Scientific Research

A Survey of *Escherichia coli* O157:H7 Virulence Factors: The First 25 Years and 13 Genomes

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Abstract

Escherichia coli O157:H7 is a human pathogen that was first identified from a foodborne outbreak in 1982, and in the 25 years that followed, many new strains were identified and emerged in numerous outbreaks of human disease. Extensive research has been conducted to identify virulence factor genes involved in the pathogenesis of *E. coli* O157:H7 and many genome sequences of *E. coli* O157:H7 strains have become available to the scientific community. Here, we provide a comprehensive overview of the research that has been conducted over the first 25 years to identify 394 known or putative virulence factor genes present in the genomes of *E. coli* O157:H7 strains. Finally, an examination of the conservation of these 394 virulence factor genes across additional genomes of *E. coli* O157:H7 is provided which summarizes the first 25 years and 13 genomes of this human pathogen.

Keywords

1. Introduction

Escherichia coli O157:H7 is a human pathogen that was first identified from a foodborne outbreak involving

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ground beef in Michigan, USA in 1982. This organism sickened a number of individuals and led to more severe conditions such as Hemolytic Uremic Syndrome (HUS) and renal kidney failure. The O157:H7 serotype of *E. coli* was the first strain assigned to a pathotype that came to be known as Enterohaemorrhagic *Escherichia coli* (EHEC) due to symptoms of sickened individuals, which typically include bloody diahaerria. Since the initial identification of an O157:H7 strain in a hamburger outbreak in 1982 that sickened 47 individuals with 33 requiring hospitalization [1], numerous strains of *E. coli* O157:H7 have emerged in foodborne outbreaks ranging from various meat products (although typically ground beef) to organic produce (including various leafy greens such as lettuce and spinach). In 1996, the largest outbreak occurred to date occurred in Sakai City, Japan which sickened 5,000-12,680 people, requiring 398 - 425 hospitalizations, and mainly involved school children [2] [3] [4]. The implicated food in the Sakai City outbreak was contaminated radish sprouts. Surveillance of *E. coli* O157:H7 has since increased dramatically and scientific efforts have intensified to understand the virulence determinants of this pathogen, and to implement control parameters in efforts to reduce the incidence of *E. coli* O157:H7 outbreaks. Despite these efforts, outbreaks due to EHEC still occur.

Michigan outbreak strain ATCC 43895, also called EDL933, was the first *E. coli* O157:H7 genome to be sequenced [5]. Shortly thereafter, the Sakai City strain was also sequenced (RIMD) [6]. Since these hallmark accomplishments, scientists have sought to identify virulence factors through genome comparisons, to understand variations that relate to the observed differences in occurrence and virulence, and to understand the overall evolution of this pathogen. With the advent of whole genome comparisons, many interesting differences have been brought to light. Recently, a large number of additional strains belonging to the O157:H7 serotype have become publicly available. New strains and their associated genomes have emerged nearly monthly from foodborne outbreaks and also from community-associated outbreaks involving locales such as swimming pools, petting zoos, and daycare centers. In this work, we have conducted a thorough examination and identified known and putative virulence factors in the published literature for *E. coli* O157:H7 and insight derived from orthologous genes from other pathogenic members of the Enterobacteriacaea, as this family is thought to have derived from a common ancestor. We attempt to provide a current update of virulence factors and genome variations with a goal of understanding variations that correspond to epidemiology and geographical differences.

Virulence factors can be broadly subdivided as "known" or "putative" (**Figure 1**). Since this human pathogen does not evoke the same disease responses in other mammalian hosts, no ideal experimental animal model exists, and therefore much of the insight is derived using mouse models, and other various assay methods. The types of virulence factors that have multiple testable methods are involved in attachment and adhesion, and also for puta-
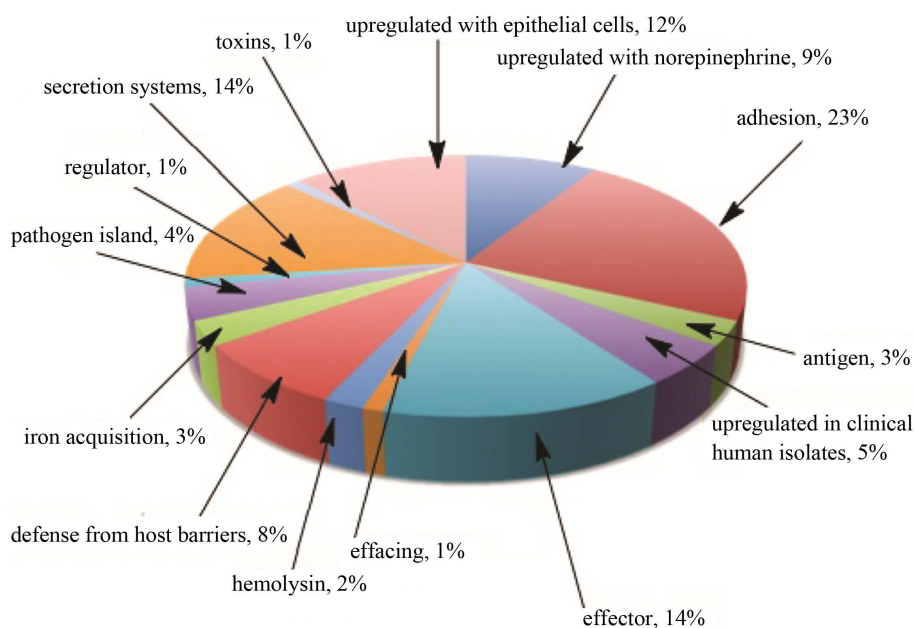

Figure 1. Known or putative virulence factors for *E. coli* O157:H7 separated into categories (n = 394 genes).

tive effectors molecules via protein translocation into human tissue cell cultures. Here, we provide a summary of all recently derived insight for virulence factors genes and those genes associated with the organism's ability to cause disease in the host. All annotations from this comprehensive literature survey are accessible in the ASAP database, and represent one of the most thorough overviews of *E. coli* O157:H7 from 1982 through 2007 which represents the first 25 years of research since this organism was first recognized as a human pathogen from an outbreak that occurred in 1982.

Overall this approach to identify candidate putative or known virulence factors has dramatically increased the numbers of identified genes to include 394 coding sequences, and 5 pseudogenes, representing ~7.3% (394/5401) of the complete genome of the EHEC strain (EDL933) (Supplemental data 1). The most abundant categories of these are genes involved in adhesion or effector molecules, since numerous experimental methods exist for assays using human tissue cell lines (Supplemental information 1). This is a dramatic increase from the previous review of Spears *et al*. [7] that provided a summary of *E. coli* O157:H7 virulence genes. It should be noted that some of the genes identified in this work as putative virulence factors, are involved in regulation and metabolism and have used recent experimental gene expression data from comparisons of human clinical isolates and isolates of bovine origin. It may be that some genes encode proteins that may have multiple functions, pleiotrophic effects, or increase the competitive abilities of the organism to compete with intestinal flora in the host, and are therefore included. There is a great deal of work that remains to fully understand the ability of *E. coli* O157:H7 to cause human disease, and this work provides new additional insight about candidate virulence genes and the variation and conservation of these 394 genes in the 13 new genomes that have been sequenced through 2007.

2. Colonization Factors

"To adhere is essential"—author unknown.

2.1. Adhesion

The adherence ability of *E. coli* O157:H7 is pivotal to its transmission from fresh produce items and cattle to its eventual human host. The ability to adhere to the human intestine is an early component of EHEC infection. Adhesion is one category of virulence factors that has made much experimental progress, due to the ability to conduct adherence assays that determine if a mutation strain of a certain gene affects the ability of the organism to adhere to human tissue culture cell lines such as epithelial intestinal cells. The most obvious genes involved in adherence are those that encode the machinery to produce fimbria. In addition to fimbrial genes, there are many genes that have been implicated to be involved in adherence, yet in many of these cases it is undetermined how these genes are involved in the process of adherence. These genes are included in this review since they play a role in the EHEC's ability to adhere to cell tissue cell lines and are therefore identified as virulence factors or putative virulence factors.

There are 16 predicted gene clusters or operons in the genome of *E. coli* O157:H7 strain EDL933 that contain genes either known or predicted to be involved in fimbrial assembly [8] (**Table 1**). It is unknown whether genes in different loci can function with gene products of other clusters/operons to produce fimbria, therefore this review effort is not limited to loci that encode a complete fimbrial operon, and also includes standalone genes identified based on protein sequence similarity. Of the 16 gene adhesion clusters/operons, the work of Low *et al*. [8] used promoter fusion assays and demonstrated that only four fusions (loc4, loc7, loc8, and loc9) demonstrated promoter activity above the baseline level in laboratory growth conditions. Yet the other gene clusters/operons should not be overlooked, since many of the genes contained in these loci have experimental evidence for gene mutant phenotypes demonstrating a role in adhesion. Loci that contain these genes are loc5, loc7, loc12, loc13, loc14, MAT, and *ppdD* (**Table 1**). In addition, loc7 was the only adherence gene cluster that had both promoter activity demonstrated experimentally, and was found to be up-regulated in clinical human isolates of EHEC compared to isolates of bovine origin. It is unclear what the precise role of the loc7 operon may serve for EHEC pathogenesis in humans, as this orthologous operon is conserved in many other *E. coli* genomes including the two non-pathogenic *E. coli* K-12 strains, *Salmonella* spp., and *Shigella* spp. In EHEC strains genes contained in loc7 have the most supporting experimental evidence to indicate a possible point of control to target for future prevention of the ability of O157:H7 to attach and adhere to the intestinal tract of humans. Collectively, 10 of the 16 adhesion loci have at least one line of experimental evidence to indicate that they are expressed or

Table 1. Known or putative virulence factor genes involved in attachment and adhesion of *E. coli* O157:H7 EHEC strains.

Gene cluster or operon	Product	Reference
(*loc*1)		
Z0020	predicted fimbrial-like adhesin protein	[8]
Z0021	predicted protein	[8]
Z0022	putative usher protein	[8]
Z0023	putative chaperone protein	[8]
Z0024	putative type-1 fimbrial protein	[8]
YadCKLMhtrEecpDyadN (*loc*2)		
yadC	putative fimbrial protein	[8]
yadK	putative fimbrial protein	[8]
yadL	putative fimbrial protein	[8]
yadM	putative fimbrial protein	[8]
htrE	putative fimbrial usher protein	[8]
ecpD	putative fimbrial chaperone protein	[8]
yadN	putative fimbrial protein	[8]
SfmACDHFfimZ (*loc*3)		
sfmA	predicted fimbrial-like adhesin protein	[12] [13]
sfmC	pilin chaperone, periplasmic	[12] [13]
sfmD	predicted outer membrane export usher protein	[12] [13]
sfmH	predicted fimbrial-like adhesin protein	[12] [13]
sfmF	predicted fimbrial-like adhesin protein	[12] [13]
fimZ	predicted DNA-binding transcriptional regulator	[8]
ybgPQDgltA (*loc*4)		
ybgO	predicted fimbrial-like adhesin protein	[8]
ybgP	predicted assembly protein	[8]
ybgQ	fimbrial biogenesis outer membrane usher protein (pseudogene)	[8]
ybgD	predicted fimbrial-like adhesin protein	[8]
gltA	citrate synthase?	[8]
ycbQRTUVF (*loc*5)		
ycbQ	predicted fimbrial-like adhesin protein	[8]
ycbR	predicted periplasmic pilin chaperone	[8]
ycbT	predicted fimbrial-like adhesin protein	[8]
ycbU	predicted fimbrial-like adhesin protein (pseudogene)	[8]
ycbV	predicted fimbrial-like adhesin protein	[8]
ycbF	predicted periplasmic pilin chaperone	[8]
*loc*6		[8]

Continued

Z1534	putative chaperone	[8]
Z1535	predicted fimbrial-like adhesion protein	[8]
Z1536	putative usher protein	[8]
Z1537	putative chaperone	[8]
Z1538	putative pilin subunit	[8]
Z1539	predicted protein	[8]
***csgGFEDBAC* (*loc*7)**		[8]
csgG	curli production assembly/transport component, 2nd curli operon	[66]
csgF	predicted transport protein	[66]
csgE	predicted transport protein	[66]
csgD	putative 2-component transcriptional regulator for 2nd curli operon	[8]
***ABH*-0285339**	hypothetical protein	[8]
csgB	curlin nucleator protein, minor subunit in curli complex	[8]
csgA	cryptic curlin major subunit	[66]
csgC	predicted curli production protein	[8]
***FmlABydeSRfmlD* (*loc*8)**		
fmlA	major subunit of F9 fimbriae	[8]
fmlB	F9 fimbriae chaperone	[8]
fmlC	F9 fimbriae usher protein	[8]
ydeS	putative F9 fimbriae protein	[8]
ydeR	putative F9 fimbriae protein	[8]
fmlD	F9 fimbriae adhesin	[8]
Z3276, *yehBCDE* (*loc*9)		[8]
Z3276	putative fimbrial protein	[8]
yehB	predicted outer membrane protein	[8]
yehC	predicted periplasmic pilin chaperone	[8]
yehD	predicted fimbrial-like adhesin protein	[8]
yehE	predicted protein	[8]
Z3596/Z3597/Z3598/*yfcSUV* (*loc*10)		
Z3595	hypothetical protein	[8]
Z3596	putative minor fimbrial subunit	[8]
Z3597	putative minor fimbrial subunit	[8]
Z3598	putative minor fimbrial subunit	[8]
yfcS	predicted periplasmic pilus chaperone	[8]
yfcU	putative fimbrial usher	[8]
yfcV	predicted fimbrial-like adhesin protein	[8]

Continued

YraHIJ, Z4501, Z4502, (*loc*11)		[8]
yraH	predicted fimbrial-like adhesin protein	[8]
yraI	predicted periplasmic pilin chaperone	[8]
yraJ	predicted outer membrane protein	[8]
Z4501	hypothetical protein	[8]
Z4502	hypothetical protein	[8]
ABH-0285237	putative transposase	[8]
***lpfABCC'DE* (*loc*12)**		[9] [11]
lpfA	putative major fimbrial subunit	[9] [11]
lpfB	putative fimbrial chaperone	[9] [11]
lpfC	putative fimbrial usher	[9] [11]
lpfC'	putative fimbrial usher	[9] [11]
lpfD	putative fimbrial protein	[9] [11]
lpfE	putative fimbrial subunit	[9] [11]
***lpf2ABCDD'* (*loc*13)**		[10] [11]
lpf2A	putative major fimbrial subunit	[10] [11]
lpf2B	putative fimbrial chaperone	[10] [11]
lpf2C	putative fimbrial usher	[10] [11]
lpf2D	putative fimbrial protein	[10] [11]
lpf2D'	putative fimbrial protein	[10] [11]
***fimBEAICDFGH* (*loc*14)**		
fimB	tyrosine recombinase/inversion of on/off regulator of fimA	[8]
fimE	tyrosine recombinase/inversion of on/off regulator of fimA	[8]
fimA	major type 1 subunit fimbrin (pilin)	[8]
fimI	fimbrial protein involved in type 1 pilus biosynthesis	[8]
fimC	periplasmic chaperone, required for type 1 fimbriae	[8]
fimD	outer membrane usher protein, type 1 fimbrial synthesis	[100]
fimF	fimbrial morphology	[8]
fimG	minor subunit of type 1 fimbriae	[8]
fimH	minor fimbrial subunit, D-mannose specific adhesin	[8]
ecpEDCBAR	*E. coli* common pilus	
ecpE	predicted chaperone	[15]
yagW/ecpD	putative fimbrial chaperone protein	[15]
ecpC	predicted usher protein	[15]
ecpB/matC		[15]
ecpA/matB	Mat fimbrillin	[15]

Continued

ecpR/matA		[8]
HcpABC (ppdD)	Hemorrhagic coli pilus	
hcpA	type IV major pilin subunit	[14]
hcpB	conserved protein with nucleoside triphosphate hydrolase domain	[14]
hcpC	assembly protein in type IV pilin biogenesis, transmembrane protein	[14]
Not in operons		
fliC	flagellar filament structural protein (flagellin)	[9] [17]
dsbA	periplasmic protein disulfide isomerase I	[22]
Iha	irgA homolog adhesion	[18]
***efa*-1'**	efa1_1 and efa1_2 are truncated fragments of efa-1, and are collectively referred to as efa-1'	[20]
ompA	outer membrane protein A (3a; II*; G; d)	[10]
wcaM	predicted colanic acid biosynthesis protein	[103]
Z4321 ABH-0027995	putative PagC-like membrane protein	Unpublished Sequence Analysis
gadE	acid-induced positive regulator of glutamate-dependent acid resistance	[25]
yhiF	predicted DNA-binding transcriptional regulator	[25]
yeeJ	Putative adhesin	[24]
Z1536	Putative usher protein	Unpublished Sequence Analysis
Z5029	Putative adhesin	[6]
Z5223	Putative fimbrial chaperone	[6]

involved in adhesion, yet strains need to be developed with all 16 loci deleted, and then through a systematic addition of each loci individually to the mutant strain would permit the investigation of the role in adhesion of each individual fimbrial loci.

The best-characterized loci involved in adhesion are the long polar filament operons *lpf*1 (loc12) and *lpf*2 (loc13). The *lpfABCC'DE* operon was characterized by Torres *et al*. [9] where they showed that this polyci-stronic operon is present in O157:H7 strains as well as the ancestral O55:H7 strains. Generation of a *lpfA* mutant strain resulted in a decreased ability to adhere to human HeLa cells indicating that the *lpf* loci is involved in ad-herence to human tissue cells. A second *lpf* operon, *lpf2ABCDD'*, was also characterized and found that a *lpfA2* mutant strain had decreased adherence to Caco-2 cells, yet no difference was observed in adherence to HeLa cells in a Fas Actin Staining (FAS) assay [10]. To further investigate the role of *lpf* for adherence to animals, Torres *et al*. [11] found that both *lpf*A1 and *lpf*A2 mutants displayed diminished ability to persist in the intestine of infected 6-week-old lambs. In the same study, they determined that *lpf*1 was expressed in response to temper-ature (37˚C), growth phase (late-logarithmic and stationary growth phase), pH of the growth medium (pH 6.5), and also osmolarity (0.2 M NaCl), whereas the *lpf*2 operon was expressed in conditions of late-logarithmic phase of growth and also during iron starvation [11]. Collectively it seems that the *lpf* fimbriae may have roles for adherence during different environmental cues, yet follow up studies should focus on the generation of an*lpf*1 and *lpf*2 double mutant strain to systematically determine the role of each in adherence and EHEC viru-lence.

The *sfm* operon (loc3), consists of 5 genes, *sfmACDHF* which are predicted to encode the machinery to as-semble fimbria on the outer surface of the cell (**Table 1**). This fimbrial gene cluster is important for disease, since they are involved in the attachment of the organism to the host's intestinal epithelial layer in the small in-

testine [12] [13]. In *Salmonella* strains, there are 13 fimbrial operons present in the genome, yet only two of these operons, *fim* and *agf* have been shown to mediate expression of fimbrial filaments on the cellular surface, which may indicate a similar role for these genes in *E. coli* EHEC strains.

The *fim* operon from *Salmonella* is orthologous to the *sfm* operon in *E. coli* O157:H7. Humphries *et al*. [12] revealed that in *S. enterica* serotype *Typhimurum* that out of the 13 predicted fimbrial operons identified in the genome, the gene product for *fimA* was the only one expressed and detected by Western blotting. Furthermore in animal studies with chickens, a *fim*D mutant of *Salmonella* increased its ability to enter the bloodstream (bacteraemia), yet modified the organism's ability to cause disease in the reproductive tract, which consequently led to a decline in egg shell contamination in laying hens [13]. Combined, these studies highlight the *fimBEAICDFGH* operon as a target for various control parameters to control dissemination of *Salmonella* in poultry products. There is a possibility that similar regulation factors and cellular machinery exist in *E. coli* O157:H7 for fimbrial filaments.

In addition the *hcpABC* operon, which was recently found to encode the hemorrhagic coli pilus, generation of a *hcpA* mutant strain of *E. coli* O157:H7 led to decreased adherence to Caco-2, T84, HT-29, HeLa, Hep-2, and MBDK human cell lines compared to the parent strain in cell adherence assays and also when tested with porcine and bovine gut explants [14]. Another locus found to be involved in adhesion to human tissue cells is the operon *ecpEDCBAR* (*E. coli* common pilus). An *ecpE* mutant strain that represented an operon mutant for the *ecp* loci displayed reduced adherence to HEp-2 and HeLa cells compared to the parent strain in celladherence assays [15]. Further experimentation is required to examine which fimbrial operons are expressed in *E. coli* O157:H7 strains, since identification of expressed fimbrial filaments represent a target for vaccine development.

Many additional gene clusters/operons predicted to encode additional machinery for fimbrial assembly have been identified based on protein sequence similarity and include loc1 (Z0020/Z0021/Z0022/Z0023/Z0024), loc2 (*yadCKLMhtrEecpDyadN*), loc4 (*ybgOPQD*), loc5 (*ycbQRTUVF*), loc6 (Z1534/Z1535/Z1536/Z1537/Z1538/Z1539)**,** loc8 (*FmlABydeSRfmlD*), loc9 (Z3276/*yehBCDE*), loc10 (Z3596/Z3597/Z3598/*yfcSUV*), and loc11 (*yraHIJ/* Z4501/Z4502/ABH-0285237). Of these predicted fimbriae genes, a random mutagenesis library found that the *ycbR* mutant (loc5) lost the ability to adhere to Hep-2 cells [16], yet all of the other lack experimental evidence to further support their role in EHEC pathogenesis. There are some genes not in clusters that are predicted to encode for fimbrial machinery based on protein sequence similarity and include Z4321, Z1536, Z5029, and Z5223, and it is unknown if these are expressed, or work in concert with other fimbrial machinery in *E. coli* O157:H7 strains.

Other genes and clusters not thought to encode for fimbrial machinery, but that have mutant phenotypes that displayed reduced adhesion to human tissue cell lines were *fliC*, *iha*, *efa*-1', *ompA*, *dsbA*, *wcaM*, *yeeJ*, *yhiF*, and *gadE*. *fliC* is involved in the generation of flagella and has been found to affect the ability of the cell to adhere to HeLa tissue culture cells [9]. In addition, a *fliC* mutant of STEC O113:H21 strain displayed reduced virulence in a mouse model [17]. Combined these studies reveal that *fliC* may play a role in virulence in human infections. The gene *iha* (*irgA* homolog adhesion) was found to confer reduced adherence to HeLa cells in a mutant strain and additionally when *iha* was introduced into non-pathogenic *E. coli*, the resulting strain had the ability to adhere to kidney cells [18]. In addition the mutant strains for the genes *toxB* and *efa*-1'(*efa*1_1 and *efa*1_2 are truncated fragments of *efa*-1, and are collectively referred to as *efa*-1') displayed diminished adherence phenotypes to HeLa cells as compared to the parent strains. In O157:H7 strains, the *efa* gene is disrupted and is represented as two truncated fragments, yet in O157:H⁻ strains it encodes a large (>3000 AA protein) [19]. It is unknown if the *efa* gene product is involved virulence of H⁻ strains, yet both truncated fragments demonstrated a role in adherence and an *efa*_1 and *efa*_2 double mutant strain had even greater reduction in adherence than the single *efa*_1 or *efa*_2 mutant strains [20]. The gene *ompA* produces an outer membrane protein that plays a role in adherence in a HeLa cell adherence assay [21]. Finally, the gene *dsbA* was found to play a role in biofilm formation and virulence since a mutant strain for *ompA* displayed reduced adherence to PVC surfaces and HT-29 epithelial cells [22].

The *wcaM* gene is part of the colonic acid operon (wca), and is involved in thermal tolerance and acid stress in *E. coli* O157:H7 strains [23]. In *Salmonella Typhimurium* strain BJ2710, a *wcaM* mutant generated a reduced thickness of biofilm to Hep-2 cells, indicating that the *wcaM* gene in *E. coli* O157:H7 may also play a role in adhesion to human cells. *yeeJ* was initially identified as a gene that produces an adhesin antigen based on sequence homology to *E. coli* surface adhesion antigen 43 that was found to play a role in biofilm formation [24].

Although a *yeeJ* mutant strain displayed no difference in biofilm formation on PVC microtiter plates or in fermenters, the predicted protein produced by *yeeJ* contains 13 bacterial immunoglobulin-like domains that may mediate the interaction with the host. Therefore the need to test the interaction with human tissue cells in vitro is warranted to determine the role of this gene in adhesion and possibly virulence of *E. coli* O157:H7 strains.

The *yhiF* gene encodes for a transcriptional regulator, and the *gadE* (*yhiE*) gene produces an acid-induced positive regulator. The contribution of these genes to virulence of EHEC strains may not be direct, but rather through the regulation of other virulence genes, since *yhi*F and *gadE* mutant strains displayed increased adherence to Caco-2 intestinal epithelial cells as compared to the parent strain [25]. In addition, there is a homologous gene from *Clostridium difficile*, *toxB*, which is present in the genome of *E. coli* O157:H7 strains that may contribute to EHEC adherence to epithelial cells. Characterization of the function of *toxB* demonstrated that mutant strains displayed a reduction in adherence to Caco-2 cells, thought to occur through the promotion, production, and/or secretion of type III secreted effector proteins [26]. A separate study determined that a *toxB* mutant formed less EspA filaments, secreted less EspD, and displayed a reduction in adhesion to HeLa cells further supporting the role of *toxB* in adherence [20]. Overall, the work in the area of virulence factors involved in adhesion has increased tremendously, yet still more work is required to further the understanding to the point where it is feasible to design and test control parameters to prevent adherence to human cells.

2.2. Attachment and Effacement

The difference between virulence genes classified as adherence compared to attachment and effacement, is that the latter causes a well-known characteristic of attaching and effacing (AE) lesions on the host epithelial cells. AE lesions are not found to be essential to cause bloody diarrhea and/or HUS in the humans, yet surveys of *E. coli* O157:H7 strains found that most strains found to cause these disease stages in humans contain intact genes encoding products conferring AE lesions. To date there have only been two virulence genes identified to cause AE lesions, one that affects humans (*eae*) and the other affects porcine hosts (*paa*). The *eae* gene is found in the locus of enterocyte effacement (LEE), which has been found in most *E. coli* O157:H7 strains and by definition in all enteropathogenic *E. coli* strains (EPEC). Recently the work of Deng *et al*. [27], utilized a relative of *E. coli*, the mouse pathogen *Citrobacter rodentium*, to further elucidate the roles of many of the genes contained in the LEE island. In the case of *eae*, it was found that an *eae* mutant of *C. rodentium* was avirulent in a mouse model assay [27]. It is well established that *ler* is a regulator for *eae* expression, yet recently the work of Nadler *et al*. [28] determined that the transcriptional expression of *eae* is also affected by *ydeOP* and *evgA*.

The other gene involved in AE lesion formation is *paa* (porcine A/E-associated gene). In the work of Batisson *et al*. [29] screening of a random mutagenesis library in non-enterotoxigenic porcine *E. coli* O45 led to the identification of a mutant that did not induce typical lesions in a pig ileal explant model. The authors make the correlation that the presence of *paa* and *eae* sequences in porcine *E. coli* O45 strains is important for generation of AE lesions. The protein sequence similarity is 100% identical for *E. coli* (EHEC) EDL933 and Sakai strains and is found intact in seven additional genomes of *E. coli* O157:H7, which may suggest that this gene may play a role in AE formation in a human host.

3. Effectors

Bacterial effector proteins are part virulence mechanisms of many microbial pathogens, and in the case of *E. coli* O157:H7, much attention has been focused on these proteins that are injected into host cells, often via type three secretion systems (TTSS) (**Figure 2**). Initially the first effectors identified in *E. coli* O157:H7 were found in the LEE locus and much of the understanding of these virulence factor genes was elucidated using the mouse pathogen *C. rodentium*. With complete genomes available for *E. coli* O157:H7, recent attention has focused on identifying homologous new candidate effector genes based on similarity searching using known effector gene/protein sequences. Initial research was followed up by investigation of tagged-putative effector proteins and their ability to translocate into human tissue culture cells. Here we provide an update on the genes that encode effectors and putative effectors virulence factor genes (**Table 2**). Although the mode of action of these effector proteins once inside of the host varies, effectors represent a large percentage of the virulence genes identified as candidates in *E. coli* O157:H7 due to their identification via sequence similarity and can be further examined with *in vitro* assays for that examine protein translocation into human tissue culture cells.

Table 2. Effectors known and predicted in *E. coli* O157:H7 EHEC strains.

Effector	Product	Reference
espA (**LEE**)	secreted protein EspA	[27] [30] [104]
espB (**LEE**)	secreted protein EspB	[27] [30] [104]
espD (**LEE**)	secreted protein EspB	[27] [30] [104]
espF (**LEE**)		[27] [30] [104]
espG (**LEE**)	LEE-encoded type III secreted effector	[27]
espH (**LEE**)	LEE-encoded type III secreted effector	[27] [104]
espZ (**LEE**)	translocated effector protein	[27] [38] [104]
tir (**LEE**)	translocated intimin receptor protein	[26] [27] [36] [104]
map (**LEE**)	mitochondrial associated type III secreted effector protein	[26] [27] [36] [104]
*espF*2-1'/*tccp*2	non-LEE-encoded type III secreted effector	[104]
espJ	translocated type III secretion system effector	[96] [104]
espK	non-LEE-encoded type III secreted effector	[104]
*espL*1	predicted non-LEE-encoded type III secreted effector	[104]
*espL*2	non-LEE-encoded type III secreted effector	[104]
*espL*3'		
*espL*4	predicted non-LEE-encoded type III secreted effector	[104]
*espM*1	predicted non-LEE-encoded type III secreted effector	[104]
*espM*2	non-LEE-encoded type III secreted effector	[104]
espN	non-LEE-encoded type III secreted effector	[104]
*espO*1-1	non-LEE-encoded type III secreted effector	[104]
*espO*1-2	predicted non-LEE-encoded type III secreted effector	[104]
*espR*1	predicted non-LEE-encoded type III secreted effector	[104]
*espR*2'		[104]
*espR*3	predicted non-LEE-encoded type III secreted effector	[104]
*espR*4	predicted non-LEE-encoded type III secreted effector	[37] [104]
espV'		[104]
espW	non-LEE-encoded type III secreted effector	[104]
*espY*1	non-LEE-encoded type III secreted effector	[104]
*espY*2	predicted non-LEE-encoded type III secreted effector	[104]
*espY*3	predicted non-LEE-encoded type III secreted effector	[104]
*espY*4	non-LEE-encoded type III secreted effector	[104]
*espY*5'		

Continued

|		
*espX*1	predicted non-LEE-encoded type III secreted effector	[104]
*espX*2	non-LEE-encoded type III secreted effector	[104]
*espX*3'		
*espX*4	predicted non-LEE-encoded type III secreted effector	[104]
*espX*5	predicted non-LEE-encoded type III secreted effector	[104]
*espX*6	predicted non-LEE-encoded type III secreted effector	[104]
*espX*7	non-LEE-encoded type III secreted effector	[104]
nleA	non-LEE-encoded type III secreted effector	[41]
*nleB*1	non-LEE-encoded type III secreted effector	[42] [104]
*nleB*2-1	predicted non-LEE-encoded type III secreted effector	[104]
*nleB*2-2'		[104]
nleC	non-LEE-encoded type III secreted effector	[27] [104]
nleD	non-LEE-encoded type III secreted effector	[104]
nleE	non-LEE-encoded type III secreted effector	[44] [104]
nleF	non-LEE-encoded type III secreted effector	[27] [104]
*nleH*1-1	non-LEE-encoded type III secreted effector	[104]
*nleH*1-2	non-LEE-encoded type III secreted effector	[104]
*nleG*2-1'		[104]
*nleG*2-2	non-LEE-encoded type III secreted effector	[104]
*nleG*2-3	predicted non-LEE-encoded type III secreted effector	[37] [104]
*nleG*2-4'		[104]
*nleG*3'		[104]
*nleG*3_1		
*nleG*3_2		
*nleG*5-1	non-LEE-encoded type III secreted effector	[104]
*nleG*5-2	predicted non-LEE-encoded type III secreted effector	[104]
*nleG*6-1	non-LEE-encoded type III secreted effector	[104]
*nleG*6-2	predicted non-LEE-encoded type III secreted effector	[104]
*nleG*6-3'		[104]
*nleG*7	non-LEE-encoded type III secreted effector	[104]
*nleG*8-1	non-LEE-encoded type III secreted effector	[37] [104]
*nleG*8-2	non-LEE-encoded type III secreted effector	[104]
*nleG*9'		
tccP	Tir-cytoskeleton coupling protein (TccP)	[45] [46] [47] [49] [50] [99]
*tccp*2/*espF*2-1'		[49]
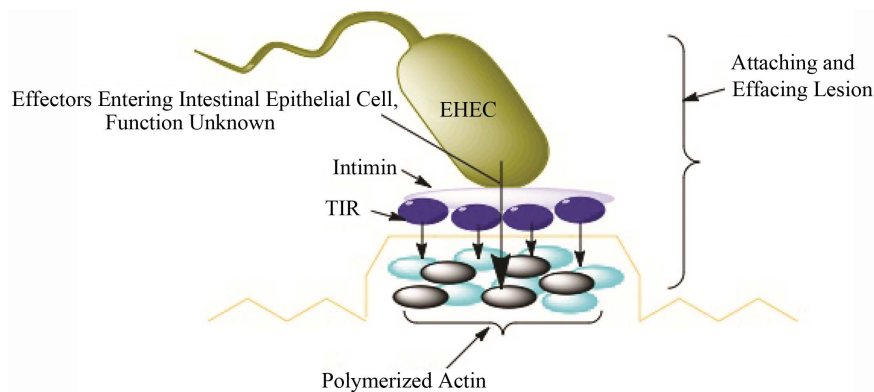
Figure 2. Schematic of the effector entering the intestinal epithelial cell.

3.1. Locus of Enterocyte Effacement

The LEE pathogenicity island has been the subject of much recent research, and since it is found also in the mouse pathogen *C. rodentium*, an organism for which there is an established experimental model to study the involvement of virulence genes in a mouse host, Deng *et al.* [27] mutagenized all 41 coding sequences that comprise the entire LEE locus, and tested each mutant strain in mice. The results of this work are extensive and further elucidate the association of many of these genes to cause virulence in the mouse model as an effort to better understand the role of the LEE locus in human EHEC illness. Since many of the effectors in pathogenic *E. coli* are injected into the host via type three secretion systems, the LEE locus was the center of the initial identification of effectors, in part since there is also an extensive repertoire of genes that encode a functional TTSS adjacent to the effectors genes. There are currently six genes identified that encode effector protein in the LEE island, and in the following section an overview of the current work is provided.

3.2. Locus of Enterocyte Effacement Effectors

In the work of Crane *et al.* [30] an *espF* mutant was found to display reduced lethality to human tissue culture cells yet retained the adherence phenotype similar to the wild-type strain, and they determined that the expression of EspF in HeLa cells is toxic in a dose-dependent manner. It was also determined that EspF secretion is reliant on the TTSS machinery and that another LEE gene, *cesF*, had a significant effect on the translocation of EspF, as a *cesF* mutant had reduced amounts of EspF translocated into human tissue cells [31]. Additional LEE TTSS gene products were found to interact with EspF and CesT [32]. More supporting evidence for the role of *espF* in virulence came from the work of Deng *et al.* [27], which found that an *espF* mutant in *C. rodentium* had attenuated virulence in a mouse host.

The *tir* gene encodes a translocated intimin receptor protein, which translocates into human tissue cells [33], and also integrates into the host cell membrane and binds intimin to promote bacterial adhesion to host cells [34]. Mutant strains lacking a functional *tir* gene show greatly reduced adhesion to HeLa cells, do not induce the formation of actin filaments by HeLa cells [34], and show reduced ability to invade HeLa cells [35]. In animal models, *tir* mutant strains are avirulent in mice thus further supporting its role in the ability to cause human illness [27].

Map (mitochondrial associated type III secreted effector protein) is required for *E. coli* O157:H7 invasion of host cells. There are numerous experiments to support the role of map in virulence, since mutant strains show a reduced ability to invade HeLa cells [35], are found to be required for filipodia formation [36], and were found to have decreased competitive abilities in calf intestine [37], thus illustrating that Map is important in the ability of *E. coli* O157:H7 to persist in bovine hosts, and likely to cause disease in human hosts.

There are numerous other effector genes that have been to play an important role in the ability to cause disease in mouse models, and these include: *espH*, *espZ*, and *espG* [27] [38] [39]. Additional roles of the espG gene in virulence have been demonstrated with experiments that found that the encoded protein, EspG, binds to tubulin which causes localized microtubule depolymerization resulting in actin stress fiber formation through an unknown mechanism [40].

3.3. Non- Locus of Enterocyte Effacement Effectors

Aside from the work on LEE effectors genes, there are numerous candidate virulence factor genes thought to function as effectors in host environments and many of these have been determined through assays that demonstrated effector protein translocation in human cells and include: *espY*4, *nleB*1, *espW*, *nleG*8-2, *espM*2, *espR*4, *nleA*, *nleH*1-2, *espM*1, *nleG*2-2, *nleG*6-1, *nleG*5-1, *espK*, *espX*2, *espY*1, *nleG*8-1, *nleD*, and *nleH*1-1 [33] [41]. Studies with gene mutants in mice animal models have also identified some non-LEE effectors as virulence genes such as: *nleA*, *nleB1*, *nleF*, *nleC*, and *nleD* [27] [42] [43]. Additional virulence genes that produce effectors were identified through a combination of research approaches that examined adherence phenotypes of mutant strains to HeLa cells, and also in the ability of mutant strains to persist in calves, and in conjuncture provide further support of these virulence genes in *E. coli* O157:H7 such as *espR*4, *nleG*8-1, and *nleG*2-3 [37]. With a lack of experimental evidence, there are a number of candidate effector virulence genes that have been identified based on sequence homology to known effector genes and these include: *espX*6, *espX*5, *espX*4, *espL*4, *nleE*, *espR*3, *espL*1, *espR*1, *nleG*5-2, *nleG*6-2, *espN*, *espX*7, *nleB*2-1, *espY*3, *espY*2, *espX*1, and *nleG*2-3 [33] [44].

One of the most studied effector molecule genes is *tccP* which encodes for the Tir-cytoskeleton coupling protein (TccP), and this virulence gene has been shown to be present in 100% of EHEC O157:H7 strains from around the world (n = 365) based on PCR amplification, yet some variation in the size of the *tccP* gene was noted to range from 700 to 1150 bp [45]. TccP is also required for EHEC-induced actin polymerization, Nck-like (EPEC) activity that facilitates interactions of Tir and actin-signaling molecules, and cooperates with Tir to induce actin polymerization at the site of bacterial attachment [46]. With regards to the protein sequence of TccP, the number of proline-rich repeats in TccP directly correlates with the binding affinity to N-WASP, and the N-terminal amino acid residues 1 - 21 are required for TccP translocation into HeLa cells, while the N-terminal amino acid residues 1 - 181 are required for actin polymerization of epithelial cells and during *in vitro* assays [47]. *tccP* mutant strains were found to compete equally well with the parent strain in mixed oral infection experiments of lambs and calves, thus do not play a significant role in *E. coli* O157:H7's competiveness to colonize mammalian hosts [48]. Even though the first genome sequence for *E. coli* O157:H7 was for strain EDL933 by Perna *et al*. 2001, another group resequenced the *tccP* gene of *E. coli* O157:H7 strain EDL933 and found that its sequence is identical to that of the *E. coli* O157:H7 Sakai strain (*i.e.*, 1014 bp and five and a half [rather than six and a half] proline-rich repeats), thus correcting initial genome sequencing errors [49]. The two genes *tccP* and *espJ* constitute an operon, but *espJ* expression was not regulated by Ler [50].

A second gene similar to *tccP*, called *tccP*2, has also received much research attention, since it was found that most clinical non-O157 EHEC isolates carry a functional *tccP*2 gene that encodes a secreted protein that can complement a *tccP* mutant, and that 90% of *tccP*2-positive non-O157 EHEC strains contain a Tir protein that can be tyrosine phosphorylated [49]. These results suggest that TccP2 is a functional equivalent to TccP and can be used by O157 and non-O157 EHEC to trigger actin polymerization via the Nck pathway [49].

4. Secretion Systems

4.1. Type Two Secretion Systems

E. coli O157:H7 strains harbor a large plasmid termed pO157, and this plasmid contains a number of genes thought to contribute to the virulence in humans such as the hemolysin (*ehxCABD*), a periplasmic bifunctional catalase/peroxidase (*katP*), an extracellular serine protease (*espP*), and homologue to a toxin from *Clostridium difficile* thought to contribute to cell adherence to epitheial cells (*toxB*). The pO157 plasmid also has an operon containing 13 ORFs that comprise a type II secretion system known as the *etp* cluster (**Table 3**). The type II secretion system encoded by the *etp* operon has been found to contribute to the virulence of *E. coli* O157:H7 strains by secreting a zinc metalloprotease protein called StcE. In addition, strains cured of the pO157 plasmid had reduced secretion of EspA, EspB, and Tir, and re-introduction of mini-pO157 plasmid composed of the *toxB* gene and the *ori* regions restored production and secretion of the effectors EspA, EspB, and Tir [25]. It was also determined that a *toxB* mutant formed less EspA filament, secreted less EspD, displayed reduced adhesion to HeLa cells, and colonized at a similar rate to the wild type strain [20]. Collectively, it is evident that there are obvious advantages to strains of *E. coli* O157:H7 to maintain and express the genes on the pO157 plasmid, since every strain with a sequenced genome in this review harbors this plasmid, and has highly conserved sequence homology for all of the putative or known virulence factors genes contained within.

Table 3. Type two and three secretion system genes known and predicted in *E. coli* O157:H7 EHEC strains.

Gene cluster or operon	Product	Reference
Type two secretion system *etpCDEFGHIJKLMNO etpC*	type II secretion protein C	[62] [101]
etpD	type II secretion protein D	[62]
etpE	type II secretion protein E	[62]
etpF	type II secretion protein F	[62]
etpG	type II secretion protein G	[62]
etpH	type II secretion protein H	[62]
etpI	type II secretion protein I	[62]
etpJ	type II secretion protein J	[62]
etpK	type II secretion protein K	[62]
etpL	type II secretion protein L	[62]
etpM	type II secretion protein M	[62]
etpN	type II secretion protein	[62]
etpO	type II secretion protein	[62]
Type three secretion system		[51] [103]
eprK	putative lipoprotein of type III secretion apparatus	[51]
eprJ	putative Type III secretion apparatus protein	[51]
eprI	predicted protein (pseudogene)	[51]
eprH	predicted type III secretion system inner membrane ring protein (pseudogene)	[51]
*ORF*6 (**Z4184**)	predicted protein	[51]
epaS	putative integral membrane protein-component of type III secretion apparatus	[51]
*epaR*2	C-terminal fragment of type III secretion system inner membrane R protein (pseudogene)	[51]
*epaR*1	N-terminal fragment of type III secretion system inner membrane R protein (pseudogene)	[51]
epaQ	type III secretion apparatus protein	[51]
epaP	putative integral membrane protein-component of typeIII secretion apparatus	[51]
epaO	type III secretion apparatus protein	[51]
eivJ	type III secretion apparatus protein	[51]
Z4192	hypothetical protein	[51]
eivI	type III secretion apparatus protein	[51]
eivC	type III secretion apparatus protein	[51]
eivA	type III secretion apparatus protein	[51]
eivE	putative secreted protein	[51]
eivG	type III secretion apparatus protein	[51]
eivF	putative regulatory protein for type III secretion apparatus	[51]

Continued

*ecs*3735 (*Z*4199)	Outside of the studied loci	[51]
*ecs*3736	Outside of the studied loci	[51]
*ecs*3737	Outside of the studied loci	[51]
LEE encoded type three SS		
*ORF*29 (*Z*5102)	LEE-encoded predicted type III secretion system factor	[27]
escF	LEE-encoded type III secretion system component	[27] [28]
sepL	LEE-encoded type III secretion system component	[27] [84]
escD	LEE-encoded type III secretion system component	[27] [28]
cesF	chaperone for type III secretion of EspF	[27] [58]
escQ	LEE-encoded type III secretion system factor	[27]
*orf*16	LEE-encoded predicted type III secretion system factor	[27]
*orf*15	LEE-encoded predicted type III secretion system factor	[27]
escN	LEE-encoded type III secretion system factor	[27] [88]
escV	LEE-encoded type III secretion system factor	[27] [88]
*orf*12	LEE-encoded predicted type III secretion system factor	[27]
escI	LEE-encoded predicted type III secretion system component	[27]
escJ	LEE-encoded type III secretion system factor	[27] [28]
sepD	LEE-encoded type III secretion system component	[27] [89]
escC	type III needle complex subunit	[27] [56]
cesD	LEE-encoded type III secretion system factor	[27] [55]
escU	LEE-encoded type III secretion system factor	[27]
escT	LEE-encoded type III secretion system factor	[27]
escS	LEE-encoded type III secretion system factor	[27]
escR	LEE-encoded type III secretion system factor	[27]
escL	LEE-encoded predicted type III secretion system factor	[27]
*orf*4	LEE-encoded type III secretion system factor	[27]
cesA	LEE-encoded chaperone This doesn't belong	[27] [32]
escE	LEE-encoded type III secretion system factor	[27]
*rorf*1	LEE-encoded type III secretion system factor	[55]

4.2. Type Three Secretion Systems

Type III secretion systems (TTSS) are part of the main machinery that *E. coli* O157:H7 cells use to adhere and permit translocation of effectors into host environments (**Table 3**). Many genes are involved in the assembly of the TTSS machinery and here we provide a summary of those genes and other homologous genes thought to also produce functional TTSS components. Some of these TTSS genes are found in the LEE region of the genome, but many also exist in other regions of the *E. coli* O157:H7 genomes. The genes *eprK*, *eprJ*, *eprH*, *epaSR2*, *eprR*1, *epaQ*, *epaP*, *epaO*, *elvJ*, *elvI*, *elvC*, *elvA*, *elvE*, *elvG*, and *elvF* are involved in the production of functional TTSS in *E. coli* O157:H7 and are believed to be involved in virulence [51]. Strains that were *eprHIJK* null mutants were injected intraperitoneally into 1-day-old chicks in addition to *eprH* null mutants, and these mutants lost the ability to adhere to HEp-2 cells *in vitro* supporting a role in virulence of strains of *E. coli* O157:H7 [51] [52].

4.3. Locus of Enterocyte Effacement Regions

There are also numerous genes that play a role in the TTSS systems in *E. coli* O157:H7 strains and many of these are found in the LEE region of the genome. These genes have either been examined through experimentation or through sequence homology to be candidate virulence factors genes. Of these, the gene *escC* produces the type III needle complex subunit, the genes *sepL*, *escD*, *escQ*, encode TTSS components, and *escF*, *escN*, *escV*, *Orf*12, *escJ*, *escU*, *escS*, *escR*, *escL*, *Orf*4, *escE*, *and Rorf1* produce TTSS factors (**Table 3**).

Then there are also numerous genes contained in the LEE genomic region that are predicted to be TTSS components based on sequence homology and include genes for type III secretion system components such as *escI* and *sepD*, and also the genes *Orf*29, *Orf*16, *Orf*15, and *cesD* that are predicted type III secretion system factors.

Many of these LEE region TTSS virulence genes have supporting experimental evidence to support their role in virulence. Among these the *escQ* product forms the ring within the basal body of the LEE-encoded type-III secretion system and EscT interacts with EscU as a structural component in the structural machinery of the LEE-encoded type-III secretion system [53]. Mutant stains deficient for *escQ* fail to secrete type III effectors and are avirulent in mice [27], have decreased competitive abilities in calf intestines, do not adhere to HeLa cells, and do not secrete EspD [37].

There are also virulence genes demonstrated to be involved in virulence using assays of mutant strains that exhibited phenotypes that fail to secrete type III effectors and/or be strains that are now avirulent in mice such as *escU*, *escT*, *escS*, *escR*, *escL*, *Orf*4, *escE*, and *eesA* [27]. The latter of these, *cesA*, is required for proper translocation of type III proteins EspA and EspB, and mutant strains showed impaired filament formation and an inability to induce lesions on and lyse host cells in addition to failing to secrete EspA and EspB [32]. The LEE-encoded chaperone CesA has also been found to interact with EspA; as it was experimentally show to co-crystallize with EspA [54]. Another gene, *Rorf*1, which produces a LEE-encoded type III secretion system factor interacts with EspD was shown to bind with EspD in yeast two-hybrid assays [55]. Furthermore, *escC*, *escD*, and *escJ* mutant strains were unable to produce the TTSS apparatus, and thereby the secretion of the Esp proteins and Tir effector was abolished. These results indicate that EscC, EscD, and EscJ are required for the formation of the TTSS apparatus [56].

5. Toxins

There are two main sets of gene clusters that are found in *E. coli* O157:H7 genomes that encode the Shiga-Like-toxin A and B protein components and are known as *stx*1*AB* and *stx*2*AB*, and is established that there is an association of Stx toxins and disease in humans [57]. The first strains identified contained one copy of each of these toxin clusters, and were the subjects of immense experimentation. Of the two toxins, Stx2 toxin was found to be 1000 times more toxic than Stx1 based on assays in baboons [58], and Stx2 is 1000 times more cytotoxic to human renal microvascular endothelial cells than Stx1 [59]. In addition, studies determined that the Stx1 toxin has a higher binding affinity to the human GB3 receptors, therefore competing with Stx2 toxin binding in human hosts [60]. Many new outbreak strains of *E. coli* O157:H7 were found to lack the *stx*1*AB* gene cluster, but instead carry two copies of *stx*2*AB* gene clusters that may differ in a few amino acids and have been classified based on classes of *stx*2a, *stx*2b, *stx*2c, *stx*2d, *stx*2e, and *stx*2f. We feel this is important information to help the scientific community better understand the severity of human EHEC disease, since in *E. coli* O157:H7 strains without *stx1AB*, the more potent Stx2 and/or Stx2c toxins will bind to host GB3 receptors and start to cause human disease, which may offer an explanation regarding the epidemiological observations that the more recent emerging strains caused a higher rate of HUS and lethality based on the total numbers of individuals sickened in the outbreaks.

6. Interaction with Host Factors

There are many ways that *E. coli* O157:H7 virulence genes contribute to interactions with hosts (**Table 4**), and here we provide a summary of the various ways that experiments have determined how these genes may play a role in human disease. The gene *stcE* was disrupted and this mutant strain was three-fold lower than the wild type strain in forming actin bundles on HEp-2 cells [61]. The gene *espP* produces a protein that contributes to the bloody diarrhea in many patients suffering from *E. coli* O157:H7 infections through its role as a serine protease that may degrade host protein [62].

Table 4. Known or putative genes involved in interaction with host factors, survival though host barriers, LEE-island non-effectors, or regulators in *E. coli* O157:H7 EHEC strains

Gene cluster or operon	Product	Reference
Interaction with host factors *stcE*	Zinc metalloprotease	[61] [102]
espP	Serine protease	[62]
ehxC	acyltransferase	[65]
ehxA	enterohemolysin (EHEC hemolysin)	[65]
ehxB	ABC transporter ATPase	[65]
ehxD	hemolysin transport protein	[65]
chuS	heme oxygenase	[67]
chuA	outer membrane heme/hemoglobin receptor	[67]
TolC	Transport channel	[63]
sapABCDF		
sapA	predicted antimicrobial peptide transporter subunit	[68]
sapB	predicted antimicrobial peptide transporter subunit; membrane component of ABC superfamily	[68]
sapC	predicted antimicrobial peptide transporter subunit; membrane component of ABC superfamily	[68]
sapD	peptide transport system ATP-binding protein	[68]
sapF	peptide transport system ATP-binding protein	[68]
afuABC		
afuA	periplasmic ferric iron-binding protein	[64]
afuB	putative permease component of transport system for ferric iron	[109]
afuC	putative ATP-binding component of a transport system	[109]
L7004	putative hemolysin expression modulating protein	[5]
kdtA	3-deoxy-D-manno-octulosonic-acid transferase (KDO transferase) endotoxin system	[105]
Island with iron transport cluster		
Z4382	putative iron compound-binding protein of ABC transporter family	[5]
Z4383	putative iron compound permease protein of ABC transporter family	[5]
Z4384	putative iron compound permease protein of ABC transporter family	[5]
Z4385	putative ATP-binding protein of ABC transporter family	[5]
Z4386	putative iron compound receptor	[5]
Survival through host defense barriers		
katP	bifunctional catalase/peroxidase	[80]
terZABCDEF		
terZ	putative phage inhibition, colicin resistance and tellurite resistance protein	[78]
terA	putative phage inhibition, colicin resistance and tellurite resistance protein	[78]
terB	putative phage inhibition, colicin resistance and tellurite resistance protein	[78]
terC	putative phage inhibition, colicin resistance and tellurite resistance protein	[78]

Continued

terD	putative phage inhibition, colicin resistance and tellurite resistance protein	[78]
terE	putative phage inhibition, colicin resistance and tellurite resistance protein	[78]
terF	putative phage inhibition, colicin resistance and tellurite resistance protein	[78]
ureABCDEFG		
ureA	putative urease structural subunit A (gamma)	[74]
ureB	putative urease structural subunit B (beta)	[74]
ureC	putative urease structural subunit C (alpha)	[74]
ureD	putative urease accessory protein D (pseudogene)	[74]
ureE	putative nickel metallochaperone	[74]
ureF	putative urease accessory protein F	[74]
ureG	putative GTP hydrolase	[74]
wcaM	predicted colanic acid biosynthesis protein	[103]
LEE-island non-effectors/non-TTSS		
cesD2	Predicted chaperone	[27] [82]
cesT	molecular chaperone for Tir	[27] [55]
etgA	Predicted lytic transglycosylase	[27] [37]
Regulators		
qseE	sensory histidine kinase in two-component regulatory system with QseF	[90]
qseF	DNA-binding response regulator in two-component regulatory system with QseE	[90]
ler	locus of enterocyte effacement (LEE)-encoded regulator	[27] [91] [92]
grlA	LEE-encoded positive regulator of transcription	[27]
grlR	LEE-encoded negative regulator of transcription	[27]
cspG	DNA-binding transcriptional regulator	[66]
phoB	DNA-binding response regulator in two-component regulatory system with PhoR (or CreC)	[66]
ykgA	predicted DNA-binding transcriptional regulator	[66]

Iron is an essential element required for microorganisms and is involved in the coordinative centers of many Fe-S interactions for proteins within the cell. The ability to scavenge iron from animal hosts from haem or haemoglobin has been implicated as a role in virulence in *E. coli* O157:H7. The *E. coli* EDL933 strain lacks classical systems to acquire iron such as aerobactin, and cannot utilize transferring or lactoferrin as iron sources. This highlights the importance for other iron acquisition mechanisms *in vivo* and the most likely sources to obtain iron in the host are from haem and haemoglobin. Therefore the genes involved in uptake and release of the haem from the haemoglobin molecule were investigated for their putative involvement in virulence for this enteric pathogen. The *tolC* gene encodes a transporter for hemolysin [63], and the gene cluster *afuABC* complements the inability of an *E. coli aroB* null mutant to import ferric iron that may modulate aspects of virulence and the uptake of iron [64] [65].

The genes in the *ehxCABD* operon produce a cytotoxin or RTX toxin that is involved in the production of enterohemolysin [63]. Iron transporter genes such as *feoABC* are also active in the roles of iron acquisition in bovine and possibly in human hosts [66]. The *chuS* and *chuA* genes are found in *E. coli* O157:H7 strains and are involved in heme/hemoglobintransport and binding, respectively. The *chuSA* genes are present in the UPEC

strains UTI89, 536, and F11, the EHEC strains EDL933 and Sakai, and EAEC strain O42. However, these genes are absent from all other *E. coli* strains examined, including the commensal strain HS and the laboratory-adapted commensal strain K-12 MG1655 [67].

Genomic regions unique to human pathogenic strains of *E. coli* but not found in non-pathogenic strains have been called genomic islands. There is one genomic island that has been used as genetic identifier for *E. coli* O157:H7 and consists of the following genes: Z0608, Z0609, Z0615, Z0634, Z0635, Z1542, Z1543, Z1544, Z1545, Z1546, Z1547, Z1548, and Z1549. There are also genes and a genomic island with a ABC iron transport system identified as being involved in iron acquisition based on sequence homology and include: the gene referred to as *L7004* which is a putative hemolysin expression modulating protein, and the genomic island containing genes thought to encode iron compound-binding proteins of an ABC-like transporter (Z4382), an iron compound permease protein (Z1965, Z4383, Z4384), and ATP-binding proteins of the ABC transporter family (Z1964, Z4385), and an iron compound receptor (Z4386).

In plant hosts, there are genes found in *E. coli* O157:H7 that are orthologous to genes contained in other related plant-associated enterobacteria, and the *sapABCDF* operon (sensitivity to antimicrobial peptides) is one example of this, since a sap operon mutant strain of *Erwinia chrysanthemi* displayed reduced virulence in potato tubers [68], and the sap locus is at least as important as the pectate lyases and more important than the T3SS for causing disease on inoculated potato tubers and chicory leaves [69] [70]. Since *E. coli* O157:H7 strains harbor this operon and outbreaks are occurring on produce items more frequently, future studies should address the role of these genes to associated with plant hosts.

7. Survival through Host Barriers

As a foodborne pathogen, with a minimum infectious dose ID_{50} of ~100 cells, *E. coli* O157:H7 strains possess various protective mechanisms to survive passage through numerous host barriers (**Table 4**). Some of the host barriers through which *E. coli* O157:H7 strains must persevere include passage through the acidic gastric fluid of the stomach (pH~2 - 3), and coping with oxidative stress from hydrogen peroxide produced from host defense mechanisms such as phagocyte engulfment. While many various genes involved in acid tolerance mechanisms exist, such as *dps*, *gadED*, and the regulator *rpoS*, this review will focus on the operon encoding the genes to produce the enzyme urease. In other pathogenic bacteria that survive passage or inhabit the lining of the stomach, the ability of the cell to breakdown urea to form ammonia has been shown to be an effective means for microbes to deal with acidic conditions. This has been illustrated in pathogens that are acquired via an oral route and/or inhabit the mucosal lining of the stomach such as *Yersinia enterocolitica* and *Helicobacter pylori* [71], such as *Brucella abortus* where Sanfrigari *et al.* [72] characterized the role of urease in virulence and determined that urease-mutants were killed more efficiently during transit through the stomach, further illustrating the survival advantage for bacterium to possess urease activity. In a survey of 294 *E. coli* strains not of the O157:H7 serotype, it was determined that 2 out of 294 (0.68%) expressed urease activity [71]. Interestingly, a survey of human clinical isolates of EHEC found that only one out of 23 (4.347%) patient-isolates tested positive for urease activity [73]. It was later determined that the lack of urease activity was attributed to a specific one-base substitution in the *ureD* gene that results in a premature stop codon and a truncated 232 amino acid protein, and that the missing 42 amino acids are required for a functional urease enzyme [74]. It is unclear why less than 5% of the *E. coli* O157:H7 strains surveyed contain this radiated mutation, as it would clearly seem advantageous to maintain the full functional product, yet since there are strains that exist with the full length functional *ureD*, a serotype may emerge in future outbreaks with a minimum infectious dose less than 100 cells due to the protection to acidic conditions afforded by urease activity. In the case of the EDL933 strain [5] there are two copies of the *ure* operon on pathogenicity islands OI43 and OI48, and both copies contain the *ureD* mutation, yet it is unknown if possessing two copies confers an advantage for survival through the gastric fluid of the stomach of the human host. Since urease genes are found in numerous lineages of bacteria in the environment, the opportunity for acquisition of a functional *ureD* gene product exists, and may represent a possible emergence of a hypervirulent as a threat to human health.

On the two pathogenicity islands mentioned, where strain EDL933 contains two copies of the *ure* operon, there are also two copies of the *ter* operon that encodes proteins that afford resistance to the toxic element tellurite. Originally tellurite was used therapeutically for the treatment of leprosy, tuberculosis, dermatitis, cystitis, and eye infections prior of the discovery of the first antibiotics (*i.e.*, penicillin). It was determined that tellurite

had an inhibitory action on coliform bacteria [75], and potassium tellurite has been used for this purpose in a variety of bacterial growth media. It has been demonstrated in *E. coli* O157:H7 that the *ter* operon does confer bacterial resistance to tellurite, yet it was demonstrated that the second copy in strain EDL933 did not result in an increase in tellurite resistance as compared to strains with only one copy [76]. Tellurite has not shown to be a biologically essential element and potassium tellurite in concentrations as low as 1 ug/ml is toxic to most microorganisms [77]. The exact mechanism of toxicity of tellurite is not understood, yet has been attributed to the strong oxidizing capabilities that may interfere with various cellular processes. The exact mechanism of tellurite resistance attributed to the *ter* operon is also not understood, and may function in an unidentified pleiotrophic role. The work of Valkova *et al*. [78] determined that in uropathogenic *E. coli* strains that the *ter* operon contributes to oxidative stress caused by hydrogen peroxide and confers protection against macrophage phagocytic activity. Therefore it seems that the *ter* operon may confer protection to peroxide stress or from host defenses such as macrophage phagocytic engulfment and if having two copies of the ter operon in the genome confers additional advantage for survival during oxidative stress, yet this has not been determined in *E. coli* O157:H7.

Protection from oxidative stress is crucial for survival against host defense mechanisms, such as macrophages, and in *E. coli* O157:H7, there are 3 copies of genes that encode catalase/peroxidase proteins, namely *katE*, *katG*, and *katP*. The latter, *katP* is found on the large plasmid called pO157, and it has been shown that strains with this plasmid are more virulent and that the *katP* gene product is one of the genes that encode the enzymes that breakdown hydrogen peroxide, thus limiting the deleterious effects on the cell [79]. In addition, the operon *lpxCDABHKLM* is involved in the production of external cellular antigens that are involved in evading the host immune system, and therefore believed to play a role in virulence in the human host [80].

8. Locus of Enterocyte Effacement Island Non-Effectors

There are numerous genes that are part of the LEE island that do not function as effector molecules, but have been examined as virulence factors in other ways (**Table 4**). For example, the gene *orf*29 which encodes a predicted type III secretion system factor is thought to play a role in virulence since *orf*29 mutant strains are found to be avirulent in mice and fail to secrete type III effectors [27]. The gene *escF* encodes a type III secretion system factor component, and *escF* null mutant strains do not secrete EspA, EspD, or EspB, fail to disrupt host cell attachment to growth container walls, and are unable to translocate Tir into host cells [79]. In addition to studies with *escF*, a *cesD*2 mutant strain of *E. coli* O157:H7 showed reduced virulence in a mouse model of infection, and show delayed adhesion of Hep-2 cells [27]. Additionally, *cesD*2 mutant strains show reduced EspD secretion and slightly reduced EspB secretion, which is believed to disrupt the required maximal secretion of the virulence factors EspB and EspD for *E. coli* O157:H7 pathogenesis [80]-[82].

The *espA*, *espB*, *espD*, and *sepL* gene products are thought to be involved in virulence in many ways, since mutant strains of *espA*, *espB*, *espD*, or *sepL* are avirulent in mice [27], shows decreased human tissue cell lethality due to reduced adherence to host cells [80], and fail to disrupt host cell attachment to an attachment assay conducted in growth container walls that indicating that type III secretion system is not functioning properly [28]. In addition, disruption of *espD* creates a mutant that has decreased competitive abilities in the calf intestine and do not adhere in a HeLa cell assay [37]. The gene product EspA is a structural component of the needle complex sheath [56], and the production of EspA filaments is how EHEC O157:H7 adhere to the leaf epidermis of arugula, spinach, and lettuce [30]. The *sepL* gene product is required for type III secretion system function and adherence to host cells [83], and *sepL* mutant strains do not secrete EspA, EspB, and EspD proteins, yet shows increased secretion of Tir and NleA [30]. Also, *escD* mutant strains do not secrete Tir, EspA, EspD, or EspB under conditions that cause secretion in wild-type cells [56].

The gene for *eae* encodes an intimin adherence protein and the presence of *eae* and *stx*2*AB* genes in EHEC isolates is considered to be a predictor for the ability to cause hemolytic-uremic syndrome (HUS) in infected individuals [56]. *eae* mutant strains are avirulent in mice [27], and show a loss of the ability to adhere to Caco-2 human tissue cells [84] [85], thus supporting the role of *eae* in the pathogensis of *E. coli* O157:H7 strains.

The gene for *cesT* encodes a molecular chaperone for Tir [55], and *cesT* mutant strains are avirulent in mice [27], fail to translocate Tir into host cells, and can bind to human Hep-2 cells, but does not induce lesions. CesF is a chaperone for the type III secretion of EspF, and *cesF* mutant strains show greatly reduced ability to translocate EspF into host cells, show reduced levels of intracellular EspF, show an inability to reduce the transepithelial resistance of monolayer of T84 cells [86] and creates a mutant strain with decreased competitive abilities in calf intestine [37].

There are also genes predicted as being involved in TTSS systems that display reduced virulence in mice such as *Orf*12, *Orf*15, and *Orf*16 and also show a decrease in secretion of effector molecules such as EscQ, EscN, EscV, EscI, EscJ, and EscC [27]. EscQ mutants have decreased competitive abilities in calf intestine, do not adhere in a HeLa cell assay, and do not secrete EspD [37]. Mutant strains for EscN fail to secrete Tir [55], and show an inability to translocate Tir into HeLa cells [87] [88]. EscV mutants are avirulent in mice, fail to secrete type III effectors [27], show an inability to translocate Tir into HeLa cells, do not induce the formation of actin filaments by HeLa cells [88], fail to disrupt host cell attachment to growth container walls, indicating that type III secretion system is not functioning properly [28], creates a mutant that has decreased competitive abilities in calf intestine, and does not secrete EspD [37]. *escJ* mutant strains do not secrete Tir, EspA, EspD, or EspB under conditions that cause secretion in wild-type cells [56], and also fail to disrupt host cell attachment to growth container walls, indicating that type III secretion system is not functioning properly [28].

SepD is a LEE-encoded predicted type III secretion system component, and *sepD* mutant strains are avirulent in mice [27], do not induce the formation of actin filaments by HeLa cells, and show an inability to secrete EspB and EspF [89], yet show increased secretion of Tir and NleA [85]. *escC* mutant strains show an inability to translocate tir into HeLa cells, do not induce the formation of actin filaments by HeLa cells [88], have decreased competitive abilities in calf intestine, and does not secrete EspD [37]. This *escC* mutant also fails to disrupt host cell attachment to growth container walls, indicating that type III secretion system is not functioning properly (Nadler *et al.* 2006), and *escC* mutants do not secrete Tir, EspA, EspD, or EspB under conditions that cause secretion in wild-type cells [56].

CesD is a LEE-encoded predicted type III secretion system factor, and mutant strains are avirulent in mice [27], fails to disrupt host cell attachment to growth container walls, indicating that type III secretion system is not functioning properly [28], show a reduced secretion of EspB and reduced phosphorylation of Tir, likely due to reduced EspD secretion, and mutants show altered patterns of actin disruption on host cell and do not secrete *espD* [55]. Finally, *etgA* encodes a predicted lytic transglycosylase, and mutant strains show reduced virulence in mice [27], creates a mutant that has decreased competitive abilities in calf intestine, does not adhere in a HeLa cell assay, and has reduced secretion of EspD [37].

9. Regulators

Gene products that are transcriptional regulators are difficult to assign as virulence factors. Typically regulators dictate the expression of global physiological genes, or are more specific in which genes they regulate. In this section we summarize the regulators that affect the expression of known or putative virulence factors, and also mention some that were determined to be unregulated in isolates of clinical human origin in comparison to isolates of bovine origin. The two-component regulators *qseEF* (quorum-sensing *E. coli* regulators E and F) have been found to regulate the known virulence factor gene *tccP* [90]. *qseEF* is part of an autoinducer-3 (AI3)/epinephrine/norepinephrine signaling system, where *qseE* produces the sensor kinase and *qseF* produces the response regulator, that when combined activates the transcription of the effector molecule gene *tccP* which promotes actin polymerization during attaching and effacing (AE) lesion formation on intestinal epithelial cells [90]. It was determined using the Flourescent actin staining technique (FAS) that *qseE* or *qseF* mutant strains exhibited a loss of the ability to form pedestals or to form AE lesions on Hela cells [90].

Another regulator, *ler* (locus of enterocyte effacement (LEE)-encoded regulator) is located in LEE island and has been found to be a global regulator of a myriad of virulence genes contained in the LEE locus (*tir*, *eae*, and the operons LEE1, LEE2, LEE3, and LEE4), genes found on the pO157 plasmid (*espP*, *hly*, and *tagA*), and chromosomal genes such as *stx* and *espC* [31]. More recently, Torres *et al.* [11] also determined that *ler* upregulates the expression of the *lpf* operon (loc12) and acts as an antisilencer of another global regulator known as *h-ns*. In studies with a *ler* null mutant strain of *E. coli* O157:H7, the mutant strain lost the ability to form AE lesions on Hep-2 cells [31]. It was determined in the *E. coli* O157:H(-) STEC strain 95SF2 strain that a mutation that resulted in a Ile57 Thr substitution resulted in a defective Ler gene product, which resulted in a strain that exhibited no AE lesions and had reduced adhesion to Hep-2 cells [91] [92]. In addition, a *ler* mutant strain of *Citrobacter rodentium* was found to be avirulent in a mouse model [27], thus further demonstrating the role of *ler* in *E. coli* O157:H7 virulence.

The product of *grlA* is a is thought to be involved in virulence since it is a LEE-encoded positive regulator of transcription and a *Citrobacter rodentium* null mutant is avirulent in mice and fails to express Tir and EspB, and

positively regulates the LEE-encoded type III secretion system [27]. In contrast, the *grlR* gene was found to be a LEE-encoded negative regulator for the production of LEE-encoded type III secretion systems [27].

In a study comparing the expression differences of *E. coli* O157:H7 strains of human clinical origin and of isolates of bovine origin, it was determined that the following three genes encoding regulators were up-regulated in the human clinical isolates *cspG*, *phoB*, and *ykgA* [66]. There is no evidence that indicates that any of these regulates genes thought to play a role in virulence, yet they may regulate fitness genes, that play a role in a competitive advantage against human intestinal flora, or play a role in the ability to cause disease in humans.

10. Genes Associated with Human Clinical versus Bovine Isolates

Prior to whole genome sequencing, the work of Kim *et al.* [93] used an octomer-based genome scanning technique to evaluate genome diversity among *E. coli* O157:H7 isolates, and they determined that two distinct lineages were observed, with lineage I containing strains isolated from clinical samples from humans, and lineage II grouped together isolates of bovine origin. More recently the work of Dowd and Ishizaki [66] used DNA microarrays to test for mRNA expression differences between lineage I and lineage II isolates of *E. coli* O157:H7. The strains used in this study for the lineage I sample set were all clinical isolates from humans and were composed of strain EDL933 from the 1982 Michigan ground beef outbreak [6], FRIK 533 a patient isolate from a Milwaukee outbreak from the Wisconsin state hygiene lab harbored in the culture collection of Dr. Charles W. Kaspar (Food Research Institute Kaspar), and fda518 a strain presumably from the Food and Drug Administration. The strains of bovine origin representing the lineage II sample set were farm isolates ne037 presumably from Nebraska, FRIK 2000 also referred to as strain 767-8-1 [94], FRIK 1985 also referred to as strain 396-2-2 [95]-[105].

Strains were tested at similar optical densities during stationary phase in anaerobic conditions, and mRNA was hybridized to a microarray representing 610 genes, which were selected based on their being associated with virulence or regulation of putative virulence factors. Of the 610 genes on the array, 179 genes were significantly up or down regulated between the two lineages. The genes that were significantly up-regulated in the human clinical Lineage I set and have other supporting evidence that they are known or putative virulence factors were genes involved in production of urease (*ureA*_2 and *ureB*_2), the shiga toxin II subunit A (*stx2A*), a number of flagellular biosynthesis genes (*fliCTP*), a protein associated with tellurite resistance (*terW*), a putative virulence protein (*pvp*/Z5816), and a genes from the fimbria loci (loc7) termed curli (*csgGFEA*). Some of the genes identified as up regulated in lineage I are thought to associate with GTP binding, metabolism, nitrogen metabolism, and regulation of transcription (Table 5).

In the isolates of bovine origin (lineage II) there were a number of genes with other supporting evidence to support their role in virulence of *E. coli* O157:H7 such as *espP*, *toxB*, *etpHOIMNKL*, *fimbrial subunits usher*2, *fimberal*4, *chaper*2, and *fimbsubI*. Based on gene ontology based analysis, other lineage II genes up regulated appear to be involved in peptidase activity, transferase activity, and DNA binding activity. It is of interest to note that of these genes, *espP*, *toxB*, and *etpHOIMNKL* are all found on the pO157 plasmid. It may be that pO157 and the genes contained on it may provide a competitive advantage in environments associated with the bovine host.

Overall, this work provides unique insight to the grouping of isolated strains into two distinct lineages lineage I in humans, and lineage II in cows. The virulence factors shown to be up regulated in lineage I were consistent with previous findings to support their role in virulence in the human host, yet other contradictory findings were determined where genes were up regulated in strains of bovine origin that were thought to play a role in colonization and pathogenesis of humans. Although these results are not conclusive, they help to provide additional information for ultimately understanding differences of strain-to-strain putative and known virulence factors.

11. Gene Expression during Interaction with Human Epithelial Cells

There are numerous genes that were found to be up regulated in the presence of human epithelial cells (intestinal cells), which is important since *E. coli* O157:H7 must attach to the human intestinal region to enable other virulence mechanisms with the human host such as toxin secretion, effacement, secretion of effector proteins, etc. These include the following genes: *ilvC*, *leuB*, *actP*, *gltB*, *betB*, *puuB*, *entD*, *bioC*, *bioD*, *chuV*, *gltD*, *fepC*, *mltD*, *dppD*, *gnd*, *waaY*, *exbB*, *chuU*, *wbdP*, *betA*, *rplB*, *wzx*, *nuoG*, *chuW*, *glyA*, *lspA*, *fadE*, *astD*, *acrB*, *entE*, *wbdO*, *chuT*, *entF*, *per*, *wbdQ*, *wzy*, *aceF*, *fadB*, *fcl*, *gmd*, *manC*, and *gltA*. It is unclear what the specific role of these

Table 5. Genes up regulated in human isolates vs. bovine isolates of *E. coli* O157:H7 EHEC strains.

Gene cluster or operon	Product	Reference
csgGFEA		
csgG	curli production assembly/transport component, 2nd curli operon	[66]
csgF	predicted transport protein	[66]
csgE	predicted transport protein	[66]
csgA	cryptic curlin major subunit	[66]
fliCTP		
fliC	flagellar filament structural protein (flagellin)	[66]
fliT	predicted chaperone	[66]
fliP	flagellar biosynthesis protein	[66]
yjhS	conserved protein	[66]
Z5816	putative virulence protein	[66]
rfaH	DNA-binding transcriptional antiterminator	[66]
pldA	outer membrane phospholipase A	[66]
IbpA	heat shock chaperone	[66]
ibpB	heat shock chaperone	[66]
feoB	fused ferrous iron transporter, protein B: GTP-binding protein and membrane protein	[66]
hydN	formate dehydrogenase-H, [4Fe-4S] ferredoxin subunit	[66]
trxC	thioredoxin 2	[66]
yejH	predicted ATP-dependent helicase	[66]
yegW	predicted DNA-binding transcriptional regulator	[66]
mdtI	multidrug efflux system transporter	[66]
ycjZ	predicted DNA-binding transcriptional regulator	[66]
hslJ	heat-inducible protein	[66]
ydeA	predicted arabinose transporter	[66]
ureA	putative urease structural subunit A (gamma)	[66]
ureB	putative urease structural subunit B (beta)	[66]
stx2A	shiga toxin II subunit A (enzymatic component of the toxin)	[66]
cspG	DNA-binding transcriptional regulator	[66]
ybbK	predicted protease, membrane anchored	[66]
ABH-0024489	putative membrane spanning export protein	[66]
phoB	DNA-binding response regulator in two-component regulatory system with PhoR (or CreC)	[66]
sbmA	predicted transporter	[66]
ykgA	predicted DNA-binding transcriptional regulator	[66]

genes in attachment/adhesion may be, but many are genes that encode the central metabolic machinery, and may play a role in catabolism of substrates accessible during interaction with epithelial cells [106].

12. Gene Expression in the Presence of Norepinephrine

Our bodies produce the compound norepinephrine during stressful conditions such as bacterial infection. These genes were up regulated in the presence of norepinephrine, which indicates that they may be involved in protecting the bacteria from the host's immune system, since the bacteria has sensed that the human host is responding to its presence. Some of these genes that up regulated in the presence of norepinephrine include: *marB*, *qseC*, *cpdA*, *hemD*, *def*, *rcsC*, *umuD*, *recB*, *slyA*, *slp*, *phoQ*, *rcsA*, *lpxP*, *gadA*, *degQ*, *nlpE*, *gcvA*, *rffT*, *plsC*, *osmB*, *rffD*, *relA*, *rstA*, *hemC*, Z2122, *rpoN*, *fpr*, *malG*, Z1931, *qseB*, *wza*, *gadB*, *fhuD*, Z2071, *cspH*, and Z6090. It is unclear what the specific role of these genes in attachment/adhesion may be, but many are genes that encode the central metabolic machinery, and may play a role in catabolism of substrates accessible during interaction with epithelial cells [107].

13. Insights from Recently Sequenced O157:H7 Strains (Table 6)

After the sequencing of the first two genomes of *E. coli* O157:H7 (strains EDL933 and Sakai), 11 more *E. coli* O157:H7 strains had their genomes sequenced prior to 2008. Among this set are numerous isolates (n = 7) from the 2006-spinach outbreak that sickened individuals from 24 states. Also it has been noted that all spinach isolates (n = 194) contains a single mutation in the *agaF* gene that was also found in other "recent" clinical outbreak strain which disrupts the organisms ability to utilize N-acetyl-D-galactosamine as a carbon source [108], thus showing that more recent outbreak strains seem to be emerging with new gene mutations.

We conducted BLASTP analysis of all 394 virulence factor genes identified in this review from the EDL933 strain against all 12 other genomes of *E. coli* O157:H7 (**Table 7**). Out of the 394 known or putative virulence genes identified in this review, 200 of these genes are present in all 13 *E. coli* O157:H7 genomes and produce full-length gene products with 100% protein sequence similarity to those present in the genome of the EDL933 strain. Of the remaining 194 known or putative virulence factors, there are instances when the EDL933 gene did not match a full length gene product or was absent in some of the 12 additional genomes analyzed (**Figure 3**). There are also numerous times when the EDL933 gene product did match a full-length gene product, but had a protein sequence similarity of 80% - 99% (**Figure 3**). This analysis has provided a new perspective to try to understand the evolution of these more recent emerging strains and we have identified numerous mutations that warrant future studies (**Table 7**).

Table 6. The first 13 genomes sequenced of *E. coli* O157:H7.

| Strain	Outbreak Year	Source
Escherichia coli **EDL933**	1982	human isolate: hamburger
Escherichia coli **EC4042**	2006	human isolate: spinach outbreak
Escherichia coli **EC4045**	2006	Isolate from bagged spinach
Escherichia coli **EC4076**	2006	human isolate: spinach outbreak
Escherichia coli **EC4113**	2006	Isolate from bagged spinach
Escherichia coli **EC4115**	2006	spinach from field
Escherichia coli **EC4196**	2006	bovine isolate spinach
Escherichia coli **EC4206**	2006	bovine isolate spinach
Escherichia coli **EC4401**	2006	Human isolate taco bell outbreak
Escherichia coli **EC4486**	2006	Human isolate: taco bell outbreak
Escherichia coli **EC4501**	2006	Human isolate: taco john outbreak
Escherichia coli **EC869**	2002	human isolate: outbreak from beef
Escherichia coli **Sakai**	1996	clinical isolate: radish sprout outbreak
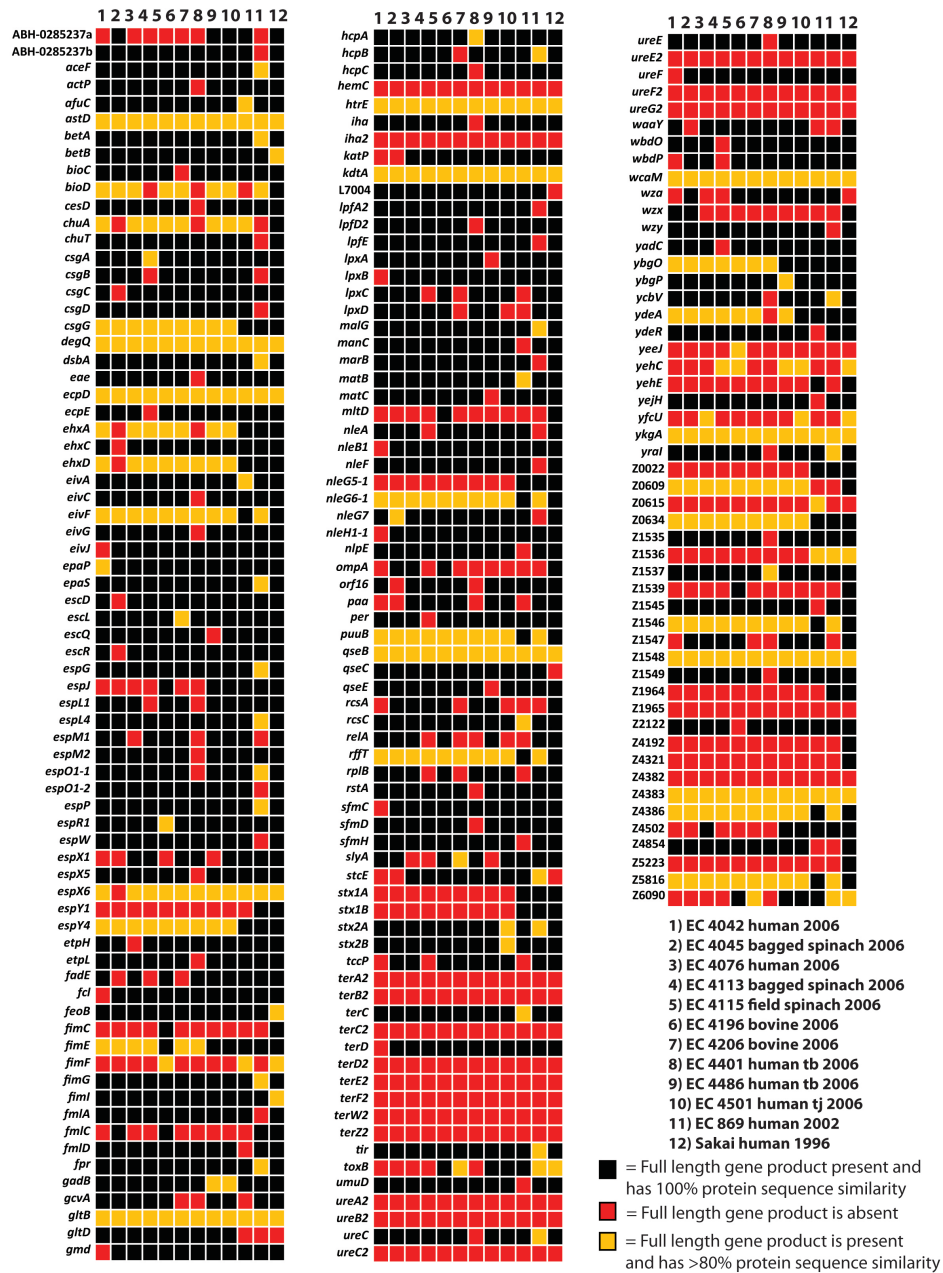
Figure 3. Gene presence and sequence variation of 194 know or putative virulence factors.

Of the 194 virulence factor genes with variation in gene presence and sequence are summarized, there are numerous new hypotheses that can be generated. The questions that remain are: 1) what differences in recent produce-associated strains help to better understand their emergence on produce items;, and 2) what differences help to explain the increased levels of pathogenesis and lethality observed in some of these outbreaks such as the 2006 bagged spinach outbreak? One observation is that the emerging strains all lack the *stx1AB* toxin cluster, but harbor both the *stx2AB* and *stx2ABc* clusters in their genomes. It is well established that Stx2 toxin is much more potent to mammalian hosts in comparison to Stx1, and this has been demonstrated with injection of Stx toxins into baboons, and also through the observation that *Escherichia coli* O157:H7 strains that express Stx2 alone are more neurotropic for gnotobiotic piglets than are isotypes producing only Stx1 or both Stx1 and Stx2 [98]. Therefore strains emerging with these genomic properties may demonstrate increased capabilities to cause bloody diarrhea, HUS, renal kidney failure, and possibly death in humans.

Table 7. Genes with radiated mutations in known or putative virulence factors genes from recent produce-associated outbreak strains of *E. coli* O157:H7 EHEC strains.

Gene cluster	Full product conserved in produce-associated outbreak strains (AA/AA)	Product
ehxD	Lys208Ile	hemolysin transport protein
ehxA	Glu157Ala	enterohemolysin (EHEC hemolysin)
*espY*4	Ile379Thr & Pro397Ser	non-LEE-encoded type III secreted effector
*nleG*5-1	Pro11Ser & Pro204Lys	non-LEE-encoded type III secreted effector
*nleG*6-1	8 AA changes	non-LEE-encoded type III secreted effector
*espY*1	5 AA deletion	non-LEE-encoded type III secreted effector
csgG	Pro76Thr	curli production assembly/transport component, 2nd curli operon
ykgA	Thr187Ile	predicted DNA-binding transcriptional regulator
ycbF	Arg60Ser	
ydeA	Trp360Leu	predicted arabinose transporter
eivF	His163Gln	putative regulatory protein for type III secretion apparatus
*stx*1A	Absent	shiga-like toxin 1 subunit A
*stx*1B	Absent	shiga-like toxin 1 subunit B
*stx*2Bc	Present	shiga-like toxin2c subunit B
espJ	mutation leads to a premature stop codon at position 181AA/217AA	translocated type III secretion system effector

In addition, the *E. coli* O157:H7 strains EC4042, EC4045, EC4076, EC4113, EC4196, and EC4206 all have the same *espJ* gene mutation that leads to a premature stop codon of the full-length protein for the effector EspJ (AA position 181). Previous studies indicated that mutant strains deficient for *espJ* were found to exhibit a non-clearance phenotype in infected C3H/HeJ mice and that *espJ* mutants persist in infected lambs much longer than wild type [95]. It may be that the truncated *espJ* gene product produced by these strains may affect the human host's ability to clear *E. coli* O157:H7 from the intestinal tract. It is of interest that only one of the 2006 spinach outbreak strains EC4115 has the functional full *espJ* gene and was isolated from a spinach plant in the agricultural environment [96], yet mutations of *espJ* may influence the dynamics of colonization of humans and bovine [97]. Therefore these emerging pathogenic strains of *E. coli* O157:H7 may have the capability to persist longer in mammalian hosts and subsequently produce more Stx2 toxins, which may help to explain the more severe complications that were observed in this clade of *E. coli* O157:H7.

14. Conclusion

The first 25 years of research studying *E. coli* O157:H7 has identified 394 genes that are now identified as known or putative virulence factors, and examination of the conservation of these virulence genes has provided new hypothesis regarding the *E. coli* O157:H7 strains that have been emerging in outbreaks, many of which are associated with fresh produce items such as lettuce and spinach. This review is the most comprehensive summary of the experimental literature from the period of 1982-2008. Since 2008, many new outbreak strains have emerged, and genome sequences of these new strains are being generated within a matter of hours with new next-generation sequencing technologies. In the next few years there will be >1000 genomes sequenced for *E. coli* O157:H7 and other EHEC strains, which will represent an unprecedented amount of information for scientists to unravel to truly understand Enterohaemorrhagic *E. coli* and human disease. Therefore, this analysis focused on the first 13 genomes of *E. coli* O157:H7 strains, and represents a template for others to follow as more EHEC genomes are analyzed and compared to determine similarities and differences of more strains. Many putative or known virulence factor genes identified in this review are involved in metabolic machinery and since

genome-scale metabolic models for *E. coli* O157:H7 EDL933 and Sakai have recently been generated and published [109]-[111]; this new computational modeling approach of examining the global metabolic capabilities will shed new light on post-genomic similarities and differences in newly sequenced strains. In summary, the first 25 years of research studying *E. coli* O157:H7 has been extensive and informative, and hopefully the next 25 years will help to develop new control strategies to prevent future EHEC outbreaks and determine a medical cure to treat human EHEC disease.

Acknowledgements

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Supplemental Information 1

Adhesion: The genes that produce these proteins produced by the bacteria are involved in the ability to attach to the epithelial tissue in the intestine. Loss of these virulence factors decreases the organism's ability to attach and cause severe disease in the host.

Antigen: The proteins produced from these genes are located on the outside of the bacterial surface of cell, and are important for the human hosts immune system to recognize the foreign bacteria.

Up regulated in clinical isolates: These genes were found to be up regulated in strains that caused foodborne outbreaks and were isolated from sick patients compared to strains that were isolated from a bovine host and had not caused human foodborne outbreaks.

Effacing: The proteins produced from these genes are involved in causing lesions to the epithelial tissue, resulting in hemorrhaging and the production of bloody diarrhea in the host.

Effector: The proteins produced from these genes are secreted into host cells, often causing damage and degradation of components of the host cell.

Hemolysin: The proteins produced from these genes can breakdown hemoglobin, a common component of human blood.

Host Barriers: These genes produce proteins that are involved in protecting the bacteria from host defenses such as the acidity in the stomach to survive gastric passage to reach the intestines where the organisms attach and promote disease.

Iron acquisition: The proteins produced from these genes are involved in scavenging iron from the host in various mechanisms.

Genomic island: These genes are located on a genomic island, that is unique to human pathogen strains of *E. coli* and not found in non-pathogenic strains.

Regulator: These genes produce proteins that regulate the expression of other genes, some of which are virulence factors.

Secretion: The proteins produced from these genes comprise the bacterial machinery that secretes proteins, such as effectors, which once secreted into host cells can cause damage and degradation of the host cell.

Toxins: The proteins produced from these genes are toxins, which are responsible for causing severe disease such as kidney failure and in some cases death in humans.

Up regulated in the presence of human epithelial cells: These genes were found to be up regulated in the presence of human epithelial cells (intestinal cells), which is important since *E. coli* O157:H7 must attach to the human intestinal region to enable other virulence mechanisms with the human host such as toxin secretion, effacement, secretion of effector proteins, etc.

Up regulated in the presence of norepinephrine: Our bodies produce the compound norepinephrine under stressful conditions such as the bacterial infection. These genes were up regulated in the presence of norepinephrine, which indicates that they may be involved in protecting the bacteria from the host's immune system, since the bacteria has sensed that the human host is responding to its presence.