



New Virulence Factors of Enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 in Dairy Food Processing

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Abstract

Enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 is well-characterized as an important food-borne pathogen worldwide and causes human diseases such as diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome (HUS) by producing shiga-like toxin (Stx). It has been reported that a number of dairy foods, including cheese, can act as the source of EHEC O157:H7 infections. In addition to the toxicity of Stx, recently it has been indicated that EHEC O157:H7 possesses virulence factors related to attachment, quorum sensing, and biofilms. Moreover, these novel virulence factors might become critical points to be considered in the future production of food derived from animals. Here, we review the evidences that support these insights on new virulence factors and discuss the potential mechanisms mediating the pathogenesis of EHEC O157:H7 in the dairy food industry.

Keywords: dairy foods, EHEC O157:H7, attachment, quorum sensing, biofilms, virulence

Enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 is a high-virulence microorganism found in ground beef and milk products. Therefore, ruminants serve as critical reservoirs for the transfer of this pathogen to humans (Griffin *et al.*, 1991). Through these routes, EHEC O157:H7 becomes a causative agent of hemorrhagic colitis (HC), hemolytic uremic syndrome (HUS), and thrombocytopenic purpura (TTP). Generally, symptoms for HC include bloody diarrhea and stomach cramps. Similarly, HUS causes bloody diarrhea, acute renal failure, and kidney failure (Ruggenti *et al.*, 1998). Furthermore, TTP has been shown to result in neurological disorders

and kidney failure (Ruggenti *et al.*, 1998).

Notably, EHEC O157:H7 has been associated with a number of food-borne outbreaks (Hudson *et al.*, 1997). EHEC O157:H7 leads to a characteristic pathology in large intestinal epithelial cells known as attaching and effacing (A/E) lesion. The Locus of Enterocyte Effacement (LEE) region is a pathogenicity island that encodes specific proteins involved in the formation of A/E lesions (Sperandio *et al.*, 2002). Since 1982, food-borne outbreaks of EHEC O157:H7 have occurred in several areas in Japan, Europe, and the United States (Bean *et al.*, 1996).

A variety of other foods, such as ready-to-eat fruit, salad, and apple cider have also been involved in outbreaks of EHEC O157:H7 as well as animal recourse foods from

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bovine (Mead *et al.*, 1999). Typically, human diseases due to EHEC O157:H7 are linked with the consumption of food or water that has been contaminated with virulent EHEC O157:H7-containing bovine feces. The ability of EHEC O157:H7 to survive in a low pH environment, as well as in refrigerated products, makes it an organism of concern especially because the infective dose for EHEC O157:H7 is very low (less 100 cells; Spika *et al.*, 1986). In 2003, 6,584 samples of ground beef products were analyzed and 20 samples (0.3%) were found to be positive for EHEC O157: H7 (FSIS, 2004). Fortunately, human diseases resulting from EHEC O157:H7 significantly declined in 2003, which otherwise was steady from 1996 to 2002 (MMWR, 2004). This was probably the result of an FSIS notice issued in October 2002 for the manufacturers of raw ground beef products that they must reassess their Hazard Analysis Critical Control Points (HACCP) plans regarding this pathogen (MMWR, 2004). Presently, beef processing plants do not distribute lots of raw ground beef unless microbiological tests performed at the plant give negative results for EHEC O157:H7 (MMWR, 2004).

Milk and dairy foods are equally important and potential sources for human EHEC infections. In 1999, over 11% of the total number of reported cases of infection caused by EHEC O157:H7 in England were due to dairy products (Vernozy-Rozand *et al.*, 2005). Moreover, recent outbreaks of EHEC O157:H7 occurred among consumers of five Southwestern states in the United States in 2010 with a total of 41 patients. Notably, a majority of patients reported consuming complimentary samples of aged Gouda type cheese produced with raw-milk (McCollum *et al.*, 2012). Therefore, given that many regional or local cheese specialties throughout Europe are manufactured from unpasteurized milk, there is growing concern that these products may pose a threat for transmitting pathogens such as EHEC O157:H7 to the consumer. Here, we will review the novel virulence factors of EHEC O157:H7 in regards to dairy food safety, focusing on their specific characteristics and interactions with the host as well as target genes clusters.

1. Shiga-like toxin and EHEC virulence

It was well-established that shiga-like toxin (Stx) produced by EHEC O157:H7 is the main virulence factor for human EHEC infections. Interestingly, EHEC O157:H7 strains have acquired bacteriophages encoding two component AB-5 toxins.

Moreover, there are several variants of shiga toxins with Stx1 being virtually identical to the enterotoxin produced by *Shigella dysenteriae* type 1 (Smith *et al.*, 2002). On the other hand, multiple variants of Stx2 have been characterized including Stx2c, d, e, f, and g. Among them, Stx2 and Stx2c have significantly strong correlation with the human infection (Bidet *et al.*, 2005). Structural studies of Stx1 and Stx2 have identified key points to distinguish the differences in their structure (Fraser *et al.*, 2004).

Importantly, the five B-subunits direct cell-binding specificity and allow translocation of the A-subunit into the cell. In the case of Stx1 and Stx2/2c, the B-subunit binds to the glycolipid receptor Gb3/CD77 (Lingwood, 1996), driving the entry of the A-subunit, which is then cleaved to produce an A1 peptide with *N*-glycosidase activity that represses protein synthesis via cleavage or degradation of the 28S ribosomal RNA. One possibility why cattle colonized with EHEC O157:H7 do not exhibit clinical symptoms compared to the potentially devastating impact due to colonization in humans is the difference between the Gb3 receptor distribution in humans and cattle (Pruimboom-Brees *et al.*, 2000). Gb3/CD77 exists on human endothelial cells (which line blood vessels), but this is not the case in cattle. Thus, any toxin reaching these cells does not have the same hemorrhagic consequences in both hosts. Significantly, Gb3/CD77 is present on human kidney glomerular cells, thus, causing severe damage to this organ as a critical outcome of human EHEC infection, such as HUS (Adler and Bollu, 1998). In humans, trafficking of Stx from the site of infection in the gastrointestinal (GI) tract occurs, at least in part, by associating with granulocytes that infiltrate in response to the infection (te Loo *et al.*, 2001). Conversely, cattle do have some Gb3/CD77-positive cells that are present in intestinal crypts and this possibly prevents the entry of Stx (Hoey *et al.*, 2002). Therefore, while both humans and cattle are colonized by EHEC O157, the difference in Gb3 receptor distribution in the hosts acts as the key determining factor for severe outbreaks in human.

2. Attachment and EHEC virulence

As stated, EHEC O157:H7 colonizes the GI tract and produces a potent Stx, resulting in HC and HUS in a significant proportion of infected people (Nataro and Kaper, 1998). Interestingly, the EHEC O157:H7 causes A/E lesion, a histopathological and characteristic formation on the intestinal

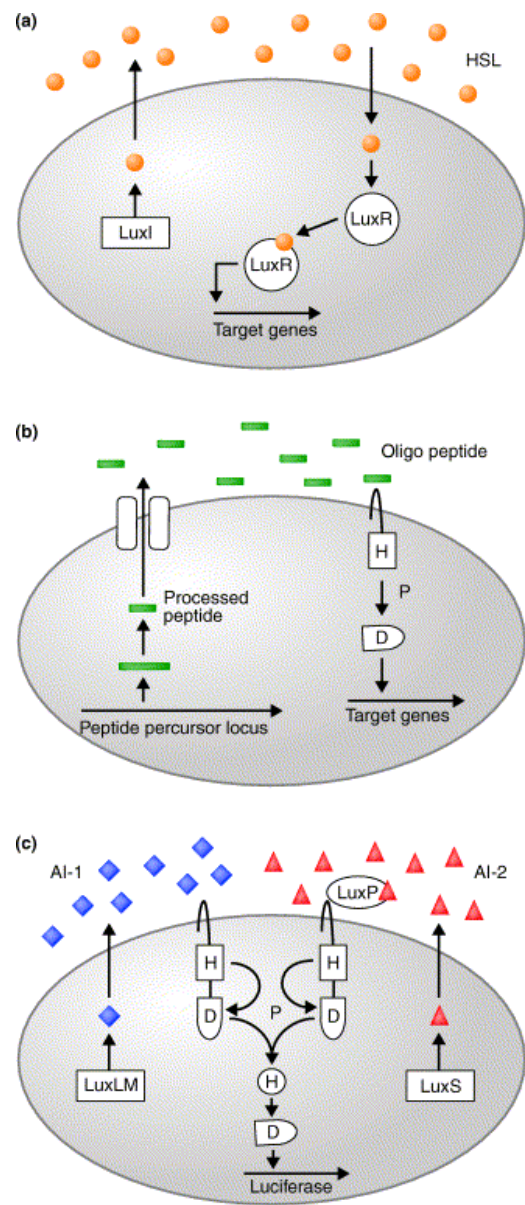
epithelial cells. This lesion is characterized by the destruction of microvilli and the rearrangement of the cytoskeleton to form pedestal-like structures that cradle the bacteria individually.

The gene cluster involved in the formation of the A/E lesion exists on a pathogenicity island termed the Locus of Enterocyte Effacement (LEE) region (Elliot *et al.*, 1998). The LEE region contains (1) *sep* and *esc* genes encoding a type III secretion system (Jarvis *et al.*, 1995); (2) the *eae* gene encodes an adhesion protein, named intimin, that is responsible for the initial bacterial attachment to the surface of epithelial cells (Jerse and Kaper, 1991); (3) the *espABD* genes that encode extracellular proteins involved in the type III secretion system. This includes EspABD, which is recognized as an important component that facilitates pore formation on the epithelia cell surface and thus completes the delivery system of proteins from the EHEC O157:H7 into the cytoplasm of the host cell (Knutton *et al.*, 1998); (4) the *tir* gene encoding the translocated intimin receptor, a partner for intimin (Kenny *et al.*, 1997); and (5) the *ler* gene (LEE-encoded regulator) that encodes a positive regulator of LEE genes (Mellies *et al.*, 1999). Based on the sequence analysis results of the conserved sequence of LEE for EHEC O157:H7, 41 open reading frames (ORFs) were identified that are highly conserved at the levels of DNA and protein for the type III secretion genes, but were more complex compositions for the *esp*, *eae*, and *tir* genes partially. In addition, the majority of the LEE genes were described to be in five primary polycistronic operons identified as LEE1 through LEE4 and *tir* (Elliot *et al.*, 1999).

3. Quorum Sensing and EHEC virulence

Quorum sensing (QS) system is a critical cell-to-cell signaling mechanism that refers to the ability of bacteria to sense or respond to chemical signal molecules, known as autoinducers (AIs), under culture conditions in a dose dependent manner. Importantly, these AIs can be produced and detected by bacteria of the same, or different, species. When an AI concentration reaches a concentration-dependent threshold, the neighboring microorganisms sense and respond to this chemical molecule, resulting in an alteration of their specific gene expression. This characteristic phenomenon allows bacteria to act as a collective unit, as opposed to individual cells performing individual functions.

Until now, the QS circuit has been a well-established feature



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Fig. 1. Several typical QS systems among bacteria. (a) In most Gram-negative bacteria, LuxI-type enzymes catalyze the formation of species-specific HSLs (circles), which are detected by LuxR-type transcriptional regulators. (b) Oligopeptides (bars) mediate quorum sensing in Gram-positive bacteria, and two-component phosphorylation cascades are used for signal transmission. (c) *V. harveyi* quorum sensing circuit. LuxLM synthesizes AI-1 (diamonds) and LuxS is required for production of AI-2 (triangles). LuxP is the periplasmic AI-2-binding protein. Signal transduction occurs by two-component phosphorelay. In (b) and (c), the conserved phosphorylation sites on the two-component proteins are indicated as H (histidine) and D (aspartate). P denotes phosphorylation (adapted from Xavier and Bassler, 2003).

of the bioluminescent bacterium *Vibrio harveyi* (Fig. 1). *V. harveyi* makes a typical Gram-negative-type homoserine lactone (HSL) AI, identified as AI-1. Additionally, *V. harveyi* produces and detects a second AI, identified as AI-2. This alternate type of AI is produced by a remarkably wide variety of Gram-negative and Gram-positive bacteria, and in every case production requires a protein called LuxS. Unlike for AI-1, the biosynthetic pathway and chemical intermediates in AI-2 production, and possibly the AI-2 molecule itself, are identical in all AI-2-producing bacteria studied to date. These findings have led to the important possibility that AI-2 is a 'universal' signal molecule, which functions in inter-species bacteria-to-bacteria communication (Xavier and Bassler, 2003) as compared to AI-1, which functions in intra-species signaling. The structure of *V. harveyi* AI-2 was recently determined; the molecule is a novel furanosyl borate diester with no similarity to other AIs. Interestingly, EHEC O157:H7 also produces AI-2 in some environmental conditions. Notably, EHEC O157:H7 could generate another AI named AI-3, whose synthesis depends on the presence of LuxS. These uncharacterized AIs may indicate the possibility of inter-kingdom communication molecules between the bacteria and host signaling systems (Sperandio *et al.*, 2003).

For bacterial pathogenesis, QS has been suggested to aid the disease process by allowing bacteria such as EHEC O157:H7 to appropriately modulate expression of virulence factors that might stimulate an immune response before the infection has progressed and occurred. As described by De Kievit *et al.* (2001), utilizing the mechanism of bacterial communication via QS, food-borne pathogens can accumulate a high cell density before virulence factors are fully expressed, and in so doing, the bacteria are able to make a united assault and produce an abundance of virulence factors to overwhelm the host's defense mechanisms. This simple concept may be suitable to explain the important role of QS in extra-intestinal infections where the pathogen encounters low levels of competing microbiota. On the contrary, under the complex microbial populations of the GI tract, QS may play other roles in illnesses. As described below, QS has been hypothesized to signal transcription of EHEC O157:H7 virulence factors once they arrive in the intestinal tract, as well as to signal transcription of *Vibrio* environmental survival components following expression of virulence factors but before environmental release via fecal samples. Therefore, although

QS has been described in numerous food-borne pathogens of the GI tract, in many cases the role of QS in the pathogenesis of the outbreaks caused by these organisms is still unclear. However, a number of reports for QS in enteric pathogens have been reported that have yielded new insights into the mechanisms of outbreaks and illnesses (Table 1).

The recent discovery of QS in human pathogens has led to considerable interest in developing new concepts for therapeutic applications to interfere with these signaling molecules involved in bacteria-bacteria communications. Indeed, instead of antibiotics that kill pathogenic bacteria directly, a compound that inhibits the QS mechanism could be employed to suppress the specific regulation of the virulence genes responsible for the food-borne disease. Such a QS-based approach is particularly promising to combat increased resistance of some bacteria to conventional antibiotics, and encouraging results in animal models have been obtained with *P. aeruginosa* (Camara *et al.*, 2002) and *S. aureus* (Mayville *et al.*, 1999). However, the development of anti-QS therapy may primarily influence normal microbiota, such as the high numbers of commensal microorganisms present in the GI tract.

4. Biofilms and EHEC virulence

A biofilm is a sessile-type microbial community consisting of cells that are irreversibly attached to a surface via an extracellular polymeric matrix produced by the cells (Donlan and Costerton, 2002). Currently, there is a generalized model for biofilm formation and development. First, microorganisms attach to an abiotic (i.e. glass, steel, or plastic) or biotic surface (i.e. teeth, mucus layer); second, microorganisms transform from planktonic status to a tightly immobilized status; third, the microcolony is formed and a continuous consortium of microorganisms build a three dimensional architecture of biofilm, which is considered a mature biofilm; finally, biofilms release and disperse cells into the environment to attach to new locations (Branda *et al.*, 2005). The most apparent difference between biofilm and planktonic styles of microorganisms is the mobility and architecture of the microorganisms. Importantly, the biofilm has distinct architectural features (Stoodley *et al.*, 2002), such as water channels that allow the diffusion of necessary nutrients, including oxygen and carbon for cell growth.

Under environmental conditions, many bacteria thrive better

Table 1. Summary of quorum sensing systems described in enteric bacteria (adapted from Kaper and Sperandio, 2005)

| Bacterial species | QS system | Autoinducer(s) | Regulated phenotype(s) |
|------------------------------|----------------------|-------------------------------------|--------------------------------|
| EHEC and EPEC | Qse/LuxS | AI-3 | TTS, flagella, and motility |
| | Lsr/LuxS | AI-2 (<i>R</i> -THMF) ^a | AI-2 uptake by Lsr |
| | SdiA | AHLs (not self-produced) | ? |
| <i>Salmonella</i> sp. | Qse/LuxS | AI-3 | ? |
| | Lsr/LuxS | AI-2 (<i>R</i> -THMF) | AI-2 uptake by Lsr |
| | SdiA | AHLs (not self-produced) | Rck |
| <i>Vibrio cholerae</i> | System 1 (CqsA/CqsS) | CAI-1 | TCP, CT, HA protease, biofilm |
| | System 2 (LuxS) | AI-2 (furanosyl-borate diester?) | TCP, CT, HA protease, biofilm |
| EAEC | Qse/LuxS | AI-3 | ? |
| | Lsr/LuxS | AI-2 (<i>R</i> -THMF) | ? |
| | SdiA | AHLs (not self-produced) | ? |
| | Other? | ? | AggR-regulated virulence genes |
| <i>Enterococcus faecalis</i> | Cyls | CyILs | Cytolysin production |
| | FsR | Peptide | Gelatinase, serine protease |
| | LuxS | AI-2 | ? |
| <i>Yersinia</i> sp. | Qse/LuxS | AI-3 | ? |
| | Lsr/LuxS | AI-2 | ? |
| | YenR/I | AHLs | ? |
| | YpsR/I | AHLs | Flagella and motility |
| | YtbR/I | AHLs | Flagella and motility |
| <i>Shigella flexneri</i> | Qse/LuxS | AI-3 | Expression of VirB? |
| | Lsr/LuxS | AI-2 | Expression of VirB? |
| <i>Campylobacter jejuni</i> | LuxS | AI-2 | Motility |
| <i>Vibrio vulnificus</i> | LuxS | AI-2 | Protease, hemolysin |
| | SmrC | ? | Protease, hemolysin, virulence |
| <i>V. parahaemolyticus</i> | LuxMR (OpaR) | AI-1 (AHL?) | TTS |
| | LuxPQS | AI-2 (furanosyl-borate diester?) | ? |

^a *R*-THMF, (2*R*,4*S*)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran.

within biofilms rather than as free-living planktonic cells (Watnick and Kolter, 2000). Biofilms are found in diverse locations such as human dental plaques and the GI tract (Probert and Gibson, 2002), leaves and root surfaces of plants (Ramey *et al.*, 2004), biocorrosion or biofouling of industrial surfaces (Videla and Herrera, 2005), and food processing equipment and facilities (Kim *et al.*, 2006). Several advantages for living in biofilms are as follows: (1) biofilm cells can survive with limited nutrient resources, due to decreased bacterial growth rates in biofilms; (2) cells in the biofilm are less susceptible to harsh conditions, especially exposure to antibiotics and other antimicrobial agents, due to the

extracellular polymeric matrix of biofilms (Mah and O'Toole, 2001); (3) for pathogens, cells within a biofilm have a higher gene transfer rate than planktonic cells because they are in close contact with other cells (Hausner and Wuertz, 1999).

Some researchers have reported differential gene expression between sessile and planktonic cells (Resch *et al.*, 2005; Schembri *et al.*, 2003). Early evidence of differential gene expression within a bacterial biofilms using DNA microarray technology, suggested that the expression of up to 38% of the *E. coli* bacterial genome might be affected by biofilm formation (Prigent-Combaret *et al.*, 1999), although other

studies reported only a small proportion of the genome was affected (1 to 15%) (Schembri *et al.*, 2003; Beloin *et al.*, 2004). These studies have generated hope that it might be possible to identify a common universal gene-expression pattern within bacterial biofilms. In addition, our two dimensional gel electrophoresis showed only 26 genes (5.8%) were differentially up- or down-regulated under biofilm conditions among the 450 detected spots, with some genes encoding periplasmic oxidative defense proteins including copper, zinc superoxide dismutase (SodC), and thiol peroxidase (Tpx) were strongly induced in the EHEC O157:H7 biofilm culture conditions as compared to the planktonic cells (Kim *et al.*, 2006) (Fig. 2).

Up to now, many approaches have been proposed to prevent bacterial biofilm formation. The most extensively studied is material surface-coating methods. An example is a photocatalytic titanium dioxide (TiO₂) surface, which has potential as a self-cleaning technology to reduce the adhesion of *Deinococcus geothermalis* cells (Raulio *et al.*, 2005). Also, antibiotic coating systems are effective in interfering with

biofilm formation, such as the application of ciprofloxacin-releasing polymer in blocking *Staphylococcus epidermidis* biofilms (Niemela *et al.*, 2006). Application of antimicrobial compounds has also been employed in industry. For example, weak hydrogen peroxide-based disinfectants have had success in inhibiting biofilm formation in dental unit waterlines (Tuttlebee *et al.*, 2002). Physical methods using ultrasound could improve the penetrations of gentamycin into biofilms of *P. aeruginosa* and *E. coli* (Carmen *et al.*, 2004). Recently, a research report showed that an efficient way to prevent *P. aeruginosa* biofilm formation is by releasing ciprofloxacin from specific coatings materials by ultrasonic control (Norris *et al.*, 2005). Other novel approaches are also being examined, such as utilizing bacteriophages to degrade extracellular polymeric matrix (Hughes *et al.*, 1998). Interestingly, (5Z)-4-bromo-5-(bromomethylene)-3-butyl-2(5H)-furanone (furanone), from the marine alga *Delisea pulchra*, also inhibits *E. coli* biofilm formation without inhibiting its growth (Ren *et al.*, 2005). Moreover, released exopolysaccharide (r-EPS) isolated from *Lactobacillus acidophilus* could prevent biofilm formation by a wide range of Gram-negative and -positive pathogens including EHEC O157:H7 (Kim *et al.*, 2009). These numerous applications may lead to the development of novel food-grade materials for selective control of microbial biofilms.

In conclusion, the presence of EHEC O157:H7 in milk used in the production of dairy foods including cheese poses a risk to consumers, even though it is present in low amounts. Therefore, it is imperative to manufacture dairy foods with highest EHEC O157:H7 bacteriological safeguards by taking these new virulence factors into consideration.

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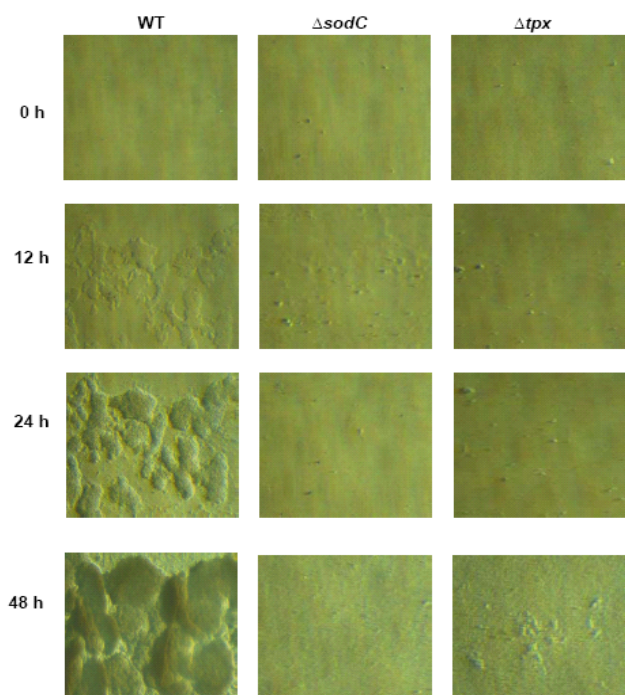


Fig. 2. Quantification of EHEC O157:H7 biofilm formation using glass capillary tubes and a continuous flow system. The biofilms developed by wild-type, *sodC*, and *tpx* mutants were observed at different time points, as indicated (adapted from Kim *et al.*, 2006).

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