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# *E. coli* O157:H7 virulence factors and the ruminant reservoir

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## Abstract

**Purpose:** This review updates recent findings about *E. coli* O157:H7 virulence factors and its bovine reservoir. This Shiga toxin (Stx)-producing *E. coli* belongs to the Enterohemorrhagic *E. coli* (EHEC) pathotype causing hemorrhagic colitis. Its low infectious dose makes it an efficient, severe, foodborne pathogen. Although EHEC remains in the intestine, Stx can translocate systemically and is cytotoxic to microvascular endothelial cells, especially in the kidney and brain. Disease can progress to life-threatening hemolytic uremic syndrome (HUS) with hemolytic anemia, acute kidney failure, and thrombocytopenia. Young children, the immunocompromised, and the elderly are at the highest risk for HUS. Healthy ruminants are the major reservoir of EHEC and cattle are the primary source of human exposure.

**Recent findings:** Advances in understanding *E. coli* O157:H7 pathogenesis include molecular mechanisms of virulence, bacterial adherence, type three secretion effectors, intestinal microbiome, inflammation, and reservoir maintenance.

**Summary:** Many aspects of *E. coli* O157:H7 disease remain unclear and include the role of the human and bovine intestinal microbiomes in infection. Therapeutic strategies involve controlling inflammatory responses and/or intestinal barrier function. Finally, elimination/reduction of *E. coli* O157:H7 in cattle using CRISPR-engineered conjugative bacterial plasmids and/or on-farm management likely hold solutions to reduce infections and increase food safety/security.

### Keywords

*E. coli* O157:H7; Shiga toxin; type three secretion; hemorrhagic colitis; hemolytic uremic syndrome; inflammation

## INTRODUCTION

Shiga toxin (Stx)-producing *Escherichia coli* (STEC, also referred to as verotoxin-producing, VTEC) belong to one of six *E. coli* pathotypes associated with diarrhea (1). Enterohemorrhagic *E. coli* (EHEC) form a subset of STEC that cause hemorrhagic colitis.

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Motile, non-sorbitol-fermenting *E. coli* O157:H7 is the major EHEC serotype associated with severe food- and waterborne illness and has a low infectious dose (<700 CFU) (2–4). Healthy cattle are the main reservoir of *E. coli* O157:H7, but the bacterium is also found in fecal samples of many asymptomatic animals including goats, deer, sheep, pigs, turkeys, and other fowl (5). Undercooked or unpasteurized foods of bovine origin are the main source of human infection. Other important sources of transmissions are fresh fruits and vegetables contaminated with feces through irrigation or other agricultural practices, contaminated potable or swimming waters, or direct contact with animals or infected people (5, 6).

After ingestion, *E. coli* O157:H7 colonizes the lower intestine (7). Limited oxygen levels, low levels of magnesium, and nutrient availability, particularly of ammonia and ethanolamine, are host cues that enhance colonization and virulence of the pathogen (8–11). *E. coli* O157:H7 attaches to intestinal mucosal cells through attaching and effacing lesions (A/E) characterized with effacement of microvilli (12). Once an infection is established, Stx damages intestinal epithelia causing hemorrhagic colitis with abdominal tenderness and pain (6). If the disease progresses, Stx gains systemic access and is cytotoxic to microvascular endothelial cells of other organs, especially the kidney and brain. (3). Life-threatening hemolytic uremic syndrome (HUS) with hemolytic anemia, acute kidney failure, and thrombocytopenia may develop (6). Young children (<5 years old), the immunocompromised, and the elderly (>65 years old) are at the highest risk for HUS. Certain lineages, especially clade 8, of the O157:H7 serotype correlate with an increased probability of HUS development in children (13). Initial data suggest that lineage correlation with HUS may also exists for less vulnerable populations but are not yet fully identified (14). This review updates recent findings about *E. coli* O157:H7 virulence factors and its healthy bovine reservoir.

## SHIGA TOXIN

Stx is the main determinant of STEC virulence. Two clinically important Stx variants, Stx1 and Stx2, and their subtypes are identified (4). Severe infection correlates with Stx2, particularly Stx2a, and levels of toxin expression (15). Stxs are encoded on lambdoid phages integrated into the chromosome and their expression is coregulated with prophage induction (16). Phage subtype determines the amount of toxin expressed. The hypervirulent clade 8, associated with the highest HUS risk, carries the phage subtype with the highest Stx2 production (15–17). Antibiotic therapy or bacteriocins produced by other members of the intestine microbiota may initiate prophage induction and worsen the clinical outcome. Similarly, phage-susceptible commensal intestinal *E. coli* can indirectly amplify toxin production (18).

Stxs are cytotoxic enzymes with RNA N-glycosidase activity. Albeit antigenically distinct, Stx1 and Stx2 share enzymic action and are $AB_5$ toxins (a catalytically active subunit A and a homopentamer of B subunits). The B subunits mediate binding to the target cell receptor, glycolipid globotriaosylceramide (Gb3 or CD77), engulfment of the holotoxin, and its sequestration in extracellular vesicles (EVs) (19, 20). The catalytic A subunit recruits ribosomes by binding to the conserved elongation factor binding C-terminal domain of ribosomal P stalk proteins (21). Next, it depurinates a specific adenine base on 28S

rRNA, inactivating 60S ribosomal subunits, and halting protein translation (22). Stx1/Stx2 structural differences in both subunits changes toxin affinity to the cellular receptor or the ribosomes and may partially explain the increased potency of Stx2-producing *E. coli* O157:H7 to induce HUS (23, 24).

Mechanisms of Stx secretion, delivery to host cells, and distribution to extra-intestinal tissues remain unclear. Stx is released during bacterial cell lysis (17). *In vitro* studies also suggest that Stx is secreted in association with outer membrane vesicles (OMVs) and delivered to host cells by dynamin-dependent endocytosis (3). Neutrophil-assisted paracellular transmigration, transcellullar nonspecific macropinocytosis, and specific transcytosis, translocate Stx across the gastrointestinal epithelial barrier into underlying tissues (25–27). Translocated Stx interacts with blood cells, activates them, and induces release of EVs that contain sequestered Stx that can disseminate to distant tissues through the bloodstream (19, 28–30). Susceptibility of tissues and cells to Stx damage depends on the cell surface levels of the Gb3 receptor (31). Prominent levels of Gb3 on endothelial cells correlates with the amount of damage of renal and cerebral endothelial tissue during HUS (32).

Upon binding to Gb3, Stx induces membrane compression, long-membrane reorganization of lipid packing, and engulfment (33). Stx uptake utilizes both clathrin-dependent and -independent mechanisms (34, 35). Stx-containing endosomes are directed to the Golgi by retrograde intracellular transport bypassing late endosomal and lysosomal degradation. From there, Stx reaches the endoplasmic reticulum and the A subunit is released into the cytosol to target the ribosomes (35). Inhibition of Stx-Gb3 interactions and intracellular trafficking of the enzyme is the focus in ongoing development of therapies against Stx-mediated kidney failure (36–39).

## ADHERENCE to HOST CELLS

Adherence to host cells is a pivotal step for *E. coli* O157:H7 colonization. A chromosomal pathogenicity island, called the locus of enterocyte effacement (LEE), encodes the most significant bacterial adhesion factors, including the structural components of the type 3 secretory system (T3SS) and the effector proteins delivered by this system. These include intimin, regulators, and chaperons. Chemical (intestine metabolites, nutrient availability) and physical (adhesive force generated from bacterial adherence to epithelial cells and fluid shear generated by intestinal motility and transit) cues activate LEE-encoded virulence genes (8, 40, 41). This allows for colonic localization and relocation of the pathogen from the intestinal lumen to the surface of intestinal epithelial cells during colonization (41, 42). LEE-dependent interaction is specific and requires two bacterial proteins: intimin, present in the bacterial outer membrane, and Tir (translocated intimin receptor). Tir is delivered into the host cell via T3SS and inserts into the host cell plasma membrane. Upon Tir-intimin binding, another T3SS effector, EspFu, remodels actin to form a characteristic pedestal beneath the bacterium (43, 44). Distinctive A/E lesions result by destruction of nearby microvilli (effacement). Interestingly, this process is modified by intestinal commensals such as *Bacteriodes thetaiotamicron* and *Lactobacillus* strains. *Bacteriodes* strains modify the metabolic landscape by releasing proteases (45, 46) and *Lactobacillus* strains generate

a localized reduced environment (45–48). Hence, host-specific microbiota differences may contribute to distinct infection susceptibilities and disease outcome.

The initial contact of *E. coli* O157:H7 with enterocytes involves non-intimin adhesion factors acting in accordance with temporal suppression of flagella expression. Curli fimbriae-associated amyloid fibers, assembled on the bacterial surface, congribute to *E. coli* O157:H7 biofilm formation and attachment to both human and bovine cells (49–52). Curli expression and motility are conversely regulated by the PchE transcriptional factor. This suggests an important role of curli in the transition from motile cells to the cells associated and attached to the intestinal surface (51, 53, 54). The roles of other proteinaceous and non-proteinaceous molecular determinants of adhesion and colonization have been well characterized and reviewed (53, 55–58).

The requirement of intimin for both human infections and cattle colonization makes it a potential target for *in vivo* antimicrobial control using engineered CRISPR-cas9 (clustered regularly interspaced short palindromic repeats) expressing an intimin guide RNA (gRNA). Such systems introduce sequence-specific lethal double-strand breaks in the bacterial chromosome (59). Our laboratory and others engineered a broad host-range conjugative plasmid using a highly conserved 20 nucleotide region of intimin found in all EHEC and enteropathogenic *E. coli*. The rationale uses a bovine commensal *E. coli* carrying this plasmid to be fed to cattle as a probiotic. This strain will amplify the CRISPR-cas9 conjugative plasmid by lateral transfer broadly to the Gram-negative gastrointestinal tract (GIT) microbiome. In theory, all intimin-bearing strains will be specifically killed upon acquisition of this engineered conjugative plasmid. Mating *E. coli* CRISPR-cas9 kills EHEC wild-type strains in vitro, but not EHEC deleted for intimin, showing the system is specific. More interesting is that wild-type strains that survive exposure to the conjugative plasmid have mutations in the 20-nucleotide intimin target sequence. Because the sequence chosen for the gRNA is so highly conserved, mutations in this sequence, while conferring resistance to the antimicrobial CRISPR system, will likely result in attenuation or loss of intimin function *in vivo* [unpublished observations and (59)].

## TYPE THREE SECRETION SYSTEM

T3SS are molecular syringes used by Gram-negative bacteria to deliver effector proteins into host cells and hijack functions (45, 60). Detailed structural descriptions of EHEC T3SS and associated regulatory components are well described (4, 61, 62). T3SS needle complexes deliver eight LEE-encoded and numerous prophage encoded effector proteins into the host cell to promote intimate adhesion, cytoskeleton rearrangement, inhibition of phagocytosis, modulation of innate immunity and apoptosis (63, 64). T3SS functionality is defined by the ability of the bacterium to sense and respond to cues, host cell physiology, and cohabitating microbiota. Interestingly, the genes required for production of the Stx receptor Gb3 and other sphingolipids are also necessary for translocation of T3SS effectors into host cells (63). Efficient translocation via *E. coli* O157:H7 T3SS also depends on the host microbiota. Symbiotic *Bacteroides thetaiotamicron* secretes proteases that cleave the T3SS translocon. The cleavage enhances effector translocation and formation of A/E lesions on epithelial cells (45). This finding is a novel paradigm that provides a molecular mechanism to describe how

commensal species affect pathogenic processes. Table 1 summarizes the functions of major T3SS effector proteins in *E. coli* O157:H7.

## INFLAMMATION

The primary site of human *E. coli* O157:H7 infection is the epithelial lining of the terminal ileum and colon (114). Upon infection, epithelial cells secrete antimicrobial factors including proinflammatory cytokines: TNF-α, IL-1, and chemoattractants: IL-8, MIP1-α, MCP-1 (115). *E. coli* O157:H7 flagellin and LPS are the most potent *in vitro* inducers of inflammation acting via TLR5 and TLR4, respectively (116–118). Other factors inducing weaker responses are Stx and hemorrhagic coli pilus (119, 120). Activation of these pathways triggers inflammatory cell infiltration in the lamina propria and transmigration of inflammatory cells across the intestinal epithelium into the lumen. Uncontrolled liberation of pro-inflammatory factors results in tissue damage and impairment of the epithelial barrier, escalating inflammation. Inflammation response is controlled by NF-κB and mitogen-activated protein kinases (MAPKs) at both transcriptional and post-transcriptional levels. Molecular mechanisms of activation and signaling cascades are well described (115).

The outcome of robust inflammation, especially during early stages of infection, is pathogen clearance. *E. coli* O157:H7 has T3SS-dependent and -independent mechanisms to inhibit innate signaling pathways and downregulate inflammation (115). Highly specific and diverse roles of T3SS effectors in these processes are summarized in Table 1. Deacetylation of lipid A is a T3SS-independent mechanism to reduce TLR4 activation (121, 122). *lpxR*, encoding 3′-*O*-deacylase, is positively regulated by Pch and Ler, necessary for expression of LEE genes and timely co-activation during colonization. Hexaacylation to tetraacylation of lipid A reduces TLR4 stimulation, activation of p38 MAPK, phagocytosis, and phagosome maturation (122). In addition, Havira *et.al* recently suggests that Stx suppresses the cytosolic LPS sensing pathway in macrophages and reduces pyroptosis (120). This is in contrast to previous finding by Platnich et. al (123). Ambiguity on the role of Stx in cross talk between canonical and noncanonical proinflammatory responses warrants more investigation.

HUS-associated kidney and brain tissue damage is induced by Stx cytotoxicity and acute systemic inflammatory responses. Elevated biomarkers: lipocalin 2, IL-8, IL-10, and neopterin are observed in HUS; however, mechanisms of activation are not fully understood (124–126). *E. coli* O157:H7 infection activates tissue-resident macrophages that mediate CXCR2-dependent neutrophil recruitment and kidney injury (127). A recent study by Lee et. al shows that Stx-containing exosomes derived from peripheral blood mononuclear cells and macrophages have significantly increased proinflammatory effects on proximal tubular cells (29). Finally, activation of platelets by Stx and platelet-bound LPS via TLR-4 may contribute to thrombotic microangiopathy in target organs (118).

Several synthetic and natural compounds that alleviate *E. coli* O157:H7-induced inflammatory responses and/or intestinal barrier dysfunction are studied in animal models (128–131). In addition, DHEA, a critical metabolite in cholesterol metabolism, reduces inflammatory responses by blocking the activation of p38 MAPK and NF-κB pathways (132, 133). Proadrenomedullin N-terminal 20 peptide (PAMP), a potent hypotensive peptide

expressed in GIT epithelium, reduces damage of intestine mucosa and serum inflammatory cytokines in challenged mice (134, 135). Finally, the effect of selected microbiota species on tight junction maintenance to reduce/prevent GIT damage by *E. coli* O157:H7 are reported (136, 137).

## RUMINANT RESERVOIR

### *E. coli* O157:H7 carriage in cattle

Healthy ruminants are the major reservoir of EHEC and cattle are the primary source of human exposure. *E. coli* O157:H7 colonizes individual animals spordaically and traniently with varying durations. The bacteria predominately inhabit the colon and recto-anal junction mucosa (RAJ) (138, 139). RAJ colonization is highly associated with *E. coli* O157:H7 in bovine feces and fecal concentration correlates with herd prevalence and long term carriage (140–142). Strategies to control bovine *E. coli* O157:H7 shedding to prevent and reduce transmission in a herd includes: (i) vaccination with *E. coli* O157:H7 variants (LEE- and Stx-negative strains), (ii) protein supplements in feed, (iii), bacteriophage thearapies, (iv) pre- and probiotic treatments, (v) farm managment practices, and (vi) feed containing CRISPR-cas9-engineered phage or conjugative plasmids (59, 143–148).

Shedding of *E. coli* O157:H7 is widespread and transient (149). Conditions that enhance acquisition and subsequent clearance of this bacterium from the ruminant GIT are complex and poorly understood, involving the host, the microbe, and the environment. Among the environmental conditions, temperature appears to be an important factor. Cattle carriage is seasonal with elevated colonization during the summertime. This is epidemiologically import because *E. coli* O157:H7 human infections also increase during this period (150). Similarly, positive fecal samples increase during wintertime warm vs. cold periods (151). Interestingly, seasonal prevalence in cattle is not dependent on seasonal changes in intrinsic determinants such as hormone or intestinal microbiota fluctuations, but rather increased seasonal oral exposure to *E. coli* O157:H7 (152).

Transmission and persistence of *E. coli* O157:H7 in cattle herds depends on a small percentage of cattle called ‘supershedders’. These animals shed 100 to 1000 times more *E. coli* O157:H7 than average shedders (142, 153). Composition of GIT commensals differs between supershedders and nonshedders, suggesting that the RAJ microbiota may be influenced by or affect by *E. coli* O157:H7 (138, 154–157). *E. coli* O157:H7 regulates gene expression for assimilation of carbon and nitrogen, the stress response, and respiration to survive various GIT conditions (158, 159). This regulation allows succesful passage through the GIT, competiton for nutrients, and colonization (160). Understanding the complex interactions involving gene regulation, microbial competition, host response, and the RAJ environment requires further investigation (161).

### Colonization factors

EHEC in ruminants are commensals and do not cause disease. In contrast to humans, Stx is not cytotoxic in cattle because the GIT lacks the toxin receptor, Gb3. Stx is not required for colonization in cattle challenged by rectal application (162). However, Stx2a

may increase the efficiency of animal-to-animal *E. coli* O157:H7 transmission among orally challenged cattle (163). Clearance of *E. coli* O157:H7 from cattle correlates with cecal and distal colonic cell proliferation (164). Stx2 restricts regeneration and turnover of the GIT epithelium which may maintain *E. coli* O157:H7 in a herd (163). In addition, the role of complex molecular and cellular interplay of Stx, epithelial, and immune cells in bovine colonization needs more investigation (143).

RAJ colonization is mediated by LEE-dependent and LEE-independent adherence factors (142, 165–167). Expression of nearly all LEE genes is induced by rectal digesta and intimin-Tir interactions promotes *E. coli* O157:H7 colonization in cattle (158, 162, 168). LEE-independent adherence factors include genes located on plasmid pO157, (169), the O-antigen, the flagellar regulatory system (not functional flagella), fimbriae, and pili (49, 142, 169–174).

*E. coli* O157:H7 is generally considered a non-invasive, extracellular human pathogen colonizing mucosal surfaces. However, cellular internalization of some *E. coli* O157:H7 strains is observed in cattle and correlates with biofilm formation and bovine colonization persistence (175). This invasive phenotype (~5% of *E. coli* O157:H7 strains) is conferred by curli fimbriae that form extracellular amyloid structures and are induced by the bovine RAJ environment (49, 158).

Strain diversity and their persistence promote horizontal transfer of elements like Stx bacteriophage. This may lead to the emergence of new, potentially more pathogenic strains colonizing cattle (176). Combinatorial strategies using culture, serology, and PCR methods to identify STEC that pose a greater food safety threat are necessary (177).

## CONCLUSIONS

This review compiles the current understanding of the most prominent EHEC serotype, O157:H7. Stx type, expression levels, and systemic translocation are key predictors of disease severity. The intricate interplay of Stx, T3SS effectors, and the microbiome are key to understanding regulation of the host inflammatory response. Finally, the role of adherence factors such as curli, intimin and Tir in both the host and the cattle reservoir and their potential as an intervention target are discussed.

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**Key Points**

- *E. coli* O157:H7 is a virulent foodborne pathogen that is especially dangerous to those in the age extremes and the immunocompromised.
- Virulence factors include Stx, adherence molecules, T3SS and its effectors that cause A/E lesions in the GIT and systemic cytotoxicity.
- Control of inflammation is key to bacterial growth and host survival.
- Understanding the relationship between *E. coli* O157:H7 and cattle has and will continue to improve food safety.

**Table 1.**

Pathogenesis-related functions of T3SS effectors in *E. coli* O157:H7

| Effector | LEE-encoded | Pathogenesis-related function | Reference |
|---|---|---|---|
| Tir | + | Receptor for intimin; mediates adhesion to host cells and formation of A/E lesions; inhibits TAK1 activation and proinflammatory cytokine production | (65, 66) |
| Map | + | RhoGEF mimic; induces actin reorganization and formation of filopodia; promotes colonization of the small intestine | (67, 68) |
| EspF | + | Promotes apoptosis; inhibits *cis*-phagocytosis; disrupts tight junction; inhibits internalization into epithelial cells; coordinates membrane remodeling; suppresses inflammation; promotes colonization of the intestinal tract | (68–74) |
| EspG | + | Inhibits phagocytosis; disrupts protein secretion; promotes pedestal formation and colonization of the small intestine | (68, 75–77) |
| EspH | + | RhoGEF inhibitor; facilitates elongation of actin pedestals; promotes colonization of the intestinal tract | (68, 78, 79) |
| EspZ | + | Regulates T3SS secretion; reduces infection-associated cytotoxicity; regulates formation of actin pedestals | (80) |
| EspB* | + | Reorganizes actin filaments | (81) |
| NleA/EspI | - | Inhibits cellular protein secretion; reduces formation of NLRP3 inflammasome | (82, 100) |
| NleB | - | Glysosyltransferase; inhibits NF-κB-dependent host innate immune responses | (83–85) |
| NleE | - | Methyltransferase; inhibits NF-κB activation | (86) |
| NleF | - | Inhibits apoptosis; affects intracellular trafficking | (87–89) |
| NleH1 | - | Serine/threonine kinase; suppresses expression of a subset of NF-κB target genes | (90–92) |
| NleH2 | - | Serine/threonine kinase; mildly stimulates NF-κB and MAPK activity | (64, 92) |
| NleC | - | Zinc metalloprotease; suppresses NF-κB and MAPK activation and inhibits proinflammatory cytokine production | (86, 93, 94) |
| NleD | - | Zinc metalloprotease; inactivates MAPK | (95) |
| NleL | - | E3 ubiquitin ligase; disrupts JNK pathway and NF-κB signaling; modulates pedestal formation | (96–98) |
| NleGs | - | E3 ubiquitin ligases | (99) |
| EspJ | - | ADP ribosyltransferase; inhibits *trans*-phagocytosis | (101–103) |
| EspL2 | - | Modifies host cell membrane; supports efficient colonization | (104) |
| EspK | - | Increases persistence of O157:H7 in the intestines of orally inoculated calves | (105) |
| EspM1<br>EspM2 | - | Guanine exchange factors; induce formation of stress fibers and repress formation of actin pedestals | (106–108) |
| EspFu/TccP | - | Facilitates actin polymerization, pedestal formation, and cell-to-cell transmission | (44, 109–111) |
| YspY1 | - | Interacts with proteins involved in apoptosis and cell cycle regulation | (101) |
| YspY3 | - | Localizes to and extends pedestal region | (112) |
| Cif | - | Induces cell cycle arrest | (113) |

(+)LEE-encoded effector

(−)non-LEE-encoded effector

(*)EspB is classified as a translocator and effector