, in investigations of the role of ECP in the adherence of enteroaggregative *E. coli* (EAEC), another enteric pathovar, to epithelial cells, the pilus was shown to play a role in adherence only in strains lacking the aggregative adherence fimbriae, the major adherence factor described for EAEC (4, 35). Further, it has been proposed that the simultaneous production of ECP and the type IV bundle-forming pilus (BFP), one of the major adhesion factors of EPEC, plays a synergistic role between these two adhesion factors during microcolony formation on the surface of epithelial cells (68). Overall, it is clear that ECP is expressed by pathogenic and commensal *E. coli* to potentially interact with the intestinal epithelium. However, more experimental data are warranted to examine the thus far inconclusive findings with respect to the role of ECP as an accessory adherence factor for STEC interaction with epithelial cells.

F9 FIMBRIAE

Ruminants, particularly cattle, are the primary reservoir for STEC. In this host, STEC strains are able to adhere to and colonize the intestinal tract without inducing disease. A putative fimbrial operon was identified in mutagenesis studies designed to find the adhesion factors involved in STEC colonization in cattle (23, 99). This operon was found to be chromosomally encoded in the O-island 61 (OI61) of STEC O157:H7 strain EDL933 (49). Further characterization revealed that this operon is most closely related at the nucleotide level to F1 fimbriae from *E. coli* and other *Enterobacteriaceae*. Furthermore, its expression in the *E. coli* K-12 strain resulted in the production of visible fimbriae, of about 1 to 2 μm in length, extending from the bacteria and able to form longer bundles different from flagella (49). The fimbria, designated F9, was found to be involved in the adherence to bovine epithelial cells and to the bovine ECM protein fibronectin, but not to bovine gastrointestinal tissue explants. In addition, inactivation of the main subunit of F9 fimbriae in the STEC O157:H7 strain resulted in findings that F9 fimbriae apparently did not have a role in colonization in weaned cattle, especially at the terminal rectum (49), the predominant colonization site of STEC O157:H7 in cattle (50). Interestingly, uropathogenic *E. coli* (UPEC) also carries the F9 fimbriae, and even though the F9 fimbrial operon is widely distributed in UPEC isolates, no association of the fimbriae with cellular adherence has been found (98). Therefore, F9 fimbriae appear to be an interesting adherence factor in pathogenic *E. coli*, but the precise contribution to colonization of the intestinal epithelia remains to be determined.

E. COLI YCBQ LAMININ-BINDING FIMBRIAE

Genome sequence analysis of the prototype STEC O157:H7 strains (36, 65) also revealed the presence of an putative operon named *ycbQRST*, which shares homology with the family of F17 fimbrial genes found in human uropathogenic and DEC strains (54). In STEC O157:H7, peritrichous, flexible fine fibers, detected by transmission electron microscopy, were observed when the bacteria were grown exclusively in Minca minimal medium (71). Further purification of these flexible fibers showed that they were composed of an 18-kDa protein matching the predicted protein encoded by the *ycbQ* gene. The recombinant protein has the ability to bind to the ECM protein laminin but not to fibronectin or collagen IV, and the authors renamed the fimbriae *E. coli* YcbQ laminin-binding fimbriae or ELF. Further characterization of ELF in STEC adherence showed that an *elfA* mutant significantly reduced adherence to epithelial intestinal cells as well as to cow and pig intestinal explants, compared to that of the wild-type and complemented STEC strains. These *in vitro* observations suggested that ELF might have a role in STEC adherence to cells; however, the environmental conditions triggering the production of this fimbria and its specific role in the colonization of the human or animal intestinal tract needs further investigation.

OTHER STEC FIMBRIAE: SFP FIMBRIAE

Sorbitol-fermenting (SF) STEC O157:H$^-$ strains have emerged as important causes of diarrhea and HUS in Germany and in other areas of Europe (2, 3, 62). These SF STEC O157:H$^-$ strains possess a virulence plasmid (pSFO157) that differs significantly from the pO157 plasmid commonly found in STEC O157:H7 strains. Analysis of the pSFO157 plasmid led to the identification of a cluster of six genes, designated *sfpAHCDJG*, with homology to the *pap* genes encoding P fimbriae in uropathogenic *E. coli* (13). Expression of the *sfp* genes in *E. coli* K-12 was associated with the appearance of fimbriae and resulted in a mannose-resistant hemagglutination phenotype (13). The plasmids of STEC are highly variable genetic elements (14), and surveys of STEC strains of various serogroups and other DECs revealed the *sfpA* and *sfpDG* regions to be restricted to SF STEC O157:H$^-$ (13, 30). The contribution of Sfp fimbriae to the adherence of SF STEC O157:NM strains to human intestinal epithelial cells has been investigated, and the data showed that the expression of Sfp fimbriae is induced under anaerobic conditions (resembling those of the natural site of infection) and that induction of Sfp fimbriae correlates with increased adherence to Caco-2 and HCT-8 cells (58).

STEC AUTOTRANSPORTERS

EhaA, EhaB, and EhaJ autotransporters. Autotransporter proteins belong to the type V secretion system described for bacteria (38). In Gram-negative pathogens, several autotransporter proteins of diverse functions have been identified. Proteins secreted via this pathway contain in their primary sequence four functional domains that direct their transport across the bacterial membrane, finally routing them to the cell surface; the domains consist of the signal sequence, the passenger (α) domain, the linker region, and the translocation β domain (for a review, see reference 38). By using conserved autotransporter motifs as a search tool, researchers have identified several autotransporters in the STEC O157:H7 EDL933 genome called Enterohemorrhagic *E. coli* autotransporters or Eha (101). The autotransporters EhaA, EhaB, and EhAJ, belonging to the AIDA-I (adhesin involved in diffuse adherence) autotransporter family, have been implicated in attachment to biological and abiotic surfaces (24, 100, 101). PCR screening of STEC strains showed that EhaA and EhaB autotransporters are highly prevalent (97% and 93%, respectively), in contrast to EhaJ, which was found in only 27% of STEC strains. Individual expression of EhaA, EhaB, and EhaJ on an *E. coli* K-12 background resulted in their location at the cell surface and mediating biofilm formation on abiotic surfaces; EhaB and EhaJ appear to promote adhesion to ECM proteins, while EhaA promoted adhesion to primary epithelial cells of the bovine terminal rectum (24, 100). However, the inactivation of *ehaA*, *ehaB*, and *ehaJ* genes individually in wild-type strains did not affect biofilm formation. Future studies to elucidate the function of these autotransporter proteins in colonization and virulence are required to provide some clues about their role in STEC adherence to the intestinal tract.

EspP rope-like fibers. A family of autotransporters known as serine protease autotransporters of *Enterobacteriaceae* (SPATES) has been described for STEC strains; these include EspP of STEC O157:H7 (22) and PssA in STEC O26:H$^-$ (99). These autotransporters display an array of distinct biological properties, including adhesins, hemagglutinins, cytotoxins, or enzymatic activity with different substrate specificities (38). In the case of EspP, it was demonstrated that this pO157 plasmid-encoded SPATE contributes to the adherence of STEC to bovine primary rectal cells and colonization of the bovine intestines (22). The authors showed that adherence of STEC O157:H7 to such cells was significantly impaired by the *espP* mutation but restored upon the addition of highly purified exogenous EspP cytotoxin (22). Further, a genome-wide transposon mutagenesis revealed that the EspP autotransporter is directly involved in biofilm formation and was also important for adherence to T84 intestinal epithelial cells,

which may indicate that this SPATE protein plays a role in tissue interactions *in vivo* (67). Recently, an unusual phenotype was described for the EspP autotransporter; this phenotype was also displayed by an EPEC strain expressing the EspC SPATE (104). The latter study showed that the EspP proteins oligomerize to form megastructures, called ropes, which possess adhesive and cytopathic activities on host epithelial cells. The isogenic STEC *espP* mutant does not produce ropes, and recombinant EspP expressed in *E. coli* K-12 strain led to rope formation (104). The *E. coli* ropes showed binding to Congo red dye and thioflavin T, which is a common characteristic of highly aggregative and insoluble human amyloid fibers and curli fibers. Further, it was also found that EspP ropes served as a substratum for bacterial adherence and biofilm formation, and additionally, it protected bacteria from antimicrobial compounds (104). This interesting new phenotype associated with the SPATE proteins warrants further experimentation to define whether the ropes play a biologically significant role during human infection or colonization of the animal reservoir.

Sab autotransporter. Analysis of the hypervirulent LEE-negative STEC O113:H21 strain revealed the presence of another autotransporter which was involved in adherence of STEC to abiotic surfaces and epithelial cells (39). PCR analyses using primers to detect the presence of putative adhesin genes in O113:21 strains with lower (strain MW10) and higher (strain 98NK2) adherence to Henle 407 cells revealed the presence of one open reading frame (ORF) present exclusively in 98NK2. Further characterization indicated that this ORF encodes a 146-kDa protein with features characteristic of an autotransporter protein, which was designated the STEC autotransporter mediating biofilm formation or Sab (39). By using a collection of LEE-positive and LEE-negative STEC strains, researchers found the *sab* gene in a subset of LEE-negative strains, while no LEE-positive STEC strains were found to possess this gene. The expression of Sab at the cell surface in the wild-type strain 98NK2 was found to be low, but it was enhanced when this protein was overexpressed by using a multicopy plasmid. Expression of Sab in an *E. coli* K-12 strain possibly indicates its involvement in adherence to HEp-2 epithelial cells but not to human colonic HCT-8 cells. In addition, purified Sab protein was shown to bind epithelial cells and, taken together, the data point to Sab as an adherence factor that might be involved in adherence to enterocytes of those STEC strains that lack the ability to form A/E lesions.

Cah autotransporters. Genome sequence analysis of STEC O157:H7 prototype strain EDL933 led to a finding of two identical ORFs, with predicted protein products similar to the AIDA-I autotransporter proteins (95). Further analysis of the predicted protein sequences revealed the presence of consensus domains found in several calcium-binding proteins and sharing 68.5% identity with antigen 43, an autotransporter participating in bacterial aggregation and biofilm formation (37). This observation has led to naming the proteins calcium-binding antigen 43 homologous or Cah. The recent evaluation of *cah* genes in a STEC collection found that the *cah* gene was present in 17 of 18 STEC O157:H7 strains (94%) and 6 of 33 STEC non-O157 strains (18%) (10). Expression of Cah on *E. coli* DH5α produced an autoaggregation phenotype, absent when the *cah* gene is inactivated, indicating that Cah promotes bacterium-to-bacterium interaction (95). *E. coli* DH5α strain expressing the Cah protein displayed a reduced adherence to tissue-cultured cells compared to that for *E. coli* DH5α transformed with the vector alone or the mutated *cah* gene. Inactivation of the two *cah*-coding sequences in the prototype STEC strain 86-24 resulted in a significant reduction in the ability of the bacteria to form biofilms (95). Interestingly, no changes in the ability of prototype EHEC strain 86-24, compared to that of the double mutant strain, to adhere to Caco-2 cells, alfalfa sprouts, and seed coats were observed (89). These studies indicated that Cah plays a role in bacterial autoaggregation, but not in adherence of STEC to cells.

OTHER STEC ADHESINS

Flagella. The role of flagella and/or flagellin in the adherence and intestinal colonization of STEC strains has remained controversial. Initial observations indicated that purified H7 flagella produced by many STEC O157 strains associated with human disease do not adhere to HeLa cells, and H7-specific antibodies do not significantly inhibit the adherence of STEC O157:H7 *in vitro* (34, 75). A subsequent study further investigated the adhesive properties of H7 flagella and the abilities of STEC O157:H7 flagella to bind bovine mucus, mucin proteins, and ECM proteins (26). The authors found that H7 flagella and the flagellin monomers can bind mucins and also freshly isolated bovine mucus. Deletion of the *fliC* gene STEC O157:H7 caused the bacteria to become significantly less adherent to bovine intestinal tissue than the parental wild-type strain, which may indicate that H7 flagella possess adhesive properties (26). Further studies, using bovine terminal rectal primary epithelial cells in examining the role of H7 flagella in epithelial adherence, have confirmed that H7 flagellum acts as an adhesin to bovine intestinal epithelium and supports its involvement in the initiating step for colonization of the cattle reservoir (53).

In other *in vivo* animal models, flagella have been shown to be required for persistence of a *stx*-negative STEC O157:H7 strain in the intestines of specific-pathogen-free chicks (8). However, it has been proposed that flagella may not be essential for STEC pathogenesis in humans, because, for example, nonmotile SF STEC O157:H$^-$ strains are associated with up to 40% of HUS cases in Germany and are an emerging problem in Europe overall (44). But in the case of STEC O157:H7 flagella, it has been shown that inoculation of H7 flagellin into the lumen of human colon xenografts containing an intact human epithelium resulted in upregulation of epithelial cell proinflammatory chemokines, accompanied by a subepithelial influx of neutrophils (57). Further, isogenic STEC flagellar mutants did not significantly upregulate neutrophil and dendritic cell chemoattractants, irrespective of Stx production. Whether or not H7 flagellum acts as an adhesin during human intestinal colonization remains to be elucidated; however, the data support the role of flagellin, and perhaps the recently identified contribution of Stx (40), as the major STEC O157:H7 factor(s) that directly upregulates proinflammatory chemokine production in the human colon epithelium *in vivo* (57).

Immunoglobulin-binding protein G. Several distinct patters of adhesion have been reported for DECs as well as STEC strains. One adherence pattern, designated chain-like adhesion (CLA), was reported for *E. coli* cells that attach to HEp-2 cells and form chain-like aggregates and that were isolated from humans with or without diarrhea (33). Interestingly, certain strains of LEE-negative STEC strains, isolated from humans with or without bloody diarrhea, adhered to HEp-2 cells in a CLA pattern (51). Using a Tn*5* transposon mutagenesis, it was shown that the gene essential for the CLA phenotype in LEE-negative STEC O91

strain encodes a protein (named EibG) similar to the *E. coli* immunoglobulin-binding (Eib) proteins EibA, -C, -D, -E, and -F (51). The immunoglobulin-binding proteins were first identified in the *E. coli* standard reference collection as a group of proteins with immunoglobulin-binding activity (72, 73). In the case of the STEC O91 EibG, it was found that this protein has dual roles: it binds human IgG and IgA and also participates in bacterial adherence to host epithelial cells (51). A recent study further evaluated whether Eib proteins bind to the Fc portion of IgG in a nonimmune manner and concluded that these proteins do indeed bind human IgG Fc and that IgG Fc receptors are present in *E. coli* strains (48). Finally, the prevalence of the *eibG* gene and its allelic variations, as well as their correlation with the CLA phenotype, was evaluated with a large collection of STEC strains (56). The *eibG* gene was identified in 36 STEC strains belonging to 14 serotypes, and these strains were found to possess a total of 21 different alleles clustered into three *eibG* subtypes (*eibG*-α, *eibG*-β, and *eibG*-γ) (56). Further, it was demonstrated that strains expressing EibG-α and EibG-β displayed a typical CLA phenotype, with formation of long chains on both human and bovine intestinal epithelial cells, whereas strains with the EibG-γ subtype adhered in short chains, forming a pattern which was designated atypical CLA (56).

Efa-1/ToxB/LifA. Another strategy that has been used to identify new adherence factors in STEC is the analysis of those highly adherent strains. Transposon-based mutagenesis analysis of a clinical isolate of STEC strain serotype O111:H$^-$, which displayed high levels of adherence to cultured Chinese hamster ovary (CHO) cells, led to identification of a 9,669-bp ORF that encodes a predicted 365-kDa protein (61). A reduced binding to CHO cells was observed, compared to that for the wild-type strain, after inactivation of this ORF, but no changes in the A/E lesion phenotype were observed. This ORF was designated enterohemorrhagic *E. coli* factor for adherence 1 or *efa-1* and found to be present in EPEC and in non-O157 STEC strains (61). In EPEC, Efa-1 was reported to be 97.4% identical to the LifA protein (also called lymphostatin), which inhibits the proliferation of mitogen-activated lymphocytes and the synthesis of proinflammatory cytokines, such as IL-2, IL-4, IL-5, and gamma interferon (IFN-γ) (1, 46). In STEC O157:H7, a truncated version of the *efa-1* gene is present in the chromosome, and some researchers have suggested that the truncated Efa-1 protein might share some properties with the full-length Efa-1 (78). A homologue of the *efa-1/lifA* gene is also present on the pO157 large plasmid, and the gene has been designated *toxB*. The ToxB protein exhibits 28% amino acid identity to the Efa-1/LifA protein and contributes to the adherence to cultured epithelial intestinal cells, which has been linked to the ToxB-induced production and/or secretion of type III secreted proteins (83). However, in animal models, ToxB is not required for intestinal colonization of calves or sheep (78), and no lymphostatin-like activity has been associated with this protein in STEC O157:H7 strains (1). In general, several properties have been associated with the Efa-1/LifA/ToxB proteins, and recent studies have cited the presence of a motif or domain that might be associated with cell adherence, lymphostatin activity, or induction of secretion of type III effectors in the STEC strain (19). Therefore, the elucidation of functional domains in the Efa-1/LifA/ToxB protein will be helpful in understanding the multiple functions of this versatile protein in STEC pathogenesis.

CONCLUSION

This paper reviews information accumulated for a diverse set of fimbrial and afimbrial adhesins that participate in the different stages of colonization and/or persistence of STEC O157 and non-O157 strains.

Cumulative evidence indicates that in the human intestine, STEC O157:H7 strains interact with the epithelia, and this initial interaction is mediated by adhesins other than intimin. Because Lpf is regulated and expressed under conditions inducing expression of the LEE gene products, it is feasible to propose that this fimbria interacts with the intestinal mucosa, perhaps via ECM proteins or other unidentified receptors (Fig. 1A). Further, the role of Lpf in colonization has also been demonstrated for non-O157 strains. Upon this initial interaction, STEC intimin binds to its bacterial and eukaryotic receptors and the A/E lesion is established. Although the role of flagella in human intestinal adhesion has not been clarified, it is evident that these surface structures participate in the host inflammatory response. The production of Stx, combined with rearrangements of the host cytoskeleton and induction of an inflammatory stage, favors translocation of the Stx toxin to the bloodstream, resulting in modulation of the severity of the intestinal damage, but also contributing to the development of HUS.

In the case of the bovine intestine or other sites where the bacteria are known to persist (vegetables such as lettuce and spinach), the data indicate that STEC O157 and non-O157 strains expressed a wide variety of fimbrial and afimbrial adhesins, some of which (ECP, HCP, Eha autotransporters, etc.) might play a key role in persistence in the ruminant reservoir or in the formation of biofilms in other cell surfaces (Fig. 1B). For example, it has been shown that HCP interacts with ECM proteins and flagella bind to mucins in the bovine intestine and that HCP and ECP also participate in the formation of stable biofilms.

To this end and due to the variety of adhesins present in STEC strains, we still need to increase our understanding of (i) when and how these adhesion factors are expressed under defined environmental conditions, permitting the bacteria to interact with the host cell, (ii) the cellular receptors involved in these interactions, and (iii) the participation of other pathogenic processes induced by the bacteria, such as inflammation, during intestinal colonization. Considering the difficulties of studying the association of STEC adhesins with the human and bovine gut mucosa *in vivo*, the development of alternative methods, such as humanized mice or bovine *in vitro* organ culture models, combined with *in vivo* imaging systems to visualize real time infections, could provide further information about bacterial pathogenesis, host specificity, and the host response.

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