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Escherichia coli O104:H4 Pathogenesis: an Enteroaggregative *E. coli*/Shiga Toxin-Producing *E. coli* Explosive Cocktail of High Virulence

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ABSTRACT A major outbreak caused by *Escherichia coli* of serotype O104:H4 spread throughout Europe in 2011. This large outbreak was caused by an unusual strain that is most similar to enteroaggregative *E. coli* (EAEC) of serotype O104:H4. A significant difference, however, is the presence of a prophage encoding the Shiga toxin, which is characteristic of enterohemorrhagic *E. coli* (EHEC) strains. This combination of genomic features, associating characteristics from both EAEC and EHEC, represents a new pathotype. The 2011 *E. coli* O104:H4 outbreak of hemorrhagic diarrhea in Germany is an example of the explosive cocktail of high virulence and resistance that can emerge in this species. A total of 46 deaths, 782 cases of hemolytic-uremic syndrome, and 3,128 cases of acute gastroenteritis were attributed to this new clone of EAEC/EHEC. In addition, recent identification in France of similar O104:H4 clones exhibiting the same virulence factors suggests that the EHEC O104:H4 pathogen has become endemically established in Europe after the end of the outbreak. EAEC strains of serotype O104:H4 contain a large set of virulence-associated genes regulated by the AggR transcription factor. They include, among other factors, the pAA plasmid genes encoding the aggregative adherence fimbriae, which anchor the bacterium to the intestinal mucosa (stacked-brick adherence pattern on epithelial cells). Furthermore, sequencing studies showed that horizontal genetic exchange allowed for the emergence of the highly virulent Shiga toxin-producing EAEC O104:H4 strain that caused the German outbreak. This article discusses the role these virulence factors could have in EAEC/EHEC O104:H4 pathogenesis.

INTRODUCTION

In May 2011, an outbreak caused by *Escherichia coli* of serotype O104:H4 spread throughout Germany (1). The next month, France also reported a cluster of *E. coli* O104:H4 infections (2). A total of 46 deaths, 782 cases of hemolytic-uremic syndrome (HUS), and 3,128 cases of acute gastroenteritis were officially attributed to this new clone of enterohemorrhagic *E. coli* (EHEC) (last update from European Centre for Disease Prevention and Control, 27 July 2011). Most or all victims (although diagnosed in different countries in Europe) became infected in Germany or France. The phenotypic and genotypic characterization of the *E. coli* O104:H4 indicated that the isolates from the French and German outbreaks were common to both incidents. Fenugreek seeds imported from Egypt, from which sprouts were

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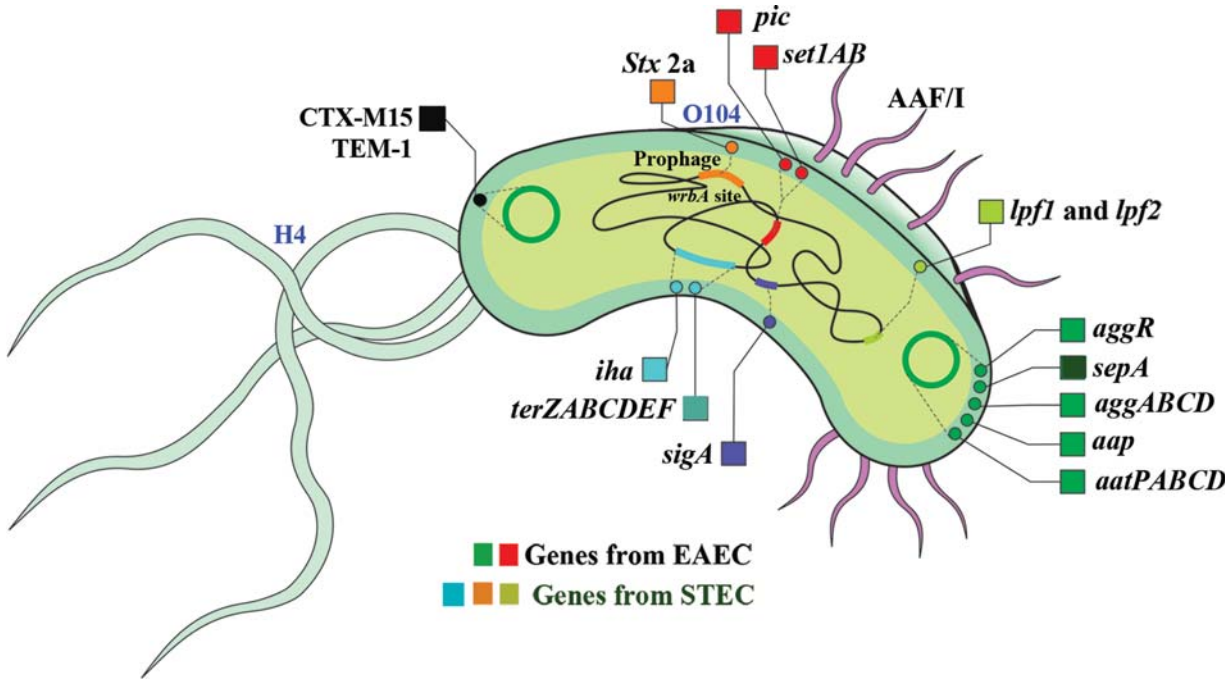
grown, were implicated as a common source. However, there is still much uncertainty about whether this is truly the common cause of the infections, as tests on the seeds did not allow the detection of any *E. coli* isolate of serotype O104:H4.

This large outbreak was caused by an unusual EHEC strain that is most similar to an enteroaggregative *E. coli* (EAEC) of serotype O104:H4. A significant difference, however, is the presence of a prophage encoding the Shiga toxin (Stx), which is characteristic of EHEC strains (3–5). This combination of genomic features, associating characteristics from both EAEC and EHEC, represents a new pathotype (Fig. 1). Because typical EAEC strains are isolated primarily from humans, the origin of this outbreak may not be zoonotic. That was recently confirmed by two surveys in Germany and France. No evidence of the Stx-producing *E. coli* O104:H4 outbreak strain or EAEC was found in cattle feces in northern Germany, the hot spot of the 2011 HUS outbreak area (6). Similarly, French cattle were not a reservoir of the highly virulent enteroaggregative Stx-producing *E. coli* of serotype O104:H4 (7). Recent identification from sporadic cases of HUS in France of EHEC clones similar to the one responsible of the outbreaks (8) suggests that the EHEC O104:H4 pathogen has become endemically established in Europe and very likely in the human population.

One burning question is what makes this outbreak EHEC O104:H4 strain so dangerous? One explanation is that this strain (in short, an EAEC with a phage coding for Stx type 2) is a better colonizer of the gut (Fig. 2). The enhanced adherence of this strain to intestinal epithelial cells might facilitate systemic absorption of Stx and could explain the high frequency of progression to HUS. It is believed that EAEC of serotype O104:H4 is by itself an emerging serovar that has acquired an original set of virulence factors (Fig. 1).

FIGURE 1 Hybrid characteristics of *E. coli* O104:H4 outbreak strain (EAEC/STEC). Schematic representation of the genes harbored by *E. coli* O104:H4; the main genes from EAEC or STEC are highlighted: *stx2* (coding for Stx 2), *pic*, *sigA*, and *sepA* (coding for the SPATE proteins); Pic, protein involved in intestinal colonization; SigA, a homolog of Pet, with cytotoxic activity; SepA, a colonization factor of *Shigella*), *set1AB* (coding for ShET1, a holotoxin AB5), *iha* (coding for Iha, a STEC adhesin that is an IrgA homolog), *aggR*, *aggABCD*, *aap*, *aatPABCD* (genes from EAEC plasmids coding for transcription regulator, AAF/I, dispersin, and dispersin transporter, respectively), *lpf1-2* (coding for Lpf of STEC), *terZABCDEF* (coding for a cluster for Tellurite resistance), CTX-M15 and TEM-1 (antibiotic resistance genes). SigA and SepA are SPATEs detected mainly in *Shigella* sp. doi:10.1128/microbiolspec.EHEC-0008-2013.f1



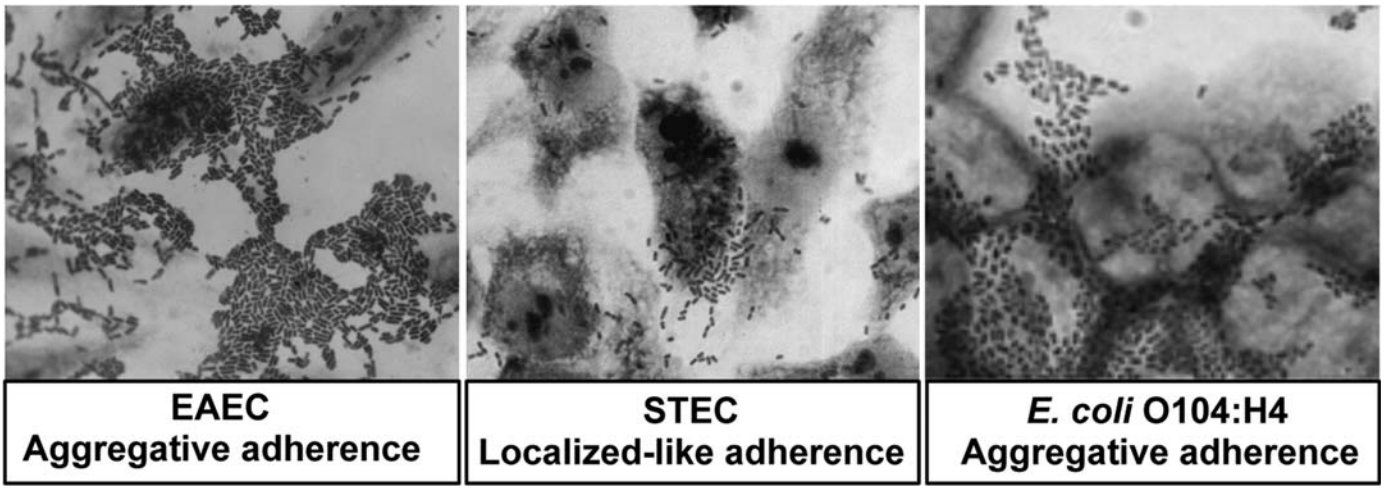


FIGURE 2 Adherence patterns of EAEC, STEC, and *E. coli* O104:H4 outbreak strain to epithelial cells. Subconfluent epithelial cell cultures are infected with the different bacterial strains. Cells are fixed and stained with Giemsa stain. From Scalesky et al. 1999. *Infect Immun* **67:**3410; Paton et al. 2001. *Infect Immun* **69:**6999; and Martina Bielaszewska. http://ecdc.europa.eu/en/press/events/Documents/22-231111-Breakthroughs-in-molecular-epidemiology-Bielaszewska.pdf. doi:10.1128/microbiolspec.EHEC-0008-2013.f2

EAEC strains of serotype O104:H4 contain a large set of virulence-associated genes regulated by the AggR transcription factor (Fig. 1). They include the pAA plasmid genes encoding the aggregative adherence fimbriae (AAF), which anchor the bacterium to the intestinal mucosa (the aggregative, so-called stacked-brick, adherence pattern on intestinal epithelial cells) and induce inflammation, as well as a protein-coat secretion system (Aat) that secretes the protein dispersin (9). A switch of the virulence plasmid (pAA) together with the type of AAF could be an additional explanation for the higher virulence of this outbreak strain (Fig. 2). Indeed, the outbreak EHEC O104:H4 strain is similar to EAEC O104:H4 strain 55989, isolated in the late 1990s from a patient with persistent diarrhea in the Central African Republic, and to EHEC O104:H4 strain HUSEC041 that was associated in 2001 with very few HUS cases in Germany. Interestingly, EHEC O104:H4 strain HUSEC041 carries the plasmid encoding AAF/III (also present in EAEC strain 55989 in a different size). In contrast, outbreak EHEC O104:H4 isolates acquired a new plasmid, encoding AAF/I, and lost the AAF/III-encoding plasmid (3, 4). AAFs are encoded in high-molecular-weight plasmids (designated pAA), and their biogenesis employs the chaperone-usher secretion pathway (10–12). These fimbriae are members of the Dr superfamily of adhesins that includes strains of uropathogenic (UPEC) and diarrheagenic *E. coli* with a genetic organization consisting of chaperone, usher, and pilin subunits (13, 14). AAF/I are flexible, bundle-forming fimbriae (15), and the genes responsible for their biogenesis are located in two regions of the plasmid: region 1, containing the genes that encode the pilin, chaperonin, and usher, and region 2, containing the regulator-encoding gene (designated *aggR*) (10, 15, 16).

EAEC strains of serotype O104:H4 also produce a variable number of serine protease autotransporters of *Enterobacteriaceae* (SPATEs) implicated in mucosal damage and colonization. The type V secretion pathway enables a family of proteins to reach the surface with a very limited number of accessory secretion factors because most information necessary to the translocation process is contained within the secreted protein itself. These proteins, which can carry out their own transport to the outer membrane, are autotransporter proteins. The current isolate diverges from common EAEC isolates in the number and nature of their SPATE proteases (Fig. 1), namely Pic, SigA, and SepA (3–5). Rasko and colleagues speculate that the combined activity of these SPATEs, together with other EAEC virulence factors, accounts for the increased uptake of Stx into the systemic circulation, resulting in the high rates of HUS. The *pic* gene has a unique characteristic among the autotransporter proteins since there are two oppositely oriented genes in tandem within the *pic* (*she*) gene, *set1B* and *set1A* (17), which encode the two subunits of the ShET1 toxin (18); this gene is also present in *Shigella flexneri* and UPEC. Pic is a mucinase (19) that has recently been shown to promote mucous secretion in the gut (20), and is responsible for the mucoid diarrhea that is a classic symptom of *Shigella* sp. and EAEC infection.

Interestingly, EC55989 contains three copies of this gene, two on the chromosome (intact, EC55989_4682 and EC55989_3279) and one on the EAEC plasmid (55989p, truncated), all of which are conserved in the German outbreak strain. In addition, a fourth *pic* gene is present in the EAEC plasmid of the outbreak strain (but missing from EC55989) that seems to be intact. The outbreak strain also encodes SigA, a SPATE that cleaves the cytoskeletal protein spectrin, inducing rounding and exfoliation of enterocytes (21). The third SPATE, SepA, is associated with increased *S. flexneri* virulence (22), but its function is unknown.

As mentioned before, EAEC strains of serotype O104:H4, as a hybrid clone, also produce Stx2 and an adhesion-siderophore called Iha; both proteins are found with high prevalence in Stx-producing *E. coli* (STEC), including EHEC (Fig. 1). The ability of STEC to cause severe disease in humans is mainly associated with the production of Stx; two distinct groups, Stx1 and Stx2, with similar biological activity but different immunogenicity are well known (23). Members of the Stx1 group are antigenically similar, whereas those of the Stx2 group are heterogeneous and comprise several variants or subtypes (24). An interesting finding that highlights the relevance of Stx2 is that Stx2 has been epidemiologically more associated with severe disease in humans than Stx1 (25). Moreover, the *stx2d* activatable subtype has been associated with high virulence and the ability to cause HUS (26). On the other hand, Iha was first described as an adhesin in an EHEC O157:H7 strain and was named IrgA homolog adhesin, based on its homology to the IrgA enterobactin siderophore receptor of *Vibrio cholerae* (27) and its ability to confer epithelial cell adherence capability to a nonadherent K-12 strain when expressed from a multicopy plasmid (28). Recently, Iha was determined to have a dual-function urovirulence factor for *E. coli* clonal group A strains and other pathogenic *E. coli* strains. However, it still needs to be determined if the siderophore receptor activity, the adhesion phenotype, or both are important for the enhanced in vivo persistence of Iha demonstrated within the urinary tract (29).

E. coli O104:H4 OUTBREAK STRAIN AS LOCUS OF ENTEROCYTE EFFACEMENT (LEE)-NEGATIVE STEC

The *E. coli* O104:H4 outbreak strain has been compared to typical EHEC outbreaks; however, EHEC only refers to a clinical condition, and the virulence factors of EHEC and STEC can be different. STEC strains isolated from humans with specific clinical signs are called EHEC (30). In humans, some strains cause severe inflammation of a section of the large intestine accompanied by hemorrhage of the intestinal mucosa and severe diarrhea (hemorrhagic colitis) or HUS, which can lead to kidney failure and even death. Thus, the STEC strains that cause these clinical pictures are designated as EHEC (31). EHEC strains are considered to be a subset of STEC, but our knowledge of STEC comes from outbreak investigations and studies of *E. coli* O157 (EHEC) infection, which was first identified as a pathogen in 1982. The non-O157 STEC serogroups are not nearly as well understood, partly because outbreaks caused by them are rarely identified. As a whole, the non-O157 serogroup is less likely than *E. coli* O157 to cause severe illness; however, some non-O157 STEC serogroups can cause the most severe manifestations of STEC illness (32).

Pathogenic STEC strains require additional virulence factors enabling adherence, for example, factors that permit colonization of intestinal epithelial cells (Fig. 2). EHEC strains contain an arrangement of virulence genes that contribute to their pathogenesis, such as the LEE pathogenicity island (PAI), the virulence plasmid pO157, and *stx*1 and/or *stx*2. LEE carries all genes necessary for the formation of the attaching and effacing (A/E) lesion (33). The A/E histopathology is characterized by effacement of the brush border microvilli, intimate bacterial adherence to the enterocyte apical plasma membrane, and the accumulation of polymerized actin beneath the attached bacteria (34). All A/E pathogens carry the LEE PAI (35) that encodes gene regulators (36, 37), the adhesin called intimin (38), a type III secretion system (T3SS) (35), chaperones (39, 40), and several secreted proteins, including the translocated intimin receptor called Tir (41). Upon contact with epithelial cells, EHEC injects a variety of effectors into the cells to modulate cellular functions involved in the host defense response, the dynamics of the cytoskeleton, and the maintenance of tight junctions. A major target of virulence factors is the cellular signaling cascade involved in the construction and modulation of the cytoskeleton and microfilaments (42). While the bacteria remain mostly extracellular in the lumen of the gut, the T3SS effectors of A/E pathogens access and manipulate the intracellular environment of host cells. The effectors subvert various host cell processes, which enable the bacteria to colonize, multiply, and contribute to the disease. Thus, two key factors encoded by LEE include the adhesin intimin (*eae*), which binds to Tir (*tir*), and both are essential for the intimate attachment of the bacteria with the cytoplasmic membrane of the host cell (33).

LEE appears to confer enhanced virulence, since LEE-positive STEC strains are much more commonly associated with HUS and outbreaks than LEE-negative STEC strains (43). EHEC has controversial definitions, most of them indicating that EHEC is a STEC harboring the *eae* gene, or a STEC implicated in the illness, or a STEC that has the same clinical, epidemiological, and pathogenic characteristics. These definitions are features of the EHEC O157 serogroup, but most of the STEC strains have been designated as non-O157 strains. Persons with non-O157 STEC infection usually have less severe illness, and non-O157 STEC strains include many serogroups (~400) with varying virulence, some typically causing only mild diarrhea and others causing HUS and death; non-O157 isolates predominantly have Stx1. Additionally, there are regional variations in prevalence; however, six serogroups of STEC have been associated with 70% of the food safety illnesses in the United States (O26, O45, O103, O111, O121, and O145) and have been termed the "Big Six." U.S. Department of Agriculture's Food Safety and Inspection Service considers these six most frequent serogroups adulterants in ground beef, materials intended for ground beef production, and other nonintact beef products. The Food Safety and Inspection Service plans to test for these adulterants using a PCR protocol that initially targets the detection of Stx genes, *stx1* and/or *stx2*, and the intimin (*eae*) gene, and then tests for the six O groups. Besides *stx* genes and adherence factors, increasing evidence shows that differences in virulence between pathogenic and nonpathogenic bacterial strains can be attributed in part to virulence genes located in PAIs (43). PAIs usually contain blocks of virulence genes and are greater than 10 kb (44). Several PAIs have been identified and characterized in STEC. For instance, the chromosomal LEE PAI was identified in *E. coli* O157:H7 strain EDL933 (45). However, some LEE-positive STEC serotypes have never been associated with disease, and some LEE-negative STEC serotypes can cause HUS and outbreaks, indicating that virulence factors other than those in LEE may contribute to pathogenesis of STEC (43). Thus, in addition to the distribution of different PAIs (O island [OI]-122, OI-43/48, OI-57, high-pathogenicity island) and their virulence genes in STEC, the association of the PAIs and individual virulence genes with STEC seropathotypes linked to severe diseases and outbreaks was evaluated. Most OI-122, OI-43/48, and OI-57 virulence genes (*pagC*, *sen*, *nleB*, *efa-1*, *efa-2*, *terC*, *ureC*, *iha*, *aidA-1*, *nleG2-3*, *nleG6-2*, and *nleG5-2*) were highly prevalent in *eae*-positive STEC, but they were largely absent in *eae*-negative STEC, with the exception of *pagC* and *iha* (46). Phylogenetic analysis revealed that *iha* genes from *eae*-positive STEC had high similarity (99.6%), whereas they had lower sequence similarity (91.1 to 93.6%) with *iha* genes from *eae*-negative STEC, indicating that *iha* from *eae*-positive and *eae*-negative STEC may have evolved independently or have different origins (46). Indeed, it has been reported (47) that *iha* was carried by a 33,014-bp PAI in STEC serotype O91:H– strains (*eae*-negative). In addition, *iha* was found in pO113 plasmid of STEC serotype O113:H21 (*eae*-negative) (48). Furthermore, some of these factors can participate in bacterial regulation; it has been reported that lysogeny with Stx2-encoding bacteriophages represses T3SS in EHEC. Deletion of Stx2 phages from EHEC strains increased the level of T3SS whereas lysogeny decreased T3SS. A model is proposed in which Stx2-encoding bacteriophages regulate T3SS to coordinate epithelial cell colonization that is promoted by Stx and secreted effector proteins (49).

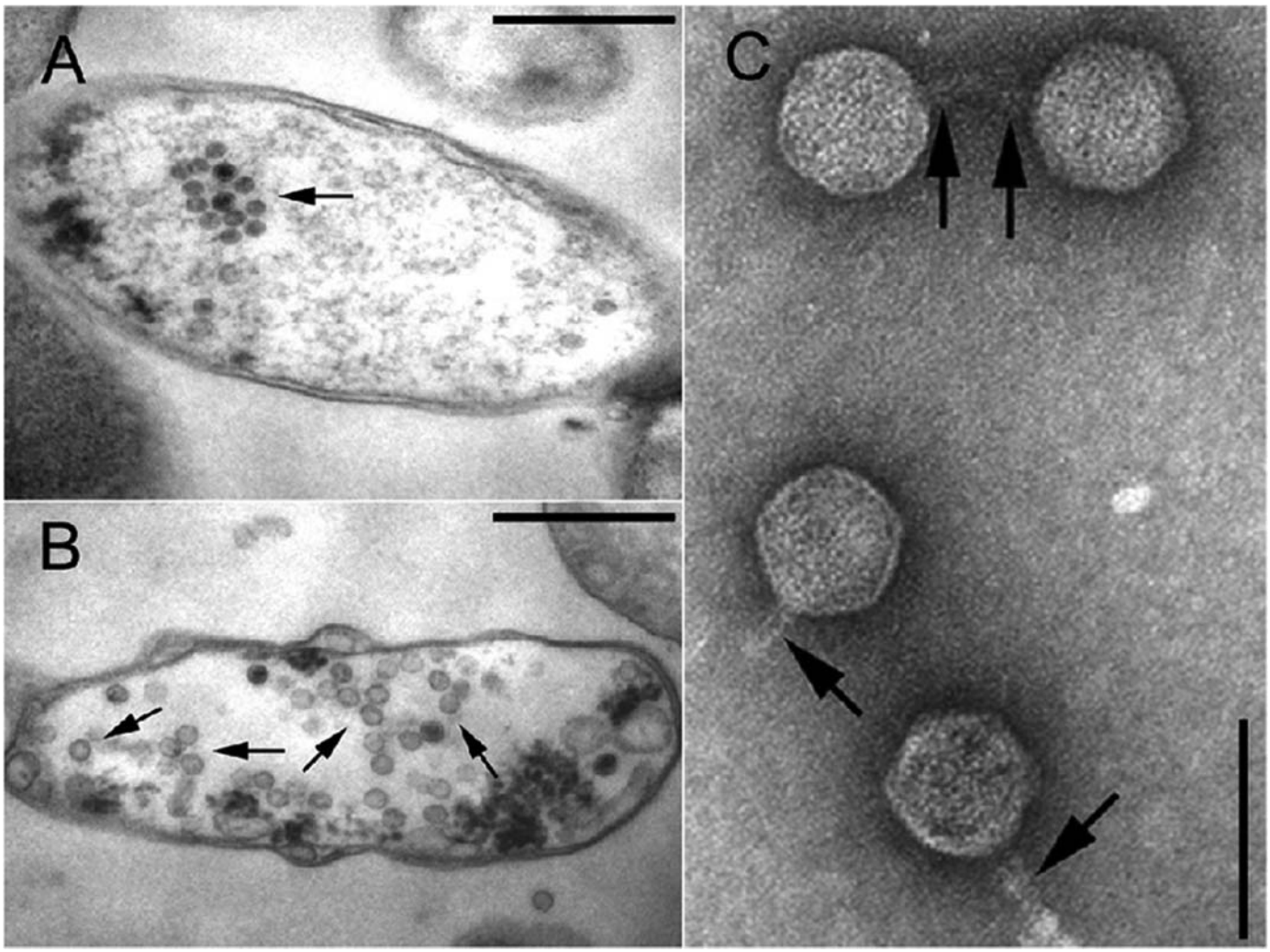
Interestingly, *E. coli* O104:H4 represents a pathogenic STEC paradigm shift, since it lacks the classic EHEC markers (the LEE PAI and the pO157) but possesses Stx2a and EAEC virulence factors, including an AAF (Fig. 1 and 2). Thus, the 2011 *E. coli* O104:H4 outbreak of hemorrhagic diarrhea in Germany is an example of the explosive cocktail of high virulence and resistance that can emerge in this species. In other words, *E. coli* O104:H4 is an *eae*-negative STEC (LEE-negative STEC) that does not possess a classical EHEC plasmid (pO157). To be pathogenic, a strain must have the necessary properties to cause disease in humans. These properties are called virulence factors. In the case of *E. coli* O104:H4 strains, their STEC-EAEC hybrid characteristics might contribute to make this outbreak EHEC O104:H4 strain so dangerous. Thus, EAEC strains of serotype O104:H4 contain a large set of virulence-associated genes regulated by the AggR transcription factor and other virulence factors encoded in EAEC plasmid. These include, among other factors, the pAA plasmid genes encoding the AAF, which anchor the bacterium to the intestinal mucosa, and the aggregative adherence pattern on intestinal epithelial cells (Fig. 2), also called stacked-brick pattern.

Stx2

The primary virulence factor in systemic host responses produced by clinical isolates of STEC is Stx2, but some isolates produce both Stx1 and Stx2, or rarely, only Stx1 (50, 51). Genes for Stx are located on a bacteriophage (a virus of bacteria) that is associated with all pathogenic

STEC strains (Fig. 3). Numerous other factors produced by STEC are believed to act locally in the intestine rather than systemically as do the Stxs. Stx includes two major immunologically distinct forms (Stx1 and Stx2), with minor variants of Stx2 (Stx2a to h). In contrast to these genotypic differences, Stxs share many properties, including molecular structure, enzymatic activity, receptor specificity, and intracellular trafficking. All Stxs possess an AB5 structure with an enzymatically active A subunit of approximately 32 kDa in noncovalent association with five identical B subunits, with each B subunit being approximately 7.7 kDa in size (52). X-ray crystallographic analyses of Stxs have shown that the pentameric B subunits form a ring with the carboxy terminus of the A subunit interdigitated within the central pore. The A subunit has *N*-glycosidase activity, and the B subunit binds to a membrane glycolipid, globotriaosylceramide (Gb3) (Fig. 4). The association of *E. coli* Stxs with diarrhea-associated HUS was established in 1985 (33). In addition, Stx1 and Stx2 do not target exactly the same tissues and organs although both bind Gb3 and are capable of causing diarrhea-associated HUS (53, 54). Injection of animals with Stx1 or Stx2 results in preferential damage to organs including kidney

FIGURE 3 Transmission electron microscopy (TEM) of Stx2 phage (P13374) induction from lysogenic strain *E. coli* K-12 strain TPE2364 (C600) infected with phage lysates of *E. coli* O104:H4 strain CB13374. (A, B) Ultrathin sections of two bacterial cells (TPE2364) with maturating virion particles within the cytoplasm indicated by arrows (bars, 500 nm). (C) TEM of CsCl-purified, negatively stained phage (P13374) particles released by strain TPE2364 (bar, 100 nm). Short tails (arrows) and a hexagonal head are shown. From Beutin et al. 2012. *J Virol* **86**:10444. doi:10.1128/microbiolspec.EHEC-0008-2013.f3



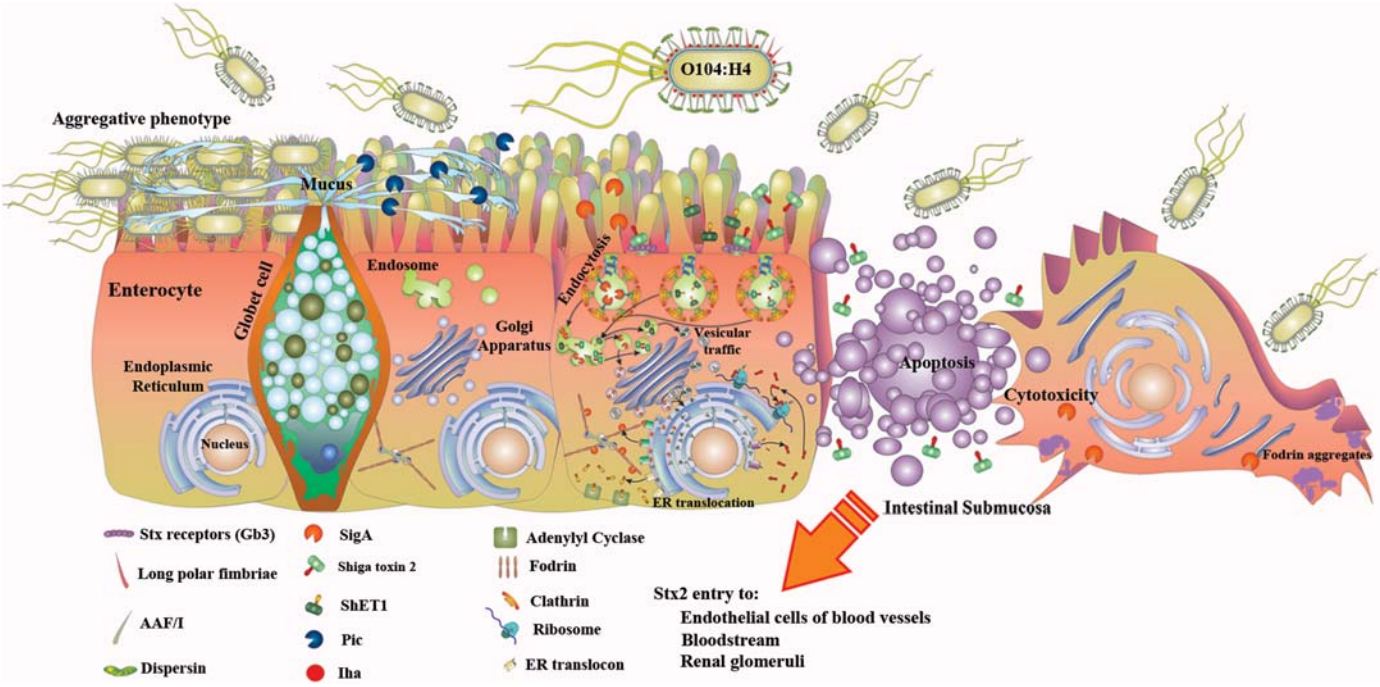


FIGURE 4 Schematic representation of *E. coli* O104:H4 virulence factors and their targets on the mucosal epithelium. The targets and virulence factors are extrapolated from their known function in other pathogens, and the action mechanism for ShET1 is hypothetical. doi:10.1128/microbiolspec.EHEC-0008-2013.f4

and lung. Stx1 appears to target the lung whereas Stx2 prefers the kidney (55).

The Stx receptor is a major determinant and of central importance to diarrhea-associated HUS kidney disease (56). Gb3 is synthesized in the Golgi apparatus of select eukaryotic cells and is transported to the plasma membrane where it resides in the outer leaflet with its trisaccharide moiety facing outward and the hydrocarbon ceramide (C-16 to C-24) moiety noncovalently arranged within the plasma membrane. The binding subunit of Stx specifically recognizes the terminal alpha-1,4-digalactose of the trisaccharide. A molecule of Stx contains five binding (B) subunits, each capable of binding one or more molecules of Gb3, resulting in cooperative high-affinity binding of Stx to cells. The importance of Gb3 in Stx action is evident from cell culture and animal studies where the absence of Gb3 eliminates the response to Stx (57). Gb3 may also be referred to as CD77 or the Pk blood antigen. Structure/function studies suggest that each toxin molecule may express 10 to 15 Gb3-binding sites per B-subunit pentamer (58), explaining the high affinity (dissociation constant [K_d] $\approx 10^{-9}$ M) of toxin binding. All Stxs, with the exception of one Stx2 variant called Stx2e, bind Gb3; Stx2e shows preferential binding to the glycolipid globotetraosylceramide. Structural differences in the toxin receptor also contribute to toxin susceptibility. Gb3 is heterogeneous, displaying variability in fatty acid chain length, degree of bond saturation, and hydroxylation. Expression of Gb3 isoforms with long-chain, unsaturated fatty acids was associated with increased toxin sensitivity (59). It has been demonstrated that long-chain, unsaturated Gb3 isoforms were more likely to induce negative membrane curvature, leading to Gb3 clustering and formation of tubular invaginations (60). Finally, Gb3 association with lipid rafts was necessary for efficient intracellular transport of Stx1 B subunits (61). Polyunsaturated fatty acid incorporation into cell membranes, known to disrupt lipid rafts, protects cells from Stx intoxication (62). Thus, much or perhaps most of the Gb3 in a plasma membrane may not be reactive with Stx. This cryptic Gb3 may be a function of direct interaction of the Gb3 ceramide moiety with other membrane components, including cholesterol, other glycolipids, fatty acids, or proteins (63, 64). This possible mechanism helps explain why a tissue often appears to bind much less Stx compared to the total amount of Gb3 that is extractable from the tissue.

Most diarrhea-associated HUS is related to young children although individuals of all ages can develop this syndrome. A simple explanation is that more Gb3 is expressed in the kidneys of young people. However, this has proven not to be the case as there is more Gb3

extractable from kidneys of adults versus children (65, 66). An alternative explanation is that pediatric kidney expresses more Gb3 on cells that are more involved in biological responses leading to diarrhea-associated HUS. It is interesting that only pediatric kidney expressed Gb3 in glomeruli (66). Another concept, as mentioned above, is that the plasma membrane microenvironment nearby Gb3 dictates the biological response to Stx. Membrane Gb3 expression is a critical determinant of toxin sensitivity; cells expressing low Gb3 levels are sensitized to toxicity by increased membrane expression of toxin receptors, whereas cells selected for loss of Gb3 expression are resistant to Stxs (59, 67).

To be an effective protein synthesis inhibitor, Stx must reach the cytoplasm to access ribosomes (Fig. 4). Stx uses a retrograde transport pathway to reach the endoplasmic reticulum (ER), an intracellular compartment rich in membrane-associated ribosomes and containing the cellular machinery necessary for protein translocation into the cytoplasm. Following binding and cross-linking of Gb3, Stx is internalized by clathrin-dependent or clathrin-independent mechanisms (60, 68). Following binding to Gb3 on target eukaryotic cells, the Stx-receptor complex is internalized and locates within endosomes. Rather than moving to lysosomes for degradation, the complex is transported in a retrograde manner through the Golgi apparatus to the ER (69). The resulting A1 and A2 fragments are linked through a disulfide bond; the latter fragment maintains association with the B pentamer. In the reductive environment of the ER, the disulfide bond is broken, and the 27-kDa A1 fragment translocates across the ER membrane, a process termed retrotranslocation. By this process, mammalian cells may recognize Stx delivered to the ER as misfolded proteins and activate the ER stress response, and the A1 fragment uses the ER-associated protein degradation pathway to reach ribosomes. To avoid the proteasome, A1 fragments contain few (Stx/Stx1) or no (Stx2) lysine residues (52), which may minimize ubiquitination, and A1 fragments associate with ribosomal proteins, which may inhibit proteasomal transport (70). Once the enzymatically active A1 subunit is released into the cytoplasm, it inactivates the eukaryotic ribosome by removal of a single adenine base from 28S rRNA within the large (60S) ribosomal subunit (71), acting as a highly specific *N*-glycosidase. This is an irreversible process that renders the ribosome defective for interaction with eukaryotic peptide elongation factor for binding of aminoacyl-tRNA and elongation of nascent peptide (72), resulting in inhibition of protein synthesis.

Stx can exert its effects on eukaryotic cells by one of three known mechanisms: (i) Inactivation of ribosomes and inhibition of cytoplasmic protein synthesis can result in cell death (73). (ii) Stx-dependent generation of the depurinated 28S rRNA in ribosomes initiates a signal-transduction response known as the ribotoxic stress response that leads to activation of cytokines, chemokines, or other factors that result in numerous events including apoptosis (Fig. 4) of the affected cell (74). The signaling pathways activated in the ribotoxic stress response include the mitogen-activated protein kinases (MAPK), such as p38MAPK (74, 75). Remarkably, these activities appear to be due to other than inhibition of protein synthesis. (iii) Receptor binding of Stx holotoxin or its B subunit alone can initiate a cytoplasmic signal-transduction cascade different from the ribotoxic stress response-activated pathway (76). Conserved features of Stx-induced apoptosis include routing active toxin to the ER where prolonged signaling through the ribotoxic and ER stress responses may activate apoptosis; alterations in the balanced expression of pro- and antiapoptotic Bcl-2 proteins; a rapid activation of caspase 8, which, in turn, activates both caspase-dependent and mitochondrial-dependent apoptotic signaling pathways; and lack of signaling through Fas and tumor necrosis factor receptors to trigger apoptosis. Complement activation products have been detected in the serum and plasma of patients with HUS, and an in vitro study showed that Stx2 not only damages the kidney directly but also indirectly via complement in two ways. First, it activates complement, and second, it delays the functions of its control protein factor H on the cell surface, both known to damage the kidney (52).

Thus, Stx can exert different responses in a cell type-specific manner. The final result of these events can be cell death (apoptosis, necrosis) or inflammatory responses in cells that remain viable, and perhaps other intermediate responses that can be due to the Stx type, the host cell type, and/or the bacterial genetic background. STEC is known to cause hemorrhagic enterocolitis and HUS. Stx plays a role in the occurrence of blood in the feces and in HUS by its action on the endothelial cells of blood vessels in the intestinal submucosa and in the renal glomeruli (Fig. 4). Epidemiologically, Stx2 seems to be more important than Stx1 in development of HUS, which was also seen in the *E. coli* O104:H4 outbreak strain. The action of Stx is not limited to inhibition of protein synthesis. Stx induces macrophages to express tumor necrosis factor alpha (tumor necrosis factor-alpha), interleukin (IL)-1 beta, and IL-6 in vitro. These cytokines and lipopolysaccharide (LPS)

are reported to increase the susceptibility of cells to Stx. A variety of cells such as tubular epithelial cells may be targets for Stx-mediated apoptosis. Apoptosis is considered to contribute to the pathogenesis of HUS caused by STEC (77). HUS is primarily due to the production and translocation of Stxs across the intestinal epithelial barrier (52). HUS may manifest after STEC is no longer detectable in the stool. Following toxin-mediated damage to colonic blood vessels, Stxs may enter the bloodstream although free toxins have not been detected in the circulation of patients with HUS. The risk of progression to extraintestinal complications is increased in patients infected with STEC expressing Stx2, either alone or in combination with Stx1 or Stx2c (78). The kidneys and central nervous system are most frequently damaged by Stxs. HUS is a constellation of hematological, renal, and neurological complications that develops in 10 to 15% of patients with hemorrhagic colitis. Complications include thrombocytopenia and hemolytic anemia with schistocytes (fragmented erythrocytes) present in blood smears. The characteristics of acute renal failure, which may follow STEC infection, include oliguria or anuria, swollen glomerular endothelial cells detached from the basement membrane, intraglomerular fibrin deposition, and thrombotic microangiopathy. Mesangiolysis or mesangial hyperplasia has been described in some HUS cases. Renal tubular injury may be present but is not a consistent finding late in the course of HUS (79). Approximately 66% of patients with HUS require dialysis. Central nervous system involvement may present as lethargy, irritability, seizures, paresis, and coma. Long-term sequelae include renal insufficiency, hypertension, hyperactivity and distractability, and insulin-dependent diabetes mellitus. Mortality from HUS is 3 to 5%. There is variability in signs and symptoms following ingestion of STEC. For example, patients may present with acute renal failure in the absence of bloody diarrhea (79).

Other events that may influence the pathogenesis of STEC are the phage origin of Stx and Stx variability (Fig. 3). Stx phages display extensive genetic mosaicism; however, genes encoding the Stx A and B subunits are generally located downstream of the antiterminator Q and the P′R promoter. As a consequence of this orientation, toxin genes are late genes optimally expressed upon induction of the lytic cycle. STEC may possess cryptic lambdoid prophages that serve as sources for recombination events, yielding novel toxin-converting phages, and Stx phages expressing new tail assemblies may expand the host range of toxin-producing organisms (80). Lysogenic conversion to the toxigenic phenotype may occur if recipient bacteria display phage receptors and possess integration sites within the genome (Fig. 3). Thus, Stx phages are responsible for the dissemination of *stx* genes in *E. coli* and other enteric bacteria. Following induction, Stx phages can infect other bacteria in vivo and in vitro. Stx phages may be considered to represent highly mobile genetic elements that play an important role in the expression of Stx, in horizontal gene transfer, and hence in genome diversification (81). On the other hand, purified Stx alone is capable of producing systemic complications, including HUS, in animal models of disease. Stx2a is more potent than Stx1. Epidemiologic studies suggest that Stx2 subtypes also differ in potency. Indeed, by examining protein synthesis inhibition using Vero monkey kidney cells and inhibition of metabolic activity using primary human renal proximal tubule epithelial cells, it was found that Stx2a, Stx2d, and elastase-cleaved Stx2d were at least 25 times more potent than Stx2b and Stx2c; in vivo this potency was also assessed in mice. Stx2b and Stx2c had potencies similar to that of Stx1, whereas Stx2a, Stx2d, and elastase-cleaved Stx2d were 40 to 400 times more potent than Stx1 (82). Furthermore, by using the classification for seropathotypes (A to E), it has been found that the *stx*(2) variant was mainly associated with strains of seropathotype A, whereas most of the strains of seropathotype C possessed the *stx*(2-vhb) variant, which was frequently associated with *stx*(2), *stx*(2-vha), or *stx*(2c). Levels of *stx*(2) and *stx*(2)-related mRNA were higher in strains belonging to seropathotype A and in those strains of seropathotype C that express the *stx*(2) variant than in the remaining strains of seropathotype C. The *stx*(2-vhb) genes were the least expressed (83). Another complicating factor in diarrhea-associated HUS is that antibiotics are not recommended in the earlier phases, i.e., before the appearance of bloody diarrhea because STEC bacteria respond to some antibiotics by producing excess Stx (84, 85).

Iha

The chromosomally encoded adherence-conferring protein Iha, a homolog of *V. cholerae* IrgA (28), is one of a range of novel adhesins identified among STEC strains (Fig. 4). Iha was first characterized in *E. coli* O157:H7, but it is distributed widely among LEE-negative and LEE-positive STEC strains and UPEC (28, 86). In a study in which the difference did not reach statistical significance, it was reported that an *iha* deletion mutant of O157:H7 STEC was impaired in adherence to HeLa cells. But there was a highly significant increase in adherence to both HeLa and MDBK cells when *iha* was expressed from a plasmid in a nonpiliated recombinant

E. coli host (28). The potential importance of this adhesin lies in the high prevalence (91%) of *iha* in STEC belonging to different seropathotypes and the presence of multiple *iha* copies in some strains (28, 87).

In the UPEC strain CFT073, Iha functions as a urovirulence factor by contributing to colonization in a mouse urinary tract infection model (88). Additionally, Iha from UPEC strain UCB34 functions as a catecholate siderophore receptor in *E. coli* K-12. The capacity of Iha to transport siderophores is TonB-dependent, whereby the protein complex TonB/ExbB/ExbD provides the energy required for active transport (29, 89). Furthermore, *iha* expression is regulated by the protein Fur, a ferric uptake regulator (90). In the presence of iron, dimerized Fur binds to *iha* promoter regions through a sequence-specific protein-DNA interaction and represses *iha* transcription. Under iron-limiting conditions, Fur is unable to interact with the DNA, and as a consequence, *iha* transcription is derepressed (29, 90). Iron is an important environmental cue, and iron-limiting conditions induce virulence-related genes in a number of pathogens (91, 92). Free iron levels are typically low at mucosal surfaces, due to binding by host lactoferrin, and therefore the induction of iron-scavenging mechanisms is an important bacterial in vivo survival strategy (92). Recently, it was found that bacterial growth under conditions simulating colonic, but not ileal, short-chain fatty acid (SCFA) concentration increases *iha* expression in three tested STEC strains, with the strongest expression detected in LEE-negative STEC O113:H21 strain 98NK2, as expression of *iha* in O157:H7 STEC strain 98NK2 is subject to Fur-mediated iron repression. However, exogenous iron did not repress *iha* expression in the presence of colonic SCFAs in either 98NK2 or O157:H7 strain EDL933. Moreover, exposure to the iron chelator 2,2′-dipyridyl caused no further enhancement of *iha* expression over that induced by colonic SCFAs. These findings indicate that SCFAs regulate *iha* expression in STEC independently of iron. Increased expression of *iha* under colonic but not ileal SCFA conditions possibly may contribute to preferential colonization of the human colon by STEC (93).

Thus, Iha is a potentially important accessory virulence factor for several pathotypes of *E. coli*, including STEC, UPEC, and avian-pathogenic strains (28, 86, 92, 94–96), functioning as an adhesin and, at least for UPEC, as a catecholate siderophore receptor (29). This latter function is consistent with the fact that *iha* is induced under iron-limiting conditions and is subject to Fur-mediated repression in iron-replete environments in both UPEC and O157:H7 STEC strains (29, 90) and in the hypervirulent LEE-negative STEC strain 98NK2.

Long Polar Fimbriae (Lpf)

E. coli O157:H7 possesses two Lpf operons, *lpf1* and *lpf2*, both of which contain genes closely related to the Lpf of *Salmonella enterica* serovar Typhimurium (97). Expression of *lpf1* and *lpf2* is induced during the late exponential-phase growth in tissue culture media at pH 6.5 and 37°C or under iron-restricted conditions (98), and has been found to influence *E. coli* O157:H7 adherence to cultured epithelial monolayers (99, 100). The first locus (*lpf1*) is located in an O157-specific island (OI-141) of approximately 5.9 kb, and inserted in the *yhjX-yhjW* intergenic region (in relation to the *E. coli* K-12 chromosome). The *lpf1* operon contains six genes (*lpfABCC′DE*) similar in sequence and gene order to the *Salmonella lpfABCDE* genes (101). 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 (101). 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 and displayed a diffuse adherence pattern (87, 101). A recent study showed 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 proteins was observed for an *lpf* mutant (*lpf1*) strain (102). This study demonstrated that Lpf1 and extracellular matrix proteins interact, and their interaction may contribute to STEC O157:H7 colonization of the gastrointestinal tract. The second *lpf* operon, the *lpf2* locus, is approximately 6.8 kb and located in OI-154; it is inserted in the *glmS-pstS* intergenic region (98). The *lpf2* locus contains five genes (*lpfABCDD′*) but lacks an *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 (98). However, a previous study using a random transposon mutagenesis showed that an insertion in the *lpfD2* gene caused increased bacterial adherence to HeLa cells (103). 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 (98). 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 (98).

Homologs of *lpf* genes have also been identified for non-O157 STEC strains, and their role in adherence has also been explored (Fig. 4). It was 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 (104). Sequencing analysis demonstrated that the STEC O113:H21 strain possesses an *lpf* operon (also referred as *lpf*O113) containing four genes (*lpfABCD* genes) found at the same chromosomal location as the STEC O157:H7 *lpf2* locus (OI-154) (104). 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 homologs may promote interbacterial interactions (104).

Due to the relatively subtle effects of *lpf* mutations on adherence in vitro, coupled with the divergent findings from in vivo or organ culture experiments, the precise role of Lpf in *E. coli* O157:H7 adherence remains somewhat unclear. Therefore, the role of the *E. coli* O157:H7 *lpf* loci was further tested in an infant rabbit model, which mimics the diarrhea and gut pathology, including the histopathological A/E lesions, seen in patients with STEC infection. By performing competition experiments between *E. coli* O157:H7 and an isogenic *lpf1 lpf2* double mutant, it was found that the mutant was outcompeted in the ileum, cecum, and midcolon of rabbits, confirming that Lpf contributes to intestinal colonization (105). Unexpectedly in this study, it was observed that the *lpf1 lpf2* double mutant showed an increased adherence to colonic epithelial cells in vitro, and transmission electron microscopy revealed curli-like structures on the surface of this mutant. Interestingly, deletion of *csgA* per se did not appear to affect intestinal colonization. Therefore, in addition to conclusively demonstrating that Lpf contributes to *E. coli* O157:H7 intestinal colonization, the authors indicated that the regulatory mechanisms controlling expression of Lpf and curli are interconnected (105).

E. coli O104:H4 OUTBREAK STRAIN AS EAEC

As mentioned before, the O104:H4 outbreak strain represents a rare combination of EAEC characteristics and Stx2 expression; only sporadic cases of disease associated with Stx-producing EAEC have previously been described in Germany (106, 107), France (108, 109), the United Kingdom (110), the Republic of Georgia (13), and Japan (111). EAEC was first described in 1987, based on the characteristic adherence phenotype with cultured HEp-2 cells (stacked-brick appearance on epithelial cells) (Fig. 2). This biological test still remains the gold standard of diagnosis, but it does not distinguish between pathogenic and nonpathogenic strains. Subsequently, a number of virulence factors have been described for EAEC strains and include adhesins (e.g., AAF/I to III), heat-stable enterotoxin, transporters, and other secreted proteins (e.g., the serine protease autotransporter Pic, which has mucinase activity), as well as multiple factors contributing to EAEC-induced inflammation. However, none of these factors are found in all EAEC isolates, and no single factor has ever been implicated in EAEC virulence. Thereby, EAEC isolates are genetically a heterogeneous group of *E. coli* strains (112). EAEC strains were first associated with persistent diarrhea in infants from developing countries; since then they have increasingly been linked as a cause of acute and persistent diarrhea in young infants and children in developing and industrialized countries, in individuals infected with human immunodeficiency virus, as a cause of acute diarrhea in travelers from industrialized regions, and with foodborne outbreaks. A major effect of EAEC infection is on the malnourished children in developing countries (112). Thus, EAEC without Stx causes diarrhea in persons who reside in developed countries, such as the United Kingdom (113, 114), Switzerland (115), and Japan (116), although EAEC is more commonly associated with acute and persistent (>14 days) diarrhea of infants, children, human immunodeficiency virus-positive persons, and travelers to developing countries (112, 117). EAEC isolates are a highly heterogeneous group of bacteria (118, 119), and each isolate carries a particular subset of EAEC-associated virulence genes; no single virulence factor is consistently associated with EAEC pathogenesis. The addition of *stx2* to the EAEC virulence gene repertoire, however, has led to a pathogen that has the capacity to cause disease on a large scale with a potentially deadly outcome due to the possibility of the development of HUS (Fig. 4).

AggR and AggR-Regulated Genes (AAF/I, Dispersin, Dispersin Transporter)

AggR is a member of the AraC/XylS family of bacterial transcriptional activators (10, 120), exhibiting the greatest levels of amino acid identity with the CfaD (68%), Rns (66%), and CsvR (62%) regulators of

enterotoxigenic *E. coli* (10). Proteins belonging to this family are defined by a conserved 99-amino-acid C terminus domain and regulate diverse cellular functions including metabolism, stress response, and the synthesis of virulence factors. Multiple epidemiologic studies suggest that strains expressing AggR are more likely to cause diarrheal disease than those without it, proposing the term "typical EAEC" to describe strains harboring the AggR regulon (121, 122). A number of AggR-regulated genes have been described previously in archetype EAEC strain 042. The genes encoding AAF were the first found to be regulated by AggR (10), followed by *aap* (encoding the dispersin surface protein) (123) and the Aat secretion system, which is required for transport of dispersin to the bacterial surface (124). AggR also activates expression of the Aai type VI secretion system (T6SS) in strain 042 (125), though the role of Aai in EAEC virulence remains unknown.

Electron microscopy studies demonstrated different fimbriae in several EAEC strains (126–128). The best characterized are AAF I, II, and III, which are responsible for the expression of aggregative adherence (15, 17, 129). AAFs are encoded in high-molecular-weight plasmids (designated pAA), and their biogenesis employs the chaperone-usher secretion pathway (11, 12, 15). These fimbriae are members of the Dr superfamily of adhesins that includes strains of UPEC and diarrheagenic *E. coli* with a genetic organization consisting of chaperone, usher, and pilin subunits (13, 14). Expression of AAF genes requires AggR (10); this protein regulates the genes involved in fimbrial biogenesis for both AAF/I and AAF/II. AAF/I are flexible, bundle-forming fimbriae, and the genes responsible for their biogenesis are located in two unlinked regions of the plasmid: region 1, containing the genes that encode the pilin, chaperonin, and usher, and region 2 (separated by 9 kb), containing the regulator-encoding gene (designated *aggR*) (10, 15, 16). *aggR* is located in an open reading frame of 794 bp that encodes a protein with a predicted molecular size of 29.4 kDa. The cloned *aggR* gene is sufficient to complement a region 1 clone to confer AAF/I expression, while an *aggR* mutant is negative for AAF/I expression.

Genomic studies of the pAA plasmid revealed a small open reading frame just upstream of the *aggR* gene in most EAEC strains. This gene was found to encode a 10-kDa secreted protein that was recognized by the sera of volunteers fed strain 042. A null mutant of this gene revealed a unique hyperaggregative phenotype, and scanning electron microscopy of the bacterial strains showed collapse of the AAF onto the bacterial cell surface. The protein product of this gene was therefore termed antiaggregation protein (Aap), nicknamed dispersin (123). Dispersin is secreted to the surface of EAEC strains and binds noncovalently to LPS of the outer membrane. Data suggest that the mechanism of dispersin's effect may be mediated predominantly through its ability to neutralize the strong negative charge of the LPS, so that the AAFs, which carry a strong positive charge, are free to splay out from the surface and bind distant sites (130). Interestingly, the secretion of dispersin is dependent on the presence of an ABC transporter complex also encoded on plasmid pAA (124). The genes encoding this transporter (the Aat complex) correspond to the site of the previously cryptic aggregative adherence probe. As both dispersin and the Aat complex are under the control of AggR, the latter protein is emerging as the central regulator of virulence functions in EAEC.

The AggR regulon is not restricted to the pAA plasmid. It has been found that in addition to the plasmid-encoded genes, AggR regulates a chromosomal operon inserted on a PAI at the *pheU* locus (125). AggR activates the expression of chromosomal genes, including 25 contiguous genes (*aaiA–Y*), which are localized to a 117-kb PAI inserted at *pheU*. Many of these genes have homologs in other gram-negative bacteria and were recently proposed to constitute T6SS. AaiC was identified as a secreted protein that has no apparent homologs within GenBank. EAEC strains carrying in-frame deletions of *aaiB*, *aaiG*, *aaiO*, or *aaiP* synthesized AaiC, but AaiC secretion was abolished. Cloning of *aai* genes into *E. coli* HB101 suggested that *aaiA–P* are sufficient for AaiC secretion.

Recent studies in the Nataro laboratory have revealed that AggR positively regulates its own expression in a complex fashion. AggR binds directly and specifically to two sites flanking the *aggR* promoter. Additionally, *aggR* promoter was found to be positively regulated by the DNA-binding protein FIS and negatively regulated by the global regulator H-NS. EAEC present in the mouse intestine possessed relatively high levels of *fis* promoter and *aggR* promoter activity and a low level of *hns* promoter when compared with in vitro experiments. The data provide significant insights into the regulation cascade leading to *aggR* expression in the mammalian intestine during EAEC infection (131). A recent study showed that there are at least 44 AggR-regulated genes in the genome of EAEC strain 042. Twenty-five of the 44 genes were previously known to be so regulated, identified as part of the Aai T6SS (16 genes), the dispersin secretion system (5 genes), and the AAF/II fimbrial biogenesis system (4 genes). Sixteen of the 44 genes

are predicted to encode hypothetical proteins, and only 5 of these genes showed homology to other genes encoding known bacterial proteins, suggesting new virulence-related functions (132).

AggR has also a role in the inflammatory response against EAEC. Jiang et al. (121) reported that IL-8 levels were higher in feces of patients infected with *agg*R- or *aafA*-containing strains compared with those infected with strains negative for these factors. Recently, it was also shown that EAEC strains harboring *aggR*, *aggA*, and *aap* were more likely to cause IL-8 induction of >4,100 pg/ml from nonpolarized HCT-8 intestinal epithelial cells than EAEC negative for those genes (133). In fact, polarized T84 intestinal cells were found to release IL-8 even when infected with strain 042 mutated in the major flagellar subunit FliC. IL-8 release from polarized T84 cells was found to require the AggR activator and the AAF fimbriae, and IL-8 release was significantly less when cells were infected with mutants in the minor fimbrial subunit AafB (134).

SPATEs (Pic, SepA, SigA)

SPATEs and other autotransporters use a type V secretion system for export to the extracellular space (135, 136). The autotransporters contain all the information necessary for passage through the inner membrane and the outer membrane. To mediate its own secretion, an autotransporter contains three functional domains: an N-terminal signal sequence, an extracellular passenger domain, and a C-terminal β-barrel domain. The signal sequence initiates Sec-dependent transport across the inner membrane and is proteolytically removed in the periplasmic space. The C-terminal domain forms a β-barrel pore in the outer membrane, which facilitates the delivery of the passenger domain to the extracellular space. The passenger domains of some autotransporters remain anchored to the extracellular face of the outer membrane, but SPATEs are released from the bacterial cell by proteolytic nicking of a site between the β-barrel pore and the passenger domain. The mature, secreted SPATEs are 104- to 110-kDa toxins that contain a typical N-terminal serine protease catalytic domain followed by a highly conserved β-helix motif, which is present in nearly all autotransporters (135, 137). Although the general process of SPATE secretion is understood, the details of many events in SPATE biogenesis (the chaperone function in the periplasm, the mechanism of β-barrel insertion into the outer membrane, the translocation pathway across the outer membrane, the proteolytic release of the mature protein from the outer membrane, etc.) remain unresolved (135).

It has been proposed that SPATEs can be divided phylogenetically into two distinct classes, designated 1 and 2 (138). Class 1 SPATEs are cytotoxic in vitro and induce mucosal damage on intestinal explants (Fig. 4). Although the actions of class 1 SPATEs are not fully understood, several have been shown to enter eukaryotic cells and to cleave cytoskeletal proteins (21, 139, 140) while the class 2 SPATEs induce mucus release, cleave mucin, and confer a subtle competitive advantage in mucosal colonization (20, 141, 142) (Fig. 4). A study to determine the prevalence of SPATEs in EAEC was performed by seeking 10 genes encoding serine protease autotransporter toxins in a collection of clinical EAEC isolates. Eighty-six percent of EAEC strains harbored genes encoding one or more class I cytotoxic SPATE proteins (Pet, Sat, EspP, or SigA). Two class II noncytotoxic SPATE genes were found among EAEC strains: *pic* and *sepA*, each originally described in *S. flexneri* 2a. Using a multiplex PCR for five SPATE genes (*pet*, *sat*, *sigA*, *pic*, and *sepA*), the authors found that most of the *Shigella* sp. isolates also harbored more than one SPATE, whereas members of most other *E. coli* pathotypes rarely harbored a cytotoxic SPATE gene. SPATEs may be relevant to the pathogenesis of both EAEC and *Shigella* spp. (143).

Pic is localized in the EAEC chromosome (118, 141), and it was found to be identical to a protein termed Shmu (*Shigella* mucinase), which is encoded on the *Shigellashe* PAI (144). Through functional analysis of Pic, it was found that its proteolytic site is involved in Pic mucinase activity, serum resistance, and hemagglutination. Phenotypes identified for Pic suggest that it is involved in the early stages of the pathogenesis and most probably promotes the intestinal colonization (141, 144). Pic binds mucin, and this binding was blocked in competition assays using monosaccharide constituents of the oligosaccharide side chains of mucin. Moreover, Pic mucinolytic activity decreased when sialic acid was removed from mucin. Thus, Pic is a mucinase with lectin-like activity that can be related to its reported hemagglutinin activity (19). Recently, it was shown that Pic induces hypersecretion of mucus, which was accompanied by an increase in the number of mucus-containing goblet cells (Fig. 4). This finding is in accord with one of the hallmarks of EAEC: the formation of biofilm, which comprises a mucous layer with immersed bacteria in the intestines of patients. Interestingly, an isogenic *pic* mutant (EAEC Δ*pic*) is unable to cause this mucus hypersecretion. Furthermore, purified Pic was also able to induce intestinal mucus hypersecretion. Thus, Pic mucinase is responsible for one of the

pathophysiologic features of the diarrhea mediated by EAEC (20). It has also been shown that Pic protease promotes intestinal colonization and growth in the presence of mucin, suggesting a novel metabolic role for the Pic mucinase in EAEC colonization. Interestingly, it has been found that Pic targets a broad range of human leukocyte adhesion proteins. Substrate specificity is restricted to glycoproteins rich in O-linked glycans, including CD43, CD44, CD45, CD93, CD162 (P-selectin glycoprotein ligand 1), and the surface-attached chemokine fractalkine, all implicated in leukocyte trafficking, migration, and inflammation. Additionally, exposure of human leukocytes to purified Pic results in polymorphonuclear cell activation, but impaired chemotaxis and transmigration; Pic-treated T cells undergo programmed cell death (145).

Besides Pic, another SPATE from class II found in *E. coli* O104:H4 is SepA. In 1995, Benjelloun-Touimi et al. (22) described a Tsh homolog designated SepA (for *Shigella* extracellular protein), which is the major extracellular protein of *S. flexneri* (22). In 1994, the first SPATE was described as a temperature-sensitive hemagglutinin (Tsh) in avian-pathogenic *E. coli*, which causes disseminated infections in birds (146). Investigation of the proteolytic activity of SepA by using a wide range of synthetic peptides found that SepA hydrolyzed several of these substrates and that the activity was inhibited by the serine protease inhibitor phenylmethylulfonyl fluoride (147). Several SepA-hydrolyzed peptides were described as specific substrates for cathepsin G, a serine protease produced by polymorphonuclear leukocytes that was proposed to play a role in inflammation. However, unlike cathepsin G, SepA degraded neither fibronectin nor angiotensin I and had no effect on the aggregation of human platelets. The presence of *sepA* on the virulence plasmid, as well as the recognition of SepA by the sera of monkeys infected with *Shigella* sp., suggested that SepA might be involved in *Shigella* pathogenicity (22). However, construction and phenotypic characterization of a *sepA* mutant suggested that SepA is required neither for entry into cultured cells nor for intracellular dissemination. Nevertheless, the *sepA* mutant demonstrated a reduced ability to induce both mucosal atrophy and tissue inflammation in the rabbit ligated ileal loop model, indicating that SepA may play a role in tissue invasion, although this hypothesis remains to be elucidated (22).

On the other hand, a SPATE of class I has been reported in O104:H4, SigA. An initial report showed that the *she* PAI, which contains the *pic* (*she*) gene, also contains a gene encoding a second immunoglobulin A protease-like homolog, *sigA*, lying 3.6 kb downstream and in an inverted orientation with respect to *pic* (144). Functional analysis showed that SigA is a secreted temperature-regulated serine protease capable of degrading casein. Experiments similar to those used with Pet (another SPATE of EAEC) revealed that SigA is cytopathic for HEp-2 cells, suggesting that it may be a cell-altering toxin with a role in the pathogenesis of *Shigella* infections. Indeed, it was found that SigA binds specifically to HEp-2 cells and degrades recombinant human αII spectrin (α-fodrin) in vitro and also cleaves intracellular fodrin in situ, causing its redistribution within cells, suggesting that the cytotoxic and enterotoxic effects mediated by SigA are likely associated with the degradation of epithelial fodrin (21) (Fig. 4). Furthermore, SigA was at least partly responsible for the ability of *S. flexneri* to stimulate fluid accumulation in ligated rabbit ileal loops (148). In the case of Pet, it is a cytoskeleton-altering toxin (136), because it induces contraction of the cytoskeleton, loss of actin stress fibers, and release of focal contacts in HEp-2 and HT29/C1 cell monolayers, followed by complete cell rounding and detachment. Interestingly, Pet cytotoxicity and enterotoxicity depend on Pet serine protease activity (149). It has been shown that Pet enters the eukaryotic cell and that trafficking through the vesicular system is required for the induction of cytopathic effects. Thus, after clathrin-mediated endocytosis, Pet undergoes a retrograde trafficking to the endoplasmic reticulum to be translocated into the cytosol (150, 151). Finally, an intracellular target, α-fodrin (αII spectrin), has been found for Pet. Pet binds and cleaves epithelial fodrin (between M1198 and V1199) in vitro and in vivo, causing fodrin redistribution within the cells, to form intracellular aggregates as membrane blebs (139).

Shigella Enterotoxin 1 (Set1)

ShET1 toxin is a subunit toxin encoded by *setA* and *setB*, which are thought to form an oligomeric toxin consisting of a single 20-kDa SetA protein associated with a pentamer of five 7-kDa B subunits (SetB) (18). ShET1 appears to induce intestinal secretion via cyclic AMP and cyclic GMP; however, the precise mechanism of action and detailed biochemistry remain inconclusive. Unusually, the *setAB* genes are encoded within the *pic* gene but on the complementary strand and thus have the same prevalence characteristics and disease associations as *pic* (141). However, the role of the ShET1 in EAEC pathogenesis has not been studied, even though it can work on adenylyl cyclase (Fig. 4). In the case of the *S. flexneri* 2a strain, culture filtrates cause significant

fluid accumulation in rabbit ileal loops, when the bacteria are grown in iron-depleted medium. Also, testing in Ussing chambers showed a greater rise in potential difference and short circuit current with such filtrates compared with the medium control. Ultrafiltration and gel exclusion size fractionation of M4243 filtrate revealed that the activity was in the fraction of approximately 60 kDa. It is thought that ShET1 is elaborated in vivo, since it elicits an immune response and may be important in the pathogenesis of diarrheal illness due to *S. flexneri* (152).

CONCLUSIONS

The genotypes, phenotypes, and phylogeny of the outbreak isolates demonstrate that the *E. coli* O104:H4 outbreak strain is a clone that combines virulence potentials of two different pathogens: STEC and EAEC. Thus, EAEC of serotype O104:H4 is by itself an emerging serovariant that has acquired an original set of virulence factors. All shared virulence profiles combining typical STEC (*stx2*, *iha*, *lpf*O26, *lpf*O113) and EAEC (*aggA*, *aggR*, *set1*, *pic*, *aap*) loci and expressed phenotypes that define STEC and EAEC, including production of Stx2 and aggregative adherence to epithelial cells (Fig. 4). Additionally, isolates displayed a high antibiotic resistance, specifically those related to β-lactamase.

EAEC strains of serotype O104:H4 contain a large set of virulence-associated genes regulated by the AggR transcription factor (Fig. 1). These include the pAA plasmid genes encoding the AAF, which anchor the bacterium to the intestinal mucosa and induce inflammation (Fig. 2), and a protein-coat secretion system (Aat), including its secreted protein, dispersin. A switch of the virulence plasmid (pAA) together with the type of the AAF could be an additional explanation for the higher virulence of this outbreak strain. Other adhesion factors (Iha, Lfp) could help this outbreak strain as a synergistic or initial adhesion factor to guarantee an efficient adhesion and perhaps Stx delivery. Additionally, EAEC strains of serotype O104:H4 produce a variable number of SPATEs, implicated in mucosal damage and colonization. It allows speculation that the combined activity of these SPATEs, together with other EAEC virulence factors, accounts for the increased uptake of Stx into the systemic circulation, resulting in the high rates of HUS (Fig. 4).

Although more studies are needed to explain the increased virulence, *E. coli* O104:H4 shows that mixed virulence profiles in enteric pathogens introduced into susceptible populations can cause serious outbreaks and have terrible consequences for infected people.

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I declare no conflicts of interest with regard to the manuscript.

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