MINIREVIEW

Weapons of mass destruction: virulence factors of the global killer Enterotoxigenic *Escherichia coli*

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Abstract

Enterotoxigenic *Escherichia coli* (ETEC) is the most common cause of food and water-borne *E. coli*-mediated human diarrhoea worldwide. The incidence in developing countries is estimated at 650 million cases per year, resulting in 800 000 deaths, primarily in children under the age of five. ETEC is also the most common cause of diarrhoea among travellers, including the military, from industrialized nations to less developed countries. In addition, ETEC is a major pathogen of animals, being responsible for scours in cattle and neonatal and postweaning diarrhoea in pigs and resulting in significant financial losses. Studies on the pathogenesis of ETEC infections have concentrated on the plasmid-encoded heat-stable and heat-labile enterotoxins and on the plasmid-encoded antigenically variable colonization factors. Relatively little work has been carried out on chromosomally encoded virulence factors. Here, we review the known virulence factors of ETEC and highlight the future for combating this major disease.

Introduction

Currently, there are six recognized categories of pathogenic *Escherichia coli* that can induce diarrhoea in infected humans via different strategies: enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAEC), enterohaemorrhagic or shiga-toxin-producing *E. coli* (EHEC/STEC), enteroinvasive *E. coli* (EIEC), diffusely adhering *E. coli* (DAEC) and enterotoxigenic *E. coli* (ETEC). ETEC is the most common cause of *E. coli*-mediated human diarrhoea worldwide (Gaastra & Svennerholm, 1996). Human ETEC infections are contracted by consumption or use of contaminated food and water and presents as a sudden onset of secretory diarrhoea that is usually self-limiting but can lead to dehydration due to loss of fluid and electrolytes (Qadri *et al.*, 2005). This pathogen is particularly common in the developing world where an estimated 650 million cases of ETEC infection occur each year, resulting in *c.* 800 000 deaths mostly in young children. Additionally, it poses a significant problem for travellers and military personnel visiting countries where ETEC is endemic (WHO, 1999; Qadri *et al.*, 2005). In addition to the high level of morbidity and mortality associated with human ETEC infection, ETEC also has important financial implications for the farming industry where it is a major pathogen of cattle and neonatal and postweaning piglets (Gaastra & Svennerholm, 1996; Nagy & Fekete, 1999).

Principles of ETEC infection

ETEC, like all other bacterial pathogens, pursue a common basic strategy mandated by host defence mechanisms and millions of years of coadaptation. This strategy has been outlined as follows: (1) adherence to host cells; (2) multiplication within the host; (3) evasion of host defences; and (4) damage to the host (Mims *et al.*, 2001). These steps are usually mediated by a number of proteinaceous virulence factors (Fig. 1). Previous investigations have identified the plasmid-encoded colonization factors (CFs) and one or more plasmid-encoded enterotoxins that induce a secretory diarrhoea as the major determinants of ETEC virulence (Yamamoto & Yokota, 1983; Echeverria *et al.*, 1986). However, several chromosomally encoded virulence factors have recently been identified and implicated in ETEC virulence. The roles of these virulence factors in the pathogenesis of ETEC-mediated disease are discussed below.

Adhesion and invasion

CFs are proteinaceous surface structures that allow bacteria to attach to the intestinal mucosa (Fig. 1). Loss of CFs from

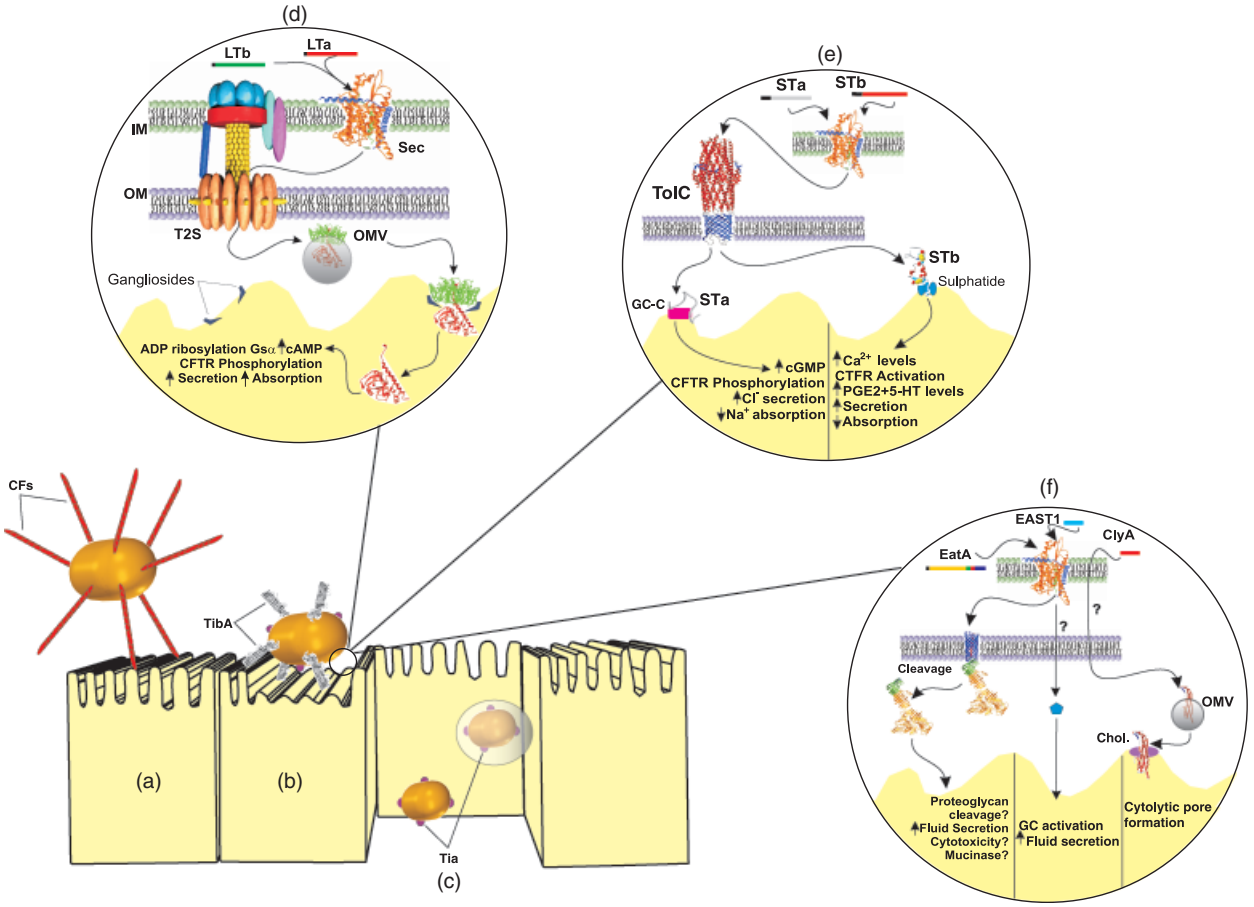

Fig. 1. Schematic model of ETEC infection. (a) After ingestion, the bacteria adhere to the intestinal epithelial cells via their surface-expressed proteinaceous CFs. The different CFs account for the species specificity of strains. (b) Surface-exposed adhesins such as TibA and Tia are likely to increase the initial interaction mediated by the CFs. The outer-membrane adhesin *tia*, encoded within a pathogenicity island, binds to heparin–sulphate proteoglycans on eukaryotic cell surfaces. The glycosylated form of the autotransporter TibA directs the bacteria to bind to a specific receptor on epithelial cells. In the absence of glycosylation, TibA promotes aggregation of a bacterial population and the formation of a biofilm, an important virulence factor in many pathogens. Adherence through these and other outer membrane proteins would increase the proximity of the bacteria to the target host cells. (c) In addition to adherence and toxicity, it has been suggested that ETEC may invade host cells, existing as free cells in the cytoplasm or within cellular vacuoles. The outer membrane protein *tia* has been shown to induce ETEC invasion of epithelial cells, suggesting that internalization could play a role in ETEC pathogenicity. Depending on the virulence factors possessed, the bacteria secrete heat-labile (d) and/or heat-stable toxins (e). (d) LT toxin subunits are translocated across the inner membrane (IM) via the SecYEG translocon (Sec) before forming its AB_5 structure in the periplasm. After type II secretion (T2S) across the outer membrane (OM), it binds to LPS on the cell surface and is associated with outer-membrane vesicles (OMV). The B subunits bind to the gangliosides on the host cell and the A subunit enters the cell, leading to an increase in cAMP. The increase in cAMP leads to phosphorylation of CFTR, resulting in increased fluid secretion and malabsorption that manifests as diarrhoea. (e) The pre-pro-peptide of STa is translocated across the inner membrane through Sec. In the periplasm, the propeptide requires DsbA to form disulphide bonds before secretion through TolC. After extracellular cleavage, STa binds to the guanylate cyclase C receptor (GC-C), leading to an increase in cGMP. An increase in cGMP leads to phosphorylation of CFTR, resulting in increased fluid secretion that presents clinically as diarrhoea. Similarly, STb is translocated through Sec to the periplasm where DsbA catalyses formation of disulphide bonds. After secretion through TolC, the toxin binds to sulphatide on the host cells, leading to an increase in Ca^{2+}. The influx of Ca^{2+} activates calmodulin-dependent protein kinase II, which opens an intestinal ion channel and may also activate protein kinase C and consequently activation of CFTR. The increased Ca^{2+} may lead to the formation of intestinal secretagogues prostaglandin E2 (PGE_2) and 5-Hydroxytryptamine (5-HT), which induce water and electrolyte transport out of intestinal cells. Such fluid and ion transport results in the secretory diarrhoea associated with ETEC infection. (f) Additional putative ETEC virulence factors include: (1) the serine protease autotransporter EatA, which could have mucinase activity or cleave proteoglycans on the host cell surface. EatA has been shown to contribute to fluid secretion although the mechanism is as yet unresolved; (2) EAST1, the *Escherichia coli* heat-stable toxin 1, is translocated across the inner membrane in a Sec-dependent fashion; however, the mechanism of outer membrane transport remains unknown. In *in vitro* models, EAST1 interacts with the host cell and stimulates guanylate cyclase, leading to increased fluid secretion; and (3) the haemolysin ClyA, which requires positive regulation by SlyA, traverses the inner and outer membranes by an unknown mechanism and is associated extracellularly with outer membrane vesicles. The protein has been shown to interact with cholesterol moieties (Chol.) in the eukaryotic cell membrane and to oligomerize to form pores in the lipid bilayer, thereby inducing cytotoxicity.

bacteria leaves them unable to colonize and cause disease (Gaastra & Svennerholm, 1996). In addition, expression of recombinant CFs in nonadherent *E. coli* can induce adherence to eukaryotic cells (Taniguchi *et al.*, 2001; Jay *et al.*, 2004). While CFs are clearly essential to disease initiation, there is accumulating evidence that additional chromosomal loci, e.g. *tia* and *tib*, are involved in interactions with and/or invasion of the host cells, and in bacterial cell–cell interactions that could promote aggregation and biofilm formation; the ability to aggregate and form biofilms can be an important part of bacterial pathogenesis (Parsek & Singh, 2003). It is possible that the CFs are responsible for initial 'long-range' contact with the host cell, after which the *tia* and *tib* loci increase the intimacy and strength of the interaction between the bacterium and host cell membrane (Fig. 1).

CFs

The genes for the structural subunits and the transport and assembly proteins required for the biogenesis of CFs are usually encoded in operons. These DNA fragments have a lower GC content and codon usage than normally associated with *E. coli* and are generally flanked by insertion sequences and transposons (Gaastra & Svennerholm, 1996; Wolf *et al.*, 1997). While some strains possess multiple CFs, most ETEC strains have no known CF, either because it is novel and as yet unidentified or has been lost in laboratory subculture (Levine *et al.*, 1984; Valvatne *et al.*, 2002).

More than 20 CFs with distinct molecular weight subunits have been identified and characterized (Table 1). The types of CF are subdivided by their antigenicity, molecular weight, N-terminal amino acid sequence of the major subunit and also structural morphology, either fimbrial, fibrillar, helical or nonfimbrial (Table 1) (Gaastra & Svennerholm, 1996; Torres *et al.*, 2005). To reduce confusion, a new nomenclature was introduced to classify human CFs designating them as coli surface antigens (CS) and a number corresponding to the chronological order of identification, with the exception of CFA/I (Gaastra & Svennerholm, 1996). The CFA/I, CFA/II and CFA/IV groups are the most prevalent CFs worldwide. CFA/I is a uniform rigid rod fimbrial structure composed of a single antigenic type of fimbria. CFA/II consists of CS3 alone or in combination with CS1 or CS2. CFA/IV consists of CS6 alone or in combination with CS4 and CS5 (Gaastra & Svennerholm, 1996). There are a number of less well-characterized CFs and new ones are likely to be identified in the future (Table 1). Animal CFs are distinct from those in human isolates and to distinguish them, the nomenclature gives them an F number. The most common of these are F4, F5 and F6, also referred to as K88, K99 and 987P fimbriae (Gaastra & de Graaf, 1982).

Table 1. Characteristics of human ETEC colonization factors*

CF designation	CS number	Morphology	MW (kDa)
CFA/I	CFA/I	Fimbrial	15.0
CFA/II	CS1	Fimbrial	16.5
CFA/II	CS2	Fimbrial	15.3
CFA/II	CS3	Fibrillae	15.1
CFA/IV	CS4	Fimbrial	17.0
CFA/IV	CS5	Helical	21.0
CFA/IV	CS6	Nonfimbrial	14.5/16.0
CS7	CS7	Helical	21.5
CFA/III	CS8	Fimbrial	18.0
2230	CS10	Nonfimbrial	16.0
PCF0148	CS11	Fibrillae	
PCF0159	CS12	Fimbrial	19.0
PCF09	CS13	Fibrillae	27.0
PCF0166	CS14	Fimbrial	15.5/17.0
8786	CS15	Nonfimbrial	16.3
CS17	CS17	Fimbrial	17.5
PCF020	CS18	Fimbrial	25.0
CS19	CS19	Fimbrial	16.0
CS20	CS20	Fimbrial	20.8
Longus	CS21	Fimbrial	22.0
CS22	CS22	Nonfimbrial	15.7

*Information derived from Gaastra & Svennerholm (1996) and Pichel *et al.* (2000).

The majority of the CFs are synthesized via the chaperone-usher-dependent pathway. In general, these CFs are encoded in a four-gene operon consisting of a periplasmic chaperone, a major fimbrial subunit, an outer membrane usher protein and a minor subunit. The minor subunit is located at the tip of the fimbriae, with the N-terminal half of the protein responsible for binding to the host cell receptor (Anantha *et al.*, 2004). This system is exemplified by the CS1 fimbriae encoded on the pCoo virulence plasmid of ETEC strain C921b-1, where CooA represents the major subunit, CooB the periplasmic chaperone, CooC the outer membrane protein and CooD the minor subunit; of note, others have used CsoA, B, C and D designations to describe the same proteins (Jordi *et al.*, 1991). The chaperone-usher pathway and the biogenesis of the CS1 fimbriae have been described in detail elsewhere (Sakellaris & Scott, 1998; Thanassi *et al.*, 2005). In brief, the periplasmic chaperone CooB associates with the CooA and CooD subunits to prevent degradation and premature polymerization and delivers the subunits to the CooC usher (Voegele *et al.*, 1997). CooC inserts into the outer membrane whence a CooB–CooD complex initiates the assembly of the CS1 fimbriae by binding of CooD to CooC and release of the CooB chaperone. A CooB–CooA complex shunts the minor subunit through the usher to the cell surface through addition of the major subunit. The pilus grows further by release of the chaperone and the sequential addition of multiple copies of the CooA protein (Froehlich *et al.*, 1994;

Voegele *et al.*, 1997). Interestingly, a dual chaperone system has been discovered for some ETEC-associated CFs e.g. the CS5 pilus. CS5 is encoded on an operon consisting of six essential gene products (*csfA–F*). CsfA is the major subunit, CsfC is the outer membrane usher protein, CsfD is the minor subunit and CsfE is an outer membrane protein involved in pilus length determination. While CsfB is the chaperone for CsfA, CsfF acts as a chaperone for CsfD and possibly also for the delivery of CsfE to the outer membrane (Duthy *et al.*, 2001, 2002).

In contradistinction to the CFs mentioned above, longus and CFA/III are both synthesized as Type IV pili in a process analogous to the Type II secretion pathway (Peabody *et al.*, 2003). CFA/III forms a long semi-flexible structure between 5 and 10 μm. In contrast, longus is situated at the poles and can be over 20 μm in length. Longus is often detected in strains producing other CFs as well as those with no known CFs. The mechanism of Type IV pilus assembly requires the presence of accessory genes, and the process of biogenesis has been described adequately elsewhere (Nudleman & Kaiser, 2004). Briefly, the CFA/III operon consists of 14 *cof* genes, including CofA, the major pilin, and CofP, the prepilin peptidase. CofP processes CofA and the other minor precursor subunits on the cytoplasmic side of the membrane to produce the mature subunits, and the accessory proteins are involved in the secretion of these subunits across the cell envelope, anchoring and polymerizing the subunits into the pilus organelle (Pugsley, 1993). The CFA/III system bears homology to the *Vibrio cholerae* Tcp pilus, leading other researchers to speculate that they have a common ancestor (Taniguchi *et al.*, 2001).

No matter whether the CFs are synthesized as Type IV pili or via the chaperone-usher pathway production of large surface appendages, such as the CFs, it is energetically expensive and is therefore tightly regulated by the bacterium. The expression of the CS1 pilin operon is silenced by the nucleoid protein H-NS and is positively regulated by an AraC family transcriptional regulator Rns (Murphree *et al.*, 1997). Homologues of Rns have been identified for many of the other fimbriae. The mechanism of action of these regulators has been reviewed previously (Martin & Rosner, 2001).

Once the CFs are expressed and located on the cell surface, the adhesive moiety can interact with the cognate receptor on the host cell surface. The receptors for CFs have not been fully characterized but some are known to be glycoconjugates in the eukaryotic cell membrane; these glycoconjugates have a range of oligosaccharide structures that can confer upon ETEC species tissue and cell specificity (Lindahl & Wadstrom, 1984; Blomberg *et al.*, 1993; Erickson *et al.*, 1994). An example of a characterized receptor is the glycolipid ganglioside NeuGc-GM3 for ETEC K99 fimbriae, which are associated with disease in pigs (Lanne *et al.*, 1995). However, given the diversity of ETEC CFs, there is likely to be a great diversity in the number and types of receptors for these adhesins.

TibA, a glycosylated adhesin

As mentioned previously, other non-CF chromosomally encoded adhesins have been described for ETEC. Thus, Elsinghorst & Kopecko (1992) described *tib*, a chromosomally encoded locus associated with nonfimbrial adherence of ETEC to human cells. This locus consists of four genes, *tibDBCA*, organized in a single operon. TibA, a member of the autotransporter family, is a 104 kDA glycosylated outer membrane protein found in some ETEC strains (Elsinghorst & Weitz, 1994; Lindenthal & Elsinghorst, 1999). Autotransporters are synthesized as single polypeptides that consist of (1) an N-terminal signal peptide mediating translocation across the inner membrane, (2) a passenger domain that adopts a β-helix conformation and that is the effector part of the protein and (3) a C-terminal translocation unit that facilitates translocation of the passenger domain across the outer membrane (Henderson *et al.*, 2004). *tibC* encodes a 45 kDa heptosyltransferase that glycosylates TibA by the addition of heptose residues (Lindenthal & Elsinghorst, 1999; Moormann *et al.*, 2002). *In vivo* production of a mature TibA requires the whole operon; however, the roles of TibD and TibB are as yet unknown but it has been suggested that they are involved in gene regulation (Lindenthal & Elsinghorst, 1999). Only the glycosylated form of TibA directs the bacteria to bind to a specific receptor on epithelial cells and invade (Lindenthal & Elsinghorst, 2001; Sherlock *et al.*, 2005). In addition to adherence to mammalian cells, TibA promotes aggregation of a bacterial population and the formation of a biofilm and this occurs independent of TibA glycosylation (Sherlock *et al.*, 2005). The ability to aggregate and form biofilms can be an important part of bacterial pathogenesis (Parsek & Singh, 2003).

Tia, an ETEC adhesin/invasin

In addition to TibA, Elsinghorst & Kopecko (1992) described a second chromosomally encoded adhesin designated Tia. In ETEC strain H10407, *tia* is encoded on a 46-kb pathogenicity island with a lower GC content than the rest of the genome (Fleckenstein *et al.*, 2000). Interestingly, a *tia* probe hybridized to ETEC, EPEC and EAEC strains, suggesting that it may play a wider role in *E. coli* pathogenesis (Fleckenstein *et al.*, 1996). Tia is translated with a classical signal peptide that is cleaved from the N-terminus to produce the 25 kDa mature outer membrane protein, which is predicted to have eight transmembrane amphipathic strands with four surface-exposed loops (Fleckenstein *et al.*, 1996; Mammarappallil & Elsinghorst, 2000). Interestingly,

in the initial study, Elsinghorst & Kopecko (1992) described the presence of ETEC associated with the cell membrane, within endocytic vesicles and also free in the cytoplasm of epithelial cell lines derived from the human ileocaecum and colon. Antisera recognizing Tia blocked invasion by *E. coli* expressing Tia, suggesting that Tia acts as an invasin as well as an adhesin (Mammarappallil & Elsinghorst, 2000). A 19-amino-acid peptide from one of the putative surface loops inhibited the invasion of *E. coli* expressing Tia, suggesting that this region is involved in its activity. Recombinant purified Tia protein binds to epithelial cells in a saturable concentration-dependent manner, suggesting a specific receptor for Tia. Indeed, subsequent investigations revealed that Tia binds to heparin-sulphate proteoglycans, which are abundant glycosaminoglycans located on eukaryotic cell surfaces (Fleckenstein *et al.*, 2002). Thus, contrary to earlier opinions, there is increasing evidence that ETEC, in addition to adhering to the intestinal mucosa, can also invade epithelial cells.

Cytotoxic effects

Having established close contact with the host cell, ETEC strains can elaborate one or more heat-stable or heat-labile enterotoxins (ST or LT, respectively), which induce fluid secretion and/or inhibition of absorption in the gut. These toxic effects are characteristic of ETEC infection and lead directly to the manifestation of secretory diarrhoea. However, in addition to the classical ST and LT toxins associated with ETEC-mediated diarrhoea, a number of other recently described toxins have been implicated in ETEC pathogenesis viz. EAST1, EatA and ClyA. The roles of these toxins are discussed below.

Heat-stable enterotoxins

The main heat-stable toxins (STs) can be divided into two structural, functional and antigenically distinct groups: the methanol-soluble, protease-resistant STa (or STI) and the methanol-insoluble, protease-sensitive STb (or STII). The toxins have different targets and maximal doses of STa and STb have synergistic effects on fluid accumulation in a pig intestinal loop model, suggesting they have different mechanisms of action (Peterson & Whipp, 1995).

STa toxins have been categorized according to the host from which the specific ETEC strain was isolated. STh was produced by an ETEC strain infecting humans while STp was identified from a strain that infected pigs, although variants of STp have now been isolated from strains of bovine and human origin (Nair & Takeda, 1998). Both types are synthesized as a pre–pro-peptide of 72 amino acids that are processed during export to produce the mature active toxins of 18 or 19 amino acids (Fig. 1). The Sec-dependent signal sequence is removed during translocation across the inner membrane, releasing the propeptide into the periplasmic space where disulphide bond isomerase, DsbA, catalyses the formation of three disulphide bonds in the C-terminus of the propeptide before it is exported through the TolC outer membrane protein transporter. The proregion is removed to release the small mature toxin. The disulphide bond formation in the mature portion of these toxins confers their heat stability (Yamanaka *et al.*, 1998; Nair & Takeda, 1998).

STa binds to the homotrimeric guanylate cyclase C receptor (GC-C), an N-linked glycosylated protein located in the brush border of the intestine, activating its guanylate cyclase domain, which results in an increase in intracellular cGMP levels (Nair & Takeda, 1998; Vaandrager, 2002). Increased cGMP leads to activation of the cystic fibrosis transmembrane conductance regulator chloride channel (CFTR) via cGMP-dependent protein kinase II phosphorylation, resulting in enhanced salt and water secretion and inhibition of Na^+ absorption through an apical Na/H exchanger (Goldstein *et al.*, 1994; Tien *et al.*, 1994; Vaandrager, 2002). There is evidence for a second pathway for cGMP generation in human colonic cells. STa can mediate inositol triphosphate-dependent release of calcium from intracellular stores (Bhattacharya & Chakrabarti, 1998). The elevated calcium levels induce translocation of protein kinase C from the cytoplasm to the membrane where it activates guanylate cyclase leading to cGMP production (Khare *et al.*, 1994; Ganguly *et al.*, 2001; Gupta *et al.*, 2005). Recently, STa has also been shown to stimulate bicarbonate secretion independent of CFTR but the mechanism behind this is still unknown (Sellers *et al.*, 2005).

STb is mostly associated with porcine strains of ETEC but has also been found in human isolates (Lortie *et al.*, 1991; Okamoto *et al.*, 1993). The gene encoding STb, *estII*, encodes a 71 amino acid ORF consisting of a typical 23-residue Sec-dependent signal peptide, which is removed during translocation across the inner membrane releasing the 48 amino acid toxin into the periplasm. Once in the periplasm, DsbA forms four disulphide bonds within STb before it is exported through TolC (Fig. 1) (Foreman *et al.*, 1995; Nair & Takeda, 1998).

STb binds to sulphatide, a widely distributed acidic glycosphingolipid (Rousset *et al.*, 1998). Fluid secretion occurs after internalization of the toxin via the activation of a pertussis toxin-sensitive GTP-binding regulatory protein. This results in an influx of calcium through a receptor-dependent ligand-gated calcium channel activating calmodulin-dependent protein kinase II, which opens an intestinal ion channel and may also activate protein kinase C and consequently CFTR (Dreyfus *et al.*, 1993; Sears & Kaper, 1996; Fujii *et al.*, 1997). The increased calcium levels are also thought to regulate phospholipases (A2 and C) that release arachidonic acid from membrane phospholipids, leading to

the formation of intestinal secretagogues prostaglandin E2 (PGE_2) and 5-hydroxytryptamine (5-HT), which mediate water and electrolyte transport out of intestinal cells (Hitotsubashi *et al.*, 1992; Harville & Dreyfus, 1995; Peterson & Whipp, 1995). PGE_2 and 5-HT are likely to activate the enteric nervous system (Sears & Kaper, 1996; Dubreuil, 1997).

EAST1, a novel heat-stable enterotoxin

In contrast to STa and STb, the 38 amino acid EAST1 toxin is less well characterized both in terms of function and contribution to ETEC-mediated disease. While EAST1 was originally isolated from EAEC strain 17-2, it has now been identified in other pathotypes of *E. coli* including ETEC strains from human and animal sources (Yamamoto & Echeverria, 1996; Yamamoto & Nakazawa, 1997; Veilleux & Dubreuil, 2006). However, the role of EAST1 in mediating diarrhoea remains controversial; while several epidemiological studies have implicated the toxin in mediating disease (Menard & Dubreuil, 2002; Veilleux & Dubreuil, 2006), EAEC strain 17-2 failed to elicit diarrhoea in human volunteer studies (Nataro *et al.*, 1995). However, *in vivo* assays have demonstrated that EAST1 isolated from EAEC can induce a fluid accumulation response in suckling mice and in the rabbit ileal loop model. Furthermore, EAST1 demonstrates enterotoxic activity in the Ussing chamber and this activity is partially heat resistant and can be induced by a synthetic peptide consisting of amino acid residues 8–29 (Savarino *et al.*, 1991; Savarino *et al.*, 1993). EAST1 demonstrates several similarities to STa: (i) like STa, disulphide bond formation within EAST1 confers heat resistance and is necessary for function; (ii) the functional regions of STa and EAST1 demonstrate 50% identity; (iii) like STa, EAST1 activates production of cGMP; and (iv) GC-C on eukaryotic cell surfaces appears to act as a receptor for both toxins. However, anti-STa antibodies fail to abolish the enterotoxic activity of EAST1 (Savarino *et al.*, 1991; Savarino *et al.*, 1993; Menard *et al.*, 2004). The distribution and function of this heat-stable toxin have been reviewed by others (Menard & Dubreuil, 2002; Veilleux & Dubreuil, 2006).

Heat-labile enterotoxins

In contrast to EAST1, the heat-labile enterotoxins (LT) encoded by the *eltAB* operon are probably the most well-characterized virulence determinants of ETEC due to their close homology with the cholera toxin (CT) (Spangler, 1992). It is clear that production of LT contributes to the induction of diarrhoea and the effects are synergistic with the ST and EAST1 toxins, indicating that they have different mechanisms of action (Berberov *et al.*, 2004).

As members of the AB_5 family of enterotoxins, the 84 kDa mature LT is a pentameric ring of five 11.6 kDa B subunits associated with one A subunit of 28 kDa. The A subunit consists of two structurally distinct domains linked by a disulphide bond; the A1 domain possesses ADP ribosylase activity and NAD glycohydrolase activity, whereas the A2 fragment mediates interactions with the B subunits to form the AB_5 complex (Spangler, 1992).

The issue of how, or even if, LT is secreted into the extracellular environment has been somewhat controversial. LT has been reported over the years to remain in the periplasmic space or to be associated with the extracellular surface of the bacterium. However, a functional Type II secretion apparatus consisting of 11 chromosomally encoded genes (*gspC-M*) was recently detected in ETEC strains and was demonstrated to be required for LT secretion across the outer membrane (Tauschek *et al.*, 2002). The Type II secretion system in Gram-negative bacteria is a two-step process in which proteins targeted for secretion are first translocated across the inner membrane via the Sec translocon and fold into a translocation-competent state in the periplasm before the Type II apparatus translocates the proteins across the outer membrane (Pugsley, 1993; Sandkvist, 2001). Once in the periplasm, the subunits are rapidly assembled into a mature AB_5 holotoxin in a process that is dependent on DsbA (Yu *et al.*, 1992). However, secretion of LT appears to have an additional level of complexity compared with other Type II secreted proteins. Accordingly, Fleckenstein *et al.* (2000) identified a four-gene operon located on a pathogenicity island that was required for LT secretion. Deletion of *leoA*, a gene within the operon, abolished LT secretion and a strain lacking the operon accumulated LT in the periplasm. However, the exact contribution that this operon makes in LT secretion remains enigmatic.

After secretion across the outer membrane, the toxin binds to LPS on the extracellular surface of the bacteria via its B subunit (Horstman & Kuehn, 2002; Horstman *et al.*, 2004). The majority of LT activity in the extracellular environment is associated with outer membrane vesicles, on both the outside and inside of the vesicle (Horstman & Kuehn, 2000; Horstman *et al.*, 2004). It has also been suggested that the whole outer membrane vesicle is internalized by the host cell; however, it is clear that even though it is bound to LPS the B subunit remains able to bind to its mammalian cell surface receptor (Fukuta *et al.*, 1988; Horstman & Kuehn, 2002; Kesty *et al.*, 2004).

The type of cell surface receptor to which the LT toxin binds depends on the specificity of the B subunit. In this context, the LTs can be split into two groups: LT-I and LT-II. LT-I toxins are most closely related to CT with 75% similarity in the A subunit and 77% in the B subunit. Two distinct LT-II enterotoxins, LT-IIa and LT-IIb, have been

isolated with biological activities similar to LT-I but they do not cross react immunologically. While the A subunit of LT-IIa and IIb share similarity to each other and to CT and LT-I, the B subunits are not significantly homologous to B subunits of CT or LT-I (Spangler, 1992). These differences in sequence confer upon the B subunits the ability to recognize different ganglioside receptors (Fukuta *et al.*, 1988). In addition to binding to gangliosides, the B subunits of LT-IIa and LT-IIb can interact with Toll-like receptor 2-stimulating cellular activation and cytokine release (Hajishengallis *et al.*, 2005). This interaction could be partly responsible for the immunomodulatory activity of the B subunits in stimulating mucosal immunity (Rappuoli *et al.*, 1999).

Once the B subunits bind to the membrane receptor, they mediate entry of the A subunit into the host cell (Spangler, 1992). The disulphide bond connecting the A subunit domain remains unreduced until the B subunit contacts the cell surface where reduction allows the A1 subunit to enter the cell. For full toxicity, the A subunit must also be cleaved proteolytically to separate the two domains (Lencer *et al.*, 1997). Once inside the cell, the A subunit ADP ribosylates the stimulatory G protein ($G_{S\alpha}$), the stimulatory component of the adenylate cyclase complex, which leads to permanent activation of the adenylate cyclase complex and subsequent elevated intracellular levels of cAMP. This in turn leads to activation of cAMP-dependent protein kinase A and phosphorylation of chloride channels, like CFTR, to stimulate Cl^- secretion and reduced absorption of Na^+ (Spangler, 1992). In addition, the A subunit has been implicated in stimulating arachidonic acid metabolism leading to the production of the secretagogues PGE_2 and 5-HT, and in turn stimulation of intestinal secretion (Nataro & Kaper, 1998).

EatA, a serine protease autotransporter

In addition to the enterotoxins described above, several other putative toxins have recently been described, including EatA, a second autotransporter of ETEC encoded on the pCS1 of *E. coli* H10407 and in multiple clinical ETEC isolates (Patel *et al.*, 2004). EatA possesses homology to a wider group of autotransporters termed the serine protease autotransporters of the *Enterobacteriaceae*, or SPATEs, which have all been implicated as virulence factors acting as toxins and extracellular proteases (Henderson *et al.*, 2004). Indeed, EatA shares 85% similarity with SepA, a SPATE from *Shigella flexneri* (Benjelloun-Touimi *et al.*, 1995; Patel *et al.*, 2004). *In vitro* studies demonstrated that EatA possesses the same specificity as SepA for *p*-nitroanilide-conjugated oligopeptides, and that site-directed mutagenesis of the residues within the predicted serine protease catalytic triad abolished the ability of EatA to cleave the oligopeptides (Benjelloun-Touimi *et al.*, 1998; Patel *et al.*, 2004). The oligopeptides cleaved by EatA were identified as substrates for cathepsin G, a serine protease that modulates or cleaves a diverse array of extracellular products including proteoglycans and cell surface protease-activated receptors, suggesting that EatA may play a role in ETEC virulence by damaging the epithelial cell surface (Patel *et al.*, 2004). Interestingly, a role for SepA in the virulence of *S. flexneri* has been implied from a reduced ability to induce both mucosal atrophy and tissue inflammation in the rabbit ligated loop model (Benjelloun-Touimi *et al.*, 1995).

In vivo studies of an *eatA* mutant with its isogenic parent strain, in the rabbit ileal loop model of infection, indicated that there were no differences between the strains at 16 h postinfection. In contrast, the *eatA* mutant elicited less fluid accumulation at 7 h postinfection compared with the wild type, even though similar numbers of bacteria could be recovered from the loops, indicating that the differences were not due to bacterial numbers or an altered survival rate. Furthermore, at 7 h postinfection, the *eatA* mutant did not demonstrate foci of mucosal destruction and leucocyte infiltration characteristic of the wild-type organism (Patel *et al.*, 2004). These data suggest that while EatA is not absolutely required for infection, it may act to accelerate ETEC virulence.

ClyA, a pore-forming cytotoxin

Like EatA, the precise role of ClyA in ETEC-mediated disease remains speculative. ClyA, also designated HlyE and SheA, was first identified in *E. coli* K-12 but has now been detected in a range of *E. coli* clinical isolates including ETEC strains (Ludwig *et al.*, 1999). Expression of ClyA is negatively regulated by H-NS and positively regulated by SlyA, FNR and CRP (Oscarsson *et al.*, 1996; Westermark *et al.*, 2000). Confusingly, *E. coli* strains, including ETEC isolates, possessing *clyA* do not express ClyA under normal laboratory growth conditions but expression can be induced when the positive regulator SlyA is added *in trans*, suggesting that ClyA expression could be switched on during the infectious process. (Ludwig *et al.*, 2004).

Nevertheless, *in vitro* assays demonstrated that purified ClyA was able to cause haemolysis of erythrocytes, and cytotoxicity in macrophages and HeLa cells (Oscarsson *et al.*, 1999; Wai *et al.*, 2003). Further studies demonstrated that the monomeric form of ClyA consisted of a rod-shaped molecule composed of a bundle of four α-helices and that the monomers oligomerized together to form pores in lipid bilayers (Wallace *et al.*, 2000). Pore formation appears to be responsible for the toxic phenotype and is more pronounced in the presence of cholesterol, suggesting that cholesterol in the eukaryotic cell membrane acts as the receptor for the ClyA toxin (Oscarsson *et al.*, 1999).

While the toxic effects of ClyA are well defined, the mechanism of ClyA egress from the bacterial cell is still poorly understood. ClyA does not possess an N-terminal signal sequence to traverse the inner membrane, although the C-terminal amino acid residues do appear to be important to the translocation process. Once the molecule has traversed the inner membrane it appears to accumulate in the periplasm in a monomeric form. While the mechanism of ClyA secretion remains enigmatic, it has recently been demonstrated that ClyA is released from bacterial cells within OMVs. Interestingly, these OMVs possess an oligomeric form of ClyA and are toxic to eukaryotic cells (Wai *et al.*, 2003).

Future prospects for the fight against ETEC

ETEC infections are classically defined as being caused by the bacteria producing a CF and one or more specific toxins. However, the emergence of new virulence factors such as EatA and ClyA suggests that this may not be the complete story and that the possession of a varying number of virulence factors could account for the variety of disease severity that is often observed clinically with ETEC infection. Undoubtedly, the current ETEC genome sequencing projects nearing completion at The Sanger Centre, UK, and The Institute for Genomic Research, USA, will reveal new virulence factors. Indeed, the recent sequencing of the pCoo plasmid revealed the presence of *virK*, which has been implicated in *S. flexneri* pathogenesis (Froehlich *et al.*, 2005).

While much of the research on ETEC over the last several decades has focused on the plasmid-encoded factors, little was known about the ETEC chromosomal background. Recently, subtractive hybridization and optical mapping of the ETEC type strain *E. coli* H10407 showed that it was very similar to *E. coli* K-12 MG1655 with a predicted identity of 96% (Chen *et al.*, 2006). The unique regions identified included some of the known ETEC-specific genes such as *tia* and *tib*, genes of known function identified in other strains, sequences with homology to phage and prophages, hypothetical proteins and unknown proteins. The gene synteny appears to be very similar to *E. coli* MG1655 with no inversions, transpositions or rearrangements. This conforms with recent phylogenetic analysis in our lab and others, which showed that *E. coli* H10407 is a group A strain closely related to *E. coli* MG1655 (Escobar-Paramo *et al.*, 2004). However, recent work in our lab has suggested that *E. coli* H10407 is not typical of ETEC strains and that there is a rich diversity in the chromosomal background of ETEC (S.M. Turner and I.R. Henderson, unpublished data). Thus, it will be interesting to determine whether ETEC, in addition to the plasmid-encoded toxins and CFs, possess other conserved key determinants of pathogenicity.

In addition to further defining CFs and enterotoxin function, much of the current research in ETEC is concerned with establishing an effective vaccine strategy to control this disease. This review has focused on the pathogenesis of ETEC, an understanding of which is essential for vaccine development. To be effective, any vaccine must contain antigens from a broad range of prevalent ETEC types. The use of purified CFs, LT B subunits, inactivated whole cells and live-attenuated organisms have all been investigated as potential vaccines and have been reviewed elsewhere (Boedeker, 2005; Qadri *et al.*, 2005). Sequence data will undoubtedly identify potential virulence factors and aid our understanding of this important global pathogen, thereby contributing to the quest for a successful vaccination strategy.

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