Journal of Applied Microbiology 2000, **88**, 729–745

A REVIEW

Virulence factors of *Escherichia coli* O157 and other Shiga toxin-producing *E. coli*

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56/11/99: received 10 November 1999, revised 14 January 2000 and accepted 18 January 2000

1. ABSTRACT

Shiga toxin-producing *Escherichia coli* O157 are important enteropathogens causing outbreaks of haemorrhagic colitis and haemolytic uraemic syndrome. Strains of *E. coli* belonging to other serogroups also produce Shiga toxins but are less frequently isolated from cases of diarrhoeal illness despite humans having greater exposure to these organisms in food and the environment. It is generally considered that *E. coli* O157 is more virulent than these other Shiga toxin-producing *E. coli*. The question of which factors make toxigenic *E. coli* O157 more virulent is unanswered. In this review the various virulence properties of *E. coli* O157 and their incidence in non-O157 Shiga toxin-producing *E. coli* are described. The most important factors for *E. coli* O157 are the production of Shiga toxin 2 and the adhesin intimin. The role of some of the other virulence factors, such as enterohaemolysin, a serine protease (EspP) and a catalase/peroxidase (Katp), in infection may be low. Uncharacterized virulence properties such as a clostridial-like toxin and haemoglobin uptake require further study and it is likely that other virulence properties remain to be discovered.

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2. INTRODUCTION

Shiga toxin-producing *E. coli* (STEC) are now recognized as an important group of bacterial enteropathogens. There are at least 100 serotypes of *E. coli* that are capable of producing Shiga toxins (Nataro and Kaper 1998); however, of these serotypes *E. coli* O157:H7 is the most well known to both microbiologists and the general public. This organism was first recognized in 1982 following an outbreak of haemorrhagic colitis (HC) in the USA (Riley *et al.* 1983). Since then, STEC O157 have been implicated in sporadic cases and outbreaks of diarrhoea world-wide. Some outbreaks have been large, e.g. Japan where over 9000 children were infected (Michino *et al.* 1998). The organism is particularly associated with the devel-

opment of the haemolytic uraemic syndrome (HUS), which complicates about 10% of cases of *E. coli* O157 infection and which carries a mortality rate of 2–10%. In a recent outbreak in Scotland, there were 21 deaths amongst over 400 individuals infected with the organism (Ahmed and Donaghy 1998). The potentially high mortality associated with *E. coli* O157 infection differentiates this organism from other types of *E. coli* such as enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC) and enteroaggregative *E. coli* (EAggEC).

The production of Shiga toxins or Verotoxins is one of the defining characteristics of *E. coli* O157; these toxins are thought to be responsible for the principal manifestations of HC and HUS. As described above, many serotypes of *E. coli* are capable of producing Shiga toxins. Non-O157 STEC have been isolated from cases of sporadic diarrhoea and HUS with varying frequencies. Some estimates suggest that, in the USA, 25% of HUS cases are caused by non-O157 STEC (Johnson *et al*. 1996). The relative isolation rates of non-O157 STEC and *E. coli* O157 vary from study to study and are influenced both by geographical area and the detection methods employed. In general, the isolation rates of *E. coli* O157 from human infections are higher than those of non-O157 STEC (Griffin and Tauxe 1991), although in a minority of studies the situation is reversed (Law 1997). This apparent predominance of *E. coli* O157 is almost certainly genuine, but may also be influenced by the detection methods used. The commonly used detection methods involving culture on sorbitol MacConkey agar, often with an immunomagnetic separation step, are capable of detecting *E. coli* O157 only (Strockbine *et al*. 1998). Non-O157 STEC infections are often overlooked or not detected. Outbreaks attributed to non-O157 STEC have been described, but are rare compared with those due to *E. coli* O157. In a recent review, eight outbreaks caused by non-O157 STEC were described (Johnson *et al*. 1996), although data implicating STEC in some outbreaks were scanty and the source of infection was not always known. Although detection of non-O157 STEC is more difficult than detection of *E. coli* O157, it is unlikely in the current climate of awareness that major outbreaks caused by non-O157 STEC would go undetected. Therefore, it would appear that outbreaks caused by non-O157 serotypes are a rare occurrence.

While *E. coli* O157 causes most human STEC infections, the isolation rates of non-O157 STEC from foods and animal faeces are higher than those of *E. coli* O157. Canadian studies found evidence of non-O157 STEC infection in 17 and 45% of cattle yet the incidence of *E. coli* O157 in the two studies was < 1% (Johnson *et al*. 1996). A study in Germany found an incidence of non-O157 STEC in cattle of 26 of 259, whereas for *E. coli* O157 the incidence was 2 of 259 (Montenegro *et al*. 1990). Surveys of ground meats have shown detection rates of 15–40% for non-O157 STEC, whilst in most studies *E. coli* O157 was not detected (Johnson *et al*. 1996). In a UK study Smith *et al*. (1991) found that 25% of pork sausages contained non-O157 STEC but *E. coli* O157 were not detected; in another British study non-O157 STEC were isolated from 17% of raw beef samples but again *E. coli* O157 were not isolated (Willshaw *et al*. 1993).

These findings imply that humans are exposed to non-O157 STEC from food and environmental sources considerably more often than *E. coli* O157. Yet despite more frequent exposure, the incidence of non-O157 STEC infections is lower than that of *E. coli* O157 infections (Johnson *et al*. 1996). It can therefore be concluded that either *E. coli* O157 is more virulent or transmissible than other STEC or that non-O157 STEC may produce a milder form of illness that is seldom brought to medical attention.

Non-O157 STEC are not equally pathogenic; certain serogroups, e.g. O26, O103 and O111, predominate in cases of human illness (Paton and Paton 1998b) and many of the STEC serotypes isolated from cattle and foods have not been implicated in cases of human infection (Smith *et al*. 1991; Johnson *et al*. 1996). Differences in pathogenic properties between *E. coli* O157:H7 isolates have been detected, implying some heterogeneity within this serotype (Baker *et al*. 1997; Horii *et al*. 1998; Benito *et al*. 1999). There is an extensive spectrum of pathogenicity within the STEC group, ranging from *E. coli* O157 isolates with outbreak potential to non-O157 isolates that have not been associated with human disease and which are possibly non-pathogenic.

The genes encoding Shiga toxins are encoded on bacteriophages (Scotland *et al*. 1983); it is therefore conceivable that *E. coli* of any serotype can acquire the toxin genes. Current opinion suggests that the ability of an organism to produce toxin alone is insufficient for that organism to cause disease. Acquisition of toxin genes is likely to confer pathogenicity only upon an organism that contains a background of appropriate complementary virulence factors (Tarr and Neill 1996).

There are several major obstacles to identifying the important pathogenic mechanisms of *E. coli* O157 and other STEC. Firstly, the lack of suitable animal models that mimic all of the aspects of infection in humans. STEC infection of animals produces either no symptoms or watery diarrhoea and animals do not develop HC or HUS. It is therefore difficult to identify the factors that contribute to these conditions in humans. When STEC are isolated, there is currently no assay that allows differentiation of those isolates capable of causing disease from those which may not. Secondly, with other pathogenic *E. coli,* volunteer studies have proved crucial in understanding their pathogenesis (Levine *et al*. 1978, 1985; Donnenberg *et al*. 1993a; Nataro *et al*. 1995). The high risk of potentially lethal complications following STEC infections prevents volunteer studies being carried out. However, outbreaks of natural infection have provided important details on the virulence of STEC. Thirdly, STEC virulence is likely to be multifactorial and the relative contribution of the var-

ious components is unknown. It is possible that certain virulence properties may be dependent on host factors or only required under certain circumstances, e.g. low infective dose, during part of the infection process or for infection of certain animal hosts. Fourthly, only strains of *E. coli* O157 have been well characterized and studied. Because of the plethora of other STEC strains, these have generally been poorly characterized and the incidence of virulence properties in these strains is largely unknown.

One of the most important factors influencing *E. coli* O157 infections is the low infectious dose, estimated at between 20 and 700 organisms in one study (Tuttle *et al.* 1999) and less than 100 organisms in another (Willshaw *et al.* 1994). This is in marked contrast to EPEC which require an inoculum of $> 10^8$ organisms to produce experimental infections in adults (Levine *et al.* 1978, 1985; Donnenberg *et al.* 1993a). An outbreak of HUS and diarrhoea in Australia illustrated the low infectious dose of some non-O157 STEC and differences in virulence between STEC serotypes (Paton *et al.* 1996). In this outbreak, traced to infected mettwurst sausage, bacteriological and molecular analysis of the sausage revealed at least seven STEC serotypes, all present in the food in low numbers. Amongst patients with diarrhoea and bloody diarrhoea, five STEC serotypes were isolated. Among 21 cases of HUS, only three STEC serotypes were isolated, and one serotype, O111:H–, predominated. There was evidence of infection with this serotype in all of the HUS patients. These subjects each ingested several strains of STEC in low numbers, but only a minority of the strains were capable of causing disease.

In this review the potential virulence factors of *E. coli* O157 will be examined, their incidence in *E. coli* O157 and non-O157 STEC compared and possible reasons for the higher virulence of the former group will be discussed.

3. CHARACTERISTICS OF SHIGA TOXIN-PRODUCING *ESCHERICHIA COLI* O157

Firstly, it is important to describe briefly the characteristics of *E. coli* O157. Shiga toxin-producing strains of this serogroup are clonal in origin and phenotypically and genotypically very similar. The main toxin-producing serotype isolated is O157:H7, although O157:H– non-motile variants are occasionally isolated. The biochemical reactions of *E. coli* O157 are similar to other strains of *E. coli* with several important exceptions. *Escherichia coli* O157 isolates do not ferment sorbitol within 24 h and do not produce β-glucuronidase, characteristics exploited in differential media for their isolation (Strockbine *et al.* 1998). However, sorbitol-fermenting, β-glucuronidase-producing toxigenic *E. coli* O157 have been isolated, and such organisms may account for 50% of *E. coli* O157 infections in Germany (Gunzer *et al.* 1992); these organisms represent a distinct clone of *E. coli* O157 (Whittam 1998). Most isolates of *E. coli* O157 produce Shiga toxin 2 (Stx2) only; Shiga toxin 1 (Stx1) and Stx2 producers are occasionally found but isolates producing Stx1 only are rare (Griffin and Tauxe 1991). The majority of *E. coli* O157 isolates possess a large 60-MDa plasmid designated pO157 (Schmidt *et al.* 1994), a terminology used in this review. The pO157 plasmid hybridizes with a DNA probe termed CVD419, which has frequently been used to identify *E. coli* O157 and other STEC (Levine *et al.* 1987). The pO157 plasmid has been sequenced and several potential virulence factors identified (Burland *et al.* 1998). Some isolates also contain smaller plasmids; a 6·7-kb plasmid found in over half of *E. coli* O157:H7 strains is associated with a colicinogenic phenotype (Karch *et al.* 1998).

3.1 Attaching and effacing lesions

One of the most important characteristics of STEC O157 and some other STEC is the ability to produce attaching and effacing (A/E) lesions on a variety of cell types (Kaper *et al.* 1998a). These lesions are also produced by EPEC and are characterized by degeneration and effacement of intestinal epithelial cell microvilli, intimate adherence of bacteria to the epithelial cells and assembly of highly organized cytoskeletal structures in the cells beneath intimately attached bacteria (Knutton *et al.* 1989). This structure is composed of cytoskeletal components such as actin, talin, ezrin and α-actinin (Kaper *et al.* 1998a). The term enterohaemorrhagic *E. coli* (EHEC) has often been used to describe STEC frequently isolated from cases of human infection that possess the pO157 plasmid and produce A/E lesions (Nataro and Kaper 1998). STEC of serogroups O157, O26 and O111 are often classed as EHEC.

Attachment of EPEC to epithelial cells induces a variety of signal transduction pathways in the eukaryotic cell. These signals are responsible for the formation of the A/E lesions, ion secretion and bacterial invasion. In EPEC all of the genes necessary for the A/E phenotype are encoded in a 35·6-kb chromosomal pathogenicity island termed the locus of enterocyte effacement (LEE) (McDaniel and Kaper 1997). This locus has been sequenced in both EPEC (Elliott *et al.* 1998) and *E. coli* O157 (Perna *et al.* 1998) and the function of many of the genes has been deduced. The LEE of *E. coli* O157 is very similar in structure, organization and gene arrangement to the EPEC LEE although it is slightly larger (Perna *et al.* 1998). The LEE can be divided into several distinct regions. At one end are genes *EspA, EspB* and *EspD*, encoding secreted proteins required for signal transduction and A/E activity. At the other end lie the genes encoding a type III secretion apparatus, required for secretion of proteins including the products of *EspA, EspB* and *EspD*. A third

region between the others contains the *eae* gene encoding intimin, an outer membrane protein required for intimate attachment, and *Tir*, encoding the protein Tir (translocated intimin receptor) which acts as a receptor for intimin (Kenny *et al*. 1997; Frankel *et al*. 1998; Kaper *et al*. 1998a). There are other genes encoded within the locus although their precise function is not yet clear (Kaper *et al*. 1998a). The role of LEE genes in adhesion is described in detail below.

4. ACID RESISTANCE

Enterohaemorrhagic *E. coli* must pass through the acidic environment of the stomach if they are to cause gastrointestinal disease. There are several mechanisms that enable *E. coli* to resist acidic conditions, an acid-induced oxidative system, an acid-induced arginine-dependent system and a glutamate-dependent system. At pH 2·0 the arginine-dependent system provided more protection in EHEC strains than in commensal *E. coli* strains, although the glutamate-dependent system was equally effective in all strains (Lin *et al*. 1996). The oxidative resistance mechanism is dependent on the alternate sigma factor *rpoS*. This regulates genes required for existence at low pH, allowing survival at a pH below 2·5 for over 2 h. Once induced, acid resistance is stable during storage at 4 °C. Stationary phase bacteria are more than 1000 times more resistant to acid than exponentially growing organisms and do not need prior exposure to low pH to exhibit resistance.

Waterman and Small (1996) compared the survival of *E. coli* O157 and non-O157 STEC at pH 2·5. Of 38 *E. coli* O157 isolates, 29 were resistant, two showed intermediate susceptibility and seven were susceptible. Of 20 non-O157 isolates, 16 were resistant, one was intermediate and three were susceptible. There is little difference between the two groups, suggesting that acid tolerance does not explain the enhanced virulence of *E. coli* O157 compared with non-O157 STEC. Mutations in the *rpoS* gene may account for differences in acid resistance (Waterman and Small 1996) and Ferreira *et al*. (1999) noted differences in the *rpoS* gene between *E. coli* O157 isolates and laboratory *E. coli* strains. A further study showed that strains of *E. coli* belonging to serogroups O157 and O111 were more acid tolerant than serogroups O26 and O128, which in turn were more acid tolerant than strains belonging to serogroups O91 and O5 (O'Brien *et al*. 1997).

The acid resistance of *E. coli* O157 and some other EHEC may be associated with these organisms causing disease from low inocula. This may be of greater significance for waterborne infections where organisms may be directly exposed to stomach acid. For food-acquired infections acid resistance may be of lesser significance because of bacterial protection by food components. Acid tolerance will, however, enhance the survival of organisms in foods which rely on acidic pH to inactivate pathogens, such as fermented sausages, mayonnaise, yoghurt and apple juice. All of these foods are known vehicles of infection with *E. coli* O157 (Meng and Doyle 1998). Of further interest is the finding that heat stress increases the acid resistance of *E. coli* O157 (Wang and Doyle 1998), thus stressed but viable organisms present in cooked foods may survive transit through the stomach better than those in an unstressed state.

5. ADHESION

Following passage through the stomach, viable organisms must adhere to the bowel mucosa to prevent their removal by the peristaltic flow. Other pathogenic types of *E. coli*, such as EPEC and ETEC, have well-characterized adhesion structures that allow them to adhere to the bowel. It is likely that EHEC are also capable of adhering to the bowel mucosa as one of the first stages of the infection process.

5.1 Fimbrial adhesins

Karch *et al*. (1987) reported that the pO157 plasmid common to STEC O157 mediated a fimbrial adhesin that allowed bacterial attachment to Henle407 cells but not to HEp-2 cells. Further studies have failed to support these findings and have yielded variable and often conflicting data. One study found that *E. coli* O157 did not adhere to HEp-2 cells (Junkins and Doyle 1989), but others have documented adherence to this cell line (Sherman and Soni 1988; Knutton *et al*. 1989; Toth *et al*. 1990; McKee and O'Brien 1996). The presence of fimbriae in *E. coli* O157 is also conflicting (Sherman *et al*. 1987; Winsor *et al*. 1992) and the role of the pO157 plasmid in adhesion is also controversial, with decreased adhesion, increased adhesion and no change in adhesion associated with pO157 loss (Junkins and Doyle 1989; Toth *et al*. 1990; Fratamico *et al*. 1993). In several *in vivo* models of *E. coli* O157 infection (rabbits and gnotobiotic piglets) the presence or absence of the pO157 plasmid made no difference to intestinal pathology or incidence and severity of diarrhoea (Tzipori *et al*. 1989; Dytoc *et al*. 1993).

After culturing *E. coli* O157 and O26 isolates on colonization factor antigen agar, Maneval *et al*. (1997) described rigid fimbrial structures on the surface of both organisms. The fimbriae of each serogroup were antigenically distinct but each fimbrial type was widespread across the specific serogroup. Further characterization of these fimbriae is required. The recent sequencing of the 60-MDa pO157 plasmid did not identify a fimbrial gene cluster (Burland *et al*. 1998) such as those encoded on the large plasmids of EPEC and some EAggEC (bfp and AAFI clusters). It is therefore unlikely that a fimbrial adhesin is encoded on the plasmid, and therefore any adhesin(s) must be chromosomally encoded.

5.2 Role of outer membrane proteins in adhesion

Sherman and Soni (1988) showed that outer membrane (OM) preparations from an *E. coli* O157 isolate inhibited adherence of *E. coli* O157 to HEp-2 cells. Further analysis showed that antibodies specific for a 94-kDa OM protein (OMP) inhibited adhesion (Sherman *et al*. 1991), although this protein was distinct from intimin (Dytoc *et al*. 1993). Using transposon mutagenesis, Zhao *et al*. (1996) identified an OMP in *E. coli* O157 associated with adherence to Henle407 cells and the ability to colonize the caeca of chickens. Tarr *et al*. (1995) described a chromosomal *E. coli* O157 gene termed *iha* that encoded the ability of strains to adhere to HeLa cells. The gene showed homology to the *IrgA* gene of *Vibrio cholerae* which encodes an iron-regulated protein. *Iha* was found in 20 of 20 O157 isolates, four of five *eae*-positive non-O157 human isolates but not in 10 *eae*-negative meat STEC isolates.

5.3 Attaching and effacing activity—its role in adhesion

Although fimbriae and OMPs have been identified which may be associated with STEC adhesion, most studies have concentrated on intimin as a potential adhesin. There is a precedent for this as intimin was shown to be involved in both initial bacterial attachment (Hicks *et al*. 1998) and in intimate attachment (Frankel *et al*. 1998; Kaper *et al*. 1998b) of EPEC. In addition, an intimin-deficient EPEC mutant showed reduced virulence for adult volunteers (Donnenberg *et al*. 1993a).

Studies on *E. coli* O157 intimin have shown that it can also act as an adhesin for attachment to HEp-2 cells (McKee and O'Brien 1996). *In vivo* studies using calves and gnotobiotic piglets demonstrated that intimin-deficient *E. coli* O157 mutants were less virulent than the parent strain and were less able to colonize the intestine of these animals (Donnenberg *et al*. 1993b; McKee *et al*. 1995; Tzipori *et al*. 1995; Dean-Nystrom *et al*. 1998). In addition, the *eae* gene of EPEC was functionally homologous to the EHEC *eae* gene as it restored full virulence on the O157-*eae* mutant (Donnenberg *et al*. 1993b; Dean-Nystrom *et al*. 1998). Further studies in gnotobiotic piglets have shown that an *E. coli* O157 intimin-negative mutant expressing $EPEC_{intimin}$ adhered to different bowel sites than the parent isolate, suggesting that $EPEC_{intimin}$ and $O157_{intimin}$ had differing receptor binding specificities in this model (Tzipori *et al*. 1995).

Although of a similar molecular weight, the intimins of EPEC and EHEC show some differences. The proteins are 83% homologous at the amino acid level, yet the first 704 amino acids, comprising 75% of the molecule, share 94% identity while the remaining 25% of the C-terminal residues share only 49% identity (Yu and Kaper 1992). The C-terminus is the receptor-binding region and differences in this region may explain the different tissue tropisms of EPEC and EHEC intimins. The issue of different tissue tropisms is dependent upon the ability of intimin to bind to host-encoded receptors as well as Tir. Evidence for this has been conflicting, with some reports suggesting that intimin binds to host cell receptors (Frankel *et al*. 1995, 1996; McKee and O'Brien 1996) and colleagues suggesting the opposite (Liu *et al*. 1999). Recent data suggest that intimin can bind to both Tir and a host receptor. Both binding activities are located in the 280 amino acid carboxy terminal of intimin. Binding to Tir occurs through two immunoglobulin-like regions and binding to the host receptors occurs through a lectin-like region (Hartland *et al*. 1999).

5.4 Differences in enteropathogenic *E. coli* and enterohaemorrhagic *E. coli* adhesion and attaching and effacing development

Whilst many genes in the EPEC and *E. coli* O157 LEE show a high degree of homology, differences have been identified particularly in the genes encoding the secreted proteins, e.g. *EspA*, *EspB*, *EspD* and *Tir* (Perna *et al*. 1998). Whether the differences in sequences are reflected in different properties is not yet clear. It is, however, apparent that development of A/E lesions in EPEC and EHEC occurs by different pathways (Ismaili *et al*. 1998a; Kenny 1999). When EPEC make contact with eukaryotic cells, genes in the LEE are activated. The protein EspA forms a tube-like structure (translocon) through which other proteins, e.g. EspB, EspD and Tir, are thought to pass (Knutton *et al*. 1998). Similar structures are also produced by *E. coli* O157 (Ebel *et al*. 1998). In the eukaryotic cell Tir becomes phosphorylated at tyrosine residues and inserts into the eukaryotic membranes where it serves as a receptor for intimin (Kenny 1999). In this form it acts as a focus for actin accumulation, and is thought to transmit signals to the host cell to bring about a variety of physiological changes, including calcium release from intracellular stores, activation of enzymes such as phospholipase C, protein kinase C and myosin light chain kinase and changes in membrane potential (Frankel *et al*. 1998; Kaper *et al*. 1998a). The activation of these enzymes brings about the widespread changes in cytoskeletal structures and ion fluxes noted with EPEC-infected cells.

Attaching and effacing lesions caused by *E. coli* O157 develop more slowly than those of EPEC and tyrosine phosphorylation of Tir does not occur, although this is seen with STEC of serotype O26 (Ismaili *et al*. 1998b; DeVinney *et al*. 1999; Kenny 1999). There are several possible explanations

for these differences. Lack of tyrosine phosphorylation by *E. coli* O157 can be complemented by co-infection with EPEC strains (Ismaili *et al*. 1998b) but not by cloned EPEC EspB, suggesting that *E. coli* O157 strains may be deficient in some factors present in EPEC, but the deficiency is not solely the expression of EspB (Karaolis *et al*. 1997). Kenny (1999) has proposed that a single tyrosine → serine substitution, noted between EPEC and EHEC Tir molecules at position 474, may explain the difference in phosphorylation patterns. Differences in Tir secretion may also reflect differences in adhesion (DeVinney *et al*. 1999). An EPEC *sepZ* mutant has a similar phenotype to *E. coli* O157, in that it shows reduced invasion, does not phosphorylate Tir, but can produce A/E lesions. The *sepZ* gene of *E. coli* O157 shows only 71% similarity to EPEC *sepZ* (Perna *et al*. 1998). The O157:H7 LEE does not have A/E activity when cloned into *E. coli* K-12; in contrast the EPEC LEE results in A/E activity when cloned into the same strain (Elliott *et al*. 1999). Furthermore, A/E activity in K-12$_{O157\ LEE}$ could not be complemented by various EPEC LEE operons, suggesting that the O157 LEE requires a factor not present in *E. coli* K-12 for A/E activity (Elliott *et al*. 1999).

There are currently four different forms of intimin recognized on the basis of antigenic structure and DNA sequences (Adu-Bobie *et al*. 1998) although other types are likely to exist. Differences have been observed between the *eae* genes of various STEC. The intimins expressed by serogroups O26 and O111 are highly homologous but show < 60% homology with the intimin of *E. coli* O157 (Agin and Wolf 1997). There is also marked amino acid sequence and antigenic heterogeneity between the Tir molecules expressed by different STEC serogroups (Paton *et al*. 1998a). Sequence differences in intimin and Tir proteins between different STEC strains may alter the adhesive properties of various strains, and thus alter the ability to cause disease.

Differences in LEE regulation may also influence virulence of STEC. In EPEC, regulation of the LEE genes involves a plasmid-encoded locus, *PerA-C*, which activates transcription of a LEE gene (*ler*) which then upregulates transcription of other LEE genes including the secreted proteins EspA, EspB and EspD (Mellies *et al*. 1999). Strains of EHEC lack the *PerA-C* locus and LEE genes are presumably activated by *ler* only. EPEC adhesion is induced by conditions mimicking those of the small intestine, whereas for *E. coli* O157 adhesion may be stimulated by conditions such as those in the large bowel, the presumed site of infection. Thus EPEC may adhere to the small intestine merely because adhesive genes are activated in that location, whereas *E. coli* O157 colonizes the large bowel because the adhesive genes are only activated in this location. In support of this, James and Keevil (1999) reported that adherence of *E. coli* O157 to HEp-2 cells was enhanced after growth under oxygen limitation and anaerobic conditions. Considerable work is required, however, before the mechanisms and regulation of EPEC and EHEC adhesion are fully understood.

5.5 The incidence of intimin (*eae*) in non-O157 Shiga toxin-producing *E. coli*

Intimin is expressed by most, if not all, STEC O157 isolates; if this protein is important for pathogenesis then the reduced virulence of non-O157 STEC may be explained by the absence of intimin in these isolates. Barrett *et al*. (1992) compared 30 *E. coli* O157 isolates, 19 human isolates of non-O157 STEC and 26 isolates of animal non-O157 STEC. All the O157 isolates and 16 of 19 human non-O157 carried the *eae* gene; however, amongst the animal isolates only 12 of 26 had the gene. Willshaw *et al*. (1992) found that 37% of 35 non-O157 STEC had the *eae* gene. Beutin *et al*. (1995) found that only 1·4% of 208 STEC from healthy animals contained the *eae* gene. A survey of over 600 bovine non-O157 STEC revealed a 24·7% incidence of *eae* (Johnson *et al*. 1996). A further study of bovine STEC isolates showed that the incidence of *eae* in serotypes frequently isolated from human infection was 92% compared with only 27% from serotypes infrequently isolated from human infection (Gyles *et al*. 1998).

These findings suggest that the *eae* gene is frequently found in non-O157 STEC commonly isolated from humans. Animal isolates of non-O157 STEC carry the *eae* gene less frequently and this may in part explain the reduced incidence in human disease. However, several non-O157 STEC serotypes are *eae*-positive, but are associated with only sporadic cases of human illness. This would suggest that in *E. coli* O157 other properties exist in addition to intimin and Shiga toxins that contribute to pathogenicity.

5.6 Other Shiga toxin-producing *E. coli* adhesins

McKee and O'Brien (1995) described a novel attachment mechanism present in *E. coli* O157 strains. The adhesion mechanism was described as a 'log jam pattern', bacteria adhered to and lined up at the junction between HCT-8 cells (an ileocaecal cell culture). The pattern was not seen on HEp-2 cells and was independent of intimin production. The pattern was not unique to STEC, but was also produced by other pathogenic types of *E. coli*. The log jam phenotype may represent a basal adherence mechanism allowing *E. coli* to bind and colonize the intestine.

Eight isolates belonging to serotype O111:H2 responsible for an outbreak of HUS showed an aggregative adherence pattern on HEp-2 cells, a phenotype not previously found in STEC. The organisms hybridized with probes for EAggEC adherence and gave positive polymerase chain reaction (PCR) reactions with EAggEC-specific primers. None of the eight strains were positive for the *eae* gene by DNA hybridiza-

tion or PCR, and none carried the pO157 plasmid. It was concluded that the aggregative adherence ability might allow these strains to colonize the bowel as efficiently as typical *eae*-positive *E. coli* (Morabito *et al.* 1998). Paton *et al.* (1999) showed that a group of serotype O113 strains isolated from cases of HUS were capable of adhering to Henle407 cells, although they were *eae*-negative, indicating the presence of a distinct adhesin. In some *eae*-negative strains adhesins exist which promote adhesion to rabbit brush borders, HEp2 and Henle407 cells (Dytoc *et al.* 1993; Paton *et al.* 1997).

5.7 The role of lipopolysaccharide in adhesion

Several studies have shown that although lipopolysaccharide (LPS) is not directly involved in adhesion it may have an indirect effect on adhesion. Paton *et al.* (1998c) showed that antibodies to LPS blocked adherence of *E. coli* O157 to Henle407 cells, but pretreatment of Henle407 cells with LPS did not block adherence. These findings suggest that antibodies to LPS interfere with adherence through a steric hindrance mechanism. Other studies have shown that LPS-deficient mutants are hyper-adherent to HEp-2 cells *in vitro* (Bilge *et al.* 1996; Cockerill *et al.* 1996), implying that LPS may mask adhesive structures present on the bacterial surface, or that the physicochemical properties of the cell such as surface charge or hydrophobicity may be altered by lack of LPS.

Many of the discrepancies in data concerning *E. coli* O157 adherence may be due to methodological differences, e.g. bacterial growth medium, growth phase, contact time with cells and enumeration methods. It is likely that EHEC adherence is tightly regulated and that *in vitro* conditions may be suboptimal for expression of adherence mechanisms. The activation of *E. coli* O157 LEE genes by culture media in which *E. coli* have grown suggests that optimal adherence may be induced under conditions such as those occurring in the large bowel. If EHEC are large bowel pathogens then they may be expected to adhere to colonic cells; many of the cell types used in adhesion studies are non-colonic in origin and may not have the necessary receptors for adhesion. Some cell lines may therefore be inappropriate for adhesion studies with this organism.

The exact adhesion mechanisms and structures of *E. coli* O157 and other STEC are not fully understood and much work is required in this area to determine the role of intimin and other putative adhesins in the pathogenesis of *E. coli* O157 and other STEC. It is likely that other structures remain to be identified, particularly in strains that do not express intimin, as the adhesion mechanisms of this group of pathogens have received little attention.

6. SHIGA TOXINS

Once bacteria have adhered to the bowel mucosa they will grow and secrete an array of extracellular products including the potent cytotoxins known as Shiga toxins. There are two major antigenically distinct forms of toxin, Stx1 and Stx2 (O'Brien and Holmes 1987). Whilst Stx1 is homogeneous there are numerous variants of Stx2; however, Stx1 and Stx2 share approximately 60% DNA and amino acid homology but are immunologically distinct. Both toxins are compound toxins consisting of an A subunit (32 kDa) and a pentameric B subunit (7·7 kDa monomers). The B subunits form a hollow ring and the C-terminus of the A subunit is inserted into this. The B subunits mediate binding to receptors in eukaryotic cell membranes and the receptors have been identified as globotriaosylceramide (Gb3), although the receptor for one Stx2 variant, Stx2e, is Gb4. Once bound to the target cell membrane, toxin molecules are internalized by a receptor-mediated endocytic mechanism. Toxin-containing vesicles are formed; in some cells the vesicles undergo fusion with lysosomes resulting in toxin degradation. In other cells, following processing in the Golgi apparatus and endoplasmic reticulum, the A subunit is 'nicked' by a protease generating a catalytically active 27-kDa A1 fragment and a 4-kDa A2 subunit. The released A1 subunit has RNA N-glycosidase activity and cleaves a specific bond in 28S rRNA, this cleavage prevents binding of amino acyl-tRNA to 60s ribosomal units, inhibiting the peptide chain elongation step of protein synthesis leading to cell death (Sandvig and van Deurs 1996).

The importance of Shiga toxins in causing intestinal mucosal damage *in vivo* has been demonstrated. A natural pathogen of rabbits, RDEC-1 which produces A/E lesions but does not produce Shiga toxins, was converted with an Stx1 phage and the virulence of the non-toxigenic parent and the toxigenic derivative were compared in rabbits. The derivative produced more severe illness, with more serious histological lesions, including oedema and severe inflammation, than the parent non-toxigenic isolate (Sjogren *et al.* 1994).

Although the majority of strains of *E. coli* O157 produce Stx2 only, amongst non-O157 STEC the toxin phenotype is much more variable with isolates producing Stx1 alone occurring commonly. Isolates of serogroup O26 usually produce Stx1 (Scotland *et al.* 1990), isolates of serogroup O111 produce Stx1 and some produce Stx2 in addition (Willshaw *et al.* 1992; Gyles *et al.* 1998). There is considerable epidemiological evidence to indicate that STEC isolates producing Stx2 are more commonly associated with serious disease than isolates producing Stx1 or Stx1 and Stx2 (Kleanthous *et al.* 1990; Boerlin *et al.* 1999). Although Stx1 and Stx2 have similar structures and modes of action their toxicities appear to be distinct. Stx2 was 1000 times more cytotoxic than Stx1 towards human renal microvascular endothelial cells, the putative target of Shiga toxins in the development of HUS

(Louise and Obrig 1995). The increased toxicity of Stx2 was not related to a greater number of receptors for this toxin on the cell surface, indeed the reverse was true. In a mouse model, infection with an *E. coli* O157 isolate producing both Stx1 and 2 resulted in fatal cortical tubular necrosis. Death was prevented by passive immunization with a monoclonal antibody to Stx2 but not to Stx1. Challenging mice with a strain of *E. coli* K-12 carrying cloned Stx2 caused death, but *E. coli* K-12 carrying cloned Stx1 did not (Wadolkowski *et al.* 1990). Tesh *et al.* (1993) went on to show that purified Stx2 had an approximately 400-fold lower $L.D._{50}$ for mice than Stx1. The association of Stx2-producing *E. coli* with the development of HUS may reflect a preferential ability of Stx2 to damage renal glomerular vasculature. Using transformed human intestinal microvascular endothelial cells Jacewicz *et al.* (1999) showed that Stx2 was more toxic than Stx1 for these cells. The authors concluded that the increased toxicity of Stx2 to endothelial cells may be relevant to the preponderance of Stx2 producers in the pathogenesis of HC.

There are several factors that complicate our understanding of the role of toxins in disease. Firstly, there are numerous variants of Stx2 differing by only one or two amino acids in either the A or B subunit (Paton and Paton 1998b). This can affect their catalytic activity or receptor binding, respectively. Secondly, many isolates produce two or more forms of Stx2 (Schmitt *et al.* 1991), and the relative contributions of the various forms of toxin to pathogenesis are not known. One form of toxin, Stx2d, is activated by intestinal mucus, which causes a marked increase in toxicity, this may compensate for a lack of other accessory virulence components (Melton-Celsa *et al.* 1996). An animal model using streptomycin-treated mice showed differences in virulence between a laboratory *E. coli* strain containing different Stx2 variants, highlighting differences in toxicity of the Stx2 variants (Paton *et al.* 1995). Thirdly, Wagner *et al.* (1999) showed that phages from different STEC strains produced different amounts of Stx2 in an *E. coli* K-12 background. The amount of Stx2 produced by a given phage in the K-12 strain was unrelated to the amount of toxin produced by the original host strain containing the phage. This suggests that there are bacterial factors that may influence the amount of toxin produced. Interestingly, although the number of strains examined was small, four *E. coli* O157 isolates produced more toxin than the other non-O157 isolates (Wagner *et al.* 1999). The high yields of Stx2 may explain the greater pathogenicity of *E. coli* O157.

The mechanism by which Shiga toxins produced by organisms in the bowel cause renal damage is not yet known. Since *E. coli* O157 is non-invasive, systemic sequelae must involve translocation of toxin from the gut lumen to underlying tissues. Damage to the intestinal epithelium by Shiga toxin, or other inflammatory mediators, may aid translocation of the toxin to the bloodstream (Paton and Paton 1998b); gaps between cells may be a further pathway. However, Acheson *et al.* (1996) have demonstrated that toxin can move through intact epithelial cells and this may be an additional transport mechanism allowing toxin to reach extraintestinal sites. Although both Stx1 and 2 are thought to cause direct damage to human glomerular cells, there is increasing evidence that cytokines such as interleukins and tumour necrosis factor in association with bacterial LPS may be involved in the process. For a fuller discussion of this aspect, the reader is referred to the review by Paton and Paton (1998b).

Although Shiga toxins are potent cytotoxins other bacterial factors may also be involved in producing mucosal damage. When added to various cell lines such as CaCo2A cells, Stx1 and 2 move across the cell layer without obvious cytotoxic effects, but when toxin-producing bacteria are applied to the layer, toxin is transported through the layer and the layer itself is destroyed (Acheson *et al.* 1998). These findings suggest that the bacteria either produce some other toxic products or that there are some other bacterial factors that augment Shiga toxin activity. Other potential toxins are described below.

7. ENTEROHAEMOLYSIN

Beutin *et al.* (1989) found an association between Shiga toxin production and a novel haemolysin termed enterohaemolysin. Enterohaemolysin (Ehx) is characterized by the production of small turbid zones of haemolysis after 18–24 h incubation on blood agar containing washed erythrocytes. This is in contrast to *E. coli* α-haemolysin, which produces large clear zones of haemolysis often apparent after 4 h incubation and occurring with washed and unwashed erythrocytes (Karch *et al.* 1998). Furthermore *E. coli* α-haemolysin has activity against human lymphocytes whereas Ehx does not (Bauer and Welch 1996). There is approximately 60% relatedness between Ehx and *E. coli* α-haemolysin. Enterohaemolysin belongs to the RTX (repeats in toxin) family of toxins, other members of which are expressed by a variety of pathogens (Bauer and Welch 1996). Secretion of Ehx by *E. coli* O157 appears to be defective resulting in only small zones of haemolysis and lack of haemolytic activity in supernatant fluids (Schmidt *et al.* 1996). When the transport system of *E. coli* α-haemolysin was transferred to *E. coli* O157, haemolytic activity and secretion of Ehx was increased (Schmidt *et al.* 1995). It was suspected that there was a defect in one of the genes involved in transport of Ehx (Schmidt *et al.* 1995). Isolates of serogroup O103 produce a haemolysis pattern similar to that seen with α-haemolysin, but they do not contain the α-haemolysin gene. Instead it was thought that they overproduce Ehx (Schmidt *et al.* 1999). It is likely that this serogroup represents a clone in which the Ehx transport mechanism is not defective. Production of Ehx *in vitro* can be markedly enhanced by growth under conditions of low oxygen tension and anaerobic conditions (Chart *et al.* 1998); similar conditions are found in the large bowel of man and

animals, and suggest that the toxin may be produced maximally in these locations. Bauer and Welch (1996) showed that Ehx isolated from O157 strains had haemolytic activity against human red blood cells; however, Chart *et al*. (1998) found that Ehx had no effect on human erythrocytes. Further studies on this aspect are warranted.

Thus the precise role of Ehx in human infection is unknown and it is possible that the toxin may be of more relevance to infection in animals. However, the *ehx* operon is highly conserved among STEC strains suggesting that Ehx is under strong selective pressure and plays an important role in STEC survival (Boerlin *et al*. 1999)

Escherichia coli isolates of serogroups O157, O26 and O111 commonly produce Ehx and it is therefore a useful epidemiological marker for potential Stx-producing strains. Scotland *et al*. (1990) showed that there was good correlation between Ehx production and hybridization with the CVD419 probe and suggested that Ehx may be encoded on the large plasmid (pO157) carried by many *E. coli* O157. A plasmid location for the toxin was confirmed by Schmidt *et al*. (1994), and later studies have shown that the CVD419 probe hybridizes with part of the Ehx structural gene *hlyA* (Schmidt *et al*. 1995).

The incidence of Ehx in non-O157 STEC can be inferred by hybridization with the CVD419 probe. Barrett *et al*. (1992) found that 28 of 28 *E. coli* O157, 16 of 19 human non-O157 but only 13 of 26 animal STEC were CVD419 probe positive and Willshaw *et al*. (1992) found that 29 of 48 non-O157 STEC were CVD419 probe positive. Johnson *et al*. (1996) examined a group of cattle isolates and found that hybridization with the CVD419 probe occurred in 125 of 224 isolates, and this property was twice as common as the *eae* gene in this group. It is noteworthy that some serotypes, such as O22:H8 and O153:H25, which have been associated with HC and HUS hybridized with the CVD419 probe but did not carry the *eae* gene. Beutin *et al*. (1995) detected the Ehx phenotype in 128 of 208 animal STEC.

A comparison of *eae*-positive and *eae*-negative non-O157 isolated from cattle found that Ehx was detected in 98% of *eae*-positive isolates and in 36% of *eae*-negative isolates, indicating a strong linkage between *eae* and Ehx (Sandhu *et al*. 1996). Although many studies have shown that Ehx is more often found in strains associated with humans rather than animals, Boerlin *et al*. (1999) did not detect a significant difference in the frequency of Ehx between isolates from serotypes found in humans and those not found in humans (62·6 and 60·2%, respectively). There was an association with Ehx and isolates associated with severe disease in univariate analysis; however, in multivariate analysis this association was not seen, and the strong association between Ehx and intimin was thought to explain the lack of significance of Ehx in multivariate models (Boerlin *et al*. 1999). Schmidt and Karch (1996) compared O111 strains isolated from cases of HUS with those from cases of diarrhoea. The incidence of Ehx was higher in the group causing HUS (16 of 18) compared with the group causing diarrhoea (four of 18) implying that this factor may be important in the development of HUS, although the number of isolates studied was small.

Although frequently associated with isolates causing severe disease, Ehx is not essential for development of HUS and HC and its involvement in human disease is unclear. The function of Ehx is therefore unknown, there is no convincing data for a role in human infections and the toxin may be more important in assisting infection of animals.

8. HAEMOGLOBIN UTILIZATION

The action of Shiga toxins on the bowel mucosa causes damage to the mucosa and underlying blood vessels leading to bleeding into the bowel. Erythrocytes, if lysed, can then release haemoglobin, a potent source of iron for many pathogenic bacteria. Although the effect of Ehx on human erythrocytes is not yet clear, erythrocytes could also be lysed by the presence of haemolysins produced by other bowel organisms or by the action of bile salts.

Law and Kelly (1995) compared the ability of *E. coli* O157 strains and non-O157 STEC from humans and cattle to use haem and haemoglobin as sources of iron. All of the O157 isolates could use both moieties but this property was extremely rare in cattle STEC isolates (one of 17) and only three of 16 human non-O157 were able to use these compounds. The differences are highly significant and suggest a plausible explanation for the increased virulence of *E. coli* O157 compared with non-O157 isolates. Growth rates in an iron-limited environment containing haemoglobin are much higher for organisms able to use haemoglobin than for those unable to use the compound (Law *et al*. 1992). Haemoglobin in the bowel derived from the action of Shiga toxins and red cell lysis could stimulate growth of *E. coli* O157 resulting in further production of toxins, etc. Organisms unable to utilize haemoglobin will probably grow more slowly and may not secrete the same quantities of toxin.

Mills and Payne (1995) identified a 56-kDa OMP in *Shigella dysenteriae* type 1, termed ShuA, that was a potential haemoglobin receptor. *Escherichia coli* O157 synthesized a 69-kDa protein involved in haem transport in response to iron-limitation. The gene *chuA* encoding the 69 kDa protein was chromosomally encoded and was highly homologous to the *shuA* gene of *Sh. dysenteriae* type 1 (Torres and Payne 1997). Using PCR, Potters *et al*. (1999) showed that all of 13 O157 isolates reacted with two primer sets for the *chuA* gene yet only three of 58 non-O157 isolates reacted with both primer sets and a further three of 58 reacted with a single primer set. None of eight strains of O26, 10 of O103 and six of O111 were positive although two of five strains of serogroup O145 were PCR positive. The authors concluded that the restriction of *chuA* to the O157:H7 serotype may in part explain the patho-

genicity of this serotype. Stumpfle *et al*. (1999) identified a DNA segment common in laboratory and wild type *E. coli*, but absent from STEC of serogroup O157 and O145, the segment had 97·2% homology to a region in the *E. coli hemB* locus with an unknown function. Introduction of the gene into an O157 strain stimulated growth of the strain particularly in iron-depleted media containing haemin. This would suggest that the mechanisms of haem and haemoglobin utilization in *E. coli* O157 and *E. coli* O145 are distinct from those of other *E. coli*.

The high incidence of haemoglobin uptake in *E. coli* O157 coupled with the low incidence in non-O157s would indicate that this property may be important in contributing to the higher virulence of the former. Further studies are required to determine the distribution of the haemoglobin uptake phenotype in STEC of different serotypes isolated from cases of diarrhoea, HC and HUS and from animals. The generation of mutants and examination of their virulence in animal models are urgently required.

9. EXTRACELLULAR SERINE PROTEASE

Strains of EPEC and *E. coli* O157 secrete several proteins into culture supernatant fluids when grown in tissue culture media (Kaper *et al.* 1998a). Secretion of some of these proteins (EspA, EspD and EspD) is governed by a type III secretion system encoded on the LEE locus and is related to production of A/E lesions (Kaper *et al.* 1998a). Strains of *E. coli* O157, however, secrete a protein, EspP (extracellular serine protease, plasmid-encoded), that is not involved in production of the A/E lesions and is not encoded in the LEE (Brunder *et al.* 1997). EspP is a protein of MW 104 kDa which shares homology with EspC of EPEC, a secreted protein of unknown function. STEC of serotype O26:H– secrete a protein homologous to EspP (Djafari *et al*. 1997). Both EspC and EspP are known as autotransporters as they mediate their own secretion through the outer membrane. EspP shares homology with several proteins including a group of serine proteases and has proteolytic activity against a narrow range of substrates, one of which is human coagulation factor V. This surprising activity indicates that EspP secreted by EHEC may influence the blood clotting cascade. Cleavage of factor V could result in a decreased coagulation reaction leading to prolonged bleeding. Local degradation of factor V by EHEC attached to the bowel mucosa could increase haemorrhage into the gastrointestinal tract (Karch *et al*. 1998). The toxin is also cytotoxic for Vero cells (Djafari *et al*. 1997). Children suffering from EHEC infections produce an antibody response to EspP indicating its expression *in vivo* (Brunder *et al.* 1997), although this does not necessarily indicate a role in pathogenesis.

Production of EspP by *E. coli* O157 is not uniform; four of five isolates were DNA probe positive but only three of five produced EspP as detected by Western blots (Brunder *et al.* 1997). In non-O157 STEC, one of four isolates produced EspP and, in all cases, the *EspP* gene was encoded on the large pO157 plasmid. In a further study Brunder *et al.* (1999) found that the pO157 plasmid of sorbitol-fermenting *E. coli* O157:H– lacked the *EspP* gene. Schmidt *et al*. (1999) analysed a collection of STEC and noted serogroup-related associations with the *EspP* gene. None of 13 O103 isolates but all 11 O26 isolates tested carried the gene. In the case of serogroup O111, seven of eight isolates produced a weak hybridization signal under low stringency conditions. Although all of these serogroups have the pO157 plasmid there are clear serogroup-related differences in plasmid content. It was concluded that the large plasmids of STEC are not uniform genetic elements but are heterogeneous in both gene composition and arrangement (Brunder *et al.* 1999). A comparison of bovine and human isolates belonging to six major serogroups (including O157) did not show any association between EspP and disease in humans (Boerlin *et al*. 1999). The significance of EspP in disease is unclear, but its absence from sorbitol-fermenting *E. coli* O157 capable of causing both HC and HUS indicates that it is not vital to the development of the disease process in humans.

10. *CLOSTRIDIUM DIFFICILE*-LIKE TOXIN

Sequencing of the pO157 plasmid revealed a large open reading frame (L7095) encoding a 3169 amino acid protein (Burland *et al*. 1998). The protein showed strong similarities in the first 700 residues with the N-terminal domain of a toxin family known as the large clostridial toxins (LCT) that includes toxins A and B of *Clostridium difficile*. These toxins consist of a C-terminal, that functions in toxin entry into the cell, and an N-terminal glucosyl-transferase, that modifies proteins regulating cell architecture. L7095 and the LCTs show a conserved motif that has recently been shown to be essential for enzymatic activity. The similarity with LCTs suggests that L7095 may act through a similar mechanism leading to disorganization of the target cell cytoskeleton. Thus L7095 may contribute to cytotoxicity and cellular ultrastructural changes. Descriptions of the pathology of *E. coli* O157 infections show similarities to toxin-mediated *Cl. difficile*-associated colitis (Nataro and Kaper 1998) raising the possibility that the pO157 toxin may be involved in intestinal damage. Further studies are required to examine the distribution of the gene among *E. coli* O157 and non-O157 STEC. Studies are also required to assess whether a functional protein is produced and to assess its activity on various cell types and in animal models, and the potential for synergy with other toxic products

11. CATALASE/PEROXIDASE

Analysis of plasmid pO157 DNA sequences revealed a potential protein with homology to other bacterial catalase/peroxidases. The gene *KatP* encodes an 82-kDa MW protein that is a bifunctional catalase-peroxidase (Brunder *et al.* 1996). The enzyme accumulates in the periplasm of the cells, although no significant differences in resistance to the action of hydrogen peroxide were noted between the parent isolate and a plasmid-cured derivative. The role of this enzyme in virulence is unclear; it has been suggested that bacterial catalases and superoxide dismutases detoxify cytotoxic oxidants produced during the oxidative bursts of macrophages and neutrophils, thereby assisting the bacterium in escaping host defences. The enzyme could also contribute to assisting recovery of the organism from heat stresses. It would be interesting to examine the responses of parent and *KatP* mutants to various stresses.

Although *KatP* is plasmid encoded, its distribution among pO157-containing organisms is not uniform. Brunder *et al.* (1999) found that *KatP* was present in only 66% of *E. coli* O157 isolates and in 38% of non-O157 EHEC and was not found in sorbitol-fermenting *E. coli* O157. Like EspP the distribution of KatP is serotype dependent (Schmidt *et al.* 1999). Once again this demonstrates heterogeneity in the pO157 plasmid amongst different serogroups. The absence of *KatP* in sorbitol-fermenting strains of *E. coli* O157 which cause HC and HUS would suggest that the enzyme does not have a major role in virulence.

12. ENTEROAGGREGATIVE HEAT-STABLE TOXIN

Enteroaggregative *E. coli* are associated with persistent diarrhoea in children and their virulence mechanisms have been intensively investigated (see Law and Chart (1998) for a review of this subject). Several toxins appear to be produced by EAggEC, one such toxin is a heat-stable 38 amino acid enterotoxin similar to the heat-stable toxin of ETEC (Savarino *et al.* 1991). The gene for the toxin is encoded by the *astA* gene and the gene product is known as enteroaggregative heat-stable toxin (EASTI; Savarino *et al.* 1993). Examination of STEC strains showed that the *astA* gene was present in 75 of 75 O157, eight of nine O26 and 12 of 23 non-O157/O26 isolates (Savarino *et al.* 1996). In three O157 strains culture ultrafiltrates containing EASTI were enterotoxic using a rabbit ileal model. The high prevalence of the *astA* genotype in O157 isolates is striking, indeed the incidence is considerably higher than that found in EAggEC. Although EASTI stimulates fluid secretion in a rabbit model, the role of the toxin in human infection is unknown. In *E. coli* O157 infection, it is possible that EASTI contributes to the initial phase of watery diarrhoea seen in patients with HC although it is unlikely to make a major contribution to the high virulence of *E. coli* O157.

13. TYPE II SECRETION PATHWAY

A gene cluster has been identified on the pO157 plasmid that encodes a putative type II secretion apparatus. This pathway provides a mechanism of exoprotein secretion for certain Gram-negative bacteria (Burland *et al.* 1998). The significance of this gene cluster is not known as the identity of the protein(s) secreted by EHEC is not known. Karch *et al.* (1998) found that all of 50 *E. coli* O157 isolates possessed the pathway, but it was present in only 52% of 50 non-O157 EHEC. In another survey, the presence of one of the genes in the pathway, *etpD*, was detected in 13 of 13 O103 strains but not in any of the O26 or O111 strains tested (Schmidt *et al.* 1999).

14. LYMPHOCYTE INHIBITION

A study by Klapproth *et al.* (1995) showed that strains of both EPEC and *E. coli* O157 are capable of producing proteinaceous factors that bring about inhibition of lymphocyte activation and lymphokine production. In an EPEC isolate the factor(s) was (were) chromosomally encoded and also produced by mutants with defective LEE genes, suggesting that it is not encoded on the LEE. These factors may be important in modifying gastrointestinal immune responses in enteric bacterial infections. However, their significance is unclear since many patients infected with STEC produce significant levels of serum antibody to lipopolysaccharide and secreted proteins such as EspA, EspB and Tir of the infecting organism (Chart and Jenkins 1999). As these organisms do not cross the bowel mucosa it is apparent that the local immune system is operative.

15. CONCLUSIONS

It is clear that STEC are highly pathogenic and that virulence is not dependent on a single gene or gene product but is a multifactorial process. The organism can survive in water and a wide range of foods including acidic products and then remain viable after transit through the acidic environment of the stomach. Colonization of the bowel, mediated by one or more of a range of potential adhesins, then occurs. Following colonization, several toxins and other proteins are produced which may assist with survival and multiplication in the intestinal environment. Elaboration of potent Shiga toxins into the gut lumen causes intestinal damage and eventually systemic complications.

What are the minimum requirements of an EHEC to cause disease in humans? The production of Shiga toxins, in particular Stx2, is a prerequisite and the capacity to adhere to the bowel mucosa is likely to be important. These two factors

alone may be sufficient for an organism to cause disease, a theory supported by the data of Boerlin *et al*. (1999) which showed that these factors were highly related to disease in humans. Furthermore, there are numerous cases of infection caused by isolates devoid of other factors, in particular the strains of O111:H2 causing HUS which produced Stx2, showed aggregative adherence but were devoid of the pO157 plasmid and the plasmid-associated virulence attributes. However, if adherence and toxin production are the sole requirements for disease then it does not explain the lower incidence of serogroup O26 and O111 infections, as they express intimin and produce Shiga toxins. Differences in acid resistance, regulation of adhesion, receptor specificity of intimin or type of toxin (Stx1 or 2) and quantity of toxin produced may have a strong influence on the capacity of an organism to produce disease. Factors such as uptake of iron from haemoglobin, present in most strains of *E. coli* O157 but occurring with a much lower incidence in non-O157 STEC, may confer an advantage in the blood-rich environment of the toxin-damaged large bowel. Alternatively, as yet unidentified virulence factors may also be present in *E. coli* O157. There is circumstantial evidence that this may be the case; the significant difference in infective dose between *E. coli* O157 STEC and EPEC cannot be explained on the basis of known virulence properties. *Escherichia coli* O157 isolates from cattle are less virulent in a gnotobiotic piglet model than human outbreak isolates despite being matched for known virulence properties (Baker *et al.* 1997). In the USA two distinct *E. coli* O157:H7 lineages have been identified by octamer-based genome scanning. Human and bovine isolates are non-randomly distributed between these lineages suggesting that one of these lineages may be less virulent for humans or less efficiently transmitted to humans from bovines (Kim *et al.* 1999). Finally, the *E coli* O157 genome is approximately 1 Mb larger than the *E. coli* K-12 genome (Kaper *et al.* 1998a). Although Shiga toxin phage genes, the LEE and the pO157 plasmid may account for some of the additional DNA it is probable that there are unidentified genes in the O157 genome, some of which may be involved in production of disease. The relative importance of factors such as Ehx, EspP, catalase/peroxidase and the clostridia-like toxin, is not yet known although it is becoming apparent that the impact of some of these factors on disease may be relatively low. Non-O157 STEC, because of their lower virulence, may be capable of causing disease only when a high inoculum is ingested or in particularly susceptible individuals. Mild illness or asymptomatic carriage may result when low inocula are ingested.

It is not surprising that there is a wide spectrum of virulence within the STEC group and even within serogroup O157 as many of the putative virulence factors are encoded on mobile genetic elements, such as phages, plasmids and pathogenicity islands, which in turn have been subject to numerous insertions and deletions (Boerlin *et al*. 1998). Comparison of *E. coli* O157 and non-O157 STEC has shown that they probably evolved independently, as the sites of insertion of the LEE (when present in non-O157 STEC) are distinct (Sperandio *et al*. 1998; Whittam 1998).

The recent advances in molecular techniques and the identification of the genetic loci encoding many of the putative virulence properties will be extremely useful in identifying the contribution of the various factors to pathogenicity. The sequencing of the *E. coli* O157 genome and its comparison with that of *E. coli* K-12 will be crucial in identifying novel virulence attributes. *Escherichia coli* O157 mutants deficient in one or more factors, or producing distinct forms of Shiga toxins, can be assessed in both *in vitro* and *in vivo* models. Such mutants can also be compared with well-characterized non-O157 STEC, with known combinations of phenotypic properties, e.g. intimin, Ehx, EspP, catalase/peroxidase and haemoglobin utilization. The recently described canine model that mimics HUS (Fenwick and Cowan 1998) may prove a suitable *in vivo* model for assessing the virulence of such mutants and elucidating the role, if any, of these properties in disease.

The identification of factors which are crucial for production of disease may allow the development of better diagnostic tools, allowing us to detect and isolate only those organisms with disease-causing potential. Investigation of pathogenicity factors also provides insights into possible therapeutic and immunization strategies. Various trials are under way looking at vaccine development in animals and humans. In particular, intimin has potential value as a cattle vaccine, other vaccines may be based on Tir or other secreted proteins, such as EspA, EspB and EspD, as these proteins are highly immunogenic and antibodies are produced during the course of infection in humans. A problem with vaccines based on LEE-encoded proteins is that if they are effective then they will only be effective against isolates that carry the LEE region. Whilst this includes the majority of isolates associated with human illness it does not cover all disease-producing STEC.

It is now over 15 years since strains of *E. coli* O157 emerged as a human pathogen. Although great inroads have been made in our knowledge of these organisms, their toxins and accessory virulence factors there is still a great deal to learn before we can fully understand the processes leading to disease.

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