INTERACTIONS OF ENTEROPATHOGENIC AND ENTEROHAEMORRHAGIC *ESCHERICHIA COLI* WITH INTESTINAL MUCOSAE *IN VITRO*

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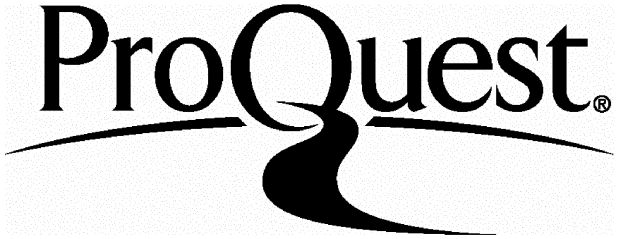
by Robert James Fitzhenry



Abstract.

Enteropathogenic *Escherichia coli* (EPEC) is associated with infantile diarrhoea and remains a major cause of paediatric morbidity and mortality in developing countries. Enterohaemorrhagic *Escherichia coli* (EHEC) is distinguished from EPEC by secretion of a Shiga-like cytotoxin, which can cause bloody diarrhoea, haemorrhagic colitis and haemolytic uraemic syndrome. EPEC and EHEC produce characteristic attaching and effacing (A/E) lesions on cultured cells and intestinal mucosae *in vitro,* and *in vivo* A/E intestinal lesions have been shown for EPEC in man, but not EHEC. The first gene to be associated with A/E lesion formation, the *eae* gene, encodes an outer membrane protein called intimin, and different intimin types have been recognised. The site of intestinal adhesion has been linked to intimin type in a pig model. Using *in vitro* organ culture (IVOC), one EHEC O157:H7 strain has been shown to produce A/E lesions on human Peyer's patches of the terminal ileum, but it did not adhere to other intestinal regions.

The work in this thesis describes the use of IVOC, electron microscopy and molecular biology a) to study the interaction of EPEC and EHEC with human intestinal mucosa, and b) to address the phenomenon of tissue tropism of EPEC and EHEC. IVOC of EPEC and EHEC serogroups expressing different intimin types, confirmed the A/E lesion capability of EHEC on human tissue, demonstrated the FAE restricted phenotype of O157:H7 and O103:H- EHEC, and showed a variable tropism in EPEC strains and O26:H11 EHEC. Intimin exchange studies confirmed its tissue tropism role. Site-directed mutagenesis of intimin established that other, unidentified, factors also contribute. Colonic A/E lesion formation was shown in a minority of IVOC's, leading to the study of factors that might promote large bowel colonisation. Passage, fimbrial expression, mannose, activation, extended IVOC, and infected HEp-2 cell co-culture did not influence tropism. The *lpf*-like operons of EHEC were chosen for analysis, as *lpf* in *Salmonella typhimurium* is reported to mediate adhesion to Peyer's patches. Deletion in *lpf*-like operons in two EHEC strains altered tissue tropism in IVOC, giving the first example of a gene outside the LEE which influences intestinal colonisation.

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Abbreviations.

AAF/I	aggregative adherence fimbriae I
AAF/II	aggregative adherence fimbriae II
A/E	attaching and effacing lesion
AI-2	autoinducer 2
BFP	bundle forming pili
bp	base pair
BSA	bovine serum albumin
Caco-2	human colonic carincoma cell line isolated from the colorectal region
Cah	calcium binding antigen 43 homologue
cAMP	cyclic adenosine monophosphate
CFTR	cyctsic fibrosis transmembrane receptor
CFU	colony forming units
cGMP	cyclic guanosine monophosphate
CHO	Chinese hamster ovary cells.
COS	African green monkey (*Cercopithecus aethiops*) kidney cell line
CT	*Cholerae* toxin
DAEC	diffusely adhering *E. coli*
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
E69	E2348/69
EAF	EPEC adherence factor plasmid
EAST1	enteroaggregative *E. coli* heat stable enterotoxin 1
eae	*E. coli* attaching and effacing
Efa1	EHEC factor for adherence
esc	*E. coli* secretion
Esps	EPEC secreted proteins
Fis	factor for inversion stimulation
FITC	fluorescein isothiocyanate
Gb_3	glycosphingolipid globotriaosylceramide
Gb_4	glycosphingolipid globotetraosylceramide

G+C	guanine and cytosine
HeLa	cervix epithelium human adenocarcinmoa cell line (Herietta Lacks)
HEp-2	larynx epidermoid human carcinoma cell line
HC	haemorrhagic colitis
H-NS	histone like nonstructural protein
Hr	hour
HUS	haemolytic uraemic syndrome
kb	kilobases
kDa	kilodalton
IVOC	*in vitro* organ culture
LB	L-broth
LCT	large Clostridial toxin
LEE	locus of enterocyte effacement
LPF	long polar fimbriae
LPS	lipopolysaccharide
LT	heat labile toxin of ETEC
MAP	mitochondria associated protein
Mb	megabases
MDa	megadalton
Mol. Wt	molecular weight
NF-κB	nuclear factor κB
ng	nanogram
nm	nanometres
NMR	nuclear magnetic resonance
NP	no passage
N-WASP	neural Wiskott – Aldrich syndrome protein
OD	optical density
ORF	open reading frame
Pas	protein associated with secretion
PBS	phosphate buffered saline
pEHEC	large plasmid present in EHEC equivalent to pO157

pg	picogram
pheU	phenylalanine
PP	Peyer's patches
QseA	quorum sensing *E. coli* regulator A
RDEC-1	rabbit diarrhoeagenic *E. coli*
rpm	revolutions per minute
Saa	STEC autoagglutinating adhesin
selC	selenocysteine
sep	secretion of *E. coli* protein
Sfp	sorbitol fermenting EHEC O157 fimbriae plasmid encoded
ST	heat stable toxin of ETEC
STEC	Shiga toxin expressing *E. coli*
Stx	Shiga toxin
T84	colonic epithelium cell line
Tir	translocated intimin receptor
TTSS	type III secretion system
µm	micrometres
UPEC	uropathogenic *e. coli*
UTEC	urinary tract *e. coli*
Vero cells	African green monkey (*Cercopithecus aethiops*) kidney cell line
VTEC	Verocytotoxin expressing *e.coli*

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I dedicate this thesis to my Mother and Father.

Section 1.0 Introduction.

1.1 General Introduction.

Escherichia coli is a genetically diverse group of bacteria exhibiting varied phenotypes within vertebrate hosts. The natural habitat of *E. coli* is the gastrointestinal tract and in humans it is the predominant facultative anaerobe of the colonic flora. Most *E. coli* are commensal bacteria but they do not exist as a homogenous group. The *E. coli* genome can undergo horizontal gene transfer from other bacterial species and in addition conserved genes within the genome, such as house keeping genes involved in metabolic functions, are polymorphic with multiple alleles found among different isolates (Pupo *et al.*, 1997). The majority of the *E. coli* strains reside within their host without causing damage but may cause harm in the immunocompromised host or when the intestinal barrier has been disrupted. Pathogenic *E. coli* can be distinguished from the commensal flora by their ability to colonise the host and cause clinically significant pathology. Virulence is a property that is dependent on the host-pathogen interaction and not an independent microbial characteristic (Casadevall and Pirofski, 2001).

The association of *E. coli* with diarrhoea in young children was first established in the 1940s (Bray, 1945) and since then five categories of diarrhoeagenic *E. coli* have been defined. These include enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* and enteroaggregative *E. coli* (EAEC). These categories were defined according to epidemiology, the presence of distinct virulence factors and pathology. These diarrhoeagenic *E. coli* will be introduced with particular attention being paid to the mechanisms underlying the pathogenesis of EPEC and EHEC. A sixth category, termed diffuse adhering *E. coli* (DAEC), has been recognised on the basis of their pattern of adhesion to HEp-2 cells (Bilge *et al.*, 1989;Nataro *et al.*, 1987), but their association with diarrhoea is unclear (Tacket *et al.*, 1990) and they will not be discussed further.

The experiments described in this thesis investigate the adhesion of EPEC and EHEC strains to human intestinal mucosae with particular attention placed on putative adhesins.

1.2 Definition of diarrhoea.

Diarrhoea can be defined as the passage of three or more liquid stools over a twenty four hour period (Gracey, 1993b;Gracey, 1993a) and can be categorised according to the duration of the illness.

1.2.1 Acute diarrhoea.

Acute diarrhoea is a self limiting characterised by the rapid onset of diarrhoea with an episode lasting between three and five days. Such episodes can occur in both children and adults and unresolved illnesses may lead to persistent (chronic) diarrhoea. Approximately 10% of acute diarrhoea cases become persistent in developing countries (Wanke *et al.*, 1991).

1.2.2 Persistent (chronic) diarrhoea.

Persistent diarrhoea is defined as abnormally loose stools for a period of fourteen days or longer. Patients with persistent diarrhoea due to infection usually present with watery or dysenteric stools containing blood, pus and mucus, whereas patients with noninfective persistent episodes present with loose or bulky stools (Gracey, 1993a;Gracey, 1993b).

1.2.3 Intractable diarrhoea.

The intractable diarrhoea of infancy syndrome defines those infants under one year old with chronic diarrhoea which persists despite intravenous fluids or elimination diets (Walker-Smith and Murch, 1999). Infective causes are excluded and congenital disorders such as microvillous atrophy may be responsible (Phillips and Schmitz, 1992). Other diagnoses include tufting enteropathy (Reifen *et al.*, 1994), autoimmune enteropathy

(Unsworth *et al.*, 1982;Unsworth and Walker-Smith, 1985) and syndromic diarrhoea (Goutet *et al.*, 1981).

1.3 Global burden of diarrhoeal disease.

Diarrhoea is one of the principle causes of morbidity and mortality among children in the developing world and the incidence of acute diarrhoeal illness is second only to upper respiratory tract infection. For children under five years of age in developing countries, data published between 1992 and 2000 suggests there is a median of 3.2 episodes of diarrhoea per child per year. WHO estimates of mortality reveal that for children under the age of five 4.9 children per 1000 die each year from diarrhoeal illness and diarrhoea accounts for 21% of all deaths of children under the age of five in developing countries (Kosek, Bern, and Guerrant, 2003). The WHO estimates that the disease burden from contaminated water, poor sanitation, and poor hygiene to be 4% of all deaths and 5.7% of the total disease burden occurring worldwide (Pruss *et al.*, 2002). In developed communities, such as exist in the U.S.A, children under five years of age have between 1.5 and 2 episodes of diarrhoea per year which shows that even in developed countries morbidity due to diarrhoea is still high (Hughes *et al.*, 1978). The WHO estimates that in developing countries diarrhoea is responsible for 2.5 million deaths per year but in a developed country such as Britain diarrhoea accounts for 6 deaths per 100000 infants (Wharton *et al.*, 1988).

Treatment of acute diarrhoeal illness is based on limiting dehydration, which can lead to a life threatening situation, with the administration of oral rehydration solutions. Antibiotic treatment is not encouraged. However, in cases of EPEC associated protracted diarrhoea the use of antibiotics may be an effective treatment (Hill, Phillips, and Walker-Smith, 1991a). Antibiotics have been shown to be important in treating cases of endemic diarrhoea in the developing world and in the developed world (Hill *et al.*, 1988;Thoren *et al.*, 1980). However prevention of diarrhoea through sanitation remains important and improving the quality of water can reduce diarrhoeal incidents by 16%, making water available can reduce the risk by 20% and good hygiene, such as washing hands with soap,

can reduce the risk of diarrhoea by 47%. Thus measures taken to educate people about hygiene and improving the quality of the water available can have a marked effect on the diarrhoeal burden (Curtis and Cairncross, 2003). Indeed, a water and sanitation program in Pakistan has shown that improving both the quality of water available and hygiene practises resulted in a 33% fall in the risk of contracting diarrhoea (Nanan *et al.*, 2003).

In addition to treatment and prevention, the development of vaccines may provide an important tool in preventing diarrhoea in children and illnesses related to diarrhoeagenic *E. coli*, such as HC. Initial studies have shown promise in animal models and may provide a means of limiting spread through reservoir hosts such as cattle (Dean-Nystrom *et al.*, 2002a;Ghaem-Maghami *et al.*, 2001). Vaccine development will benefit from an increased understanding of the mechanisms involved in the pathogenesis of diarrhoeagenic *E. coli*.

1.4 Diarrhoeagenic *E. coli*.

The identification of diarrhoeagenic *E. coli* requires that they be distinguished from the non-pathogenic commensal flora. In 1944 Kaufman (Nataro and Kaper, 1998) proposed a scheme to classify *E. coli* using serology of an isolate . According to this scheme *E. coli* are serotyped on the basis of their O (somatic), H (flagellar) and K (capsular) surface antigen profile. The K phenotype of an isolate can be conferred by several different molecular structures and the K antigen profile has been refined to exclude proteinaceous fimbriae, which are designated as F antigens (Orskov *et al.*, 1982). However, in order to define the serotype of an isolate the specific combination of O and H antigens is sufficient. *E. coli* is a clonal species and evidence for this was underlined by the recovery of diarrhoeagenic *E. coli* clones of the same serotype from separate outbreaks of diarrhoea. Serotype markers can correlate with certain categories of diarrhoeagenic *E. coli* but the serotype of an isolate is not sufficient to designate a strain as being diarrhoeagenic (Reid *et al.*, 2000;Donnenberg and Whittam, 2001).

The evolution of diarrhoeagenic *E. coli* from commensal *E. coli* strains is facilitated by commensal strains possessing the genes necessary for host interaction but not the genes

required for pathogenesis. The acquisition of gene clusters that confer pathogenic traits may occur through lateral gene transfer and pathogenic *E. coli* strains do not appear to originate from a common ancestor but evolve multiple times from several ancestors

(Buchrieser, Prentice, and Carniel, 1998;Pupo *et al.*, 1997;McDaniel and Kaper, 1997).

As well as lateral gene transfer bacteria may be prone to genetic instability and genomic rearrangements have been reported in *Yersinia pestis* (Buchrieser, Prentice, and Carniel, 1998). Therefore diarrhoeagenic *E. coli* strains have evolved with distinctive virulence mechanisms which may be used to group strains into categories. This can be achieved by using *in vitro* phenotypic assays or by detecting genes encoding virulence factors.

However common amongst diarrhoeagenic *E. coli* is the ability to colonise the intestinal mucosae, evade host defences, multiply and cause host damage. Three general diarrhoeagenic mechanisms have been identified: ETEC and EAEC produce enterotoxin, EIEC invade host cells and EPEC and EHEC adhere intimately, translocate bacterial effector proteins into the host cell produce an attaching effacing lesion (Nataro and Kaper, 1998).

In the following sections the different categories of diarrhoeagenic *E. coli* will be described by defining the virulence factors that are associated with their pathogenesis and distinguish them from other diarrhoeagenic *E. coli* (see table 1.1).

Table 1.1 Diarrhoeagenic *E. coli* categories.

E. coli category	Clinical features	Diarrhoeagenic factors	Pathology
ETEC	Watery diarrhoea	Heat stable (ST) and heat labile (LT) enterotoxins, fimbriae	Normal mucosa
EIEC	Dysentery and watery diarrhoea	Invasion of enterocytes and cellular motility	Invasion
EAEC	Diarrhoea with mucus	Plasmid encoded cytotoxin, fimbriae	Intestinal brush border damage
EPEC	Watery diarrhoea and vomiting	Fimbriae, type III secretion system, host cell effector proteins, intimin	Attachment and effacement of intestinal microvilli, enteropathy
EHEC	Watery diarrhoea, bloody diarrhoea and haemolytic uraemic syndrome	Type III secretion system, host cell effector proteins, intimin, Shiga toxin	Attachment and effacement of intestinal microvilli, haemorrhagic colitis, HUS.

1.4.1 Enterotoxigenic *E. coli* (ETEC).

The enterotoxigenic *E. coli* (ETEC) are an important cause of diarrhoea in children in the developing world and of travellers to regions where ETEC is endemic. In a study of ETEC related diarrhoea in Mexico, it was shown that acute diarrhoea was primarily due to ETEC in both Mexicans and travellers to Mexico (Bouckenooghe *et al.*, 2002). The prevalence of ETEC in certain regions may be due to many factors including the development of mucosal immunity of exposed individuals, the shedding of ETEC by immune asymptomatic individuals and the high infectious does required for infection. Thus most infants born in endemic regions will contract ETEC related diarrhoea and travellers to such regions are likely to come into contact with ETEC (Steinsland *et al.*, 2003).

ETEC are defined by their ability to produce at least one of two enterotoxins, i.e. heat stable (ST) and heat labile (LT) toxins. ETEC strains have been shown to produce diarrhoea in human volunteer studies but the characterisation of ETEC as diarrhoeagenic was based on studies of diarrhoea in piglets and fluid accumulation induced by human *E. coli* isolates in ligated rabbit ileal loops (DuPont *et al.*, 1971).

ETEC produce diarrhoea by colonising the mucosae of the small intestine and elaborating their enterotoxins. ETEC strains may express LT only, ST only or both LT and ST (Levine, 1987). Bacterial enterotoxins are defined as bacterial products that act on the mucosal epithelium of the small intestine, causing fluid secretion and profuse watery diarrhoea, without damage to the intestinal mucosae (Sears and Kaper, 1996).

1.4.1.1 ETEC heat labile toxins (LT).

The LTs of ETEC are oligomeric proteins that are closely related in structure and function to the cholera toxin (CT) produced by *Vibrio cholerae* (Sixma *et al.*, 1993). The structure of the holotoxin of LT and CT are similar and the protein sequence of both toxins is approximately 80% identical and they share similar receptors. LT can be divided into two types based on serology, LT-I and LT-II. LT-I is associated with disease in animals and humans. LT-II has been found in *E. coli* isolates from cattle and buffalo but rarely from humans and has yet to be associated with disease (Seriwatana *et al.*, 1988).

LT-I is an oligomeric protein 86kDa in size composed of one 28kDa A subunit and five identical 11.5 kDa subunits. The A subunit is responsible for the enzymatic activity of the toxin and is cleaved by proteolysis of a disulphide bridge into A_1 and A_2 peptides. The A_1 peptide has an ADP – ribosyltransferase activity which modifies membrane bound GTP binding protein and stimulates adenylate cyclase activity. Adenylate cyclase can convert ATP to cyclic AMP (cAMP) thus modulating the Na^+ and Cl^- transporters leading to an ion imbalance and loss of water into the intestinal lumen. The chloride channel that is activated by LT and CT is the CFTR (cystic fibrosis transmembrane regulator) which is

defective in cystic fibrosis. The binding of LT to the intestinal membrane results in stimulation of Cl^- secretion by intestinal crypt cells and inhibition of NaCl absorption by the villous tip cells. The increased ion content of the luminal space results in osmotic diarrhoea due to the passive diffusion of water via the paracellular pathway (Sears and Kaper, 1996). The inhibition of fluid and ion absorption from the intestinal lumen may be due to cAMP inhibition of the H^+/peptide cotransporter (Muller *et al.*, 1996). Other pathways may also be involved in LT-I induced diarrhoea, based primarily on studies using CT, such as inflammation of the intestine in response to LT-I, prostaglandin synthesis and stimulation of the enteric nervous system (McGee, Elson, and McGhee, 1993;Sears and Kaper, 1996).

LT-II is serologically different to LT-I and shows homology in it's A subunit to LT-I and CT but no homology exists between B subunits. The enzymatic activity of the A subunit is similar to LT-I but they do not possess the same host cell receptor.

1.4.1.2 ETEC heat stable toxins (ST).

In contrast to LT, ETEC ST is a heat stable monomeric nonantigenic peptide that owes its stability under heating to the presence of cysteine residues bound by disulphide bonds. It does not stimulate cAMP like LT (Tin *et al.*, 1993). ST can be divided into two unrelated peptides that differ in structure and function.

STa is produced by human ETEC isolates and is approximately 2kDa in size. STa binds to guanylate cyclase C which stimulates guanylate cyclase leading to increased intracellular levels of cGMP (Parkinson *et al.*, 1997). The result of this cGMP increase is a stimulation of Cl^- secretion and inhibition of NaCl absorption causing the secretion of fluid into the intestinal lumen. STa receptors are more common in human infants than in adults which may explain the severity and frequency of diarrhoea due to ETEC in children (Cohen *et al.*, 1988).

STb is primarily associated with ETEC strains isolated from pigs as is the K88 fimbriae (Grange *et al.*, 2002). STb has a molecular weight of 5.1 kDa and although it contains no homology to STa it does contain cysteine residues. STb does not stimulate secretion of cAMP or cGMP but it does increase intracellular calcium from extracellular sources (Dreyfus *et al.*, 1993). STb binds to sulphatide and induces histological damage of the intestinal epithelium characterised by loss of villous epithelial cells and villous atrophy (Labrie, Harel, and Dubreuil, 2002;Chao and Dreyfus, 1997).

1.4.1.3 ETEC fimbriae.

In order to produce diarrhoea ETEC must first adhere to the small intestine. This may be mediated by fimbriae or pili. Not all ETEC fimbriae have been identified but ETEC fimbriae confer the species specificity of a strain. ETEC strains expressing K99 are pathogenic for calves, lambs and pigs whilst those strains expressing K88 fimbriae are only pathogenic for pigs (Nagy and Fekete, 1999). Human ETEC strains express specific fimbriae which are termed colonisation factor antigens (CFA) and are usually encoded on plasmids. The CFA fimbriae can be divided according to their morphology (Wolf, 1997). In addition to CFAs, human ETEC isolates also produce a type IV pilus related to CFAs which has been termed Longus (Pichel *et al.*, 2002). The expression of so many different fimbriae by ETEC means that generating a vaccine is not straightforward (Jertborn *et al.*, 1998).

1.4.2 Enteroinvasive *E. coli* (EIEC).

The identification of EIEC involves showing that the bacterial isolate has the biochemical characteristics of *E. coli* and the genotypic and phenotypic characteristics of *Shigella*. Both species are closely related and molecular evidence derived from studies involving DNA hybridization, multilocus enzyme electrophoresis, and sequencing of housekeeping genes indicates that *E.* coli and all members of the genus *Shigella* belong to the same species (Lan and Reeves, 2001). *Shigella* and EIEC are distinguished by the Sereny test or guinea pig keratoconjunctivitis test, which measures the ability of strains to invade the

corneal epithelia of guinea pigs and spread to contiguous cells, with the most virulent strains causing ulcerative keratoconjunctivitis (Faundez *et al.*, 1988). EIEC are considered to be non motile but some isolates do express flagella in modified motility agar (Andrade *et al.*, 2002).

In developing countries EIEC may account for 4% of childhood diarrhoeal cases (Taylor *et al.*, 1988) but in a study of diarrhoeagenic *E. coli* in Argentina EIEC were not isolated from infants in their first 20 months of life (Quiroga *et al.*, 2000). EIEC isolates from infected patients in Germany have been shown to be carried by asymptomatic economic migrants (Beutin *et al.*, 1997).

EIEC like *Shigella*, invades the enterocytes of the large intestine. Both strains possess related invasion 140kDa plasmids which may have been acquired through lateral gene transfer (Santapaola *et al.*, 2002). Invasion results in lysis of the endocytic vacuole, intracellular multiplication, directional movement through the cytoplasm and extension into neighbouring epithelia cells. EIEC can produce a watery diarrhoea which may, during severe infections, result in an acute inflammatory colitis leading to possible intestinal cramps, bloody diarrhoea, and neurological symptoms such as lethargy, confusion, severe headache, and convulsions (Sansonetti, 1992).

In addition to invasion, EIEC produces a 63 kDa protein which may behave as an enterotoxin (Nataro *et al.*, 1995b) explaining the ability of EIEC supernatants to produce fluid accumulation in rabbit ileal loops. The expression of an enterotoxin may be the mechanism behind the watery diarrhoea associated with EIEC(Fasano *et al.*, 1990).

1.4.3 Enteroaggregative *E. coli* (EAEC).

Enteroaggregative *E. coli* (EAEC) are frequently associated with clinical cases of diarrhoea in the developing world (Scaletsky *et al.*, 2002) and have been isolated from 26% of travellers with diarrhoea. In addition, a study in Switzerland showed that EAEC is involved in significant proportion of diarrhoea cases occurring in children under five years

of age (Pabst *et al.*, 2003). In immunocompromised patients with AIDS in adults in the Central African Republic, diarrhoea has been associated with EAEC infections (Mossoro *et al.*, 2002).

EAEC is associated with persistent diarrhoea in children in the developing world and with diarrhoeal outbreaks worldwide (Cobeljic *et al.*, 1996;Smith, Cheasty, and Rowe, 1997). The clinical features of EAEC pathogenesis are defined by a watery secretory diarrhoea, absence of fever and absence of polymorphonuclear cells or gross blood in the stool. The stools contain mucus and some reports have suggested that blood may be present in the stool up to a third of patients (Cravioto *et al.*, 1990). In gnotobiotic piglets infected with EAEC a mucoid gel was seen adhering to the small intestinal epithelium (Tzipori *et al.*, 1992) and analysis of this gel showed the presence of large numbers of aggregative bacteria. EAEC can bind to mucus *in vitro* and human volunteers develop a mucoid diarrhoea (Nataro *et al.*, 1995a;Wanke *et al.*, 1990). In addition, *in vitro* organ culture (IVOC) of intestinal explants showed that EAEC can adhere to the small and large intestine and are seen in association with a thick mucus layer that was not present in uninoculated control explants (Hicks, Candy, and Phillips, 1996). A 110kDa serine protease termed pic (protein involved in intestinal colonisation), may act as a potential effector of mucus secretion by EAEC (Henderson *et al.*, 1999a).

Patients excreting EAEC strains in their stool have been shown to have high levels of interleukin – 8 in their stool. A flagellin expressed by EAEC has been implicated in IL-8 release from Caco-2 cells and susceptibility and faecal IL – 8 production correlate with certain polymorphisms in the interleukin – 8 promoter region in humans (Steiner *et al.*, 2000).

1.4.3.1 EAEC cytotoxins.

Cytotoxic effects can be attributed to EAEC infections. In rabbit and rat leal loops EAEC inoculation produces a shortening of the villi, haemorrhagic necrosis of the villous tips, and a mild inflammatory response with oedema and mononuclear infiltration of the

submucosae. In addition to these histopathological results the infection of rabbits with EAEC resulted in limb paralysis and death of the rabbits suggesting the presence of a toxin (Vial *et al.*, 1988). The IVOC of EAEC produced a cytotoxic effect on colonic explants which was characterised by the exfoliation of the enterocytes and the dilation of the crypt openings (Hicks, Candy, and Phillips, 1996). The explants also showed microvillous vesiculation which can be demonstrated using T84 cells *in vitro*. In T84 cells the vesiculation of the microvilli is accompanied by cell death and exfoliation of the cells (Nataro *et al.*, 1996). A 104 kDa plasmid encoded toxin (pet) is required for cytotoxic effects during IVOC but not during infection assays using T84 cells suggesting that EAEC cytotoxicity can occur via different pathways (Henderson *et al.*, 1999b).

In addition to the plasmid encoded toxin, EAEC produce a heat stable enterotoxin 1 (EAST1) which is homologous to STa and encoded by the *astA* gene (Menard and Dubreuil, 2002). EAST1 is expressed by nonpathogenic *E. coli* strains as well as EHEC strains (Savarino *et al.*, 1996b). Rabbit ileal loops inoculated with EAST1 containing supernatants show a rise in the level of intracellular cGMP suggesting that EAST1 may play a role similar to STa during pathogenesis (Savarino *et al.*, 1993). However, an EAST1 producing strain did not produce diarrhoea in adult volunteers which casts doubt on the role of EAST1 in EAEC induced diarrhoea (Nataro *et al.*, 1995a).

1.4.3.2 The adhesion of EAEC.

The HEp-2 cell adherence assay showed that diarrhoeagenic *E. coli* adhere to HEp-2 cells in characteristic patterns (Cravioto *et al.*, 1991a). Using this assay and molecular biology EAEC were noted for their lack of EPEC adherence factor (EAF) plasmid and their aggregative or stacked bricked adhesion pattern on HEp-2 cells and their lack of LT or ST production (Nataro *et al.*, 1995a;Nataro and Kaper, 1998).

A flexible bundle forming fimbriae, 2 – 3nm in diameter, designated aggregative adherence fimbriae I (AAF/I) mediates adhesion to HEp-2 cells and human erythrocytes by EAEC (Nataro *et al.*, 1992). The genes responsible for the expression of AAF/I are encoded on two separate regions of a 60 MDa plasmid (Nataro *et al.*, 1993). Only a minority of EAEC strain express AAF/I and a search for other factors involved in EAEC adhesion revealed another fimbria which is morphologically distinct from AAF/I. This fimbria was designated AAF/II and is 5 nm in diameter and does not bind to anti – serum raised against AAF/I. AAF/II mediates adhesion to HEp-2 cells, like AAF/I, and AAF/II is necessary for adhesion of EAEC to colonic explants during IVOC (Czeczulin *et al.*, 1997).

Adherence factors other than fimbriae have also been described for EAEC. A 58 kDa outer membrane protein expressed by EAEC has been shown to be involved in adhesion to HEp-2 cells. This protein also mediated adhesion to bovine erythrocytes but no receptor has been characterised (Monteiro-Neto *et al.*, 2003).

In addition, to adherence factors EAEC can form biofilms on intestinal mucosae and on glass or plastic surfaces when grown in cell culture medium with high sugar and osmolarity. In some strain biofilm formation is mediated by AAF although strains which do not express AFF also form biofilms. The use of transposon mutants of a prototypic strain of EAEC, O42, showed that a *yafK* gene product is required for biofilm formation and that this gene product is required for the transcription of AAF/II encoding genes. This suggests that EAEC have elaborated different biofilm forming mechanisms (Sheikh *et al.*, 2001). In addition to fimbriae, an *aap* gene product has been shown to be involved in the disaggregation of a surface coat of bacteria during IVOC that may counteract the aggregates formed by AAF. The 10kDa gene product was termed dispersin and shown to have immunogenic properties during human EAEC challenge studies (Sheikh *et al.*, 2002).

1.5 Enteropathogenic *E. coli* (EPEC).

In the 1940s Bray et al showed that cases of summer diarrhoea among infants were due to infection by *E. coli* (Bray, 1945). The term enteropathogenic *E. coli* was then used to describe *E. coli* strains associated with diarrhoea (Neter *et al.*, 1955;Neter, 1965). Further investigation of *E. coli* related diarrhoeal outbreaks led to certain O and H serotypes being associated with EPEC infections, although not all serogroups were equally pathogenic (Rowe, Gross, and Allen, 1974). Subsequently with the discovery of the enterotoxins produced by ETEC and the invasive potential of EIEC it was clear that diarrhoeagenic *E. coli* produced diarrhoea via different pathogenic mechanisms and that diarrhoeagenic *E. coli* should be grouped according to pathogenic mechanisms. Adult volunteer studies (Levine *et al.*, 1978) showed that EPEC caused diarrhoea but it was defined by exclusion, i.e. toxin negative and non – invasive. The use of small intestinal biopsy with light and electron microscopy in *in vivo* infections (Rothbaum *et al.*, 1983;Ulshen and Rollo, 1980) and subsequently molecular biological techniques on cell culture models for EPEC infection eventually led to defining EPEC according to its pathogenic characteristics.

1.5.1 Clinical aspects of EPEC related diarrhoea.

EPEC produce a watery diarrhoea which can be accompanied by a low grade fever and vomiting. Faecal leucocytes are seen in some cases and stools may be positive for lactoferrin. The diarrhoea produced by EPEC can be acute or chronic and primarily affects infants under two years of age. In children older than two years of age EPEC can be recovered from both sick and asymptomatic individuals (Levine and Edelman, 1984). In the developed world death from EPEC related diarrhoea can still occur although mortality is lower than the 30% reported in an outbreak in Kenya (Senerwa *et al.*, 1989). In infants in the developing world infection due to EPEC can be complicated by malnutrition and food intolerance (Senerwa *et al.*, 1989) and spread is by the faecal oral route (Wu and Peng, 1992). Episodes of EPEC related acute diarrhoea may become persistent, and this may be due to continuing infection and, in some cases, food intolerance has been

implicated as an aggravating factor indicating that dietary management may be important when treating EPEC related diarrhoea. Antibiotic therapy has been suggested in cases of persistent EPEC infection (Hill, Phillips, and Walker-Smith, 1991b). Oral rehydration is often used when treating diarrhoea to correct electrolyte imbalance and fluid loss but in cases where nutritional imbalance occurs total parental nutrition may be required (Fagundes-Neto *et al.*, 1996).

In addition to such clinical symptoms Ulshen and Rollo showed in 1980 that a case of EPEC related diarrhoea was characterised by a specific intestinal lesion. Proximal small bowel biopsies examined using transmission electron microscopy (TEM) revealed tightly adherent *E. coli* on the surface of the intestinal mucosae and effacement of the microvilli on the villous epithelium. This was termed an attaching and effacing lesion and is characteristic of EPEC (Ulshen and Rollo, 1980;Sherman *et al.*, 1989).

In an effort to define EPEC the following sections will focus on the pathogenesis of EPEC and the virulence factors involved in A/E lesion formation.

1.5.2 The attaching and effacing lesion of EPEC.

The histopathology produced by ETEC, EIEC, and EAEC is different to that observed with EPEC infections. EPEC produces an attaching and effacing (A/E) lesion characterised by the effacement of microvilli and intimate adherence of the bacteria to the host cell membrane. Using a pig and a rabbit model of infection it was shown that this histopathology could be reproduced by human and rabbit EPEC strains and led to the term A/E lesion being used to described part of EPEC pathogenesis (Moon *et al.*, 1983). In addition to EPEC, A/E lesions are produced by EHEC (Tzipori *et al.*, 1986), REPEC (Heczko, Abe, and Finlay, 2000), *Hafnia alvei* (which may be reclassified as part of the *E. coli* genus (Janda *et al.*, 2002)) and *Citrobacter rodentium* (Schauer and Falkow, 1993), which suggest that the genes involved in A/E lesion formation may be acquired through horizontal gene transfer and that the pathogenic mechanisms of these organisms may share common themes (Deng *et al.*, 2001).

Rothbaum et al showed in 1983 (Rothbaum *et al.*, 1983) that infants infected with 0119 EPEC showed A/E lesions in the jejunum, ileum and the rectum. TEM of jejunal biopsies showed disappearance of glycocalyx and microvilli at the areas of bacterial adherence. The bacterial membrane was observed to be in intimate contact with the host cell membrane at the site of microvillus effacement. Intracellular damage was indicated by dilatation of rough endoplasmic reticulum, mitochondrial changes, and cytoplasmic pallor. The presence of A/E lesion on the intestinal mucosae produced a reduction in the absorptive surface area of the intestinal tract leading to malabsorption of nutrients (Rothbaum *et al.*, 1982). Thus A/E lesions are involved in producing an enteropathy and defining a class of pathogenic *E. coli.*

The intimate adherence of EPEC to enterocytes was replicated in animal models and cell lines cultured *in vitro* and cytoskeletal changes beneath the adhering bacteria could be observed using TEM (Moon *et al.*, 1983;Batt *et al.*, 1987;Knutton *et al.*, 1987). Beneath the bacterium host cell interface dense actin microfilaments were observed and the bacteria were seen adhering to cellular projections which were termed pedestals. The staining of these actin rich regions using fluorescein isothiocyanate (FITC) labeled phalloidin, which binds to filamentous actin (Falcigno *et al.*, 2001;Wulf *et al.*, 1979), allowed the A/E lesion to be viewed by light microscopy (Knutton *et al.*, 1989). This allowed the bacterial genes involved in A/E lesion formation and EPEC pathogenesis to be characterised using defined bacterial mutants and light microscopy. The first gene to be associated with EPEC A/E lesion formation was the *eae* gene which codes for the 94 kDa outer membrane protein intimin. This gene was shown to be required for A/E lesion formation on cultured epithelial cells (Jerse and Kaper, 1991). The second gene to be shown to be required for A/E lesion formation was the *eaeB* gene which was later renamed EspB (Donnenberg, Yu, and Kaper, 1993). The genes involved in A/E lesion formation by EPEC are encoded on the locus of enterocyte effacement (LEE) and a summary of this pathogenicity island and

the proteins involved in A/E lesion formation and EPEC pathogenesis will be discussed in the following sections.

1.5.3 The locus of enterocyte effacement (LEE) of EPEC.

The locus of enterocyte effacement (LEE) was first identified by McDaniel et al (McDaniel *et al.*, 1995) in the EPEC O127:H6 strain E2348/69. The LEE contains 35 624 bp and DNA probes throughout this locus hybridise to EHEC O157:H7, REPEC, *H. alvei* and *C. rodentium*. However, the probes generated to this region did not hybridise with nonpathogenic *E. coli*. The guanine and cytosine (G+C) content of the LEE is 38.4% which is lower than the 51% composition of the *E. coli* K-12 chromosome. This difference in G+C content suggests that the LEE was originally acquired from a foreign source and inserted into the chromosome. The LEE is inserted at a position on the chromosome coding for the *selC* (selenocysteine) tRNA gene. This site is also a site of insertion for a virulence region associated with uropathogenci *E. coli* and therefore may be a region of the chromosome prone to insertion (Blum *et al.*, 1994). Pathogenicity islands were first described in *E. coli* (Hacker *et al.*, 1990) and the LEE is considered to be a pathogenicity island due to the presence of virulence genes, it absence in *E. coli* K-12, its insertion into the chromosome at a site which is used for the insertion of virulence genes in other pathogens and because of its distinctive G+C content with respect to the rest of the chromosome.

However, not all EPEC strain contain the LEE inserted at the *selC* locus and some strains have been isolated with the LEE inserted next to a *pheU* (gene encoding phenylalanine) gene and a third insertion may exist which has yet to be identified (Wieler *et al.*, 1997). The insertion site of the LEE differed in relation to the clonal phylogeny of the EPEC strains suggest that the LEE was inserted as a pathogenicity island at multiple times and sites during the evolution of EPEC and other LEE containing diarrhoeagenic *E. coli*.

The LEE of EPEC strain E2348/69 contains 41 open reading frames (ORFs) which encode a type III secretion system (TTSS) and the genes involved in A/E lesion formation. The

LEE genes involved in TTSS are similar to those of the *Yersinia* and *S. typhimurium* TTSS. TTSS encoded by several gram negative bacteria can transport effectors across the inner and outer membranes of the bacteria and deliver effector proteins to the surface or interior of cells (Hueck, 1998). The genes which are homologous to the *Yersinia* TTSS are termed *esc* (*E. coli* secretion) and the genes which are not homologous to the *Yersinia* TTSS genes but are involved with type III secretion are termed *sep* (secretion of *E. coli* proteins). The proteins that are secreted by the TTSS are termed Esps (EPEC secreted proteins) and those characterised to date are EspA, EspB, EspD, EspF, EspG, EspH and the translocated intimin receptor (Tir) encoded by *tir* (Kenny *et al.*, 1997b). In addition, the LEE contains the *eae* which encodes an outer membrane adhesin called intimin. As with other TTSS, cystolic chaperone proteins have been shown to be required for the translocation of secrete effector proteins. Three genes encoding chaperones have been identified *cesD* and *cesD2* (chaperone for *E. coli* secreted protein) which are chaperones for EspB and EspD (Wainwright and Kaper, 1998) (Neves *et al.*, 2003a), and *cesT* which is a chaperone for Tir (Abe *et al.*, 1999;Elliott *et al.*, 1999). The LEE also contains noncoding genetic elements which may be involved in the insertion of the LEE into the chromosome (Elliott *et al.*, 1998).

The LEE is present in human and animal EPEC strains (An *et al.*, 2000) and the LEE of EPEC strain E2348/69 is necessary and sufficient to produce A/E lesions on HEp-2 cells *in vitro* (McDaniel and Kaper, 1997). Therefore in the following sections the virulence factors involved in adhesion and A/E lesion formation will be described with particular attention to the adhesins and host cell effectors encoded by the LEE.

1.5.3.1 Intimin.

The 94 kDa outer membrane protein intimin from the prototype EPEC strain E2348/69 is encoded by the *eae* (*E. coli* attaching and effacing) gene located in the LEE upstream from the *esp* genes(Jerse and Kaper, 1991). Intimin is necessary for the intimate adhesion of EPEC to host cells and binds to the 90kDa translocated intimin receptor Tir (Donnenberg

and Kaper, 1991;Kenny *et al.*, 1997b). Antibodies to intimin are found in the colostrum of mothers and in the serum of mothers and infants in Mexico (Parissi-Crivelli, Parissi-Crivelli, and Giron, 2000a). In human volunteer studies intimin is necessary for full virulence of the EPEC strain E2348/69 (Donnenberg *et al.*, 1993). Using a mouse model of infection intimin specific antibodies were shown to prevent colonisation of the mouse colon by *C. rodentium* (Ghaem-Maghami *et al.*, 2001;Higgins *et al.*, 1999a).

Intimin proteins expressed by different bacterial strains have been typed according to their antigenicity and their sequence variation (Adu-Bobie *et al.*, 1998;Agin *et al.*, 1996;Frankel *et al.*, 1994;McGraw *et al.*, 1999;Oswald *et al.*, 2000). Certain intimin types have been associated with distinct pathogens. The prototype EPEC O127:H6 strain E2348/69 is an intimin α expressing EPEC whilst the EHEC O157:H7 strains express intimin γ. The variation in intimin type is due to the variation in sequence in the carboxy terminus of the intimin protein as the amino terminus is highly conserved across the intimin expressing strains. To date nine different intimin types have been identified and assigned the Greek letters α to ζ. The diversity of intimin types may have occurred through recombination as well as random substitution events (Zhang *et al.*, 2002). Intimin types have been shown to be related through sequence analysis with the following intimin types being closely related to one another; α and ζ; β and κ; ϵ and η; and ϵ and γ (Oswald *et al.*, 2000;Tarr and Whittam, 2002b;Zhang *et al.*, 2002). Whether the biological activities of these intimin types are related remains to be characterized.

The structure of intimin α from the prototype EPEC strain E2348/69 has been resolved by both nuclear magnetic resonance and X ray crystallography (Kelly *et al.*, 1999;Luo *et al.*, 2000). The structure produced by both methods is similar in structure to the invasin protein of *Yersinia pseudotuberculosis*, which mediates the invasion of host cells by *Yersinia*. Like invasin, intimin can bind to β_1 integrins *in vitro* but such binding activity is not necessary for A/E lesion formation (Frankel *et al.*, 1996;Kelly *et al.*, 1999;Liu,

Magoun, and Leong, 1999). The cell binding domain of intimin is located in the carboxy terminal 280 amino acids which are divided into three globular domains (Frankel *et al.*, 1994;Kelly *et al.*, 1999). The first two domains D1 and D1 (residues 1-91 and 93 181) are similar in structure to the immunoglobulin superfamily domains (IgSF). The third domain resembles a C - type lectin and is known as the C – type lectin domain (CTLD residues 183 - 280). The region of intimin necessary for Tir binding has been located to a 190 amino acid region which spans the CTLD and the IgSF D2 domain (Batchelor *et al.*, 2000). Amino acid residues within D2 and the CTLD have been implicated in binding to Tir, A/E lesion formation during IVOC and colonisation and colonic hyperplasia in mice (Reece *et al.*, 2001;Reece *et al.*, 2002b;Reece *et al.*, 2002a). The extracellular regions of intimin may be linked to the membrane bound regions via a flexible linker which suggest that there may be movement in the intimin molecule at some stage either during genesis, attachment to Tir or during post attachment events.

The variation in intimin type may have a biological role in defining the tissue tropism of strains during *in vivo* infection of a gnotobiotic piglet model or during adhesion to intestinal explants during IVOC (Phillips and Frankel, 2000;Tzipori *et al.*, 1995). These studies showed that the intimin from EHEC O157:H7 strains when expressed in an EPEC background replaced the EPEC associated tissue tropism with an EHEC associated tissue tropism. In addition these studies showed that intimin is necessary for adherence *in vivo* and during IVOC.

The binding of intimin to Tir is necessary for adhesion to cell lines. However, in addition to binding to Tir, intimin may bind to a host cell receptor. Like invasin, intimin has a CTLD which may bind to a glycoprotein. Tir is not a glycoprotein and purified intimin binds to the surface of Hep-2 cells in the absence of Tir (Frankel et al., 1994). However, this activity is was not reproduced when using HeLa cells and different methodology may be a problem in establishing such Tir independent binding by intimin

(Rosenshine *et al.*, 1996). In addition to these results, mutation of a cysteine residue at position 937 in the CTLD of intimin α from E2348/69 disrupts adhesion to HEp-2 cells and to intestinal explants during IVOC (Hicks *et al.*, 1998). However this mutation in intimin α does not prevent intimin - Tir binding in gel overlays or in the yeast two hybrid system (Hartland *et al.*, 1999). In addition to *in vitro* studies, *in vivo* studies using *C. rodentium* have shown that a mutation in cysteine 937 failed to induce a *C. rodentium* related colonic hyperplasia and a TH1 response (Higgins *et al.*, 1999b). Therefore the disruption of the CTLD of intimin due to the mutation at cysteine 937 may result in the lack of recognition of a receptor *in vivo*, which may not be detected using denatured proteins during gel overlays and in the yeast hybrid system where disulphide bond formation is unlikely (Nougayrede, Fernandes, and Donnenberg, 2003).

The CTLD domain of intimin has been mapped by X ray crystallography and unlike other CTLDs it does not contain calcium binding acidic amino acid residues. Thus intimin may not bind via carbohydrate but may bind via another form of receptor (Luo *et al.*, 2000).

The Intimin γ of EHEC O157:H7 has been shown to bind nucleolin on HEp-2 cells and this protein may act as a host cell receptor *in vivo* (Sinclair and O'Brien, 2002). It remains to be seen if nucleolin binds to the intimin expressed by EPEC strains.

Using EPEC strains deficient in Tir translocation, EPEC were shown to induce the elongation of microvilli like processes (MLPs) on HEp-2 cells. These MLPs formed cage like structures around the adherent bacteria and this was also seen following incubation of HEp-2 cells with intimin coated plastic beads. This biological activity of intimin may occur during the early stages of A/E lesion formation in a Tir independent phase (Phillips *et al.*, 2000a).

Together these results suggest that intimin is an adhesin involved in binding to host cells via its receptor, Tir, or possibly via a host cell receptor, and that it mediates tissue tropism during *in vitro* and *in vivo* infections. In addition, intimin is immunogenic and can remodel the host cell cytoskeleton.

1.5.3.2 The EPEC translocated intimin receptor (Tir).

The EPEC type three III secretion system allows EPEC to inject its own receptor, for intimin, into the host cell to enable intimate attachment to the cellular membrane, via the translocated intimin receptor (Tir). Before entering the host cell Tir is a 74kDa protein and after translocation it becomes a 90kDa protein. This change in molecular weight of Tir may be due to phosphorylation of a tyrosine residue at position 474 and serine residues at position 434 and 463 in Tir (Kenny, 1999;Warawa and Kenny, 2001). Tir is the first example of a bacterial pathogen injecting its own receptor into the host cell and Tir requires the TTSS and the Esps for translocation (Knutton *et al.*, 1998). Tir has a 156kDa chaperone CesT which binds to a 100 amino acid region at the amino terminus of Tir. This chaperone is involved in Tir secretion and the formation of the CesT – Tir multimeric complex may prevent degradation of Tir during translocation to the host cell and stabilize Tir levels in the cytoplasm (Abe *et al.*, 1999;Elliott *et al.*, 1999;Luo *et al.*, 2000).

Tir is essential for A/E lesion formation and actin nucleation in EPEC (Rosenshine *et al.*, 1992;Rosenshine *et al.*, 1996). Intimin binds to a central 107 amino acid region of Tir (Hartland *et al.*, 1999). This region of Tir is presented on the host cell surface as an extracellular loop (residues 260-362) and is flanked by two α helical transmembrane domains (residues 234-259 and 363-382). These transmembrane domains are required for incorporation of Tir into the host cell membrane. The intimin binding domain of Tir is hairpin shaped and is composed of two long α helices. These α helices mediate dimerisation of Tir in complex with intimin by forming an antiparallel four helix bundle and this helix bundle may have a biological function. The intimin binding domain is highly conserved which suggests that dimerisation and intimin binding requires a defined structure. X ray crystallography shows that Tir binds to the positively charged tip of intimin via hydrogen bonds and a salt bridge, and the amino terminus of Tir binds to α-actinin. The amino terminus of Tir (residues 1-233) and the carboxy terminus of Tir (residues 383-551) are located in the cytoplasm of the host cell underneath the plasma membrane (Goosney *et al.*, 2000;Luo *et al.*, 2000).

Once Tir is translocated to the host cell it is tyrosine phosphorylated at residue 474. This phosphorylation is essential for EPEC HeLa cell actin nucleation at the site of bacterial adherence. However, phosphorylation of tyrosine 474 is not required for correct incorporation of Tir in the host cell membrane. The phosphorylation of serine residues at positions 434 and 463 in Tir is required for the correct incorporation of Tir into the host cell membrane. The phosphorylation of serine 434 may be mediated by protein kinase A and although phosphorylation of serine 434 is not required for actin nucleation it is required for pedestal formation (Abe *et al.*, 1999;Luo *et al.*, 2000;Warawa and Kenny, 2001). Evidence exists that cytoskeletal proteins are recruited beneath adherent EPEC during the infection of cell lines but no mechanism has yet been defined for these proteins during pedestal formation. However three examples exist of the direct interaction of Tir and host cell proteins. The amino terminus of Tir interacts with α–actinin and talin, and the carboxy terminus 474 tyrosine residue binds to the host cell adaptor protein Nck located in the cytoplasm. The Src homology 2 domain of Nck can stimulate neural Wiskott – Aldrich syndrome protein (N - WASP) to trigger actin polymerisation *in vitro* by binding to phosphotyrosine residues. Thus the phosphorylated EPEC Tir tyrosine 474 binds to Nck which recruits N-WASP and the actin related protein 2/3 complex to pedestals thus mediating actin nucleation. The interaction of Tir and Nck has been located to a 12 amino acid region in the carboxy terminus of Tir (Gruenheid *et al.*, 2001;Campellone *et al.*, 2002). However EHEC O157:H7 Tir is not tyrosine 474 phosphorylated suggesting that Tir may have different activities in different strains (DeVinney *et al.*, 1999). In addition to α– actinin binding and Nck binding, which have been localized to specific areas of Tir, Tir binds to talin at its amino terminus. Talin associates with integrins in focal adhesions and is involved in transmitting extracellular signals to the cell cytoskeleton. Focal adhesions have been shown to be recruited to the site of bacterial adhesion at both the amino and carboxy termini of Tir (Freeman *et al.*, 2000). A dissociation constant was calculated for Tir - talin binding, and shown to be similar to that for talin - integrin binding, and the phosphorylation of Tir did not alter this binding constant suggesting that phosphorylation of Tir is not required for talin binding and signaling to host cells via this mechanism. As talin can interact with actin these results

suggest that actin nucleation is not solely due to phosphorylation of Tir (Cantarelli *et al.*, 2001).

Thus Tir binds to cytoskeletal proteins which may anchor the bacteria to the host cell and allow cell signaling events to occur. However, there is the possibility that these mechanisms may not be identical in all Tir expressing bacteria.

Antibodies to cytoskeletal components of the enterocyte have been used to describe the composition of the A/E lesion formed during adhesion to cell lines. The actin pedestals are composed of many actin binding proteins, membrane proteins and adaptor proteins and the typing of the proteins involved in EPEC and EHEC A/E lesions showed that differences exist in the composition of A/E lesions between EPEC and EHEC strains (Goosney, DeVinney, and Finlay, 2001). The formation of pedestals in cell lines appears to be a dynamic process in which pedestals can bend and change shape. This can results in bacteria moving along the surface of the host cell in a process driven by actin polymerization. Using video microscopy an estimate of the speed of this movement was given as 0.07 μm/s. This movement is different than that of EIEC as is located on the exterior of the cell and using *Shigella* cellular protrusions were shown to not relate to the speed of movement of a bacteria (Monack and Theriot, 2001).

In addition to cell line studies, Tir has also been shown to be essential for the virulence of rabbit enteropathogenic *E. coli* (REPEC) *in vivo* during the infection of weaned rabbits (Marches *et al.*, 2000). Tir and intimin negative mutants produced no clinical signs associated with REPEC infection. In addition, the colostrum of mothers and the serum of mothers and children with or without diarrhea, living in Sao Paulo, Brazil, contain antibodies to the intimin binding domain of Tir (Sanches *et al.*, 2000). Therefore like its ligand, intimin, Tir appears to be immunogenic.

1.5.3.3 EPEC secreted protein A (EspA).

As well as the study of intimin and Tir, the EspA protein has been the subject of extensive investigation.

The Esp A, B and D proteins are required for signal transduction in host cells and the formation of A/E lesions (Kenny *et al.*, 1996). These proteins are translated without a conventional amino terminus signal peptide but the TTSS of EPEC is similar to that of other TTSS harbouring gram negative bacteria (Jarvis *et al.*, 1995). The LEE encodes homologues of the proteins involved in the TTSS of *Shigella* and *Salmonella* which include in EPEC EscC, EscJ, EscN and EscF. However the EPEC TTSS appears to be different to the TTSS of *Shigella* and *Salmonella* in that EspA forms a filamentous structure. This EspA structure has been shown to be essential for the translocation of EspB , EspD and Tir into the host cell but not into culture supernatants and forms part of a surface organelle which forms a bridge between the bacteria and the host cell (Knutton *et al.*, 1998;Shaw *et al.*, 2001). Thus EspA is a 20kDa structural protein whose monomers interact via coiled coiled domains. Such domains allow oligomerisation of monomers via association of two or more α– helices and are present in TTSS and in the assembly of flagella (Delahay *et al.*, 1999). TEM has allowed the structure of the TTSS and its relationship to EspA to be characterised. EspA binds to EscF, the putative structural needle protein, and EscF is needed for protein secretion and the formation of EspA filaments. Thus a sheath like structure, composed of EspA, is attached to the TTSS and may extend as far as 600 nm from the bacterial surface (Daniell *et al.*, 2001b;Sekiya *et al.*, 2001;Shaw *et al.*, 2001). The three dimensional structure of EspA has been resolved and it comprises a helical tube with a diameter of 120 A enclosing a central channel of 25 A diameter through which effector proteins may be transported. The subunit arrangement corresponds to a helix with 28 subunits present in five turns of the helix and an axial rise of 4.6 A per subunit (Daniell *et al.*, 2003).

The DNA sequencing shows that the *espA* of EPEC strains shows that the *espA* of EPEC strains is approximately 65% identical. Using antiserum raised against EPEC and EHEC

EspA EPEC antiserum bound to all EPEC functional EspA filaments, except for the EPEC strain O55:H7 which is related to EHEC O157:H7. The EspA filaments of EPEC O55:H7 bound EHEC EspA and this suggests that polymorphisms exist within functional EspA filaments which may impact on EspA based vaccines strategies (Neves *et al.*, 2003b). The immunogenic properties of EspA are characterised by the presence of antibodies to EspA in the colostrum of mothers and the serum of mothers and infants in Mexico (Parissi-Crivelli, Parissi-Crivelli, and Giron, 2000b).

1.5.3.4 EPEC EspB and EspD.

As can be seen in the description of the function of EspA the three secreted proteins EspA, B and D share some functional similarity but more importantly their activities are coordinated during pathogenesis.

EspB is thought to act as an effector within the host cell and as a structural protein. HeLa cells transfected with *espB* show changes in the morphology of the cell and a reduction in stress fibre formation suggesting that it may act as a cytotoxin (Taylor *et al.*, 1998). In addition, studies have sought to establish the role of EspB in relation to EspA and EspD. Following bacterial attachment, EspB is translocated to the cytoplasm and the plasma membrane of the cell. This process requires the expression of EspA and is enhanced by intimin mediated attachment to the host cell. EspB has weak homology to the YopB and YopD proteins of *Yersinia* (21% and 17% respectively) and the structure of EspB is similar to YopD. This structure consists of a putative transmembrane domain and a predicted trimeric coiled coil domain region (Pallen, Dougan, and Frankel, 1997). EspB is required for the translocation of Tir as well as being translocated itself (Francis and Wolf-Watz, 1998). Binding of EspA to the host cell can occur in the absence of EspB but EspB is colocalised with EspA during HEp-2 cell adherence suggesting that EspB is important during translocation of effector proteins and is delivered to the host cell (Hartland *et al.*, 2000a). The sequence analysis of EspB suggests that it may contain a motif which in enzymes binds to pyridoxal phosphate but EspB does not appear to bind to pryidoxal phosphate *in vitro* and no receptor for EspB has yet been defined (Taylor *et al.*, 2001).

The role of EspB unlike EspA and EspD has been tested during infection of human volunteers. This showed that EspB is necessary for diarrhoea in 9 out of 10 volunteers. Biopsies showed that EspB was necessary for A/E lesion formation during infection and 40% of the volunteers showed a lymphoproliferative response to EspB. These results confirmed that, as in a rabbit model of infection, EspB is a virulence factor in man (Abe *et al.*, 1997). EspB is also required for activation of NF-κB in T84 cells and that this stimulates transcription of IL-8, which is recovered in the stool of patients with EPEC related diarrhoea (Savkovic, Koutsouris, and Hecht, 1997). The effect of EspB during the pathophysiology of EPEC diarrhoea has examined by using Ussing chambers to measure the change in ion transport across Caco-2 cells during EPEC infection. EPEC provokes a change in current which is linked to the modulation of electrolyte transport across the Caco-2 cells, and Esps A, B and D and intimin are required to produce this change. This suggests that all four proteins play a role during the pathophysiology of EPEC diarrhoea (Collington *et al.*, 1998).

EspB and EspD interact *in vitro* and EspD is essential for the formation of complete EspA filaments and its expression. EspD and EspB expression is mediated by the 17 kDa chaperone CesD which binds directly to EspD and is localised to the plasma membrane unlike other TTSS chaperones (Wainwright and Kaper, 1998). In addition to CesD, the LEE encodes another chaperone for EspD, CesD2. This chaperone is required for secretion of EspD but not EspB (Neves *et al.*, 2003a).

EPEC EspD is translocated into the host cell membrane (Wachter *et al.*, 1999) as predicted by its structure of two transmembrane domains and a coiled coil region. EspD is required for attachment to the host cell and EPEC induced haemolysis but intimate attachment is not required for insertion of EspD into the host cell membrane (Warawa, Finlay, and Kenny, 1999). Haemolysis of erythrocytes is correlated with possible pore forming activity (Hakansson *et al.*, 1996). EspD may interact with itself and the carboxy terminus of EspD is predicted to adopt a coiled coil conformation. Mutation of residues in the coiled coil domain reduced the number of A/E lesions formed on Hep-2 cells and reduced the

haemolysis of erythrocytes but did not affect the length of EspA filaments suggesting that EspD may contain regions with different biological activities (Daniell *et al.*, 2001a). The haemolysis of erythrocytes may be mediated by pores formed in the erythrocyte membrane by EspD and EspB. Osmoprotection studies have shown the pore size to be between 3 and 5 nm in diameter which compares favourably with pores formed by *Shigella* (2.5 nm) and *Legionella* (3 nm) (Kirby *et al.*, 1998;Blocker *et al.*, 1999). These results suggest that EspA may bind to the host cell via the EspB/D pore forming complex. The binding of EspA to EspB and EspD may occur in several ways but it is possible that like Tir, EspA binds to EspB and Esp D once they are present in the host cell plasma membrane. In addition, it is possible that EspB and EspD may interact with host cell factors and both proteins are required for the production of a cytopathic effect during the infection of HeLa cells by REPEC. The adhesion of the EspA filament structure to host cells remains to be elucidated.

1.5.3.5 EPEC EspF.

EspF is a proline rich protein encoded by the LEE and secreted by the TTSS. It is not necessary for A/E lesion formation on HEp-2 cells (McNamara and Donnenberg, 1998). EspF has been shown to produce a dose dependent change in the transepithelial resistance of T84 cells. EspF increases the permeability of the cell line monolayer and redistributes the tight junction associated protein occludin. EspF has no predicted transmembrane domains and is translocated to the cytoplasm of HeLa and T84 cells with a heterogeneous distribution indicative of a soluble cytoplasmic protein. Thus EspF may be sequestered by host cell proteins with which it interacts in order to disrupted host cell barrier function and produce diarrhoea (McNamara *et al.*, 2001). Further study of EspF has shown that strains deficient in EspF production do not kill host cells and that purified EspF has no effect of T84 cells in culture but that transfection of HeLa cells with *espF* results in apoptosis. Thus EspF appears to be an effector of cell death in epithelial cells and intracellular levels of EspF require the presence of a 14 kDa type III secreted chaperone termed CesF (Elliott *et al.*, 2002).

1.5.3.6 EPEC EspG.

The analysis of the LEE revealed that the gene encoding EspG may have homology with the gene encoding VirA in *Shigella flexneri* (Elliott *et al.*, 2001b). VirA plays a role in the invasion of *S. flexneri* but its biological activity has not been fully characterised. A cloned *espG* gene can rescue the invasion in a *S. flexneri virA* mutant suggesting that in *Shigella* both proteins have the same biological activity. However EspG has no effect on A/E lesion formation or the TTSS, but is secreted in supernatants and translocated into HEp-2 cells. However EspG is involved in the colonisation of the rabbit intestine by REPEC but it did not affect the onset of diarrhoea suggesting a peripheral role for EspG during pathogenesis. In addition to EspG encoded by the LEE, EPEC contain a second EspG homologue Orf3 on the EspC pathogenicity island (see 1.5.3.8). Deletion of the *orf3* gene and deletion of *espG* and *orf3* did not affect the adhesion of EPEC to HEp-2 cells. Thus the role of both proteins remains to be characterised.

1.5.3.7 EPEC EspH.

EspH is translocated by the TTSS into host cells where it acts as an effector protein. Once EspH is in the host cell it is located beneath adhering bacteria. COS cells overexpressing EspH produce elongated pedestals when infected by EPEC. Inactivation of *espH* in EPEC results in filopodia formation and overexpression of EspH results in the repression of filopodia and the elongation of pedestals. EspH transiently expressed by COS cells was localised to the membrane and disrupted the actin cytoskeletal structure. Thus EspH may modulate the formation of pedestals by interacting with actin - modulating proteins in the cell such as the actin related 2/3 complex (Tu *et al.*, 2003). Interestingly Tir has also been shown to interact with actin binding proteins suggesting that the actin containing structures within the cell may be modulated in several ways by EPEC. Tir appears to repress filopodium formation (Kenny *et al.*, 2002), but the filopodia repressing activity of EspH is independent of Tir (Tu *et al.*, 2003).

1.5.3.8 EPEC EspC.

EspC is a 110 kDa protein which does not require the TTSS for secretion (Stein *et al.*, 1996). The *espC* gene is present on a 15.2 kb pathogenicity island that is made up of sequences with varying G+C content that have been acquired through horizontal gene transfer events (Mellies *et al.*, 2001). There exists significant homology between the predicted EspC protein sequence and a family of immunoglobulin A protease-like autotransporter proteins which are widespread among pathogenic bacteria avian pathogenic *E. coli*, *Haemophilus influenzae* and *Shigella flexneri*, the Pic and Pet proteins of EAEC and the EspP of EHEC. EspC is not necessary for A/E lesion formation *in vitro* and is located within a second pathogenicity island in EPEC. Using rat jejunal tissue and an Ussing chamber it was show that EspC affects the short circuit current present across the tissue and therefore may act as an EPEC entrotoxin. This activity was abolished by antibodies to the plasmid encoded toxin (pet) of EAEC (Henderson *et al.*, 1999b) suggesting that EspC is a homologue of this enterotoxin. Their DNA sequences share 52% identity. However, EspC has not been assessed for its activity during IVOC.

1.5.3.9 Non Esp designated EPEC effectors.

EPEC secrete host cell effectors that have not been designated as Esps and have been assigned their own names.

1.5.3.9.1 EPEC mitochondrial associated protein (MAP).

The LEE *orf19* encodes a type III secretion effector protein MAP which is delivered into the host cell in an EspB dependent manner. This protein interacts with mitochondria and was seen as punctate accumulations in the cytoplasm and absent from the plasma membrane. MAP may cause loss of membrane potential of the mitochondria, resulting in disruption of ATP synthesis in the mitochondria. Disruption of the plasma membrane may prevent release of proapoptotic factors from the mitochondria implying a role for MAP as an anti - apoptotic factor (Kenny and Jepson, 2000). In addition to interacting with the

host cell mitochondria MAP has been implicated in host cell cytoskeletal rearrangements. Binding of EPEC to HeLa cells can induce early Tir - independent host cell events which include the formation of fillopodia at the site of bacterial adherence. Such fillopodia formation is dependent on the expression of MAP by EPEC. This function is independent of MAP mitochondrial targeting activity. MAP cell signalling inhibits pedestal formation suggesting that coordinated events must occur related to MAP, Tir and EspH during the adhesion of EPEC to host cells and pedestal formation. MAP shows that effector proteins may have dual function when inserted into the host cell. In addition, MAP is bound by the CesT chaperone which binds to Tir (Creasey *et al.*, 2003).

1.5.3.9.2 EPEC Efa1 (EHEC factor for adherence).

The EHEC factor for adherence was, as the name suggests, first described in EHEC. In EHEC strain O111:H- a 9669 bp open reading frame was shown to be required for adherence to CHO cells *in vitro* (Nicholls, Grant, and Robins-Browne, 2000). This ORF predicted to encode a 365 kDa protein was therefore designated *efa1*. Efa1 is present in all A/E lesion forming pathogens although it has not been shown to be essential for adhesion to cells in culture in all A/E lesion forming strains. However, in the absence of BFP production EPEC strain E2348/69 requires Efa1 for adhesion to CHO cells (Badea *et al.*, 2003). Efa1 is not required for actin nucleation at the site of bacterial adherence and does not affect the expression of EspA filaments. Rabbits previously infected with REPEC have antibodies to Efa1 and antibodies against the middle region (2823-3223) of Efa1 inhibit binding of EPEC to CHO cells. Thus this region of Efa1 may have cell binding activity and is predicted to have a coiled coil domain that may contribute to protein – protein interactions or binding to a host cell protein.

Efa1 has 99% nucleotide sequence similarity with the lymphostatin (*lifA*) of EPEC which is involved in inhibiting lymphoproliferation (Klapproth *et al.*, 2000) and thus this characteristic may also be shared by Efa1.

1.5.3.10 The EPEC bundle forming pilus (BFP).

The bundle forming pilus (BFP) of EPEC is encoded on plasmids which range in size from 50 – 70 MDa. During adhesion to eukaryotic cells in culture, EPEC form three dimensional microcolonies and this phenotype is referred to as localised adherence. The prototype EPEC strain E2348/69 is the benchmark for localised adherence and its plasmid which is responsible for expressing BFP can confer the localised adhesion phenotype on EPEC strains (Baldini *et al.*, 1983). Therefore the plasmids which contain the genes for BFP production were named EPEC adherence factor (EAF) plasmids. In addition to expressing BFP these plasmids are involved in the regulation of intimin and other EPEC virulence factors (Gomez-Duarte and Kaper, 1995;Elliott *et al.*, 2000).

The *bfp* operon encodes 14 genes most of which are involved in pilus biogenesis (Stone *et al.*, 1996). The *bfpA* gene encodes the major structural subunit of BFP, bundlin, which shares limited homology with the type IV pilin proteins of other bacteria. Using structural data from the X ray crystallographical analysis of other type IV proteins, the carboxy terminal region of bundlin is predicted to be surface exposed with a hydrophobic amino terminal tail buried within the core of the pilus structure in order to stabilise the overall structure (Zhang, Lory, and Donnenberg, 1994).

BFP may also play a role in the formation of bacterial aggregates. EPEC can auto aggregate but this activity is lost in a *bfpA* mutant (Vuopio-Varkila and Schoolnik, 1991). BFP may also play a role in dispersal during the infection of epithelial cells by EPEC. The *bfpf* gene is required for morphological changes in BFP from thin to thick pili as the infection proceeds. This change in morphology results in loose aggregation of the bacteria rather than tight microcolonies which may be a form of dispersal (Knutton *et al.*, 1999).

In human volunteer studies both *bfpF* and *bfpA* were shown to be involved in the development of diarrhoea in some but not all patients (70% and 87.5% of the respective test cohorts) and not all EPEC clinical isolates express BFP due to a deletion in the *bfp* operon (Bieber *et al.*, 1998;Bortolini *et al.*, 1999). However, it is clear that BFP is an

important virulence factor in EPEC pathogenesis but its mode of action remains to be further defined.

The type IV pili of other bacterial pathogens have been shown to be involved in adhesion to host cells and colonisation in humans. *Pseudomonas aeruginosa* binds to host cells via the tip of its type IV pilus and the type IV pilus of *V. cholerae* is necessary for colonisation of humans but has not been shown to bind to host cells (Herrington *et al.*, 1988;Lee *et al.*, 1994). The involvement of BFP in localised adherence has prompted its investigation as an adhesin. Due to its role in forming bacterial aggregates the use of antisera to prevent bacterial adherence during experiments may not be directly related to BFP acting as a host cell adhesin. Two reports by the same coworkers, Tobe and Sasakawa (Tobe and Sasakawa, 2001;Tobe and Sasakawa, 2002), have sought to establish BFP as an adhesin and define its role in tissue tropism *in vitro*. In their initial report they showed that EPEC expressing BFP did not adhere to established microcolonies but adhered to the host cell suggesting that BFP acts as a cellular adhesion. This does not account for the presence of other factors which may mediate adhesion of bacteria to microcolonies and target them to other regions of the host cell. The second report showed that EPEC did not adhere in similar numbers to mouse and human derived cell lines. This characteristic was dependent on the presence of the EAF plasmid but not regulatory genes present on the EAF plasmid. Further work showed that the bundlin precursor protein prebundlin bound preferentially to human derived cell lines. Therefore the second study suggests that in addition to adhesion BFP is involved in binding to species specific host cells. However no specific receptor has been found to bind BFP which remains an obstacle in it being defined as an adhesin. In addition, the EAF plasmid was shown to not be essential for adhesion to intestinal explants during IVOC but that it may have a role in bacterial aggregate formation (Hicks *et al.*, 1998). This suggest that BFP is not required for initial adhesion and A/E lesion formation on intestinal explants *in vitro*. Although BFP is appears to be required for diarrhoea production during EPEC infection of adult volunteers, not all the of volunteers developed diarrhoea when challenged with an EAF negative strain of E2348/69. These results suggest that further experiments may be needed to characterise the role of BFP in pathogenesis and adhesion to explants during IVOC (Levine *et al.*, 1985).

Several putative receptors have been suggested for BFP. Oligosaccharide receptors have been implicated in BFP binding due to their inhibition of localised adherence. These include acetyl – galactosamine, fucosylated tetra – and pentasaccharides (Cravioto *et al.*, 1991b) and lactosyl glycans (Vanmaele, Heerze, and Armstrong, 1999). However such interaction may be as result of the saccharides affecting the regulation of virulence factors rather than binding to BFP. Another receptor was suggested to be phosphatidylethanolamine which is present in the plasma membrane of eukaryotic cells. The binding of phosphatidylethanolamine has been implicated in cell death and apoptosis (Abul-Milh *et al.*, 2001). However in all cases the saturability and strength of the interaction have not been noted suggesting that a specific receptor for BFP remains to be defined.

1.5.3.11 Other EPEC pili/fimbriae.

The prototype EPEC strain E2348/69 produces BFP and type 1 fimbriae. The type 1 fimbriae produced by E2348/69 are not involved in adhesion to Hep-2 cells or Caco-2 cells (Elliott and Kaper, 1997). However, the genes involved in type 1 fimbriae expression may be involved in aggregate formation during pathogenesis or in signalling to other fimbrial operons (Schembri, Christiansen, and Klemm, 2001;Xia *et al.*, 2000). However, deletion of the LEE encoded regulator (*ler* see section 1.5.4) resulted in EPEC strain E2348/69 expressing three novel fimbriae that were described as long fine fimbriae, rigid bent fimbriae and short fine fimbriae. These fimbriae were not BFP and suggest that EPEC strains may express as yet uncharacterised fimbriae under appropriate regulatory conditions (Elliott *et al.*, 2000).

An EPEC O111:NM expresses fimbriae in addition to BFP. These fimbriae have a molecular weight of 16.5, 15.5 and 14.7 kDa. Their amino acid sequences were homologous to the P fimbriae of uropathogenic *E. coli* and the F1845 fimbriae of diffusely adhering *E. coli*. Antibodies raised against all three of the fimbriae reduced adherence to

HEp-2 cells and adherence to HEp-2 cells was abolished when antibodies to BFP were included in the assay (Giron, Ho, and Schoolnik, 1993).

Some strains of the EPEC O119 serogroup have been shown to produce thick pili, 12.5 nm in diameter, which were encoded on a 140 kb non transferable plasmid. Their relation to adherence has not been characterised.

1.5.3.12 EPEC flagella.

Certain serogoups are associated with EPEC strains and the flagellar H antigen has also been assessed for its role during adhesion to host cells (Giron *et al.*, 2002). The flagellin structural protein of EPEC is encoded by the *fliC* gene and mutation of this gene resulted in a loss of adherence to HeLa cells. Introduction of this gene into *E. coli* K-12 resulted in adhesion of the K-12 strain to HeLa cells. The flagella were shown to extend outwards from the bacteria which suggest a possible role in adhesion. In addition, motility was shown to be influences by a soluble eukaryotic product. The results were complicated by the fact that the *fliC* mutant was not restored by complementation with a *fliC* expressing plasmid and that mutation of *fliC* also resulted in a reduction in the expression of BFP (Giron *et al.*, 2002). Therefore the role of flagella in adhesion remains to be clarified.

It should be noted that *Salmonella* flagellin has been shown to activate the chemokine CCL20 which recruits immature dendritic cells (Sierro *et al.*, 2001). Dendritic cells express tight junction proteins allowing them to sample the luminal environment without disrupting the epithelial tight junctions (Rescigno *et al.*, 2001). Thus expression of flagella may allow bacteria to bind and interact with dendritic cells allowing bacterial uptake in certain bacterial species such as *Salmonella*. The flagellin of EPEC has been shown to stimulate the secretion of the proinflammatory cytokine IL-8 in T84 cells and thereby flagella may play a role in host cell inflammation (Zhou *et al.*, 2003).

1.5.3.13 EPEC toxins.

1.5.3.13.1 EPEC enteroaggregative *E. coli* heat stable enterotoxin 1 (EAST1).

EAST1 has been found in EPEC strains and the EPEC strain E2348/69 has a copy of the gene encoding EAST1 on its chromosome and its EAF plasmid. No evidence exists as yet for a role for EAST1 in EPEC infections (Savarino *et al.*, 1996a).

1.5.3.13.2 EPEC haemolysins.

E. coli can produce the 107 kDa exotoxin alpha haemolysin which increases the lethality of *E. coli* peritonitis (May *et al.*, 2000). EPEC strains can express alpha haemolysin as well as an enterohaemolysin which is genetically and antigenically distinct to alpha haemolysin (Pelayo *et al.*, 1999;Beutin *et al.*, 1988). No role has been assigned for haemolysins during EPEC pathogenesis but they may act to aggravate the disruption of the intestinal barrier by lysing erythrocytes.

1.5.3.13.3 EPEC lymphostatin.

Lymphostatin is predicted to be a 366 kDa toxin encoded by the *lifA* gene whose sequence shares 99.9% homology with *efa1*. Lymphostatin inhibits lymphoproliferation and the production of the lymphokines Il-2, Il-4 and gamma interferon. This suggests that a lymphostatin protein may function by binding to hosts cells or to lymphocytes, as described for EFa1, and inhibit lymphocyte proliferation during infection by EPEC

(Klapproth *et al.*, 2000).

1.5.4 Regulation of the LEE and quorum sensing in EPEC.

The EAF plasmid of EPEC, as well as encoding the *bfp* operon, contains four genes which make up the plasmid encoded regulator, *per*, and are defined as *perA*, *perB*, *perC* and

perD. The predicted molecular weights of the polypeptides encoded by these four genes are 24, 14.8, 10.5 and 9.4 kDa respectively. PerA shares homology with other bacterial promoters such as VirF of *S. flexneri* which is a positive regulator of a gene involved in the regulation of *S. flexneri* invasion (Sakai, Sasakawa, and Yoshikawa, 1988). PerA activates the expression of intimin and is enhanced by the expression of the proteins encoded by *perB* and *perC*. The sequence of *perD* resembles a transposable element which may have been involved in the insertion of *per*. Thus the *per* is involved in the regulation of LEE encoded genes and further work was carried out to determine how the LEE encoded genes are regulated.

The LEE is organised into five polycistronic operons. The *LEE1*, *LEE2*, *LEE3* operons encodes the components of the type III secretion system that are located in the bacterial cell and the *LEE4* operon encodes the secreted Esp proteins. The fifth operon is the *tir* operon which contains the *tir* and *eae* genes along with the Tir chaperone *cesT* (Elliott *et al.*, 1999;Mellies *et al.*, 1999). The product of the first open reading frame of *LEE1* was shown to positively regulate the expression of *LEE2*, *LEE3*, *LEE4* and the *tir* operon and was termed the LEE encoded regulator (*ler*). In EPEC the *ler* is activated by the *per* and an integration host factor which binds upstream from the *ler* (Friedberg *et al.*, 1999). Both integration host factor binding and *ler* activation are required for A/E lesion formation *in vitro*.

The 15kDa *ler* protein product is predicted to be similar to the H-NS family of DNA binding proteins. H-NS is a histone like non-structural protein which binds to strongly curved or adenine – thymine rich DNA sequences which leads to changes in the supercoiling and packing of the DNA, and alters gene expression (Hueck, 1998). It is a pleiotropic transcription factor affecting the expression of approximately 5% of the genes in *E. coli* (Atlung and Ingmer, 1997). In *Shigella* H-NS counters the upregulation activity of VirF, which is homologous to Per in EPEC. This suggest a possible mechanism of interplay between *per* and *ler*. H-NS in EPEC may act as a repressor protein of the *LEE2* and *LEE3* operons which is counteracted by the activation of *ler* (Bustamante *et al.*, 2001).

Therefore it appears that *per* activates *ler* which in turn overcomes the downregulation of the first two LEE operons by H-NS.

Due to the expression of LEE encoded promoters during the transition from the late exponential to the stationary phase it was hypothesised that the LEE operons may be regulated by quorum sensing (Sperandio *et al.*, 1999). Gene expression is regulated in response to an increase in population density in many different bacterial species. Collectively this activity is termed quorum sensing (Surette, Miller, and Bassler, 1999). This cell to cell signalling is mediated by the production of compounds known as autoinducers that allow the bacteria to sense its own population as well as the population of other bacteria within a given environment. The regulation of bioluminescence by *Vibrio harveyi* by quorum sensing was the first description of such a biological activity, which has now been shown to be present in both gram negative and gram positive bacteria (de Kievit and Iglewski, 2000). Quorum sensing in EPEC activates the expression of *LEE1* and *LEE2* whereas the expression of *LEE3*, *LEE4* and *tir* operons are regulated by *ler* and indirectly by quorum sensing, through its activation of *per*.

EPEC colonises the small intestine where the number of *E. coli* and other coliforms is much lower than in the large intestine (10^7/g in the small intestine and 10^{10}/g in the large intestine) (Sperandio *et al.*, 1999). This suggests that the presence of the *per* operon in the EAF plasmid containing *E. coli* strains such as EPEC may compensate for the lower levels of autoinducer predicted to be present in the small intestine. The autoinducer for EPEC has yet to be defined. In addition, GadX (glutamate decarboxylase) binds to *per* and is a transcriptional regulator of genes in the *per* operon. Regulation of the *per* by GadX is altered by external pH and culture media conditions and this suggests that GadX may be activated by transit of EPEC in the gut, from the gastric region to the small bowel, and allow EPEC to regulate genes involved in acid resistance and virulence (Shin *et al.*, 2001).

In addition to the *ler* and *per* another EPEC factor has been shown to regulate the expression of the LEE. The Fis protein (factor for inversion stimulation) is a member of the histone like nucleoid associated proteins due to its ability to alter DNA topology (Gille

et al., 1991). This protein activates the transcription of the *ler*, and Fis has been shown to have a similar activity in *Shigella* where it is involved in the H-NS regulation of VirF (Falconi *et al.*, 2001;Goldberg *et al.*, 2001).

Thus the *per* and the *ler* are involved in the expression of LEE encoded virulence factors. However it has been shown that deletion of the *ler* in EPEC and in EHEC leads to the expression of novel fimbriae suggesting that the *ler* may have a role in regulating LEE and non LEE genes (Elliott *et al.*, 2000). The H-NS regulatory factor is also involved in fimbriae regulation (O'gara and Dorman, 2000). In addition, quorum sensing acting via *ler* or another factor may regulate other as yet undescribed factors involved in EPEC colonisation and pathogenesis *in vivo*.

1.5.5 Defining EPEC.

As can be seen from the introduction to the virulence factors of EPEC, bacteria belonging to this category possess specific virulence traits. These virulence factors determine the phenotype of EPEC during certain assays and allow gene probes to be developed to detect the presence of EPEC during outbreaks of diarrhoea.

At the second international symposium on EPEC in 1995 a consensus was reached on the defining characteristics of EPEC. EPEC are characterised (i) epidemiologically by association with sporadic cases and outbreaks of diarrhoeal illness in young infants; (ii) clinically by a syndrome of watery diarrhoea with mucus, fever and anorexia; and (iii) pathologically by the production of attaching and effacing lesions on intestinal epithelial cells in the small and large intestine. The A/E lesion can be recognised by ultrastructural examination of cultured intestinal biopsies or epithelial cells exposed to EPEC *in vitro*, by the FAS test using cultured epithelial cells. Alternatively bacterial genes necessary for A/E lesion formation can be recognised in an *E. coli* strain using an intimin and/or EAF gene probe (Kaper, 1996). EPEC do not produce Shiga toxin (Stx) and this distinguishes them from A/E lesion forming EHEC strains. The presence of an EAF plasmid has complicated the definition of EPEC since the EAF plasmid is variably present in EPEC strains

associated with outbreaks of diarrhoea (Scotland *et al.*, 1996;Echeverria *et al.*, 1991). EPEC strains which possess the EAF plasmid are defined as being typical EPEC strains whereas those strains lacking the EAF plasmid but possessing the intimin gene are referred to as atypical EPEC strains. In addition, this implies that typical EPEC strains produce a localised adherence pattern of adhesion on HEp-2 cells due to the expression of BFP.

Furthermore EPEC can be divided into groups based on sequence variation in housekeeping genes, expression of different intimin types and the insertion site of the LEE into the bacterial chromosome (Frankel *et al.*, 1998). The EPEC groups 1 and 2 contain strains with a variety of O antigens whilst the H flagellar antigen appears to be conserved within a group. EPEC with flagellar antigen H6 are assigned to group 1 and EPEC expressing the H2 and H- flagellar antigen are assigned to group 2. In addition to the variation in intimin expressed by EPEC strains, the *bfpA* gene can show variation and two BfpA types have been assigned the Greek letters α and β. However both types are present in both EPEC groups which suggests that the EAF plasmid has spread through horizontal gene transfer recently (Blank *et al.*, 2000).

The definition of EPEC, agreed at the second EPEC international symposium, ignores any invasive ability that EPEC may have. However EPEC have been shown to invade cultured epithelial cells *in vitro* (Donnenberg, Donohue-Rolfe, and Keusch, 1990;Fletcher *et al.*, 1992;Francis *et al.*, 1991;Staley, Jones, and Corley, 1969) and O55:H7 has been shown to invade the ileum of newborn pigs (Staley, Jones, and Corley, 1969). Unlike EIEC and *Shigella,* EPEC do not multiply intracellularly or escape from a phagocytic vacuole. However an EPEC isolate has been shown to contain genes involved in invasion on its plasmid (Fletcher *et al.*, 1992). In addition, invasion assays have been used during experiments to determine whether certain genes are implicated in A/E lesion formation suggesting that, on certain cell lines *in vitro,* genes encoding factors involved in EPEC A/E lesion formation may also be involved in invasion (Donnenberg *et al.*, 1990;Goldberg *et al.*, 2001). However, EPEC do not cause dysentery or typhoid and therefore the clinical significance of *in vitro* EPEC invasion has not yet been determined.

1.5.6 Detection of EPEC.

In order to identify EPEC strains they are often characterised by the adhesion phenotype to cultured epithelial cells (Cravioto *et al.*, 1991a). The presence of localised adherence is suggestive of the presence of an EAF plasmid in a bacterial isolate. A/E lesion formation during infection of cell lines can be determined by actin staining (Knutton *et al.*, 1989). DNA probes for the *eae* gene encoding intimin as well as monoclonal antibodies to intimin are used to detect the presence of the LEE (Scotland *et al.*, 1996;Vandekerchove *et al.*, 2002). EAF DNA probes exist but strains which are localised adherence positive can also be EAF probe negative and would not detect atypical EPEC strains (Scotland, Smith, and Rowe, 1991).

Detection of EPEC can be achieved through meeting the criteria established in defining EPEC. This may involve a combination of serotyping, adhesion assays and DNA probes, and will depend on the time and resources available.

1.5.7 EPEC diarrhoea.

The pathophysiology of EPEC diarrhoea remains to be fully elucidated despite the initial characterisation of many LEE encoded virulence factors. The disruption of the epithelial barrier may occur via the formation of A/E lesions leading to a loss of microvillus absorptive surface area in the intestinal tract. This may be exacerbated by the expression of EspF which increase the permeability of tight junctions. EPEC secreted proteins such as Tir, EspH and Map are involved in signalling to the host cell cytoskeleton in order to form pedestals beneath the bacteria and therefore anchor the bacteria to the host cell. Other proteins such as EspC may act as entertoxins indicated by the ability to disrupt membrane potential in cell lines. This modulation of electrolyte transport *in vivo* is proceeds with A/E lesion formation and chlorine ions have been implicated in this process (Collington, Booth, and Knutton, 1998).

Inflammation has been noted in animal models of infection as well as in human biopsies from patients with diarrhoea (Tzipori, Gibson, and Montanaro, 1989;Ulshen and Rollo, 1980). Cotton – top tamarind monkeys develop acute colitis, an inflammation of the colon, when infected with EPEC (Mansfield *et al.*, 2001). EPEC expresses lymphostatin which suggest that regulation of lymphocytes is involved during EPEC pathogenesis. A/E lesion formation in cell culture can result in the migration of polymophonuclear cells which can stimulate chloride secretion. Lactoferrin positive (Kane *et al.*, 2003;Naidu *et al.*, 1991) stools imply that inflammation occurs during EPEC infection and inflammation and reduced intestinal surface area may be the mechanisms involved in EPEC diarrhoea.

In vivo cases of EPEC infection show small intestinal crypt hyperplastic villous atrophy (Hill, Phillips, and Walker-Smith, 1991b;Rothbaum *et al.*, 1982;Ulshen and Rollo, 1980) and cell turnover is increased to levels approaching coeliac disease (Savidge *et al.*, 1996), demonstrating epithelial cell loss and reduced surface area. Intraepithelial neutrophils are apparent in *in vivo* EPEC infections (Lewis, Walker-Smith, and Phillips, 1987), along with chronic inflammation (Rothbaum *et al.*, 1982;Rothbaum *et al.*, 1983;Ulshen and Rollo, 1980). All these phenomena would contribute to diarrhoea.

1.6 Enterohaemorrhagic *E. coli* (EHEC).

EHEC were first recognised as a new class of pathogenic *E. coli* due to two reports published in 1983 concerning outbreaks of a distinctive bloody diarrhoea syndrome. Riley et al (Riley *et al.*, 1983) isolated a rare *E. coli* serotype from the stools of patients with bloody diarrhoea and a haemorrhagic colitis (HC). Karmali et al (Karmali *et al.*, 1983b) showed that patients with haemolytic uraemic syndrome (HUS) had cytotoxin producing bacteria in their stool. They used an assay based on the sensitivity of Vero cells to cytotoxins (Konowalchuk, Speirs, and Stavric, 1977) and showed that *E. coli* culture filtrates were cytopathic for Vero cells. Subsequent to these reports the cytotoxic activity of the *E. coli* isolates was shown to be neutralised by antibodies prepared against *Shigella dysenteriae* 1 Shiga toxin (Stx) (O'Brien *et al.*, 1982) and *E. coli* O157:H7 from patients with haemorrhagic colitis produced a cytotoxin active on Vero cells (Johnson, Lior, and Bezanson, 1983). Thus it appeared that O157:H7 produced a Vero cytotoxin or Shiga - like toxin which was a common virulence factor between HC and HUS and was responsible for damage to both the intestinal tract and renal tissue (Karmali *et al.*, 1983a).

Since these outbreaks further outbreaks have been associated with *E. coli* O157:H7. A term to describe the category of *E. coli* that produce Vero cytotoxin or Shiga toxin has led to two terms being used, VTEC and STEC for verocytotoxin producing *E. coli* and Shiga toxin producing *E. coli* respectively. Both terms are equivalent and refer to *E. coli* strains which produce one or more toxins related to the Stx family. In addition, the term EHEC has been used to describe *E. coli* isolates which are implicated in cases of HC and HUS (Nataro and Kaper, 1998). EHEC generally denote *E. coli* strains that produce Stx and A/E lesions, and harbour a 60 MDa plasmid (Levine, 1987). Therefore EHEC strains are a subset of STEC strains. Strains which do not contain the LEE and/or the 60 MDa plasmid are atypical EHEC (Nataro and Kaper, 1998). In addition, the term STEC will be used for strains that express Shiga toxin but have not been implicated in an outbreak or sporadic case of HC or HUS.

1.6.1 Epidemiology of EHEC.

Surveys have been carried out to determine the presence of O157:H7 and Stx expressing strains present in *E. coli* isolates from recorded outbreaks. During the 1970s and early 1980s isolates of EHEC O157:H7 were rare in the U.S.A, the U.K. and Canada, and when bacterial strain stocks were reviewed it appears that disease due to *E. coli* O157:H7 was not under reported prior to 1982 and that this is a true emerging pathogen (Griffin and Tauxe, 1991;Riley *et al.*, 1983;Day *et al.*, 1983;Johnson, Lior, and Bezanson, 1983). In addition, Stx production in *E. coli* is not solely associated with the O157:H7 serotype and a large number of different *E. coli* serotypes have the ability to produce Stx (Karmali, 1989). This indicates that Stx positive and Stx negative strains may exist within a given serotype and that detection of Stx production is essential during outbreaks involving bloody diarrhoea.

Outbreaks of diarrhoeal illness due to O157:H7 occur as both sporadic cases and large outbreaks in the U.S.A. and Canada. In these regions, the number of sporadic cases peaks in the summer months and in the year 1985 to 1986 the number of laboratory confirmed O157:H7 infections was 8 per 100 000 persons per year (MacDonald *et al.*, 1988). In the U.S.A EHEC O157:H7 is responsible for an estimated 73 000 illnesses annually and is the most common pathogen associated with post diarrhoeal HUS (Mead *et al.*, 1999). The annual incidence of HUS in the U.S.A is approximately 3 cases per 100 000, in a population of children under five years of age. Approximately 5% to 10% of people with diarrhoea caused by O157:H7 develop HUS and the mortality rate among children with HUS is 3% to 5% (Rowe *et al.*, 1991;Siegler *et al.*, 1994). In two studies reporting cases of haemorrhagic colitis in the summer months, in the U.S.A and Canada, O157:H7 was isolated more frequently than *Shigella* and *Salmonella* (Remis *et al.*, 1984;Bryant, Athar, and Pai, 1989). In addition, to North America, EHEC is an important pathogen in Europe, Asia and Australia (Izumiya *et al.*, 1997;Willshaw *et al.*, 1994). In a study of Australian cases of HUS the O157:H7 serotype was rarely isolated in contrast to North America, Europe and Japan (Elliott *et al.*, 2001a). In the Australian review the authors noted that the epidemiology of HUS in the southern hemisphere is poorly described and that laboratory

testing for serotypes other than O157:H7 does not occur. Non – O157:H7 STEC are usually only recognised when specialist testing is carried out. Most *E. coli* O157:H7 do not ferment sorbitol within 24 hours allowing for easy screening of stool specimens whereas most non – O157:H7 strains are sorbitol fermenters. Screening for other virulence factors such as Stx may be an important way to identify non – O157:H7 STEC (McCarthy *et al.*, 2001). Therefore there may be some correlation between geographic area and the occurrence of STEC serotypes. In addition, EHEC is less frequently isolated than other diarrhoeagenic *E. coli* in developing countries (Albert *et al.*, 1995). A study of STEC prevalence in India indicated that STEC were not thought o be an important cause of diarrhoea in India (Khan *et al.*, 2002).

In a study of indigenous foodborne disease in England and Wales between 1992 and 2000, EHEC O157:H7 was seen as an important cause of disease burden, along with *Campylobacter*, *Salmonella*, *Clostridium perfringens* and *Listeria monocytogenes* (Adak, Long, and O'Brien, 2002). In 1995 there were 2 365 909 cases of foodborne disease with 21 138 hospital admissions and 718 deaths in England and Wales. By the year 2000 the incidences of foodborne disease had decreased by one million and the deaths by 238, but the hospital admissions total was similar. In 1995 in England and Wales there were 3 incidences of intestinal disease due to O157:H7 per 100 000 population and 3 incidences of disease related to non - O157:H7 STEC per 1000 000 population. Adak et al (Adak, Long, and O'Brien, 2002) state that these results taken into account with population rate indicates that the ratio of food related illness in the U.S.A. to indigenous food related illness in England and Wales is 11:1. In a study of STEC O157 isolates from human infections in England and Wales from 1990 to 1998 the annual incidence of infection due to STEC O157 was reported as being 1.6 cases per 100 000 population (Chalmers *et al.*, 1999). In England and Wales STEC O157:H7 isolates rose from 1 in 1982 to 1087 in 1997 (1998), and the incidence over the years 1997 to 2001 ranges from 768 to 1087, which is higher than the number of incidences recorded in 1995 and in the years 1990 to 1995 (2002). In the period 1992 to 1996 seven countries across Europe reported 67 outbreaks of caused by EHEC. Fifty six of these were reported by the UK (39 from England and Wales, 17 from Scotland) and eleven by the rest of Europe. Studying the incidence of EHEC related

illness in Europe is complicated by the fact that not all countries have appropriate surveillance mechanisms. EHEC infection is statutorily notifiable in three countries: Austria, Finland and Sweden. Five countries, in 1997, had established sentinel systems to monitor EHEC outbreaks: Belgium, Finland, Italy, Netherlands and the UK. This would suggest that in the UK surveillance of EHEC related illness is more rigorous than in European countries that do not have a public health laboratory system in place to monitor EHEC infections, but may not exclude real differences in incidence rate (1997a). In Scotland the incidence of EHEC related illness may be higher than in the rest of the UK and a report by the Pennington group has sought to address factors related to an outbreak of EHEC in central Scotland in 1996. This outbreak affected 496 people, 272 of whom had microbiologically confirmed O157:H7 infection and 18 died. The source of infection was home cooked meat from a local butcher. The epidemic strain was isolated from cooked steak in gravy and an unopened vacuum packed joint of beef prepared by the butcher (1997b).

STEC can be transmitted via food, as shown in the first reported cases of STEC (Riley *et al.*, 1983), and a reservoir for EHEC O157:H7 is the intestinal tract of domestic animals. STEC can be found in fresh samples of beef, pork , poultry and lamb (Martin *et al.*, 1990;Boyce, Swerdlow, and Griffin, 1995;Griffin and Tauxe, 1991;Riley *et al.*, 1983). The first outbreak of *E. coli* associated HC was due to contaminated meat from a single processing plant distributing beefburgers to multiple outlets. In several cases the beefburgers had been sold to customers undercooked (Riley *et al.*, 1983). STEC are frequently detected in the bovine faeces and in Germany it has been estimated that 50% of cattle shed STEC (Richter *et al.*, 1997). Calves may produce a watery diarrhoea but adult cattle do not exhibit disease. The presence of antibodies to Stx in the colostra of cows in Germany is between 84% and 90% (Pirro *et al.*, 1995). STEC are less prevalent in sheep than in cattle (Armstrong, Hollingsworth, and Morris, Jr., 1996) and pigs may also act as a reservoir of STEC (Booher, Cornick, and Moon, 2002). STEC may contaminate undercooked beef, unpasteurised milk, fruit juices, cheese, well water and wading pools (Swerdlow *et al.*, 1992). In an outbreak in Africa an infection by O157:H7 was due to contamination of water supplies after swelling of surface water due to heavy rains

following drought (Effler *et al.*, 2001). Contact with a farming environment is a major risk factor for O157:H7 infection in humans (O'Brien, Adak, and Gilham, 2001). However, in a study of an outbreak of O157:H7 in children in a nursery, in North Wales, U.K., person to person contact via the faecal oral route was seen as being an important cause in the spreading of the infection and therefore implies a low infectious does which as been estimated at being approximately 100 bacteria (Al Jader *et al.*, 1999;Griffin and Tauxe, 1991).

It should be noted that in cases of diarrhoeal disease due to STEC in the UK and Éire, the intimin types α, β and γ were associated with outbreaks of whilst intimin types β and ϵ were associated with sporadic cases (Jenkins *et al.*, 2003).

1.6.2 Haemorrhagic colitis and haemolytic uraemic syndrome.

STEC strains can produce HC and HUS and such *E. coli* isolates are termed EHEC. The incubation period of EHEC diarrhoea is on average between 3 and 5 days. The initial complaint is nonbloody diarrhoea and during this period vomiting may occur in about half of the patients. Within 1 or 2 days the diarrhoea becomes bloody but may resolve itself. In approximately 10% of the patients under the age of ten, the illness will progress to HUS (Tarr, 1995). Approximately 3% to 5% of children affected with HUS will die and about 12% to 30% of them will have severe sequelae including renal impairment, hypertension or central nervous system manifestations (Pickering, Obrig, and Stapleton, 1994).

The common features of HUS are endothelial damage, which causes the adhesion and accumulation of platelets, producing thrombi, and the impairment of fibrinolysis. Renal pathology includes the thickening of capillary walls and development of thrombi (Ray and Liu, 2001). Thus HUS can be summarised as a thrombogenic microangiopathy, which is believed to be precipitated by Stx absorption from the gut. Thrombogenic alleles have been studied to determine whether factors involved in coagulation may exacerbate O157:H7 related HUS. No such correlation was shown. Blood group antigens have been shown to be involved in susceptibility to *Helicobacter pylori* infections and in O157:H7

related HUS two studies have shown conflicting results. One study suggest that A and P blood group antigens are protective in O157:H7 related disease but this was not confirmed by a second study which suggest that ABO and P blood group antigens may not be protective and that the lower incident of HUS in patients with B blood group antigen during an O157:H7 outbreak in Sakai, Japan, may have been due to other factors (Shimazu *et al.*, 2000;Jelacic *et al.*, 2002;Blackwell *et al.*, 2002).

HC is characterised by patients presenting with colonic oedema, erosions, ulceration, haemorrhage and bowel wall thickening in the ascending and transverse colon. Colonic biopsy specimens show inflammation in the superficial mucosae with preservation of the intestinal crypts. Neutrophils were observed focally infiltrating the lamina propria and the findings are suggestive of ischaemic colitis and infectious colitis respectively(Griffin, Olmstead, and Petras, 1990). No A/E lesions have been detected in clinical samples and this may be due to the time of biopsy collection where A/E lesions may occur before the cytotoxic effects of Stx are manifested (Nataro and Kaper, 1998). However using IVOC, EHEC O157:H7 has been shown to produce A/E lesion on intestinal explants and antibodies to intimin, EspA and EspB are present in patients infected with O157:H7 (Phillips *et al.*, 2000b;Karpman *et al.*, 2002). Therefore elements of the LEE are involved in O157:H7 pathogenesis and HC may be due to A/E lesion formation followed by production of Stx. Alternatively, the effects of Stx may be evident at sites distant from those where EHEC colonisation is found. Based on studies involving pigs it was postulated that approximately 100 ng of Stx would induce illness in a child of between 20 and 30 kg and the faecal concentration of Stx is greater than 100pg/ml. This suggests that enough Stx is produced in the lumen to transit the intestinal tract and that Stx enduced pathology may not require bacterial adherence in the colon (Acheson *et al.*, 1996).

In a rabbit model of EHEC O157:H7 infection, A/E lesions are associated with host inflammation and neutrophil infiltrates(Elliott *et al.*, 1994). However, intestinal neutrophils are seen in less than 50% of patients with HUS but this may be due to neutrophil activation being an early and transient event and neutrophil activating cytokines

such as Il-8 and tumour necrosis factor α are present in patients with HUS (Fernandez *et al.*, 2002).

1.6.3 EHEC Shiga toxin (Stx).

Shiga toxin (Stx) is a virulence factor of STEC and is implicated in the onset of bloody diarrhoea and HUS. The Stx genes of many STEC strains are encoded by prophages of the λ bacteriophage family (Wagner *et al.*, 2002). EHEC strains produce one or two toxins designated Stx1 and Stx2. Stx1 is highly conserved but Stx2 shows sequence variation and the different variants are designated Stx2c (Schmitt, McKee, and O'Brien, 1991) and Stx2e (Marques *et al.*, 1986) for example. Stx2 is the most frequently associated with HUS (Boerlin *et al.*, 1999). Stx is a 71 kDa protein consisting of a 32 kDa A subunit and five 7.7 kDa B subunits. Thus like LT and CT, Stx has an AB_5 structural conformation (O'Brien and Holmes, 1987). Stx specifically recognises the glycosphingolipid globotriaosyl ceramide, galactose α(1-4) galactose β(1-4) glucosyl ceramide (Gb_3) located on the surface of the plasma membranes (Waddell, Cohen, and Lingwood, 1990). The B subunit mediates binding of the holotoxin to the cell surface receptor Gb_3. Binding of the B subunit to Gb_3 results in internalisation of the toxin and apoptosis (Lingwood, 1996;Jones *et al.*, 2000). The cytotoxic A subunit is an RNA *N*-glycosidase that inhibits protein translation by removing a specific adenine residue from the 28S ribosomal RNA. Stx2e uses Gb_4, globotetraosylceramide, as a receptor and is associated with pig oedema disease rather then human disease but some human isolates have been shown to express Stx2e (Muniesa *et al.*, 2000). Cattle, on the other hand, lack Gb_3 receptors on villous and surface epithelium and on endothelium in the gastrointestinal tract (Pruimboom-Brees *et al.*, 2000), apart from on crypt epithelial cells, where Stx uptake is directed towards lysosomal bodies and degraded (Sekino *et al.*, 2002), which may explain the asymptomatic carriage of O157:H7 in adult cattle. Stx2 is activated by mouse and human intestinal mucus during infection of streptomycin treated mice (Melton-Celsa, Darnell, and O'Brien, 1996) and proteolytic cleavage of the A subunit is essential for cytotoxicity of Stx1 from O157:H7 (Lea, Lord, and Roberts, 1999).

Stx1 binds to neutrophils in peripheral blood cells, suggesting that neutrophils may transfer Stx to the renal endothelial cells. The transfer of Stx to neutrophils is facilitated by the presence on neutrophils of a receptor that has a 100 fold lower affinity for Stx than the Gb_3 present on renal microvascular endotheial cells (te Loo *et al.*, 2000). In addition, neutrophil migration across the epithelial monolayer of T84 cells *in vitro* corresponded to a movement of Stx1 and Stx2 across the polarised epithelial layer. The amount of Stx translocation was proportional to the level of neutrophil migration and neutrophil migration across the intestinal barrier may allow Stx to pass into the tissues underlying the intestine and increase the risk of HUS (Hurley, Thorpe, and Acheson, 2001). Stx binding is related to the presence of Gb_3 receptors on specific cell types in rabbits and intravenous administration of Stx2 to rabbits results in cerebrovascular damage. Renal cells are more sensitive to Stx when they are pre-exposed to tumour necrosis factor α or IL-1β which induce the expression of Gb_3 and Stx binds to the renal glomerular cells of children but not of adults suggesting a developmental regulation of Gb_3 expression (van de Kar *et al.*, 1992;Lingwood, 1994). This suggests an explanation for the age - related incidence of HUS and pathogenesis in adults may require an additional step involving the stimulation of Gb_3 expression. In addition, Stx may need another factor to cause the extensive vascular lesions characterised by HUS. This is suggested by the different response of cell lines to Stx (Hurley, Thorpe, and Acheson, 2001;Obrig *et al.*, 1993), the different renal pathology associated with bacteria producing identical Stx (*Shigella dysenteriae* and *E. coli* O157:H7) and the isolation of *E. coli* strains that cause HUS but do not produce Stx (Schmidt *et al.*, 1999).

A pathogenic mechanism of Stx would involve the adhesion of EHEC to the intestinal tract and elaboration of Stx. The Stx can be translocated from the apical to the basolateral surface of the intestinal epithelium potentially releasing IL-8 (Acheson *et al.*, 1996;Thorpe *et al.*, 1999;Thorpe *et al.*, 2001). Stx and lipopolysaccharide absorbed from the inflamed intestinal tract induce microvascular cell damage and haemorrhagic colitis (Jacewicz *et al.*, 1999). Neutrophils bind Stx and transport it to the glomerular endothelial cells whose Gb_3 receptors may have been upregulated by the release of cytokines from the neutrophils (van

Setten *et al.*, 1996). This results in renal tubular injury and fibrin accumulation resulting in haemolytic uraemia and possible renal failure.

Several strategies have been elaborated for treatment of Stx related disease. However, no treatment for Stx related HUS exists in practise and preventing STEC infection is a priority.

The human serum amyloid P component can bind to Stx2 and neutralise it *in vitro* (Kimura *et al.*, 2001). However this does not prevent HUS in all EHEC infections. Monoclonal antibodies have been developed against Stx2 which neutralise cytotoxicity *in vitro* and prevented fatal cerebral damage in gnotobiotic piglets (Nakao *et al.*, 2002;Mukherjee *et al.*, 2002). Bovine colostrums may be used to improve the diarrhoea of patients with STEC infections (Huppertz *et al.*, 1999). Nitrobenzylthioinosine has been shown to alter the intracellular transport by sequestering the Stx in early endosomes, thus preventing cell damage *in vitro* (Sekino *et al.*, 2002). Multi ligand carbohydrates that bind and neutralise Stx have been developed and these ligands have the ability to protect against fatal doses of O157:H7 in mice when administered after the infection (Nishikawa *et al.*, 2002;Kitov *et al.*, 2000). In addition, recombinant bacteria expressing Stx receptor mimics have been developed which protect mice from fatal doses of STEC (Paton, Morona, and Paton, 2000). The use of antibiotics during infection by STEC is complicated by the fact that λ prophages my act as Stx converting bacteriophages (Smith, Green, and Parsell, 1983). Stimuli such as UV light are know to induce these prophages and can lead to uptake of Stx bacteriophages by surrounding bacteria. Antibiotics have also been shown to stimulate the release of Stx and Stx converting bacteriopahges from O157:H7 strains which may aggravate infections due to STEC (Matsushiro *et al.*, 1999). It should be noted that antibiotics used as growth promoters in animal husbandry can also induce the release of Stx and λ bacteriophages baring Stx from STEC strains. Thus Stx released by bacterial lysis may contribute to the development of HUS in children (Wong *et al.*, 2000).

1.6.4 The EHEC locus of enterocyte effacement.

In gnotobiotic piglets the presence or absence of Stx did not influence diarrhoea caused by EHEC O157:H7, whereas the extent and distribution of A/E lesions was more relevant in predicting intestinal symptoms in this animal model (Tzipori *et al.*, 1987). In an infant rabbit model infection with O157:H7 lacking Stx produced the same changes in ion secretion and absorption, as with infection by O157:H7 producing Stx (Li *et al.*, 1993). In this model of infection A/E lesion formation and infiltration of intestinal tissues with neutrophils was crucial to the development of diarrhoea. Therefore the genes encoded by the LEE are important in the development of diarrhoea by EHEC strains but elaboration of Stx is essential for the development of bloody diarrhoea and HC. The EPEC LEE from strain O127:H6 strain E2348/69 is necessary and sufficient for A/E lesion formation however, in contrast, the EHEC O157:H7 LEE is necessary but not sufficient to induce A/E lesion formation on HEp-2 cells *in vitro* (Elliott, Yu, and Kaper, 1999). The LEE from EHEC O157:H7 strain EDL933 has been cloned and sequenced and possesses all the genes found in the EPEC LEE in the same organisation and like EPEC the LEE of EHEC O157:H7 contains 41 open reading frames (Perna *et al.*, 1998). Overall the EPEC E2348/69 and EHEC EDL933 LEE pathogenicity islands are 94% conserved at the amino acid level and differ by less than 2% in the regions encoding the TTSS, although divergence of up to 34% is seen in genes encoding proteins that are believed to interact with the host. The EHEC LEE is also larger than the EPEC LEE but this is due to the insertion of sequences belonging to a lysogenic bacteriophage which was inserted into the LEE after the LEE was inserted into EHEC. These regions are unlikely to encode any virulence factors.

1.6.4.1 The evolution of EHEC.

Before characterising the difference between known EHEC and EPEC virulence factors, the relationship between EPEC and EHEC should be considered.

EHEC can be divided into clonal groups like EPEC. EHEC O157:H7 strains are part of EHEC group 1 and have inherited their LEE from an atypical EPEC ancestor, not harbouring the EAF plasmid and expressing the intimin γ. This may have been the closely related atypical EPEC serotype O55:H7 with the LEE inserted close to the *selC* gene in the EPEC chromosome(Rodrigues *et al.*, 1996). O55:H7 probably then acquired the Stx transduction by a Stx converting bacteriophage, which led to the presence of Stx positive O55:H7 strains. The next step would have involved the acquisition of the EHEC plasmid (see section 1.6.5.4) and a switch in somatic antigen from O55 to O157. Other steps may have involved the loss of sorbitol fermentation to create the prototype O157:H7 strains and further loss or acquisition of genes during the evolution of O157:H7 would lead to the O157 variants seen in nature, such as the O157:H- strain implicated in a HUS outbreak in Germany (Brunder *et al.*, 2001). In addition O157:H7 has been shown to be to have a 1000 fold higher mutation rate than commensal *E. coli* which may have been imported in the emergence of O157:H7 as a pathogen (Whittam, Reid, and Selander, 1998).

A second group of EHEC, EHEC clonal group 2, also contain the LEE and Stx and have been implicated in outbreaks of HC and HUS. The O26:H11 serogroup, part of EHEC group 2, is the most common EHEC strain isolated from sporadic cases of HUS in Europe (Misselwitz *et al.*, 2003). EHEC strains in group two possess diverse O and H antigens. This group also contains the O111 clone which produces both Stx and enterohaemolysin (Campos *et al.*, 1994). The emergence of EHEC group 2 is based on the acquisition of the LEE close to the *pheU* site which is found in both EPEC 2 and EHEC 2 groups (Rumer *et al.*, 2003). However both EPEC and EHEC group 2 strains may have evolved from multiple LEE insertions (Tarr and Whittam, 2002a). The EPEC ancestor of the EHEC 2 strains typically expressed intimin β and acquired the EAF plasmid to EPEC 2 whilst the EHEC 2 group originated from the strains that did not acquire the EAF plasmid and were therefore atypical EPEC (Reid *et al.*, 2000). Further steps involved the acquisition of the Stx bactriophage and the EHEC plasmid to create strains such as EHEC O26:H11. In addition, O26:H11 have been shown to contain a high pathogenicity island homologous to that contained in *Yersinia*, which is not found in O111 strains (Karch *et al.*, 1999).

In addition to the insertion of the LEE at the *selC* and *pheU* sites in the *E. coli* chromosome, a third insertion site has been identified, in a bovine STEC O103:H2 strain, at the *pheV* site which suggest that O103:H2 either arose from loss of a previous LEE pathogenicity island followed by insertion at the *pheV* site or that this O103:H2 isolate forms part of separate branch of EHEC not evolved from *E.* coli strains which contained the LEE at the *selC* or *pheU* sites (Jores *et al.*, 2001). Comparison of LEE pathogenicity islands from strains of different evolutionary lineage may elucidate the origin and dissemination of the LEE among diarrhoeagenic *E. coli.*

In the next section the difference in virulence factors between EPEC and EHEC strains will be compared. In most cases, but not all, due to the focus of research efforts and data available from sequencing projects, this comparison involves the prototype EPEC O127:H6 strain E2348/69 and EHEC O157:H7 isolates.

1.6.4.2 EHEC intimin.

The amino terminus of intimin is highly conserved across different intimin types and the sequence variation seen in the carboxy terminal 280 amino acids allows the intimin from different EPEC and EHEC isolates to be typed using antisera or PCR primers that recognise specific sequences intimin (Adu-Bobie *et al.*, 1998;Oswald *et al.*, 2000). EPEC strains from EPEC clonal group 1 express intimin α while intimin β is mainly associated with human and animal EPEC and EHEC strains belonging to their respective clone 2 groups. EHEC O157:H7 strains express intimin γ as does the related EPEC strain O55:H7. Intimin ϵ is expressed by human and bovine EHEC strains of serogroups O8, O11, O45, O103, O121 and O165. Intimin ζ is expressed by bovine STEC O84:NM, intimin ι by human STEC O145:H4 and intimin κ by human STEC O118:H5 (Oswald *et al.*, 2000). Therefore EHEC strains like EPEC strains can express different intimin types. The presence of different intimin types may be due to selection pressure and understanding the biological activity of intimin may explain how the intimin types have evolved.

The sequence diversity within the carboxy terminal cell binding region of intimin may have been influenced by natural selection as intimin is immunogenic (Adu-Bobie *et al.*, 1998). The presence of different intimin types may allow EHEC and EPEC to colonise different parts of the intestine. This was first suggested by the ability of intimin to change the site of bacterial adherence in the gnotobiotic piglet intestinal tract when cloned into a different *E.* coli backgrounds. This showed that the intimin γ of EHEC O157:H7 influences tissue tropism in a different manner than the intimin α of EPEC strain E2348/69 (Tzipori *et al.*, 1995). Thus EHEC and EPEC intimin express different intimin types which may allow them to colonise the host in different ways and this may have implications on pathogenic mechanisms. This hypothesis will benefit from studying the tissue tropism of other intimin types and studying the tissue tropism of different EPEC and EHEC strains expressing similar intimin types.

Like EPEC, the intimin γ of EHEC strains has been characterised. The region of intimin γ necessary for Tir binding has been localised to the 181 carboxy terminal amino acids which is similar to the 190 amino acids necessary for EPEC intimin α binding to EPEC Tir (Batchelor *et al.*, 2000;Liu *et al.*, 1999). The X ray crystallographic structure available for intimin α has been used to make predictions about intimin γ – Tir binding. The results showed that using the both intimin types share similarities that allow the structural data available for intimin α to be used for predicting the activity of residues within intimin γ (Liu *et al.*, 1999). The valine at position 906 in the intimin γ Tir binding region is crucial for Tir binding as is the equivalent valine at position 911 in intimin α. Tir recognition by intimin α and intimin γ is similar but some equivalent mutations in these two proteins display different phenotypes. The disruption of a disulphide bond at position 937 in intimin α did not affect the binding of this intimin type to Tir in gel overlays or the yeast two hybrid system whereas the equivalent mutation in intimin γ, disrupting the disulphide bond at position 932, resulted in lack of binding to Tir in similar assays (Hartland *et al.*, 1999;Liu *et al.*, 1999;Reece *et al.*, 2001). However both mutations failed to form A/E lesions during infection of cell lines *in* vitro. The carboxy terminal intimin and full length intimin β from EHEC differ in their binding activity to host cells (Deibel, Dersch, and Ebel, 2001). This suggests an explanation for differences using recombinant intimin

proteins during experiments and also suggests that like invasin from *Yersinia pseudotuberculosis*, intimin binding domain may be subject to control by the amino terminal portion of the protein. Structural evidence predicts that the amino terminal region of intimin α may bind to the extracellular portion of the protein via a flexible link which would allow for changes in orientation (Luo *et al.*, 2000). In addition, to gel overlays and yeast two hybrid systems recombinant intimin proteins have been used to modulate activity during *in vivo* infections of mice and during intimin exchange studies using IVOC (Phillips and Frankel, 2000;Hartland *et al.*, 2000b). This suggests that different intimin molecules do display different biological activities but that the role of different Tir types in this interaction remains to be resolved. The expression of intimin γ in an EPEC background with resultant colonisation of the explants during IVOC and colonisation of the gnotobiotic piglet intestine suggest that Tir type does not determine adhesion *in vivo* (Phillips and Frankel, 2000;Tzipori *et al.*, 1995). The intimin binding domains of EPEC and EHEC Tir are 72% identical and have the ability to recognise each other (DeVinney *et al.*, 1999) but difference in binding between the two proteins does exist. A salt bridge is formed between lysine at 927 in intimin α and the EPEC Tir glutamine at position 312. In intimin γ there is an asparagine at position 927 and a valine at position 312 which may explain the 20 fold lower affinity that intimin α exhibits for EHEC Tir when compared with intimin γ (DeVinney *et al.*, 1999;Luo *et al.*, 2000).

Like intimin α EHEC O26:H11 intimin β and EHEC O157:H7 intimin γ have been shown to adhere to eukaryotic cells lacking the Tir receptor in the plasma membrane. This suggests that intimin may have an additional receptor to Tir (Deibel, Dersch, and Ebel, 2001). No such receptor has been discovered for intimins α and β but a cell surface localised protein has been reported to be a receptor for the intimin γ of EHEC O157:H7 (Sinclair and O'Brien, 2002). This 110 kDa protein, bound to intimin γ, was isolated from HEp-2 cell extracts and identified by its amino acid sequence as being nucleolin. Intimin γ and nucleolin both colocalised on HEp-2 cells and the site of adherence of O157:H7 on Hep-2 cells is coincident with nucleolin expression on the HEp-2 cell surface. Antibodies raised against nucleolin reduced binding of O157:H7 to Hep-2 cells and this indicates that intimin γ – nucleolin interactions must occur early in the infection process before

antibodies are developed to intimin. Nucleolin is expressed in the nucleus of cells and on the surface of cells but expression levels vary with actively dividing cells expressing more nucleolin. This may explain variation in Tir independent binding results that may occur (DeVinney *et al.*, 1999;Frankel *et al.*, 1994;Liu *et al.*, 1999). Invasin binds to integrins at a site that is also bound by laminin. Nucleolin also binds to laminin and it is possible that intimin γ binds to nucleolin at the laminin binding site. The binding of intimin γ to nucleolin may activate the host cell in a similar manner to laminin which is involved in the regulation of actin microfilaments (Dogic, Rousselle, and Aumailley, 1998). Intimin α on latex beads can induce microvillous like processes on Hep-2 cells independent of Tir binding and it remains to be seen if this process involves binding of nucleolin and triggering of host cell responses similar to those generated by different laminin isoforms (Phillips *et al.*, 2000a). In EPEC Tir and EspH have been shown to modulate the actin based pedestals formed at the site of bacterial adherence and it is possible that prior to translocation of Tir that intimin is involved in host cell signalling and actin modulation. Thus intimin is involved in the adhesion of EHEC to the intestinal tract, in tissue tropism *in vivo* and in binding to a host cell receptor independently of Tir.

EHEC infection of neonatal calves can produce a watery diarrhoea and colonisation, disease and A/E lesion formation are dependent on intimin expression by EHEC strains during the infection process. This suggests that adhesion to the intestinal tract of neonatal calves is an important mechanism in the enteropathogenicity of EHEC in calves (Dean-Nystrom *et al.*, 1998). Vaccination of pregnant dams with intimin γ from EHEC O157:H7 protects suckling piglets from EHEC infection and this may be means to prevent symptomatic and asymptomatic carriage of EHEC O157:H7 in cattle and transmission to humans (Dean-Nystrom *et al.*, 2002b). Therefore intimin γ is immunogenic and intimin specific responses are protective *in vivo* and can prevent A/E lesion formation on HEp-2 cell *in vitro* (Gansheroff, Wachtel, and O'Brien, 1999;Ghaem-Maghami *et al.*, 2001).

1.6.4.3 EHEC Tir.

EPEC Tir is tyrosine phosphorylated at position 474 in the Tir carboxy terminus which resides within in the cell cytoplasm during intimin Tir binding (Kenny, 1999;Luo *et al.*, 2000). EHEC Tir is secreted as a 72 kDa protein and is present in infected HeLa cells as an 88 kDa implying that some posttranslational modification occurs to the EHEC Tir protein. This is different to EPEC Tir which is secreted as a 78 kDa protein and modified in the host cell to become a 90 kDa protein. However, the DNA sequence of the Tir chaperone, CesT, of EPEC is 97.5% identical to the CesT of EHEC O157:H7 (Elliott *et al.*, 1999). Therefore the translocation of Tir probably occurs in a similar manner in EHEC and EPEC. The change in molecular weight of EHEC Tir probably occurs by phosphorylation but is not achieved by tyrosine phosphorylation (DeVinney *et al.*, 1999;Kenny, 2001). EHEC Tir show homology (66% sequence identity) between their amino and transmembrane region but show a greater variation in their carboxy terminal regions (44% sequence identity) which in EPEC Tir is implicated in tyrosine phosphorylation and binding to Nck to initiate pedestal formation (Perna *et al.*, 1998;Gruenheid *et al.*, 2001). Nck, and the actin adaptor proteins CrkII and Grb2 (Goosney, DeVinney, and Finlay, 2001), are not localised to EHEC pedestals and EHEC Tir does not trigger actin nucleation in an EPEC background suggesting that EPEC and EHEC have evolved different mechanisms to activate the actin polymerisation process (Kenny, 2001). In contrast to the EHEC Tir, EPEC Tir can mediate actin nucleation when expressed in an EHEC background suggesting that EHEC strains may encode specific factors that facilitate the correct modification of EHEC Tir within host cells (Campellone *et al.*, 2002;Campellone and Leong, 2003).

Therefore EHEC Tir is involved in different cell signalling events when compared to EPEC Tir and additional factors may be involved in EHEC Tir based actin polymerisation.

1.6.4.4 EHEC EspA, B and D.

The Esc proteins of EPEC E2348/69 and EHEC O157:H7 show a 98%-100% sequence identity (Elliott *et al.*, 1998). Therefore it is predicted that assembly of the TTSS system will be similar in EHEC and EPEC. EHEC EspA filaments are essential for A/E lesion formation on HeLa cells (Ebel *et al.*, 1998). The 8 kDa EscF protein forms a part of the needle complex in EPEC and is attached to EspA (Wilson *et al.*, 2001). EHEC EscF has not been characterised as yet but the sequence identity (100% (Elliott *et al.*, 1998)) between the EscF of EPEC strain E2348/59 and the EscF of EHEC O157:H7 strain EDL 933 suggests that EHEC EscF will bind to EHEC EspA. EHEC EspA filaments have been visualised during non contact dependent haemolysis of erythrocytes (Shaw *et al.*, 2001). However, the secreted proteins of EHEC and EPEC, like intimin, appear to show some sequence variation. Antiserum to EHEC O157:H7 EspA does not stain the EspA filaments of the typical EPEC serotypes O127:H6 and O55:H6, but does stain the EspA filaments of O55:H7. Such polymorphism and antigenic variation implies that EspA filaments may have sequence variation due to environmental pressure. Both EHEC O157:H7 and EPEC strain E2348/69 contain the LEE inserted at the *selC* site and this suggests that changes in EspA sequences and thus antigenicty acurred after incorporation of the LEE.

The EspB of EHEC interacts with α– catenin in the presence and absence of Tir. The protein α– catenin is a 102kDa cytoskeleton protein homologous to vinculin that binds to EspB at its carboxy terminus and recruitment of α– catenin to EspB is by direct interaction of both proteins and not due to filamentous actin accumulation. It is not known how α– catenin is involved in A/E lesion formation but *Listeria monocytogenes* uses α– catenin to trigger E – cadherin induced actin rearrangement. This report of EHEC O157:H7 EspB activity is the first report of an EspB host cell binding protein, and EPEC EspB was shown to also bind α– catenin (Kodama *et al.*, 2002). The EspB protein of EPEC strain E2348/69 is 74% homologous to the EspB protein of EHEC O157:H7 strain EDL933 (Elliott *et al.*, 1998). In addition, to binding to host cell proteins sera from patients with diarrhoea due to *E. coli* O157:H7 contained antibodies to EspB suggesting that EHEC host cell targeted effectors such as EspB are still recognised by the host immune response (Li *et al.*, 2000).

The EspD protein of EHEC O157:H7 strain EDL933 has 80% homology with the EspD of EPEC strain E2348/69. EHEC EspD like the EspD expressed by EPEC is required for A/E lesion formation and the production of functional EspA filaments. The deletion of *espD* in EHEC appears to affect EspA filaments in a different way as an EHEC *espD* mutant had 30nm thick EspA filaments compared with the 50nm filaments seen in an EPEC *espD* mutant. In addition the EHEC EspD protein may interact with the cytosol as well as form part of the plasma membrane unlike the EPEC EspD protein which only forms part of the plasma membrane (Kresse, Rohde, and Guzman, 1999).

1.6.4.5 EHEC EspF.

Comparison of the LEE from EPEC strain E2348/69 and EHEC O157:H7 strain EDL 933 suggests that EHEC encode an EspF proline rich protein with 75% sequence homology and 93% amino acid identity to the EspF from EPEC (Perna *et al.*, 1998). Therefore it is possible that EHEC strains express an EspF - like protein which may alter intestinal permeability during EHEC infection.

1.6.4.6 EHEC EspG.

The predicted EHEC EspG protein has yet to be functionally characterised but the gene which encodes EspG, *rorf2/espG*, is present in EHEC O157:H7 strain EDL933 and has a 98% sequence identity with the *espG* of EPEC strain E2348/69 (Deng *et al.*, 2001;Elliott *et al.*, 1998). Therefore, as with EPEC, EspG in EHEC may have a role in the colonisation of the intestinal tract (Elliott *et al.*, 2001b).

1.6.4.7 EHEC EspH.

EHEC EspH is translocated to the host cell by the TTSS in a manner similar to EPEC. It is located beneath adhering bacteria and overexpression of the EspH protein in EHEC results in pedestal elongation at the site of bacterial adherence (Tu *et al.*, 2003). EHEC EspH was not shown to be involved in filopodia formation suggesting that EHEC and EPEC interactions with the host cell cytoskeleton are different.

1.6.4.8 EHEC protein associated with secretion (Pas).

An ORF was identified upstream of *espA* and between *eae* and *espA* on the LEE of EHEC O157:H7 strain EDL933. This ORF was essential for secretion of the Esp proteins and deletion of this ORF resulted in loss of adherence to HeLa cells and A/E lesion formation. The protein product of this ORF was termed Pas for protein associated with secretion (Kresse *et al.*, 1998). When compared to the LEE of EPEC strain E2348/69 it is possible that Pas is *escD* in EPEC which was termed *rorf12*. Both *escD* and *pas* share sequence homology to *yscD* in *Yersinia* and *spiB* in *Salmonella* (Elliott *et al.*, 1998). Both *yscD* and *spiB* are part of the TTSS (Ochman *et al.*, 1996;Cornelis, 2002).

1.6.5 Non LEE encoded EHEC virulence factors.

1.6.5.1 STEC IrgA homologue adhesion (Iha).

A gene with similarity to the iron regulated gene A of *Vibrio cholerae,* involved in *V. cholerae* colonisation of mice (Goldberg *et al.*, 1992), was identified in *E. coli* O157:H7 strains. Iha is a 67 kDa outermembrane protein that can confer adherence to HeLa cells non - adherent *E. coli* strains but is not essential for the adhesion of O157:H7 to HeLa cells. Therefore Iha may not be an adhesin on cell lines *in vitro* but may activate a cryptic adhesin in laboratory *E. coli* during *in vitro* adhesion or require *in vivo* conditions for virulence activity in STEC (Tarr *et al.*, 2000). However, *iha* is located in a novel genomic island present in non LEE harbouring STEC strain such as O91:H-. This island is located

at the *selC* locus like the LEE of EHEC O157:H7. In addition to Iha this region may contain novel STEC adhesins that allow LEE negative strains to adhere to the intestinal tract (Schmidt *et al.*, 2001).

1.6.5.2 EHEC EspP.

EHEC EspP is encoded on the pO157 of EHEC O157:H7 and O26:H11 strains and is homologous to the EspC expressed by EPEC strains (Brunder, Schmidt, and Karch, 1997). Like EspC it is a member of the type IV secreted autotransporter family and is an extracellular serine protease. Immune sera from patients with EHEC infections react to EspP and functional analysis shows that EspP is able to cleave pepsin A and human coagulation factor V thus implying a role in mucosal haemorrhage of patients with HC. However, unlike EspC of EPEC and the plasmid encoded toxin of EAEC, no cytotoxic activity has yet been attributed to EspP (Dutta *et al.*, 2002).

1.6.5.3 EHEC Efa1.

Generation of transposon mutants in an EHEC O111:H- strain, isolated from a patient with HUS, identified a gene that was involved in the adhesion of this strain to CHO cells (Nicholls, Grant, and Robins-Browne, 2000). The gene, *efa1,* was located in a 15 kb genetic locus that has a low G+C content suggesting acquisition via horizontal gene transfer (Medigue *et al.*, 1991). The gene was also present in all strains containing the LEE but was not sufficient to confer A/E lesion formation activity on a laboratory *E. coli* strain on CHO cells *in vitro*. In addition to adherence of O111:H- to CHO cells *efa1* was shown to be involved in haemagglutination and autoaggregation of O111:H- bacteria. A homologue of *Efa1* exists on the pO157 plasmid of O157:H7 and is termed *toxB* (Tatsuno *et al.*, 2001;Reid *et al.*, 2000). O111:H- does not contain a pEHEC and it may be that the presence of Efa1 on the chromosome is equivalent to the presence of *toxB* on the pO157 of O157:H7, as functional analysis of *toxB* showed that it is essential for adhesion in O157:H7 and may regulate EspA expression. In O157:H7 *efa1* is a truncated gene but this is a serotype specific characteristic (Badea *et al.*, 2003). The *efa1* is virtually identical, to

the *lifA* with 99.9% sequence identity. LifA is a lymphocyte inhibitory factor (Klapproth *et al.*, 2000) present in EPEC (see section 1.5.3.13.3) and the homology between the two suggests that the EFa1 of O111:H- may display such functionality in addition to being involved in adhesion, haemagglutination and autoaggregation of EHEC O111:H-. The EHEC O157:H7 strain EDL933 has lymphocyte inhibitory activity and this may be due to the presence of *toxB* on its pO157 and/or the presence of Efa1 (Klapproth *et al.*, 1995).

1.6.5.4 The EHEC 60 MDa plasmid (pEHEC).

A DNA probe has been used to show that the majority of EHEC O157:H7 and O26:H11 strains involved in disease harbour a 60MDa plasmid (Levine *et al.*, 1987). This plasmid is termed pEHEC or pO157 if it is harboured by an O157:H7 strain. This plasmid has been shown to encode fimbriae that are involved in adhesion to Henle 407 intestinal cells but not to HEp-2 cells or erythrocytes (Karch *et al.*, 1987b). However pO157 has been shown to not be essential for virulence in a gnotobiotic piglet model (Tzipori *et al.*, 1987). The pO157 also encodes a haemolysin operon which has similar pore forming activity to the *E. coli* alpha haemolysin (Schmidt, Beutin, and Karch, 1995;Schmidt *et al.*, 1996). The haemolysin produce by EHEC is regulated by H-NS (Scott, Melton-Celsa, and O'Brien, 2003). Antibodies to the EHEC haemolysin are found in bovine colostrum and can be used to prevent cytolytic effects on erythrocytes (Lissner, Schmidt, and Karch, 1996).

Some reports suggest that the intestinal lesions produced in EHEC O157:H7 colitis, and the toxin-mediated damage to intestinal cells in *Clostridium difficile* infections, are similar (Griffin, Olmstead, and Petras, 1990). The sequencing of the complete nucleotide sequence of PO157 derived from two clinical isolates of O157:H7 (Burland *et al.*, 1998;Makino *et al.*, 1998) revealed a large pO157 ORF of 3169 amino acids which shows strong sequence similarity to the activity-containing N-terminal domain of a toxin family known as the large clostridial toxins (LCT) that includes ToxA (2710 amino acids) and ToxB (2366 amino acids) from *Clostridum difficile*. A plasmid cured derivative of an O157:H7 isolated from an outbreak of diarrhoea in Sakai, Japan, showed delayed microcolony formation on Caco-2 cells and reduced secretion of EspA, EspB and Tir. This

activity of the pO157 plasmid resided in the *toxB* gene which influences adhesion to Caco-2 cells via possible promotion of secretion of the type III secreted proteins (Tatsuno *et al.*, 2001). The pO157 *toxB* gene is homologous to the LCT - like Efa1. Therefore *toxB* may be involved in adhesion and cell binding activity like EPEC Efa1 and it may have lymphocyte inhibitory activity like LifA in EPEC. It may mediate autoaggregation like Efa1 in EHEC O111:H- therefore influencing microcolony formation.

The pO157 of a sorbitol fermenting O157:H- strain isolated from HUS patients in Germany encodes a novel fimbriae (Brunder *et al.*, 2001). This predicted structural subunit of the fimbriae has 62% amino acid similarity to the PapA structural subunit of P fimbriae and 41% amino acid similarity with type 1 fimbriae. The sorbitol-fermenting EHEC O157 plasmid-encoded fimbriae (Sfp) was encoded by six genes present in a 323 kb fragment of the plasmid of O157:H- and the genes were organised in a similar manner to the *pap* genes encoding P fimbriae. When expressed in the laboratory strain HB101 the fimbriae are 0.4 μm long and 3 to 5 nm in diameter which is smaller than P fimbriae, which are 7nm in diameter, and smaller than type 1 fimbriae which are 1-2 μm in length and 7 nm in diameter with a 3nm tip (Gander and Thomas, 1987;Martinez *et al.*, 2000). However, these fimbriae were not seen expressed on the surface of the wild type O157:H- strain. The transformation of HB101 with the Sfp gene cluster produced fimbrial expression in HB101 which was said to not express fimbriae. The HB101-Sfp bacteria showed agglutination of erythrocytes in a mannose resistant manner implying the presence of fimbriae other than type 1 fimbriae. However HB101 does produce type 1 fimbriae (Blomfield, McClain, and Eisenstein, 1991;Elliott, Nandapalan, and Chang, 1991) when grown in conditions which promote fimbrial expression and the Sfp fimbriae were not typed with antisera and therefore is not conclusive that the fimbriae observed were Sfp fimbriae.

The pO157 of EHEC O157:H7 encodes a protein involved in biofilm formation by O157:H7 and in determining the shape of the bacteria. This protein was termed Cah, for calcium binding antigen 43 homologue (Torres *et al.*, 2002b). Antigen 43 is a self

recognising surface adhesin found in most *E. coli* strains which may protect strains against hydrogen peroxide killing (Schembri *et al.*, 2003).

A gene cluster closely related to the type II secretion pathway of Gram-negative bacteria has recently been sequenced in pO157 (Schmidt, Henkel, and Karch, 1997). This pathway encoding 13 genes provides a mechanism of exoprotein secretion for these and other pathogenic Gram-negative bacteria. However, although the operon appears to contain the necessary genes, its ability to secrete proteins has not yet been demonstrated, nor is its substrate known. Its correlation with disease is also unclear (Burland *et al.*, 1998;Elliott, Nandapalan, and Chang, 1991).

The atypical EHEC strain O113:H21 does not encode a LEE and therefore must elaborate a non A/E lesion forming mechanism to adhere to the intestinal epithelium. This strain has been implicated in HC and HUS. It contains a pEHEC which encodes a gene *saa* (STEC autoagglutinating adhesin) which can confer adhesion to HEp-2 cells on a laboratory *E. coli* strain (Paton *et al.*, 2001). Deletion of *saa* in O133:H21 results in a loss of adhesion of this strain to HEp-2 cells. There is sequence variation in *saa* genes characterised in different STEC strains however this gene may provide another means for STEC adhesion to the intestinal tract.

As described in section 1.6.5.2, EHEC also encodes EspP on the pO157. The involvement of the pEHEC in the pathogenesis of EHEC has not be fully characterised and it is important to note that the large plasmids of EHEC are not uniform genetic elements but heterogeneous in both their gene composition and arrangement (Brunder *et al.*, 1999). This implies that the pEHEC of different strains may contain different virulence factors or similar virulence factors encoded by different DNA sequences leading to possible changes in virulence phenotype.

1.6.5.5. EHEC fimbriae.

EHEC strains do not express BFP and the isolation of intimin negative EHEC strains associated with bloody diarrhoea and HUS suggests that EHEC may express a second adhesin (Dytoc *et al.*, 1994;Elliott, Nandapalan, and Chang, 1991). As the EHEC O157:H7 LEE is not sufficient to produce A/E lesions when transfected into a nonpathogenic *E. coli* strain, it is possible that adhesins are encoded by genes outside the LEE (Elliott, Yu, and Kaper, 1999).

Type 1 fimbriae are not required for EPEC adhesion to HEp-2 cells (Elliott and Kaper, 1997). UPEC utilise type 1 fimbriae to adhere to and invade bladder epithelial cells (Martinez *et al.*, 2000). In general EHEC strains do not express type 1 fimbriae and have a 16 bp deletion in the *fim* genes that prevents expression of type 1 fimbriae. In EHEC O26:H11 strains this 16bp deletion may not be present but the *fim* genes are in an off orientation which prevents type 1 fimbriae expression in some but not all O26:H11 strains (Enami *et al.*, 1999;Roe *et al.*, 2001). However, not all EHEC O157:H7 strains lack the ability to express type 1 fimbriae and an EHEC O157:H7 strain Cl-49 has been shown to express mannose sensitive type 1 fimbriae (Durno, Soni, and Sherman, 1989). Therefore attempts to use the 16bp deletion as a probe for O157:H7 strains may not be appropriate for all strains (Li, Koch, and Cebula, 1997). Type 1 fimbriae are expressed by a variety of *E. coli* strains and in commensal *E. coli* strains suggesting that some role may exist for type 1 fimbriae *in vivo* (Elliott, Nandapalan, and Chang, 1991;Stentebjerg-Olesen, Chakraborty, and Klemm, 1999). EPEC type 1 fimbriae have been shown to produce a serological response in patients with EPEC related diarrhoea (Karch *et al.*, 1987a) and in UPEC the expression of type 1 fimbriae influences the colonisation of the bladder (Gunther *et al.*, 2001). Type 1 fimbriae production is growth condition sensitive and type 1 fimbriae can be regulated by the genes involved in P fimbriae expression and H-NS (O'gara and Dorman, 2000;Xia *et al.*, 2000). P fimbriae and type 1 fimbriae have been implicated in binding during different stages of infection in a chicken model of infection (Pourbakhsh *et al.*, 1997). In addition, the FimH mannose binding protein of type 1 fimbriae can valency convert and bind to different receptors as well as mediate

autoaggregation of *E. coli* (Schembri, Christiansen, and Klemm, 2001). Therefore the expression of type 1 fimbriae is complex and their expression *in vivo* in certain EHEC strains remains to be characterised.

Curli are surface structures expressed by a variety of *E. coli* which can bind to fibronectin and laminin. Curli expression is uncommon in EHEC O157:H7 strains but can occur in human isolates in a temperature independent manner. The expression of curli by O157:H7 strains is dependent on *csgD* promoter and variations in the *csgD* promoter is associated with increased virulence in mice and increased invasion of HEp-2 cells (Uhlich, Keen, and Elder, 2002;Uhlich, Keen, and Elder, 2001). Laminin is involved in mediating actin cytoskeleton events and binding of laminin by certain O157:H7 strains may be a means to signal to the host cell cytoskeleton proteins (Dogic, Rousselle, and Aumailley, 1998).

The sequencing of the genome of EHEC strain EDL933 has allowed the search for adhesins that are not encoded by the LEE. When compared to the *E. coli* strain K-12 EDL933 has 1.34 Mb of DNA extra (Perna *et al.*, 2001). The additional regions present in the EHEC O157:H7 genome are termed O islands. 177 O islands exist and 33% of these O islands encode genes of unknown function and genes have been classified according to their homology with known virulence genes. Two regions are thought to contain operons that encode long polar fimbriae (LPF) with homology to the LPF of *S. typhimurium* . LPF have been shown to target *S. typhimurium* to murine Peyer's patches (PP) (Baumler, Tsolis, and Heffron, 1996) and have polar distribution and are 2- 10 μm long when the LPF operon is expressed in laboratory *E. coli* (Baumler and Heffron, 1995). LPF is controlled by a phase variable on/off genetic switch that regulates the expression of *S. typhimurium* LPF in different tissues and organs. The phase variation of LPF is also a mechanism by which *S. typhimurium* evades cross immunity among different *Salmonella* serotypes (Norris and Baumler, 1999). Therefore functional analysis of the operons present in EHEC that are homologous to the operons encoding LPF in *S. typhimurium* may elucidate the mechanism by which EHEC adhere to the intestinal tract and target PP explants during IVOC (Phillips *et al.*, 2000b). The first *lpf* - like operon present in EHEC O157:H7 is encoded by the 141 O island and is 5.9 kb in size. The second *lpf* - like operon

is encoded by the 154 O island and is 6.9 kb long and both LPF like operons have a different genetic organisation but both possess the *lpfA* gene which is predicted to be the major structural subunit of EHEC LPF (Torres *et al.*, 2002a). A deletion of the first *lpf*- like operon, *lpfABCC'DE*, in EHEC O157:H7 results in a slight decrease in adhesion to HeLa cells. The putative fimbrial structural subunit *lpfA* was expressed in a nonfimbriated *E. coli* strain which resulted in this strain expressing peritrichous fimbriae that were similar in morphology to type 1 fimbriae. The fimbriae reacted with antiserum raised against the LpfA subunit but were not probed with anti type 1 fimbriae antibodies. However, no LPF fimbriae were detected on wildtype EHEC O157:H7 strains and probing of western blots for LpfA with antiserum was carried out using laboratory *E. coli* overexpressing *lpfA* (Torres *et al.*, 2002a). In a second study, the LEE negative atypical EHEC strain O113:H21, isolated from outbreaks of HC and HUS, was probed for the presence of an *S. typhimurium lpf*- like operon. It was shown to contain an *lpf* operon which corresponded to the *lpf*- like operon on the 154 O island of the EHEC O157:H7 EDL933 strain. This *lpf* - like operon was also found in other LEE negative strains and in LEE positive non O157:H7 EHEC. The deletion of the *lpfA* of the *lpf*- operon in EHEC strain O113:H21 resulted in a decrease in adhesion to CHO cells (Doughty *et al.*, 2002). Therefore the *lpf* operon may be involved in adhesion of non LEE strains thus providing a means of adhesion to the intestine that is not A/E related and in addition may allow LEE expressing strains to adhere to the intestinal tract without forming A/E lesions or during the early stages of A/E lesion formation.

1.6.6 EHEC regulation and quorum sensing.

Unlike EPEC, EHEC do not harbour the EAF plasmid and therefore are not regulated by the *per*. However EHEC do contain the *ler* encoded in the *LEE1* operon and it activates the expression of the *LEE2*, *LEE3* and *tir* operons but, unlike EPEC, not the *LEE4* operon which encodes the EHEC secreted proteins (Elliott *et al.*, 2000). Deletion of the *ler* in EHEC O157:H7 results in the expression of novel long fine fimbriae which have yet to be characterised but indicate that the *ler* in EHEC is involved in the regulation of non LEE potential virulence factors. Quorum sensing in EHEC involves the *luxS* system which has

been reported to be involved in interspecies communication in bacteria and was first characterised in *Vibrio harveyi* (Surette, Miller, and Bassler, 1999). The *luxS* gene is responsible for the production of an autoinducer (AI-2) which activates a regulator to increase the transcription of the operon. A regulator that acts upstream of *ler* and is part of the quorum sensing system of EHEC and EPEC has been characterised and termed quorum sensing *E. coli* regulator A (QseA). In the presence of enough AI-2 *qseA* transcription is activated and in turn QseA activates the expression of *ler* which activates the LEE genes. However QseA is not the sensor for AI-2 which has yet to be described. Other regulators have been described that are activated by the presence of AI-2 and are involved in the activation of Stx, motility and metabolic genes (Sperandio, Li, and Kaper, 2002;Sperandio, Torres, and Kaper, 2002). Deleting *qseA* in EHEC O157:H7 produces a reduction in type III secretion activity.

The genes involved in quorum sensing and regulation of EPEC and EHEC have yet to be fully elucidated. However, quorum sensing in EPEC acts upon *per* which interacts with *ler*, and *ler* is an antirepressor for H-NS. A regulator of EHEC that has yet to be characterised in EPEC has been described which regulates the expression of EspD and intimin located on the *LEE4* and *tir* operons respectively. This regulator is termed SdiA and is part of the quorum sensing system of EHEC O157:H7 providing evidence that *LEE4* genes are also regulated by quorum sensing in EHEC (Kanamaru *et al.*, 2000).

Thus quorum sensing allows EHEC to regulate the expression of LEE encoded genes in response to the presence of AI-2 in the environment. EHEC produces pathology in the large intestine as seen in HC patients. The bacterial flora of the large intestine is dense and the AI-2 quorum sensing system in EHEC may allow EHEC which has a low infective dose (approximately 100 (Griffin and Tauxe, 1991)), to regulate its virulence factors through the AI-2 produced by the bacterial flora of the intestine. Therefore bacteria may develop AI-2 sensitive regulatory systems that have different repressor and activation genes which may allow them to express virulence factors in defined environments. An O157:H- EHEC strain isolated from an outbreak of HUS has been shown to have a mutation in *ler* responsible for the strain's poor adhesion to HEp-2 cells and thus

suggesting that other regulators may compensate for the defective *ler* (Ogierman, Paton, and Paton, 2000).

1.6.7 EHEC diarrhoea and detection.

EHEC is a heterogenous group of strains that are implicated in cases of HC and HUS. EHEC contain the genes necessary for A/E lesion formation on cell lines and in animal models of infection but an *in vivo* description of A/E lesion formation in a human EHEC infection has yet to be described. EHEC A/E lesions have been described using the IVOC assay system which provides a tool for describing the mechanism behind A/E lesion formation by EHEC O157:H7 (Phillips *et al.*, 2000b). The involvement of A/E lesions produced by EHEC during the infection process may share some of the characteristics of EPEC but A/E lesion formation and diarrhoea may be achieved in EHEC using different host cell signalling events and effector proteins. Not all STEC possess the genes necessary for A/E lesion formation but may harbour other genes involved in adhesion to the intestinal tract. Diarrhoea due to EHEC and STEC may involve a combination of adhesion and disruption of the intestinal barrier which is then exacerbated by the production of cytotoxic proteins by EHEC and STEC. Similar to EPEC, EHEC O157:H7 induce a host cell inflammatory response linked to A/E lesion formation. Cultured epithelial cells show an increased level of IL-8 in response to EHEC infection and in a rabbit model neutrophil migration in response to EHEC was blocked by the addition of anti CD-18 antibodies (Elliott *et al.*, 1994;Jung *et al.*, 1995). As described in section 1.6.3 neutrophil migration in response to EHEC may disrupt the intestinal barrier allowing Stx to come into contact with the underlying tissues. In the rabbit ileum the Gb_3 receptor is present in much higher concentrations in the villous cells than in the secretory crypt cells therefore Stx may disrupt the enterocytes and not affect the secretory cells promoting net secretion at the villous epithelium (Kandel *et al.*, 1989;Keenan *et al.*, 1986). In addition, studies have shown that intestinal cells vary in their expression of the Gb_3 receptor and that crypt cells in the bovine intestine bind Stx which is internalised, excluded form the endoplasmic reticulum and localised to the lysosomes therefore abrogating the interaction of Stx with the host cell ribosomes (Hoey *et al.*, 2003). Expressing Stx in a rabbit diarrhoeagenic *E. coli* strain and

infecting rabbits resulted in the toxin positive strain causing more severe histological lesions with vascular changes, oedema and inflammation when compared to the toxin negative strain (Sjogren *et al.*, 1994). Therefore the secretion of Stx by STEC may lead to HC or HUS. However, Stx does not increase active Cl^- secretion and rabbits administered Stx intravenously produce a nonbloody diarrhoea suggesting that other mechanisms exist for producing diarrhoea other than toxin binding to villous tips (Richardson *et al.*, 1992). Galanin receptors line the intestinal tract and are activated by NF-κB which results in Cl^- secretion via cAMP independent process. Mouse colons infected with EHEC increased NF-κB and galanin receptor expression which produced a change in epithelial electrolyte transport possibly by Cl^- secretion (Hecht *et al.*, 1999). Therefore the EHEC may induce diarrhoea via Stx and other mechanisms involving host inflammation such as A/E lesion formation which in the gnotobiotic piglet model of EHEC infection is the determining factor in ion secretion and EHEC related diarrhoea (Tzipori *et al.*, 1995). The association of different STEC strains expressing variable virulence genes with diarrhoea, HC and HUS requires sophisticated detection methods. Sorbitol MacConkey agar can be used to isolate *E.* coli O157:H7 by distinguishing colonies which do not ferment sorbitol. However, the lack of O157:H7 isolates may not imply that an EHEC strain is not involved and the association of STEC strains with certain geographical regions provides useful information on how to proceed. Stx production in the stool or antibody levels to STEC LPS may provide a means of detecting a possible STEC associated diarrhoea. Antibodies to Stx or DNA probes to detect *stx* have been developed. DNA probes which detect *eae* will not necessarily implicate an STEC strain as both EPEC O55:H7 and EHEC O157:H7 express intimin γ. DNA probes against the pEHEC will miss the strains which do not express a pEHEC. Thus it is apparent that detection of STEC strains can become complicated and in some cases require time and technical expertise. Therefore describing A/E lesions during the early stages of EHEC infections is not straight forward and cell lines, animal models and IVOC have been developed as A/E lesion models. In addition, Stx effects cannot necessarily be compared between species because of the variation in expression of Gb_3, and in the way of dealing with the translocated Stx.

1.7 Models of EPEC and EHEC infection.

EPEC and EHEC produce A/E lesions on cultured epithelial cells and EPEC A/E lesions have been observed in biopsies from patients with EPEC related diarrhoea (Ulshen and Rollo, 1980;Hill, Phillips, and Walker-Smith, 1991b;Rothbaum *et al.*, 1983). The discovery of A/E formation by EPEC was a defining characteristic of this category of diarrhoeagenic *E. coli*. The elaboration of Stx by diarrhoegenic *E. coli* led to a new category of *E. coli* being formed which was termed STEC and included the EHEC which could produce A/E lesions on epithelial cell lines *in vitro* and contained the genes necessary for A/E lesion formation in EPEC, the LEE. A/E lesions have been reported for EHEC in animal models of infection and on human explants in culture but their distribution during human infection has not been reported.

1.7.1 Cultured epithelial cell lines.

Human malignant tumour cells grown in tissue culture have been developed as a means of studying the affect of EPEC and EHEC on eukaryotic cells with respect to A/E lesion formation, host cell effectors, cytotoxin elaboration and host cell inflammatory responses. The cell lines used include human derived cell lines such as HeLa , Hep-2, Caco-2 and T84 (Cravioto *et al.*, 1991a;Rosenshine *et al.*, 1996;Collington, Booth, and Knutton, 1998) (Li *et al.*, 1999), and animal derived cell lines such as the mouse cell line CMT-93 and the bovine cell line MDBK (Tobe and Sasakawa, 2002;Torres *et al.*, 2002a).

Cell lines are used because they allow host bacterial interactions to be analysed via microscopy, fractionation of cellular compartments, extraction of bacterial host cell effectors and host cell receptors and DNA arrays.

HeLa cells and HEp-2 cells have been used to study A/E lesion formation in EPEC and EHEC. This was facilitated by the development of the FAS test which allows bacterial strains to be assessed for A/E lesion forming activity (Knutton *et al.*, 1989). In addition, the adhesion pattern of EPEC and EHEC has been used to characterise strains. The

expression of BFP by EPEC is associated with localised adherence and certain EHEC strains have been associated with a delayed localised adherence termed localised adherence - like phenotype (Francis *et al.*, 1991;McKee and O'Brien, 1995;Scotland *et al.*, 1990), or poor localised adherence (Knutton *et al.*, 2001). The HeLa cell line was the first carcinoma cell line to be maintained in long term culture and was derived from a cervix epitheloid carcinoma (Grey, Coffman, and Kubicek, 1952). The HEp-2 cell line is derived from a larynx carcinoma but the stocks present in cell line repositories are indistinguishable from the HeLa cell line by DNA profile (Macville *et al.*, 1999;Chen, 1988). Thus, although not intestinal cells HeLa cells and HEp-2 cells have been derived from mucosal sites. They are unlike Caco-2 and T84 cells as they are not fully differentiated or polarised. Caco-2 cells have been derived from a colon adenocarcinoma and can achieve enterocyte like differentiation (Zweibaum *et al.*, 1984). At late confluency the Caco-2 cells are fully differentiated and exhibit properties of polarised distal small intestinal enterocytes, with microvilli present on the apical surface. The cells also become polarised with the presence of tight junctions (Grasset *et al.*, 1984). Therefore differentiated cell lines such as Caco-2 and T84 cells are more similar to the intestinal enterocyte *in vivo* than HeLa and HEp-2 cells and may be more relevant when examining the effects of EHEC and EPEC.

However, eukaryotic host cell signalling has been implicated in regulation of flagella expression in EPEC using HEp-2 which implies that although cell lines such as Hep-2 cell may not be differentiate they have biologically activity that may resemble the *in vivo* environment(Giron *et al.*, 2002). In addition, the HT-29 colon cell line, derived from a colon adenocarcinoma, has been shown to produce matrilysin, a bacteriocidal matrix metalloproteinase, when exposed to *E. coli* (Lopez-Boado *et al.*, 2000). CHO cells derived from Chinese hamster ovaries, have been used to show conjugation between *E. coli* and mammalian cells implicating novel host bacterial interactions (Waters, 2001).

The co - culture of a Caco-2 monolayer with B lymphocytes can result in the Caco-2 cells expressing features of the follicle associated epithelium and M cells (Kerneis *et al.*, 1997). M cells in this system showed a disorganised brush border and lack of binding of Ulex

europaeus agglutinin 1. *Yersinia enterocolitica* binds to this region of the Caco-2 cell co - culture model via invasin binding of host cell integrins, which are expressed on the apical membrane of M cells in contrast to their basolateral membrane expression in villous epithelium. Thus invasin may target *Y. enterocolitica* binding to M cells resulting in invasion (Clark, Hirst, and Jepson, 1998;Schulte *et al.*, 2000). This model may allow the interaction of EPEC and EHEC with M cell like structures to be analysed which may exist as transient epithelial cell types *in vivo* (Sierro *et al.*, 2000). However, the model is capricious and not easily reproducible.

Primary cell lines have been developed in order to culture cells that have not been derived from carcinomas and to allow the establishment of fully differentiated cell lines from regions of interest. Intestinal epithelial cells may undergo apoptosis due to loss of cell anchoring when attempts are made to isolated the epithelial cells. This "anoikis" can make it difficult to maintain intestinal epithelial cells *in vitro* (Grossmann *et al.*, 2003;Rosen *et al.*, 2002). Intestinal epithelial cells can be maintained in culture for up to 48 hours but prolonged maintenance up to 10 days is difficult (Kaeffer, 2002). Bovine primary cell lines have been used to study the interaction of Stx with the intestinal epithelial cells but this technique has not been developed for A/E lesion formation analysis (Hoey *et al.*, 2003). Human duodenal epithelial cells have been isolated and maintained as spheroid like vesicles with microvilli on the exterior and a central lumen (Boxberger *et al.*, 1997). Using this technique duodenal epithelial cells were maintained for 9 to 13 days. The advantage of isolating epithelial cells is that they can be selected from areas of interest and over a short period maintain *in vivo* biologic activity and complexity with the presence of mucosal surface elements such as mucus and the glycocalyx. Isolated duodenal enterocytes have been used to study the interaction of ETEC, EPEC and EAEC epithelial cells during short term culture for up to 3 hours (Knutton *et al.*, 1984b;Knutton *et al.*, 1984a;Knutton, Lloyd, and McNeish, 1987;Knutton *et al.*, 1992).

1.7.1.1 *In vitro* organ culture (IVOC).

IVOC, like primary cell culture allows the analysis of specific regions of the intestinal tract, such as Peyer's patches. The use of explant tissue during IVOC removes the epithelial cell isolation procedures and the intestinal epithelial cells present in intestinal explants are attached to the submucosae. IVOC culture allows host bacterial interactions to be examined and incorporates factors such as mucus release, continued cell turnover and protein synthesis (Browning and Trier, 1969;Mitchell, Mitchell, and Peters, 1974). Small intestinal mucosae can be maintained *in vitro* for up to 24 hours without significant morphological damage. In order to avoid tissue degradation mucosal explants are not submerged in tissue culture medium but are maintained on a support which allows the tissue culture medium to cover the explant by capillary action and provide nutrients. Using this method it has been shown that IVOC can maintain small intestinal explants, such as adult duodenum and jejunum, for up to 48 hours with maintenance of cell proliferation and fat absorption (Browning and Trier, 1969). After 48 hours of culture microvillous shortening is observed as well as cytoplasmic vacuolation, and brush border enzymes decrease progressively with time (Mitchell, Mitchell, and Peters, 1974). Paediatric human intestinal IVOC has been used to show that EHEC O157:H7 produce A/E lesions on intestinal epithelium and that EHEC O157:H7 binds preferentially to PP explants, a possible initiation site of intestinal infection (Phillips *et al.*, 1999). IVOC has also been used to show A/E lesion formation by EHEC O157:H7 on bovine explants and the interaction of human EPEC with rabbit ileum (Batt *et al.*, 1987). During the study of host bacterial interactions explant culture maintains the complexity of the *in vivo* intestinal barrier, such as the presence of host cell receptors, glycocalyx and mucus production. The glycocalyx has been shown to be an effective mediator of particulate access to the intestinal surface and is thinner on PP that in the small or large intestine suggesting a possible physical explanation for targeting of PP by EHEC O157:H7 (Frey *et al.*, 1996;Phillips *et al.*, 2000b).

A severe combined immunodeficient murine intestinal xenograft model has also been developed using human foetal intestinal xenografts (Savidge *et al.*, 1995). The intact

segments of immature foetal intestine are surgically implanted into subcutaneous tunnels on the back of the immunodeficient mouse where they develop and remain viable over several months. Ten weeks after transplantation the xenograft shows extensive vascularisation and normal intestinal morphology, however there is some mixing of mouse and human cells in the explant. This model has been used to study the interaction of *Shigellae* with the intestine and implied a role for neutrophils in controlling the invasion of the human intestine by *Shigellae* (Zhang *et al.*, 2001). Thus this model is a combination of an explant model and an animal model and allows long term culture of human intestine which may be useful in studying EHEC and EPEC infections.

1.7.2 Animal models of EPEC and EHEC infection.

Conditions similar to that observed in the intestinal tract have been implicated in the induction of protein secretion in EPEC (Kenny *et al.*, 1997a). Infection of animals with human and animal EPEC and EHEC strains, and the use of natural animal infection models of infection such as the infection of mice by *C. rodentium* (Barthold, Osbaldiston, and Jonas, 1977;Barthold *et al.*, 1978), allows the examination of the host - bacteria interactions with reference to pathology, immunology and *in vivo* bacterial expression in the context of a living host with an intact intestine with in some cases an acquired commensal flora(Higgins *et al.*, 1999b;Higgins *et al.*, 1999a;Tzipori *et al.*, 1987;Khan and Isaacson, 2002;Hensel *et al.*, 1995). The use of animal models to study EHEC pathogenesis has identified that intimin is implicated in tissue tropism *in vivo*, is required for enteropathogenicity in calves and generates an intimin specific immune response (Dean-Nystrom *et al.*, 1998;Tzipori *et al.*, 1995) (Ghaem-Maghami *et al.*, 2001). In addition bovine ligated ileal loops have been used to suggest that EHEC O103:H2 produce enteropathogenic effects independent of intimin and Stx (Stevens *et al.*, 2002). REPEC infections of rabbits have been used to show that EPEC strains may initially adhere to PP *in vivo* and that REPEC can decrease apoptosis in the ileum and at PP(Heczko, Abe, and Finlay, 2000;Heczko *et al.*, 2001). Cattle, an important reservoir of EHEC O157:H7, lack vascular receptors for EHEC O157:H7 Stx and bovine intestine has been shown to be resistant to Stx damage (Hoey *et al.*, 2003). Greyhounds can act as a model for HUS as a

naturally occurring condition in greyhounds called iodiopathic cutaneous and renal glomerular vasculopathy is similar to HUS (Fenwick and Cowan, 1998). The greyhounds like human patients with HUS exhibit thrombocytopenia and microangiopathic haemolytic anaemia. This may be due to an STEC infection but no causal link has been made. However, EHEC can be recovered from dogs with diarrhoea (Beutin, 1999). Thus efforts have been made to develop other models of EHEC infection. Ferrets have been used to model the Stx induced kidney damage which occurs after infection of the intestine by STEC (Woods *et al.*, 2002). Mucus activation of Stx has been implicated in the virulence of EHEC O157:H7 in orally inoculated streptomycin treated mice (Melton-Celsa, Darnell, and O'Brien, 1996). Mice lack Stx receptors on their PP and in their peripheral lymph nodes suggesting that binding to PP may allow EHEC to adhere *in vivo* without causing Stx mediated damage to the host and without inducing an Stx immune response (Imai *et al.*, 2002). Infection of chickens with EHEC can produce (Beery, Doyle, and Schoeni, 1985) and a monkey model of EHEC O157:H7 has also been developed which shows that A/E lesion formation occurs in the first 12 hours of infection and is followed by the development of ulcers and surface epithelium necrosis. No renal or cerebral damage was reported (Kang *et al.*, 2001). In addition to the gnotobiotic piglet model A/E lesions are formed during the infection of cattle with EHEC O157:H7, in which the O157:H7 bacteria adheres to the lymphoid follicle dense mucosa of the terminal rectum which may allow greater dissemination of O157:H7 (Naylor *et al.*, 2003). However, very few A/E lesions were identified although a high level of O157:H7 was recovered. This suggests that adhesion in cattle may not be generally via A/E lesion formation which explains the lack of clinical symptoms in adult cattle. In both mice and neonatal lambs EHEC O157:H7 produces A/E lesions in the caecum (Wales *et al.*, 2001;Nagano *et al.*, 2003).

C. rodentium is a natural pathogen of mice and encodes the LEE, and in mice produces a transmissible colonic hyperplaisia with A/E lesions. This model has been used to demonstrate immune specific responses to intimin, the involvement of amino acid residues within intimin and intimin type during colonisation and colonic hyperplaisia and as a model of inflammatory bowl disease (Ghaem-Maghami *et al.*, 2001;Goncalves *et al.*,

2001;Reece *et al.*, 2001;Reece *et al.*, 2002b;Reece *et al.*, 2002a;Higgins *et al.*, 1999b;Higgins *et al.*, 1999a;Vallance *et al.*, 2003;Vallance *et al.*, 2002).

Thus a wide range of animal models exist which have been used to answer different questions about EPEC and EHEC mediated disease.

1.7.3 Human volunteer studies.

Human volunteer studies have been used to examine the involvement of EPEC virulence factors in diarrhoea. Due to the production of Stx and the association with HC and HUS STEC and EHEC strains are not tested in human volunteers.

To date the EAF plasmid (Levine *et al.*, 1985), intimin (Donnenberg *et al.*, 1993) and EspB (Tacket *et al.*, 2000) have all been examined for their involvement during diarrhoea in humans *in vivo*. These studies revealed that all three virulence factors are involved in EPEC diarrhoea. However, deletion of the virulence genes did not always result in prevention of diarrhoea and each studied implied that no one factor was critical for EPEC related diarrhoea. No indication was given as to the prior exposure to diarrhoeagenic *E. coli* of the volunteers although before inoculation volunteers were screened for potential bacterial and parasitic pathogens. Although prior exposure to EPEC does not appear to protect against heterologous rechallenge it does reduce the severity of subsequent EPEC related diarrhoea (Donnenberg *et al.*, 1998). In future studies it would be appropriate to test for the involvement of vaccine candidates such as EspA during human EPEC infections and regulators such as the *ler* and the quorum sensing protein QseA.

1.8 Conclusions.

EPEC and EHEC share common pathogenic themes but evolutionary pressures have produced different combinations of virulence factors in EHEC and EPEC strains. Many model systems exist to answer questions about the pathogenic mechanisms of EPEC and EHEC, but it is important to use a model system which approximates to the *in vivo* condition. This thesis will use the IVOC and electron microscopy to characterise the adhesion of EHEC and EPEC strains to human intestinal explants and correlate the tissue tropism of the strains with their known virulence factors. This may provide further information as to the A/E lesion forming ability of EHEC strains and the involvement of intimin in EPEC and EHEC tissue tropism. In addition to characterising the role played by intimin during A/E lesion formation and tissue tropism other factors involved in adhesion will be examined. In order to achieve this, the bacterial strains will be examined for fimbrial expression during various growth conditions and putative fimbrial genes will be assessed for their role in IVOC intestinal tissue tropism.

2.0 Methods and Materials.

2.1 Bacterial strains.

The EPEC and EHEC strains, listed in tables 2.1 and 2.2, were used to generate the results in sections 3, 4 and 5 and were kindly provided by the collaborators indicated in the strain descriptions. Descriptions are also included in the relevant results sections.

Table 2.1 Wildtype Bacterial strains.

Strain	Serotype	Source/Reference
EPEC		
E2348/69	O127:H6	(Levine *et al.*, 1978)
E77804	O103:H-	H. Smith, Division of Enteric Pathogens, Central Public Health Laboratory, London, U.K.
G21	O55:H6	(Adu-Bobie *et al.*, 1998;Rodrigues *et al.*, 1996)
G30	O55:H6	(Adu-Bobie *et al.*, 1998;Rodrigues *et al.*, 1996)
G35	O55:H-	(Adu-Bobie *et al.*, 1998;Rodrigues *et al.*, 1996)
G57	O55:H7	(Adu-Bobie *et al.*, 1998;Rodrigues *et al.*, 1996)
G58	O55:H7	(Adu-Bobie *et al.*, 1998;Rodrigues *et al.*, 1996)
EHEC		
85/170	O157:H7	(Tzipori *et al.*, 1987)
AGT300	O157:H7	(Torres *et al.*, 2002)
H11/8624JK	O157:H7	(Griffin *et al.*, 1988;Sperandio *et al.*, 1999)
TT12B	O157:H7	(Feng *et al*, 2001)
3801	O26:H11	J.B. Kaper, Center for Vaccine Development, University of Maryland, Baltimore, U.S.A.
PMK5	O103:H2	(Mariani-Kurkdjian *et al.*, 2001)
EAEC		
O42	EAEC O44:H18	(Nataro *et al.*, 1996)

All the strains used during the experiments described section 3, 4 and 5 were Stx negative. The laboratory is a Class II laboratory and Stx expressing bacteria need to be handled in a Class III laboratory.

E2348/69 was isolated from a case of infant diarrhoea, Taunton, Somerset, U.K. It is EAF plasmid and BFP positive and as such is considered to be the prototype typical EPEC. It produces LA on HEp-2 cells and causes diarrhoea in human volunteers (Donnenberg *et al.*, 1993).

O42 was isolated from an infant with diarrhoea in Lima, Peru, in 1983. It adhered to HEp-2 cells in an aggregative pattern that is distinctive and the HEp-2 assay (Nataro *et al.*, 1987a) and a DNA probe (Baudry *et al.*, 1990) are used as diagnostic tools for EAEC. Human volunteers administered O42 developed a watery, non-bloody diarrhoea without fever (Nataro *et al.*, 1995). It is intimin and Stx negative.

85/170 was isolated from a food handler in a Canadian nursing home. It is a spontaneous Stx negative derivative of EHEC O157:H7 strain 84-289 and is *stx1* and *stx2* negative (Tzipori *et al.*, 1987).

AGT300 was isolated in the same outbreak associated with EHEC O157:H7 strain 86-24. AGT300 is a streptomycin resistant derivative of EHEC strain 87-23 which does not produce stx2. This strain was kindly provided by A.G. Torres, Center for Vaccine Development, University of Maryland, U.S.A.

H11 and 8624JK are derivatives of the wild-type EHEC O157:H7 strain 8624, which is positive only for Stx2 expression, containing a mutation in the *stxA2* gene that inactivates Stx2 production. The 8624 strain was isolated from an HC outbreak in Walla, Walla, Washington, U.S.A. (Griffin *et al.*, 1988). These strains were kindly provided by A. Hull and J.B. Kaper, Center for Vaccine Development, University of Maryland, U.S.A.

TT12B was isolated from a Japanese patient with haemorrhagic colitis. It does not contain *stx1* and *stx2* genes and does not produce Stx. TT12B was isolated along with its progenitor strain TT12A which was identical to TT12B except that it produced Stx and had intact *stx1* and *stx2* genes. This strain was kindly provided by P. Feng, Division of Microbiological Studies, US Food and Drug Administration, Washington DC, U.S.A.

3801 was isolated from a child with bloody diarrhoea in 1995 Germany by H. Karch, Institut fur Hygiene und Mikrobiologie, Universitat Wurzburg, Germany. 3801 is *stx1* and *stx2* probe negative. This strain was kindly provided by J.B. Kaper, Center for Vaccine Development, University of Maryland, U.S.A.

E77804 was isolated from an adult male with diarrhoea in Oxford, UK. It is an atypical EPEC intimin ϵ expressing, EAF plasmid negative strain. This strain was kindly provided by H. Smith, Division of Enteric Pathogens, Central Public Health Laboratory, London, U.K.

PMK5 was isolated from a child with HUS in France(Mariani-Kurkdjian *et al.*, 2001). It is Stx1 negative due to internal deletions in *stx1A* which also removes the start codon for *stx1B*. It does not express Stx2 are probed by PCR. This strain was kindly provided by E. Oswald, Unité INRA – ENVT de Microbiologie Moleculaire, École Vétérinaire de Toulouse, France. The mutation in *Stx1A* was carried out by M.P. Stevens, Institute for Animal Health, Compton, U.K.

The EPEC strains from the O55 serogroup were isolated, between 1950 and 1993, from patients with diarrhoea in the U.S.A, Brazil and Chile. The strains were kindly provided by L. R. Trabulsi, Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, Brazil.

Table 2.2 EPEC and EHEC derivative strains.

Strain	Description	Source/Reference
EPEC		
CVD206	Intimin negative, EAF+, BFP+ E2348/69 derivative strain	(Donnenberg and Kaper, 1991;Kelly *et al.*, 1999)
JPN15	Intimin α expressing, EAF-, BFP- E2348/69 derivative strain	(Gomez-Duarte and Kaper, 1995)
CVD206(pCVD438)	CVD206 expressing the intimin α plasmid CVD438	(Donnenberg and Kaper, 1991)
CVD206(pCPG2)	CVD206 expressing intimin α V907A	Section 2.7 And section 4.3.3.4
EHEC		
ICC170	Intimin negative strain of 85-170	Professor G. Frankel CMMI, Imperial College, London, U.K.
ICC170pCVD438	ICC170 harbouring the intimin α expressing plasmid pCVD438	Professor G. Frankel CMMI, Imperial College, London, U.K.
ICC170pICC55	ICC170 harbouring a pCVD438 derivative plasmid, PICC55, expressing recombinant intimin γ	Professor G. Frankel CMMI, Imperial College, London, U.K.
ICC170pCVD438/01	ICC170 harbouring the intimin α expressing plasmid pCVD438, containing a C937A substitution	Professor G. Frankel CMMI, Imperial College, London, U.K.
ICC170(pCPG2)	ICC170 expressing intimin α V907A	Section 2.7 And section 4.3.3.4
85-170lpf1-	The EHEC O157:H7 strain 85-170 with a deletion in the *lpfA* of the first *lpf* - like operon. Cm^r	Dr A.G. Torres CVD, University of Maryland, U.S.A.
85-170lpf1/c	Strain 85-170lpf1- complemented with pBR322*lpfABCC'DE*. Ap^r	Dr A.G. Torres CVD, University of Maryland, U.S.A.
85-170lpf2-	The EHEC O157:H7 strain 85-170 with a deletion in the *lpfA* of the second *lpf* - like operon. Cm^r	Dr A.G. Torres CVD, University of Maryland, U.S.A.
85-170lpf1-2	Strain 85-170 with deletions in the *lpfA* of *lpf* - like regions 1 and 2	Dr A.G. Torres CVD, University of Maryland, U.S.A.
AGT300lpf1-	The EHEC O157:H7 strain AGT300 with a deletion in the *lpfA* of the first *lpf* - like operon. Cm^r	Dr A.G. Torres CVD, University of Maryland, U.S.A.(Torres *et al.*, 2002)
AGT300lpf1/c	Strain AGT300lpf1- complemented with pBR322*lpfABCC'DE*. Ap^r	Dr A.G. Torres CVD, University of Maryland, U.S.A.(Torres *et al.*, 2002)

Based on homology with the *S. typhimurium lpfABCDE* operon (Baumler and Heffron, 1995;Perna *et al.*, 2001), the first *lpf* - like operon, *lpfABCC'DE* is 5.9 kb in length and corresponds to the 141 O island in the EHEC O157:H7 EDL 933 genome and the second *lpf* – like region, *lpfABCDD'* corresponds to the 154 O island, which is 6.9 kb in length (Perna *et al.*, 2001).

2.1. Storage of bacterial strains

All bacterial strains were stored at -70°C in a Microbank (ProLab Diagnostics, UK). Bacterial cultures were plated onto LB agar and grown overnight at 37°C to check for appropriate colony morphology before use in experiments.

2.2 The *in vitro* organ culture assay (IVOC).

2.2.1 Ethical Approval.

Ethical approval for the taking of human intestinal mucosal biopsies from patients undergoing routine endoscopic procedure was granted to the University Department of Paediatric Gastroenterology, Royal Free and University College Medical School by the local Ethical Committee of the Royal Free Hampstead NHS Trust Hospital. The biopsies were taken under the direction of the clinician in charge after fully informed parental consent was obtained.

2.2.2 Tissue Samples.

Human intestinal biopsies were obtained during routine investigation of paediatric patients for potential intestinal disorders. All procedures were performed using Olympus PCF or Fujinon EG/EC-41 paediatric endoscopes, and took place between 8:30 and 13:00. The patients were being investigated for abdominal pain, chronic diarrhoea, failure to thrive, polyps and inflammatory bowel disease. Proximal (fourth part duodenum or duodenojejunal flexure) and distal (terminal ileum with or without Peyer's patches) small

bowel biopsies were obtained as well as colonic biopsies (caecum, ascending colon, transverse colon, descending colon, sigmoid colon and rectum). The tissue used in this study was biopsied from mucosal regions of the gastrointestinal tract which showed no disease macroscopically and were reported as histologically normal following routine examination by the histopathology department. Thus the availability of normal tissue cannot be guaranteed but is entirely dependent on the individual patient. In the majority of colonoscopies access into the terminal ileum was achieved but this was not certain for every patient as entry through the ileo-caecal valve is a difficult endoscopic manoeuvre. Thus Peyer's patch samples are the more difficult samples to obtain. The tissue samples measured approximately 3mm in diameter. Immediately after biopsy the explants were placed in 5 mls of prewarmed (37°C) and pregassed (95%O_2 5%CO_2) *in vitro* organ culture medium (see appendices). They were then transported to the laboratory for culturing.

2.2.3 Standard eight hour *In Vitro* Organ Culture.

Human intestinal biopsies were cultured as described by Phillips et al (Phillips *et al.*, 2000). Tissue samples were studied using an olympus SZ40 dissecting microscope to screen for good preservation, and intact, non-haemorrhagic, samples were placed on foam squares (approximately 0.25 cm^2) orientated with the mucosal surface facing upwards and incubated in 5cm diameter petri dishes (Merck Sterilin) containing approximately 4ml pre-warmed (37°C) IVOC medium. These dishes were placed on a rocking platform (Biometra) and incubated at 37°C in 95%O_2/5% CO_2 for eight hours. The IVOC adhesion assay was carried out by adding 25µl of bacterial culture to the mucosal surface of the explant. Unless otherwise stated the bacterial strains were grown in 5ml BHI broth in glass bijoux overnight at 37°C without agitation and the amount of bacteria contained in a 25µl aliquot of overnight culture was approximately 4 x10^7. This was verified by colony counts (see section 2.3). Bijoux (Merck) are sterile glass containers that hold up to 6ml of liquid media and were filled with 5ml of media resulting in up to 1ml of air being present during growth of the bacterial strains The medium was changed every two hours using aseptic technique to prevent bacterial overgrowth and the development of an acidic pH. As the medium cannot be completely removed from the foam support re-inoculation is not

necessary. After the eight hour incubation period, the biopsies were removed from their foam supports and washed three to five times in IVOC medium in order to remove mucus and non adherent bacteria. The biopsies were fixed in 2.5% glutaraldehyde pH 7.2, stored at 4°C and processed for scanning and/or transmission electron microscopy.

Each bacterial strain was incubated in the IVOC system at least three times per intestinal region using intestinal explants taken from different patients on repeat occasions. This is to reduce the influence of host variability on the experimental outcome. In addition, each incubation included an uninoculated biopsy sample to check for endogenous bacterial adhesion, and a positive control to check for host susceptibility to infection.

2.2.3.1 Mucus associated bacteria during IVOC.

In order to determine if bacteria were present in the mucus layer of explants during IVOC the explant associated mucus was isolated and assessed for the presence of bacteria using SEM. After the eight hour IVOC (see section 2.2.3) the mucus layer present on the explants was removed by washing in DMEM. The mucus containing DMEM solution was fixed in an equal volume of 2.5% glutaraldehyde for five minutes. It was then filter onto a 13 mm diameter membrane (Agar Scientific) and the membrane was processed for SEM as described in section 2.2.9.

2.2.4 IVOC assay with prolonged incubation time and change in frequency of medium change.

In section 3.2.5.2 assays were used to determine if prolonged incubation of bacteria and explants or changes in the medium change time points could alter adhesion to explants. The IVOC assays carried out in section 3.2.5.2 were based on the IVOC assay described in section 2.2.3 and modified as follows.

2.2.4.1 Medium change.

Two assays involved increasing the time before medium change or washing of the explants following IVOC assay. In the first assay the medium was changed after four hours incubation at 37°C and gassed with 95% O_2/5%CO_2. The explants were washed and processed for SEM after eight hours. In the second assay the medium was not changed throughout the eight hour IVOC and the explants were washed and processed for SEM at the end of the eight hour incubation. The explants were gassed with 95% O_2/5%CO_2 every two hours.

2.2.4.2 Prolonged IVOC assay.

The IVOC assay was performed as described in section 2.2.3. Instead of the assay being carried out for eight hours the medium was changed after eight hours and ten hours of incubation and the explants were incubated for a further ten hours without medium change. Thus making a total IVOC assay time of twenty hours. The explants were then washed and processed for SEM.

2.2.5 Centrifugation of bacteria onto explants.

The results in section 3.2.5.3 describe the effect of centrifuging bacteria onto explants in order to initiate the IVOC assay. This involved using two different systems to centrifuge the bacteria onto the explants.

2.2.5.1 Microfuge tube.

The first system involved using a 1.5ml microfuge tube (Anapore, Whatman). The microfuge tube is divided into two chambers separated by a 0.2μm pore filter. 850μl of IVOC medium was added to the lower chamber of the microfuge tube. 150μl of IVOC medium was placed in the upper chamber. The explants were placed on circular pieces of foam and the foam was then placed on the filter in the upper chamber. Care was taken to

avoid disruption of the explant orientation on the foam support. The upper chamber was then inoculated with bacteria. The microfuge tube was then centrifuged at 1600 rpm for 4 minutes at 37°C (ALC PKR121 R centrifuge). After centrifugation the explant and foam support were removed from the microfuge tube and placed in a 5cm IVOC dish containing pre-warmed (37°C) IVOC medium. The inoculated explants were then incubated for eight hours with a medium change every two hours as described in section 2.2.3. The explants were washed and processed for SEM.

2.2.5.2 Microtitre plate.

In the second system, the microfuge tube was replaced with a 96 well microtitre plate as it was easier to place the explant within the well than in the chamber of the microfuge tube. The explant was orientated onto the surface of a microtitre well. 100µl of pre-warmed IVOC medium was added to the well and it was then inoculated with a bacterial culture. The plate was placed in a centrifuge and centrifuged at 1600 rpm for 4 minutes at 37°C. After centrifugation the explant was removed and placed on a foam support in an IVOC dish containing pre-warmed (37°C) IVOC medium. The inoculated explants were then incubated for eight hours with a medium change every two hours as described in section 2.2.3. The explants were washed and processed for SEM.

2.2.6 Activation of bacteria prior to IVOC assay.

Each bacterial strain was cultured overnight in 5 ml of BHI at 37°C without shaking. The strains were activated by diluting the overnight culture 1:100 in 5ml of DMEM or M9CM followed by incubation at 37°C for four hours without shaking. Activation was observed as an increase in the expression of intimin when compared to intimin expression after overnight growth in BHI. Growth in DMEM has been shown in previous studies to increase the production of LEE encoded virulence factors (DeVinney *et al.*, 1999;Ebel *et al.*, 1998;Jarvis and Kaper, 1996;Knutton *et al.*, 1998;Sperandio *et al.*, 1999). The bacteria were centrifuged at 14,000 rpm for one minute and re-suspended in 50 µl of IVOC medium

immediately prior to the inoculation of the explants, and the IVOC assay was carried out as described in section 2.2.3.

The M9CM is composed of 22mM KH_2PO_4, 48mM Na_2HPO_4, 18.4 mM NH_4CL, 1mM $MgSO_4$, 8. 55mM NaCl and is complemented by the addition of 44mM $NaHCO_3$, 1mM CaCl, 0.4% glucose and 0.1% casamino acids. M9CM has been shown to produce high levels of protein secretion by EHEC (DeVinney *et al.*, 1999) and induce the expression of LEE encoded proteins by EHEC and EPEC(Abe and Nagano, 2000).

2.2.7 Passage of bacteria in BHI in bijoux prior to IVOC.

The results in section 3.2.5.5.2 describe the effect of passage of bacteria prior to IVOC assay.

Small glass bijoux (6ml) were filled with 5ml of BHI broth and used to culture the bacterial strains overnight at 37°C without shaking. The strains were sub-cultured by taking 5µl of the overnight culture and inoculating a fresh 5ml test tube containing BHI broth. After 10, 12 and 14 days passage a 25 µl aliquot of the bacterial cultures was used to inoculate explants according to the IVOC protocol outlined in section 2.2.3.

In addition colony counts (section 2.3) were made of the bacteria prior to IVOC. Passaged bacteria were also tested for their adhesion to HEp-2 cells (section 2.4) and by Western blot for intimin expression (section 2.5).

Colony counts were made of the bacterial cultures after passage for 10, 12 and 14 days in BHI broth in bijoux at 37°C. The colony counts were performed as described in section 2.3.

2.2.8 HEp-2 and IVOC co – incubation assays.

Assays were designed in order to determine if co-incubation of HEp-2 cells with transverse colon would influence adhesion to transverse colon or alter the adhesion phenotype of EPEC and EHEC on HEp-2 cells. In a second set of assays adhesion, to HEp-2 cells before incorporation into the IVOC assay, was used to determine if initial adhesion to HEp-2 cells might influence adhesion to transverse colon explants during IVOC.

Using a 24 well plate HEp-2 cells were seeded, as described in section 2.4, overnight prior to incorporation into the IVOC assay.

2.2.8.1 The T_0 T. colon + HEp-2 assay.

In the first set of assays the HEp-2 cells were washed in DMEM and approximately 200µl of IVOC medium without D-mannose, was added to each well. D- mannose has been used to prevent type1 fimbrial adhesion during adhesion to IVOC and Hep-2 cells (Elliott and Kaper, 1997;Knutton, Lloyd, and McNeish, 1987;Phillips and Frankel, 2000), and was not included as it may prevent the interaction of mannose binding adhesins with the host cells. The transverse colon explants were orientated onto the surface of the 60-80% confluent HEp-2 cells. The medium was adjusted to ensure that the explant would not be submerged in the IVOC medium but that enough medium was present to allow for irrigation of the explant. The explant on the HEp-2 cells was inoculated with 25µl of bacteria and thus the explant and the HEp-2 cells were inoculated at the same time (T_0). This was referred to as the T_0 T. colon + HEp-2 assay. The medium was changed every two hours, during the eight hour IVOC assay, and the medium at each IVOC time point was used to carry out a separate HEp-2 adhesion assay for both 3 and 6 hour time points. After eight hours of co-incubation the explant and the HEp-2 cells were removed from the T_0 T. colon + Hep2 IVOC assay and processed for SEM.

In addition a parallel well, which contained no explant but contained T_0 inoculated HEp-2 cells was incubated for eight hours and the medium at each two hour time point was used

to inoculate HEp-2 cells and acted as control for the well containing HEp-2 cells and an explant. After the eight hour incubation period the HEp-2 cells in this well were stained with phalloidin and viewed using a light microscope in order to determine the adhesion phenotype of the bacteria and their FAS status. Thus a HEp-2 adhesion assay was used to check the adhesive ability of the bacteria throughout the T_0 T. colon + HEp-2 IVOC assay, in the presence or not of an explant. In addition, a well was used to carry out an eight hour IVOC assay using transverse colon without the presence of HEp-2 cells. This explant was processed for SEM as described in section 2.2.10.

A further two wells were used as uninoculated control wells with one containing HEp-2 cells and no explant and the other containing no HEp-2 cells and an explant. The IVOC medium in the well containing the explant was changed every two hours and at the end of eight hours the explant was processed for SEM and analysed. The IVOC medium in the well containing HEp-2 cells was changed very two hours and at the end of the eight hour incubation period the cells were stained with phalloidin and checked for bacterial adhesion by light microscopy.

A standard HEp-2 cell adhesion assay as described in section 2.4.2 was carried in parallel with the assays described above.

2.2.8.2 Adhesion to HEp-2 cells followed by IVOC assay.

In the second set of assays the bacteria were inoculated onto HEp-2 cells for 3 and 6 hour incubation periods and then incorporated into the 8hr IVOC assay. The bacteria were inoculated onto HEp-2 cells as described in section 2.4. After 3 and 6 hour incubation periods the 1ml of medium containing bacteria and HEp-2 cells was removed and added to approximately 3ml of IVOC medium. This medium was used to inoculate transverse colon explants and initiate an IVOC assay as described in section 2.2.3. A control well was used to determine the adhesion pattern and FAS test of the bacteria added to the IVOC assay.

Each assay also included a negative control assay which was identical to the assays described in this section but involved no inoculum.

2.2.8.3 The use of supernatants from the IVOC medium of E2348/69 and 85-170lpf1- to influence the adhesion of 85-170 to duodenal explants.

IVOC was carried as described in section 2.2.3 using duodenal explants and the bacterial strains E2348/69, 85-170lpf1- and 85-170. At each two hour time point the IVOC medium from the E2348/69 and 85-170lpf1- incubations were filtered using a 0.2 μm micropore filter and the supernatant was used as the IVOC medium for the incubation of two separate eight hour 85-170 duodenal IVOC assays. Thus the IVOC medium from the two hour E3248/69 and 85-170lpf1- time points were used to incubate the 0 – 2 hour incubation period of the 85-170 IVOC assays and the IVOC medium from the four hour time points were used to incubate the 85-170 IVOC assays during the 2 – 4 hour time incubation period and this was continued until both IVOC assay had completed their eight hour incubation period. The explants were processed for SEM as described in section 2.2.9

2.2.9 Processing of IVOC explants for scanning electron microscopy (SEM).

The glutaraldehyde fixed explants were washed in two changes of cold (4°C) O.1M phosphate buffer containing 3% sucrose (w/v) over 90 minutes. The explants were then post-fixed in 1% aqueous osmium tetroxide for 90 minutes, washed three times in distilled H_2O over 10 minutes, and dehydrated through three 3 minute changes in 2,2-dimethoxypropane and transferred to100% ethanol with two changes over 10 minutes. The explants were then critically point dried using liquid CO_2 and an Emitech K850 critical point drier. The explants were mounted onto 10mm aluminium stubs (Agar Scientific) using silver paint and sputter coated with gold/palladium using a Polaron E5100 series II sputter coater. This provided a conductive surface over the specimen and base. The explants were then examined using a JEOL JSM 5300 SEM at an accelerating voltage of 30 kV.

2.2.10 SEM and TEM photography.

SEM photographs were taken using Ilford 120 FP4 film.

AGFA Scientia 6.5 x 9cm EM film was used for TEM. The TEM samples were examined in a JEOL JEM 1200-EX II transmission electron microscope at an accelerating voltage of 80kV.

Photographs were printed on Ilford multigrade paper using a Durst enlarger. SEM and TEM micrographs were scanned into Adobe photoshop 5.5 using a Hewlet Packard scanner (scanjet 5470c), and brightness and contrast were optimised for printing within Microsoft Word.

2.3 Colony counts.

Bacteria were cultured overnight at 37°C in BHI Broth and serially diluted in LB broth. 10 µl of bacterial suspension was then applied in triplicate to LB plates and grown overnight at 37°C. The colonies were counted and an average of the three results calculated and given as colony forming units (CFU).

2.4 HEp-2 cell culture.

HEp2 cells were isolated from tumours produced in irradiated cortisonised weanling mice after injecting with epidermoid carcinoma tissue from the larynx of a 56 year old male (American type culture collection) (Moore, Sabachewsky, and Toolan, 1954).

2.4.1 Storage and Maintenance of HEp-2 Cell Cultures.

HEp-2 cells are maintained in culture medium which consists of Dulbecco's modified Eagle's medium (DMEM) with 10% foetal calf serum (Sigma), 2mM L – glutamine, 50 000 units of penicillin and 50 mg streptomycin per litre (Sigma). The flask containing the HEp-2 cells were incubated at 37°C in a 5% CO_2 incubator (Sanyo).

HEp-2 cells were monitored using an Olympus CK2 inverted microscope and were split when confluent.

To split HEp-2 cells the HEp-2 culture medium is poured off from a 75cm^2 flask (Triple Red) and 5 ml 0.01M PBS pH 7.4 (Sigma) is used to rinse the flask. 2-3 ml prewarmed 0.1% trypsin/0.02%EDTA is added to the flask making sure to cover the cell surface. The flask is placed flat in a 37°C 5% CO_2 incubator for 4 – 5 minutes until the cells begin to lift off the surface. As much trypsin/EDTA solution as is possible is removed without losing the HEp-2 cells and the HEp-2 cells are resuspended in HEp-2 cell culture medium. The cells are counted using a haemocytometer and x10 inverted microscope objective. The cells are then used to seed 75cm^2 flasks for maintenance (approximately 10^6 cells/ml) microtitre wells for HEp-2 assays or cryo vials for storage.

HEp-2 cell aliquots containing approximately 10^6 cells/ml of HEp-2 medium are stored in liquid N_2. The aliquots are prepared by splitting the HEp-2 cells with 0.1%trypsin/0.02%EDTA (Sigma) and freezing to –70°C using the Mr Frosty system (Nalgene). For freezing after splitting the HEp-2 cells the HEp-2 cells are centrifuged at 1000 rpm for 10 minutes to remove the trypsin/EDTA solution. The HEp-2 cells are resuspended in chilled (4°C) 1ml dimethyl sulfoxide/1ml of foetal calf serum/8ml of HEp-2 medium per flask of cells. This solution is divided into 2ml aliquots and placed in the Mr Frosty container. The Mr Frosty container provides a constant cooling rate of 1°C/minute using vial holders surrounded by 100% isopropyl alcohol. Once the aliquots have reached –70°C they are then placed in liquid N_2.

HEp-2 cells are seeded from aliquots stored in liquid N_2. An aliquot is removed from the liquid N_2, wrapped in parafilm(Jencons) and sprayed with 70% industrial methylated spirits. The aliquot is then placed in running water for five minutes until it is completely thawed. The thawed cells are placed into 30ml of culture medium and centrifuged at 1000 rpm for 10 minutes to allow removal of the dimethyl sulfoxide (Sigma). The pellet is resuspended in 15 ml of HEp-2 medium and centrifuged at 1000 rpm for 10 minutes. This pellet is resuspended in 2ml of HEp-2 culture medium and used to inoculate a 75 cm^2 tissue culture flask containing between 25 and 30 ml of HEp-2 culture medium. The cells are maintained at 37°C with 5% CO_2. The HEp-2 cells are split once confluent.

2.4.2 HEp-2 cell assay.

HEp-2 cell monolayers were established on 24 well microtitre plates (Triple Red) with each well containing a 13 mm glass coverslip. The cells were seeded at a density of 5 x 10^6 cells/ml of HEp-2 medium and incubated for 24 hrs at 37°C with 5% CO_2 (Sanyo CO_2 incubator). This resulted in monolayers which were between 60 and 80% confluent. Prior to each assay the wells were washed three times with DMEM, to remove the antibiotics contained in the HEp-2 culture medium, and replaced with 1ml of DMEM with or without 0.5 % D- Mannose (Sigma). The wells were then inoculated with 25 µl of an overnight culture of bacteria grown in BHI broth (approximately 4 x 10^7 bacteria) and incubated for 3 and 6 hrs at 37°C with 5% CO_2. After 3 hours the bacteria were removed. For the 6 hour adhesion assay 1ml of fresh DMEM, with or without D-mannose, was (tense changes from first sentences) added to each well after removal of the medium present in the wells after three hours of incubation, and the cells incubated for a further 3 hrs. Once the infection period was completed the wells were washed three times with PBS to remove non-adherent bacteria. The cells were then fixed with 4% formalin/PBS pH7.4 for twenty minutes at room temperature. Once fixed the cells were washed three times in PBS and permeabilised with 0.1% Triton- X 100/PBS for four minutes. The cells were washed three times in PBS to remove detergent and stained with FITC phalloidin (Sigma) or FITC anti – phosphotyrosine (Sigma) antibody.

2.4.2.1 HEp-2 cell adhesion patterns.

The pattern of adhesion was determined under phase contrast light microscopy. The formation of microlonies on the Hep-2 cells by bacteria is termed localised adherence or LA (Nataro *et al.*, 1985;Nataro *et al.*, 1987a). Bacterial strains adhering with an LA pattern after the 3hr incubation period were termed LA. Those strains with an LA pattern after the 6hr incubation period were termed LA like or LAL (McKee and O'Brien, 1995). LAL bacteria may form occasional microcolonies after three hours of incubation but they are much less frequent than the number produced by E2348/69. Bacterial strains which adhered but did not produce microcolonies or the LA pattern after the 6hr incubation period were termed poorly adherent or PA. Bacteria adhering with the PA phenotype are few in number, but may on occasion form small groups of bacteria numbering less than five bacteria and these may be nascent microcolonies. The EPEC O127:H6 strain E2348/69 was used as a reference point when assigning the LA (Nataro *et al.*, 1987b) pattern to other bacterial strains and the EHEC O157:H7 strain 85-170 was used as a reference point when assigning the LAL pattern of adhesion to other bacterial strains. The term LAL/PA was used to describe bacteria that do not form as many microcolonies as the EHEC strain 85-170 on Hep-2 cells after six hours of incubation, but some microcolonies are present.

2.4.2.2 FAS test and antibody staining

HEp-2 cells assays were prepared as described in 2.6.2 and stained once permeabilised.

The FAS test was carried out as described by Knutton et al (Knutton *et al.*, 1989). Permeabilised HEp-2 cells are incubated for twenty minutes with 5 μg/ml FITC Phalloidin (Sigma) at room temperature. Phalloidin binds to filamentous actin and is used to recognise actin recruitment at the site of bacterial adherence by both EPEC and EHEC. The nucleation of actin by bacteria to form filamentous or F-actin, which is stained by FITC conjugated phalloidin, allows the bacteria to be divided into fluorescent actin staining (FAS) positive or FAS negative bacteria. After staining the cells were washed in sterile 0.01 M PBS pH 7.4 three times for one minute and mounted onto glass slides using

citifluor (Agar Scientific) or vecta shield (Dako Ltd) mountant. The cells were examined using a Zeiss UV microscope fitted with a Zeiss Axiocam digital camera system (Image Associates, UK).

In order to visualise the phosphorylation of tyrosine residues within Tir, permeabilised HEp-2 cells were incubated with anti-phosphotyrosine antibody at 1/50 dilution for 1 hour at room temperature. The cells were then washed five times in PBS and incubated with the secondary antibody, a swine anti-rabbit FITC conjugated IgG (Sigma) diluted 1/50 in PBS, for forty five minutes at room temperature. The cells were then washed five times in PBS and mounted on glass slides using citifluor or vecta shield mountant.

2.4.2.3 Passage and standing broth cultures (not passaged) of bacteria prior to HEp-2 cell assay.

The cultures were grown in BHI broth at 37°C without shaking.

The bacteria were passaged for 10, 12 and 14 days by subculturing 5 µl of an overnight bacterial culture into a fresh 5ml broth culture. Standing broth cultures (NP) involved inoculating 5ml of BHI broth and leaving in standing culture for 10 days. Both glass bijoux (6ml volume with 5ml broth and 1ml air) and plastic universals (25ml volume with 5ml broth and 20 ml air) were used. The bacterial cultures were then tested for their adherence to HEp-2 cells and their ability to nucleate actin. The HEp-2 assay and FAS test were carried out as described in section 2.4.2 except that D-mannose was removed from the incubation medium to assess the interactions of type 1 fimbriae or other mannose binding adhesins.

The HEp-2 cell assay was carried out in triplicate and on three separate occasions. On each occasion new bacterial isolates were used.

2.5 Western blot.

Bacteria were grown in BHI broth overnight at 37°C without agitation. For activation in DMEM the broth culture was then diluted 1/100 in DMEM and incubated at 37°C for 4 hours. The optical densities (O.D.), at λ 600 nm, of the bacterial cultures were then adjusted to yield equal absorbencies. Bacterial cultures with or without activation in DMEM were centrifuged at 14000 rpm for one minute and the pellet resuspended in 2x loading buffer (100 mM Tris – HCl pH 6.8, 200mM dithiothreitol, 4% sodium dodecyl sulphate, 0.2% bromophenol blue, 10% glycerol). An equivalent to an O.D. of 0.4 (λ 600 nm) was then loaded onto a 7.5% SDS polyacrylamide gel. Prior to loading the samples were boiled for five minutes. The electrophoresed polypeptides where then transferred onto a nitrocellulose membrane (0.2μm pore, Hybond ECL, Amersham). The membrane was blocked in 5% skimmed milk/PBS (pH 7.4) Triton X-100 0.05% solution for one hour when probing for intimin expression or the membrane was blocked in 1% BSA (Sigma) for 1 hour when probing for type 1 fimbriae or long polar fimbriae expression. After blocking the nitrocellulose membrane was washed three times for five minutes in PBS/Tween 0.05%.

In order to detect intimin expression in bacterial whole cell lysates the membrane was then incubated at room temperature for one hour with a 1/1000 dilution of a rabbit anti-intimin antibody raised against the conserved residues present in all intimin types (residues 388 – 667) (Batchelor *et al.*, 1999). In order to detect type 1 fimbriae the membranes were incubated for 1 hour with a 1/1000 dilution of rabbit anti – type 1 fimbriae antibodies (kindly supplied by J.A. Giron Center for Vaccine development, University of Maryland, U.S.A. and by C. Toma Department of Bacteriology, Faculty of Medicine, University of the Ryukyus, Okinawa, Japan). Long polar fimbriae were detected by incubating the membranes with a 1/1000 dilution of a rabbit anti – LPF antibody for 1 hour (Torres *et al.*, 2002). The membrane was then washed three times for five minutes in PBS/Tween 0.05% and swine anti - rabbit alkaline phosphatase conjugate (Sigma) was applied, for forty five minutes, to the membrane to detect binding of the primary antibody. The membrane was developed with NBT/BCIP (Sigma) in distilled H_2O.

Activation in DMEM prior to probing for intimin expression was carried out as described in previous reports (Adu-Bobie *et al.*, 1998;Knutton *et al.*, 1997;Reece *et al.*, 2001). Growth of EPEC and EHEC in shaking or standing cultures to an O.D. of between 0.4 and 1.0 (mid to late log phase) yields cultures expressing a high degree of intimin and secreted proteins (DeVinney *et al.*, 1999;Ebel *et al.*, 1998;Jarvis and Kaper, 1996;Knutton *et al.*, 1998;Sperandio *et al.*, 1999).

2.5.1 Intimin expression during IVOC.

The IVOC medium was probed for bacterial expression of intimin during IVOC. The western blot was carried out as described in section 2.5 with the following modifications. The amount of bacteria applied to the polyacrylamide gel varied from the protocol outlined in section 2.5. At each two hour time medium change and at the end of the eight hour IVOC assay, 4ml of IVOC medium containing bacteria was removed from the explant culture dish and centrifuged at 14000 rpm for one minute. The bacterial pellet was re-suspended in 2x loading buffer as described in section 2.5 and the samples were kept on ice before using them to probe for intimin expression.

The bacterial cultures probed for intimin expression during IVOC were adjusted to an O.D. of 0.1 before loading onto the polyacrylamide gel (Knutton *et al.*, 1997). The western blot was carried out as described in section 2.5.

Due to the limited availability of intestinal explant tissue, intimin expression during IVOC assay was verified using only the EPEC O127:H6 strain E2348/69 and the EHEC O157:H7 strain 85-170. These two strains were tested for intimin expression during their incubation with duodenal, PP and transverse colon explants.

2.5.2 Western blot of intimin expression after growth in M9CM.

The bacteria were grown overnight in BHI at 37°C and were diluted 1/100 in M9CM (see section 2.2.3.3) and grown for four hours in standing culture at 37°C. A western blot was then carried out as described in section 2.5.

2.5.3 Western blot of bacteria passaged for 10, 12 and 14 days in BHI broth in bijoux and universals.

Bacterial strains were grown in BHI broth and incubated at 37°C without shaking.

The strains were tested for intimin expression by western blot after passage for 10, 12, and 14 days in BHI broth in bijoux (6ml volume with 5ml broth and 1 ml air) and universals (25ml volume with 5ml broth and 20ml air). Passage involves subculturing a 5 µl of an overnight bacterial culture into 5ml of a fresh broth culture. The western blots were carried out as described in section 2.5 and the passaged cultures were tested for intimin expression before and after activation in DMEM. For the detection of intimin after passage in BHI broth an equivalent of an OD_{600} of 0.4 was loaded onto a polyacrylamide gel.

The strains were tested for intimin expression after passage on two separate occasions and for each time point.

2.6 Construction of an *eae* deletion mutant of EHEC O157:H7 strain 85-170.

The construction of the ICC170 strain was carried out in the laboratory of Professor G. Frankel, Centre for Molecular Microbiology and Infection, Imperial College, London, U.K. A summary of its construction is outlined below.

In order to introduce a deletion mutation into the *eae* gene two unique *Nru*I restriction enzyme cleavage sites in pCVD444 were used (Yu and Kaper, 1992), which are located in

coding region of the gene (figure 2.1). Following digestion and gel purification the plasmid was self ligated, resulting in a plasmid which contains an 1873bp deletion in the *eae* gene (figure 2.1). Using PCR primers EAE1F (5' TCTATTCCCGGGAATGAAAACAGATTGTGTTCTTTTGC 3'; positioned at 16001 to 16037 bp of the LEE region from EHEC O157:H7) and EAE2R [5' AGAACATTCCCGGGTACATTTCAGCAGATATTTTTCCC 3', positioned at 19759 to 19722bp of the LEE region (accession number AF071034)], containing a 5' *Sma*I site (underlined), the deleted *eae* gene and flanking DNA was recovered on a 1885bp fragment. This fragment was gel purified and ligated into a *Sma*I digested suicide vector, pCVD442. The recombinant pCVD442 was then used to electroporate EHEC O157:H7 *eae* positive strain, 85-170. Ampicillin (100 $\mu g\ ml^{-1}$) resistant colonies were isolated and then plated onto 10% Sucrose L-agar plates, grown overnight at 30°C, in order to select for double-crossover isolates. Ampicillin sensitive clones containing the *eae* deletion were identified by PCR amplification using primers EAE1F and EAE2R. A PCR product of 1885bp, expected from the *eae* deleted clones, was obtained from over 50% of the sucrose resistant and ampicillin sensitive colonies. The rest of the colonies produced a PCR product of 3758bp fragment similar to the wildtype strain.

Figure 2.1 Construction of an *eae* deletion mutant of EHEC O157:H7 strain 85-170.

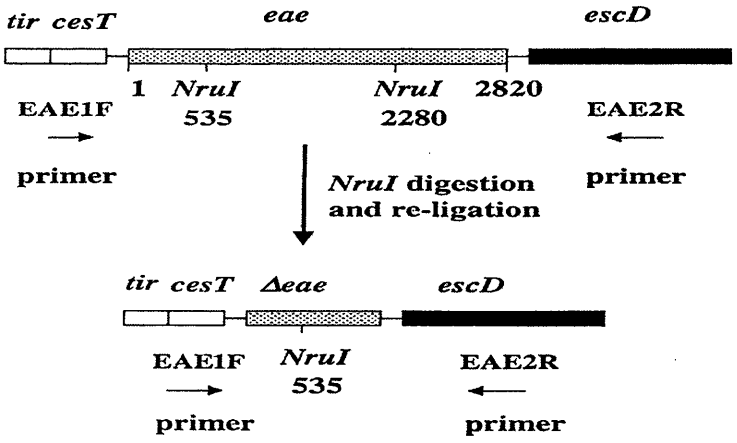


Figure 2.1 shows the deletion of the *eae* gene located in the LEE of 85-170.

2.6.1 The generation of ICC170 strains expressing different recombinant intimin proteins.

Confirmation of *eae* deletion in 85-170 was achieved by PCR. The *eae* mutation in ICC170 was complemented *in trans* with pCVD438, encoding intimin α from EPEC.(Donnenberg and Kaper, 1991). In addition, ICC170 was complemented, as controls, with pCVD438/01 encoding a biologically inactive form of intimin α in which cysteine (Cys, C) at position 937 was replaced with serine (Ser) (Frankel *et al.*, 1998), or pICC55, a pCVD438 derivative containing the receptor-binding domain of intimin γ □on a cloned intimin α backbone.(Phillips and Frankel, 2000;Hartland *et al.*, 2000) Expression of the different intimins was determined by Western blot analysis of whole cell lysates prepared from the ICC170 derivatives using a universal, broad-spectrum polyclonal intimin anti-serum, reactive with all the different intimin types (Batchelor *et al.*, 1999) as described in section 2.5. Lysates from all the recombinant strains, but not from ICC170, reacted similarly with the antiserum.

2.7 DNA Sequencing of Intimin from O55:H6 Strain G21.

Genomic DNA from G21 was isolated using the DNAeasy tissue kit (Qiagen), according to manufacturer's instructions. The 3' end of the *eae* gene encoding Int280α from O55:H6 was amplified by PCR using high fidelity *Pfu* DNA polymerase (1 cycle of 94°C, 1 min, followed by 25 cycles of 94°C, 30 secs, 50°C, 1 min, 74°C, 1min, followed by 1 cycle of 74°C for 5 mins) (forward primer 5'-GGAATTCATTACTGAGATTAAGGCT-3'; reverse primer 5'-CGGGATCCTTATTTTACACAAGTGGC-3'). The amplified DNA and the plasmid pMAL-c2 were cut using the restriction enzymes *EcoR1* and *BamH1*. The amplified DNA was inserted into cut pMAL-c2 by ligation with T4 DNA ligase. pMAL-c2 was transformed into *E. coli* XL1-Blue cells. Ampicillin (100 μg ml^{-1}) resistant transformants were randomly selected and screened for the appropriate insert. XL1-blue colonies containing the recombinant plasmid were grown overnight in LB broth, the plasmids isolated using the plasmid miniprep kit (Qiagen) and ethanol precipitation. Ethanol precipitation was carried out by re-suspending the plasmid DNA in 3M sodium

acetate pH5.2 at a 1/10 dilution. Two volumes of 100% ice cold ethanol were added and the sample left at -20°C for 30 minutes. The sample was then centrifuged at 15,000 rpm for ten minutes at 0°C and the ethanol poured off. The plasmid DNA was re-suspended in 70% ethanol, centrifuged for 2 minutes at 15000 rpm and the ethanol removed. The plasmid sample was left to dry before sequencing. DNA sequencing was performed using 1- 2μg of plasmid DNA and the Int 280 forward and reverse primers (Reece *et al.*, 2001). The sequencing was carried out by the Sequencing Centre, Department of Biological Sciences, Imperial College London.

2.7.1 Site directed mutagenesis of the carboxy terminal intimin α from O55:H6 strain G21.

Site-directed mutagenesis of intimin α was performed using the Quick-Change Site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions. Plasmid pCVD438 encoding intimin α (Donnenberg and Kaper, 1991) was used as a template. A complimentary mutagenesis oligonucleotide pair incorporating a single amino acid substitution was designed.

Forward Primer 5' CAGCTCAAGATGTGAAGAGTGGTGTTGC 3'

Reverse Primer 5' GCAACACCACTCTTCACATCTTGAGCTG 3'

Mutated plasmid containing staggered nicks was generated by extension of primers annealed to opposite strands of the denatured plasmid by temperature cycling in the presence of the high fidelity *Pfu* DNA polymerase. Synthesised DNA containing the desired mutation was selected from the original DNA template by incubation with the enzyme *Dpn*1 at 37°C for 1 hour, which cleaves the parental DNA at *dam* methylated GATC sequences leaving the unmethylated newly synthesised mutated plasmid intact. Nicks in the plasmid were repaired after transformation of 1 μl of the synthesised DNA into competent *E. coli* XL1-Blue cells. Chloramphenicol resistant transformants were randomly selected and grown overnight in LB (Sigma). Plasmid DNA was isolated using

the plasmid miniprep kit (Qiagen) and ethanol precipitation. Ethanol precipitation was carried out by re-suspending the plasmid DNA in 3M sodium acetate pH5.2 at a 1/10 dilution. Two volumes of 100% ice cold ethanol were added and the sample left at -20°C for 30 minutes. The sample was then centrifuged at 15,000 rpm for ten minutes at 0°C and the ethanol poured off. The plasmid DNA was re-suspended in 70% ethanol centrifuged for 2 minutes at 15,000 rpm and the ethanol removed. The plasmid sample was left to dry before sequencing. Correct incorporation of the mutation was monitored by DNA sequencing using an automated DNA sequencer. The mutated plasmid was then transformed into *eae* deletion mutants of EPEC, strain CVD206 (Donnenberg and Kaper, 1991), and EHEC, strain ICC170.

2.7.2 Generation of electrocompetent CVD206 and ICC170 strains and transformation with pCPG2.

CVD206 and ICC170 were grown overnight in LB at 37°C. Both strains were diluted 1/100 in LB and grown for 3 hours with shaking (250 rpm) at 37°C. The strains were centrifuged at 4,000 rpm for 5 minutes at 4°C and washed three times in ice cold H_2O. 1µl of plasmid DNA was added to 60µl of electrocompetent cells and left on ice for 2 hours. The samples were electroporated (Biorad electroporator, at 1.75V) and placed in 1ml of LB and grown for 1 hour at 37°C with shaking (250 rpm). The samples were plated out onto LB plates containing chloramphenicol (30µg ml^{-1}) as pCVD438 contains a chloramphenicol resistant cassette. Colonies were picked and assessed for expression of intimin by western blot as described in 2.5.

2.8 Protein representations using the RasMol program.

The RasMol program version 2.6 was used to generate diagrammatic representations of the structural data for intimin α and the intimin-Tir complex. The RasMol program was downloaded from the following link http://www.openrasmol.org/ and this web page provided information for familiarisation with RasMol. In order to view proteins of interest the structural data was downloaded from the NCBI database at the following web page

http://www.ncbi.nlm.nih.gov/Structure/. The protein database was searched for intimin structures and the files marked 1F02 and 1F00 were selected as these were files based on the X-ray crystallography of intimin α 280 and contained information about the two IgSF domains, D1 and D2, as well as the CTLD, D3 (Luo *et al.*, 2000). The files were saved in the PDB format. After viewing the structure the display drop down menu was used to select the cartoon option and the colour menu was used to select the structure for colouring. This allowed the α-helices (red) and the β pleated sheets (yellow) and the hairpin turns (grey) to be viewed in different colours. In order, to select and colour a residue, using the RasMol command line window, the simplest form of instructions in the RasMol Command Line window were as follows:

RasMol>select 907

RasMol>colour 907 green

Other colours can be chosen and these can be found in the RasMol user manual at the following web page http://www.openrasmol.org/doc/rasmol.html#shapelycolours. The files were saved as GIF files and later converted to BMP files for display purposes.

2.9 The use of the SIM alignment tool for protein sequences.

The SIM alignment tool for protein sequences was found at the following web page http://us.expasy.org/tools/sim-prot.html. The sequences were retrieved from the NCBI protein database which can be searched at the following web page http://www.ncbi.nlm.nih.gov/. Percentage sequence identity was noted, and the bases of interest aligned in the primary sequence and the test sequence were noted.

2.10 Transmission electron microscopy (TEM).

2.10.1 IVOC samples.

Following post-fixation in 1% aqueous osmium tetroxide and washing in distilled water(see section 2.2.9), IVOC specimens were dehydrated in 2,2-dimethoxypropane (DMP) with three changes over nine minutes and embedded in freshly made, medium hardness type TAAB resin (TAAB resin 50ml, DDSA 26 ml, MNA 22, BDMA 2ml) (TAAB Equipment Ltd, UK). Specimens were initially placed in a 50:50 TAAB resin/DMP mixture for 30 minutes at room temperature followed by placing in fresh 50:50 resin/DMP mixture for another 30 minutes. The specimens were then placed in 100% resin for two hours with the resin being replaced by fresh resin after 1 hour. Specimens were then treated with three changes, of 3 minutes each, of fresh resin at 90°C, placed in resin in a preheated silicon mould (1 hour at 60°C), orientated and allowed to polymerise at 60°C for 24 hours.

Semi-thin (0.5 μm) sections of embedded tissue were taken using a Reichart-Jung Ultracut E ultramicrotome using freshly prepared glass knives on each occasion, stained with 1% toluidine blue in 1% aqueous sodium tetraborate and examined under light microscopy. Ultrathin (0.1 μm) sections, of selected regions, were taken onto 200 mesh copper/rhodium grids and double stained with 2% aqueous uranyl acetate and lead citrate (Reynolds 1963), a staining technique which provides a uniform increase in tissue contrast. These sections were examined in a JEOL JEM 1200-EX II transmission electron microscope at an accelerating voltage of 80kV.

2.10.2 Negative staining electron microscopy.

2.10.2.1 Growth of bacterial strains prior to negative staining.

The EPEC and EHEC strains listed in table 3.1 were grown in BHI broth (Oxoid), LB (Sigma), LB agar plates (Agar N°1, Lab M), LB agar slopes and DMEM at 37°C without shaking. They were grown over varying time periods: overnight (approximately 18 hours) and passaged for 10 days. Passage involves subculturing 5 µl of overnight bacterial culture into 5ml of fresh broth culture. BHI cultures were performed in glass bijoux (6ml volume with 5ml broth and 1ml air) and universals (25ml volume with 5ml medium and 20ml air). DMEM cultures were performed in universals.

2.10.2.2 Negative staining protocol.

After growth of the bacterial cultures, formvar/carbon coated copper/rhodium 100 mesh TEM grids were floated coated surface down on a drop of overnight bacterial culture for 1 minute. A wooden toothpick was used to remove the grid from the drop and to hold one edge against filter paper to remove excess fluid. The grid was air dried for 1 minute and then placed on a drop of 1% aqueous ammonium molybdate for 10 seconds prior to removal and drying with filter paper as before. The wooden toothpick was disposed of after each sample to ensure no cross-contamination. Grids were air dried and examined using a JEOL JEM 1200-EX II transmission electron microscope at an accelerating voltage of 80kV.

Each bacterial culture was examined in duplicate on three separate occasions.

Negative staining was used to visualise the surface structures expressed by EPEC and EHEC. Fimbriae and flagella were assessed visually based on previous morphological reports. Fimbriae were seen as hollow rod-like structures and were distinct from flagella. Type 1 and 2 fimbriae are 7 – 8 nm wide and type 3 fimbriae are 4 – 5 nm wide. Type 1, 2 and 3 fimbriae may be approximately 0.5 - 2µm long and are peritrichous(Old, 1972).

Type 4 (IV) fimbriae are 10 – 20 μm long and are polar. Long polar fimbriae (LPF) are similar to type 1 fimbriae in gene sequence. They are 2 – 10 μm long and are polar when the *lpf* operon from *S. typhimurium* is expressed in the non – fimbriated *E. coli* strain ORN172, and are peritrichous when the lpf – like operon from EHEC O157:H7 is expressed in ORN172 (Baumler and Heffron, 1995;Torres *et al.*, 2002). Flagella are not rigid rod - like structures and are wider and longer than fimbriae as well as being peritrichous.

The size of the surface structures was estimated by direct measurement from printed micrographs using a measuring magnifier (x 7) fitted with a 20mm graticule (Polaron, UK).

2.10.2.3 Negative staining of bacteria during IVOC.

IVOC was carried out as described in section 2.2.3, except that due to availability of tissue the IVOC was only carried out on one occasion. The IVOC medium was removed at each two hour time point during the eight hour IVOC and bacteria were stained according to the negative staining protocol described in section 2.10.2. The bacteria were stained in triplicate and the grids observed using a JEOL JEM 1200-EX II transmission electron microscope at an accelerating voltage of 80kV.

2.10.3 Immunogold labelling of bacterial fimbriae.

After overnight growth of the bacterial cultures 1ml of the bacterial was centrifuged for 5 minutes at 1500 rpm, re-suspended in sterile phosphate buffered saline (PBS) and centrifuged for 5 minutes at 1500 rpm. The pellet was re-suspended in 100μl of PBS. Formvar/carbon coated 100 mesh nickel grids were placed coated side downwards on the surface of 50μl of bacterial suspension for 1 minute. Handling of the grids was performed using wooden toothpicks as before (section 2.10.2.2). The grids were air dried for 1 minute and blocked by placing on a drop of 2.5% bovine serum albumin, made up in 0.01 M PBS pH 7.4, for 90 seconds. The grids were washed three times in 0.01 M PBS pH 7.4

and once in H_2O making sure that the grids remained wet. Excess H_2O was carefully removed using blotting paper and the grids were incubated with the primary antibody at 1:20 dilution in 0.01 M PBS pH 7.4, for 30 minutes at room temperature. The grids were washed three times in PBS and once in H_2O and the excess H_2O was removed using blotting paper. The grids were incubated with 1:20 swine anti rabbit IgG 10nm particle gold conjugated secondary antibody made up in 0.01 M PBS pH 7.4 for 30 minutes. The grids were washed three times in PBS and once in H_2O and the excess H_2O was removed using blotting paper and then they were stained in 1% ammonium molybdate for 10 seconds. Excess stain was removed using blotting paper and the grids were allowed to air dry before viewing in a JEOL 1200 EXII TEM at an accelerating voltage of 80kV.

Grids were always prepared in duplicate and the 2.5% BSA blocking step was removed in certain incubations and the incubation of the primary antibody was increased to 1 hour.

The primary antibodies used were: anti – type 1 fimbriae antibody raised in rabbits against the type 1 fimbriae of non-pathogenic *E. coli* K-12. This antibody was a kind gift from J.A. Giron, Center for Vaccine Development, University of Maryland, Baltimore, U.S.A. anti – type 1 fimbriae antibody raised in rabbits against the type 1 fimbriae of UPEC strain LE17 (Enami *et al.*, 1999). This was a kind gift from C. Toma, Department of Bacteriology, Faculty of Medicine, University of the Ryukyus, Okinawa, Japan.

anti- lpfA antibody raised in rabbits produced by Zymed Laboratories , Inc., and supplied as kind gift from A.G. Torres, Center for Vaccine Development, University of Maryland, Baltimore, U.S.A.

2.11 The yeast and guinea pig red blood cell agglutination assay.

The assay for the agglutination of yeast and guinea pig red blood cells (GPRBC) was based on the assays described in (Enami *et al.*, 1999;Martinez *et al.*, 2000;Roe *et al.*, 2001) respectively.

For both assays the bacterial strains were either grown overnight, sub-cultured into fresh medium daily (passaged) or left in standing culture (no passage [NP]) for 10, 12 or 14 days in BHI broth (Oxoid) in sterile bijoux or universals. Bijoux (Merck) are sterile glass containers that hold up to 6ml of liquid media and were filled with 5ml of media resulting in up to 1ml of air being present during growth of the bacterial strains. Universals (Merck) are 25ml sterile plastic containers that were filled with 5ml of liquid media resulting in up to 20 ml of air being present during bacterial growth.

All bacterial strains were grown without agitation at 37^0C prior to use in the agglutination assay.

A 1% baker's yeast (Sigma) solution and 2% washed GPRBC (Sigma) solution was made up in sterile 0.01M PBS pH7.4.

For each bacterial strain after growth at 37°C, a 20μl aliquot was removed from the culture using sterile technique and applied to a glass slide. A 20 μl aliquot of the 1% yeast or 2% GPRBC solution was applied to the bacterial aliquot on the slide. The two solutions were mixed by rotating the slide gently for 2 minutes prior to determining if agglutination was present.

3.0 Adhesion of EPEC and EHEC to Cultured Human Intestinal Mucosae.

3.1 Introduction.

It has been shown by Phillips et al (Phillips *et al.*, 2000b), using IVOC and electron microscopy, that a strain of EHEC O157:H7 produces A/E lesions on intestinal mucosae and adheres preferentially to the Peyer's Patches of the distal small intestine. Cell lines have been used to reproduce A/E lesions *in vitro* and have been used to describe key factors involved in the adhesion of EPEC and EHEC to eukaryotic cells. However, the complete mechanism involved in adhesion to intestinal mucosae and A/E lesion formation, by EPEC and EHEC has yet to be described. IVOC may be used to provide a more complex template for EPEC and EHEC adhesion than cell lines due to the presence of fully differentiated enterocytes selected from various regions of the intestinal tract, a mucus and glycoclayx layer, and the presence of intestinal flora. Thus IVOC may be used to describe adhesion mechanisms which are critical to A/E lesion formation on intestinal mucosae.

Initially this section will seek to assess whether preferential adhesion to PP explants is a characteristic of EHEC O157:H7 strains by repeating the work carried out by Phillips et al using additional EHEC and extending the assessment of tissue tropism to include other areas of the intestinal tract. In addition, EHEC strains expressing intimin β and intimin ϵ, and not the intimin γ associated with EHEC O157:H7 strains, will also be used to determine whether they can produce A/E lesions on intestinal mucosae during IVOC and to define their tissue tropism. An atypical EPEC strain will also be included. The HEp-2 adhesion assay will be used to highlight any possible differences between the EPEC and EHEC strains and compare the adhesion pattern of the strains to the localised adhesion produced by the prototype EPEC strain E2348/69. The FAS test will be used to determine the ability of the strains to produce A/E lesions on HEp-2 cells.

In the study carried out by Phillips et al no adhesion of EHEC O157:H7 to transverse colon was observed using the IVOC assay. Since it has been suggested that EHEC are colonic pathogens (Nataro and Kaper, 1998), as well as causing pathology in the colon, IVOC will be used to determine the ability of the EPEC and EHEC strains to adhere to other regions of the large intestine. In addition, other factors which might influence adhesion of EHEC

EHEC to the intestine will be considered. These include the incubation time of the IVOC assay, the effect of D-mannose in the IVOC medium assessed, bacterial activation prior to inoculation of the IVOC assay and explant – HEp-2 coincubations carried out in order to determine whether adhesion to the transverse colon by EHEC O157:H7 strains can be induced.

In this chapter we hope to address questions pertinent to the adhesion of pathogenic *E. coli* to the intestinal mucosae using the *in vitro* organ culture model of infection. The bacterial strains examined are described in Table 3.1 and in section 2 (table 2.1).

3.2 Results.

3.2.1 IVOC of EPEC and EHEC.

Paediatric intestinal explants, taken from representative sections of the digestive tract (see figure 3.1), were cultured as outlined in section 2.2.3 All intestinal explants used in this study showed appropriate, histologically normal, morphological features before being placed in the IVOC system. This included an intact mucosal surface with normal villi on the small intestinal explants and crypts and no villi in the large intestinal explants. Lymphoid follicles were observed as dome structures lacking villi and crypts on their apical surface. The culturing of the intestinal explants during the IVOC assay and processing of the explants for electron microscopy produced some changes in morphology. This was noted by the shortening of the villi of the small intestinal explants when SEM images were compared with histological sections of the same intestinal region, used during routine histopathological assessment from the same patient. However such differences may be explained in part by the different tissue processing and method of examination used. Comparison of gross changes in colonic explant morphology due to IVOC was not possible to detect, although there was some oedema of the lamina propria region in IVOC samples. Inoculated and uninoculated explants showed similar surface features when examined by SEM. Extruding cells were observed as rounded enterocytes in the extrusion zone at the tips of the villi and there was no evidence of excess cell loss. No bacteria were found adhering to the uninoculated or uncultured control explants. In some cases, control samples showed occasional areas of glycocalyx thinning and microvilli exposure but this could not be correlated with any variable. The mucus recovered from the wash process before fixing the explant tissue showed the presence of bacteria (see section 2.2.3.1). Bacteria were present on the surface of mucus obtained from inoculated explants.

Figure 3.1 Representative SEM micrographs of explants used during intestinal IVOC.

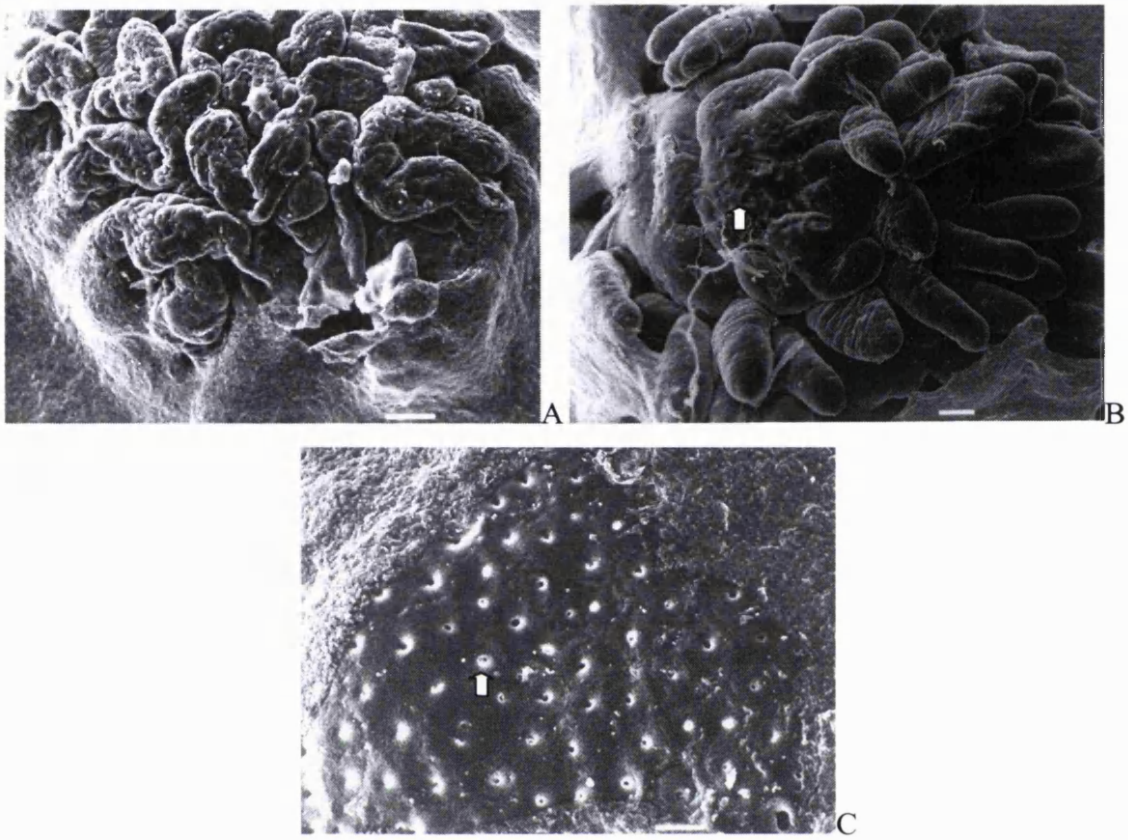


Figure 3.1 A is a micrograph of a duodenal explant with villous morphology after eight hour IVOC, bar = 100μm and the magnification is x100. Figure 3.1B shows a PP explant containing follicular associated epithelium (white arrow) surrounded by terminal ileal villi, bar = 100μm and magnification is x 75. Figure 3.1C is a micrograph of a colonic explant after eight hour IVOC, lacking the villi of the small intestine and containing an epithelium surface marked with crypt openings (white arrow), bar = 100μm and magnification is x 100.

The bacterial strains used in this study are listed in table 3.1

Table 3.1 Bacterial strains.

Strain	Serotype	Intimin type	Source/Reference
E2348/69	EPEC O127:H6	Intimin α	(Levine et al. 1119-22)
O42	EAEC O44:H18	Intimin -ve	(Nataro et al. 4761-68)
85-170	EHEC O157:H7	Intimin γ	(Tzipori et al. 3117-25)
AGT300	EHEC O157:H7	Intimin γ	(Torres et al. 5416-27)
H11/8624JK	EHEC O157:H7	Intimin γ	(Griffin PM et al)(Sperandio et al. 15196-201)
TT12B	EHEC O157:H7	Intimin γ	{Feng, Dey, et al. 2001 1 /id}
3801	EHEC O26:H11	Intimin β	J.B. Kaper, Center for Vaccine Development, University of Maryland, Baltimore, U.S.A.
E77804	EPEC O103:H-	Intimin ϵ	H. Smith, Division of Enteric Pathogens, Central Public Health Laboratory, London, U.K.
PMK5	EHEC O103:H2	Intimin ϵ	(Mariani-Kurkdjian et al. 296-301)

The adhesion characteristics of the EHEC strains studied are described in Table 3.2.

The EPEC strain E2348/69 and EAEC strain O42 adhered to the explants used during the IVOC. E2348/69 adhered to the small intestine in 26/27 explants tested. It produced an A/E lesion on transverse colon in 1/6 explants tested. In the case of adherence to small intestine, E2348/69 produced A/E lesions on 23/27 explants. Three of the explants used showed adhesion without A/E lesion formation. This was observed as bacterial adhesion without any associated changes in the mucosal surface, following extensive washing to remove non – adherent bacteria prior to fixation for SEM. In all cases the IVOC assay was carried out for eight hours. A bacterium with intimate contact with the surface epithelium will have the same properties as the sample and both will show the same shade on the grey scale produced by the black and white image on the SEM's visual monitor and on the photograph developed.

Figure 3.2 Adhesion of E2348/69 and O42 to explants during IVOC.

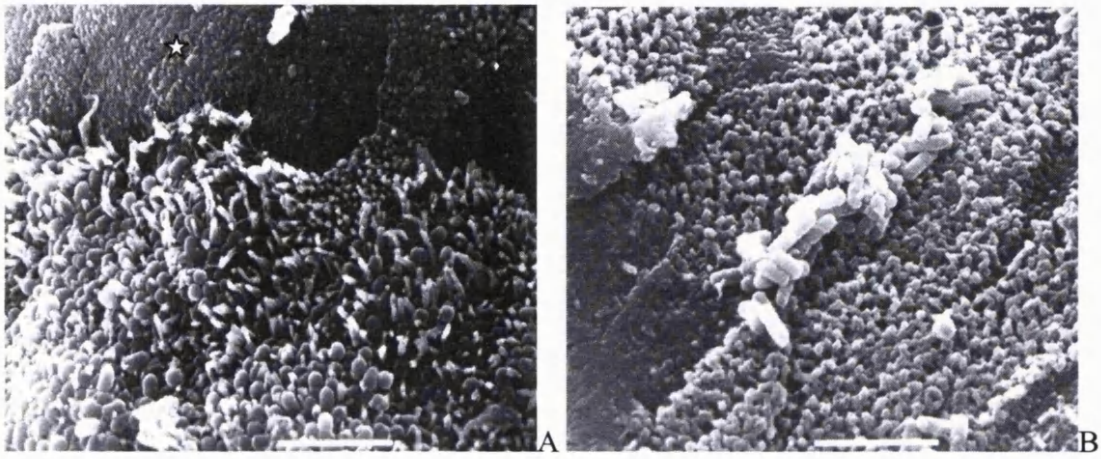


Figure 3.2A shows the typical EPEC strain O127:H6 E2348/69 producing an A/E lesion on a small intestinal explant during eight hour IVOC. The star denotes the intact epithelial surface next to an area of A/E lesion formation, consisting of glycocalyx overlying the microvilli of the intestinal enterocyte. The tips of microvilli can be seen projecting out alongside bacteria in the area of A/E lesion formation, bar = 5μm. Figure 3.2B shows the EAEC O44:H18 strain O42 adhering to a transverse colon explant during the eight hour IVOC. The tips of microvilli can be seen due to glycolayx loss, bar = 5μm.

As can be seen in figure 3.3 the microvilli and glycocalyx layer form the apical surface of the intestinal enterocyte.

Figure 3.3 SEM micrograph of a sectioned explant after IVOC.

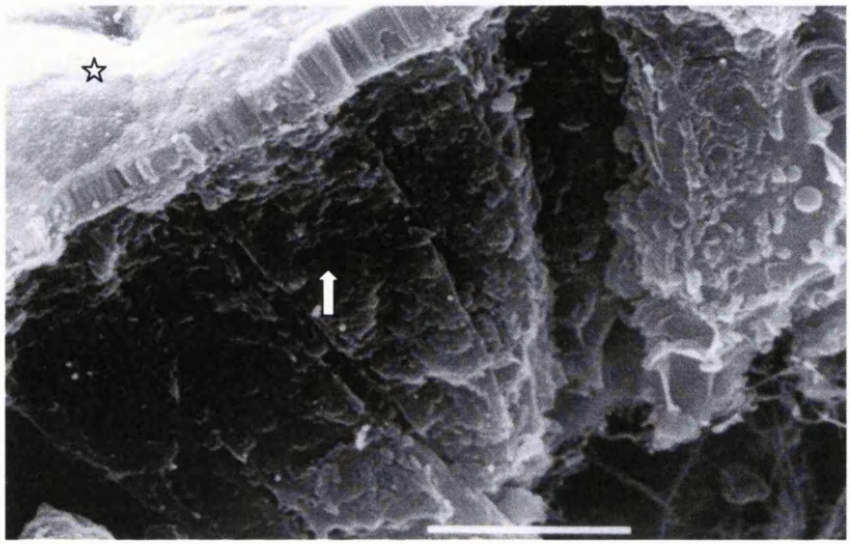


Figure 3.3 bar = 5µm.

The white arrow indicates an intestinal enterocyte and the white star indicates the mucosal surface of the intestinal epithelium.

Table 3.2 Regional adhesion of bacterial strains.

	Intestinal region									
	Small intestine				Large intestine					
Strain	D4	FAE/D4	T.ileum	PP/T.ileum	Caecum	Ascending colon	Transverse colon	Descending colon	Sigmoid colon	Rectum
85170	0/8	1/1	0/9	11/11	0/4	0/3	0/20	0/3	1/3	0/3
AGT300	0/11	NA	0/6	2/4	0/3	0/4	0/5	0/5	0/4	0/3
TT12B	2/10	1/1	0/6	7/8	0/3	0/4	1/5	0/5	0/4	0/3
H11	0/4	NA	0/4	0/4	0/5	0/3	0/4	0/3	0/3	0/3
8624JK	0/3			0/4						
3801	0/4	NA	1(+1adh)/4	1(+1adh)/3	0/5	0/3	0/3	0/3	0(+1adh)/3	1/3
E77804	3/9	2/2	0/8	2/3	0/3	0/4	0/5	0/4	0/4	0/3
PMK5	2/11	NA	0/7	5/6	0/3	0(+1adh)/4	0(+1adh)/5	1/5	0/3	0/3
Patient age (months) (median(range))	76(19–212)	103, 111, 111, 149	127(24–181)	96(34–197)	155(103–202)	103(74–179)	115(38–198)	142(46–200)	154(103–179)	142(102–172)

Note: Values correspond to A/E lesion formation as a proportion of biopsies inoculated. Adh refers to adhesion without A/E lesion formation.

D4= fourth part of the duodenum

FAE= follicular associated epithelium

T. ileum= terminal ileum

PP= Peyer's patch

NA= not available

The age is shown in months and when more than four explants were tested the age is shown as a median, with a range indicated.

The A/E lesions produced by the bacterial strains listed in table 3.1 shared similar characteristics. The A/E lesion consisted of attachment of the bacteria to the surface epithelium with loss of microvilli. In some cases this was accompanied by elongation of the microvilli and loss of glycocalyx in the area of A/E lesion formation. The bacterial microcolonies present at the site of A/E lesion formation varied in size and the A/E lesions per explant varied in number. The elongation of microvilli appeared to be a discrete cellular event as the majority of cells with no adherent bacteria showed an intact surface epithelium with an intact glycocalyx layer. However, not all microvillus elongation and glycocalyx removal was associated with A/E lesion formation. The inoculated explants showed more extruding cells than the uninoculated explants. The extruding cells had a rounded or uneven appearance and were raised above the level of the epithelial surface. The extruding cells were not just present at the tip of villi but at the base and along the length of the villi.

Figure 3.4 Area of A/E lesion formation on the tip of a duodenal villous.

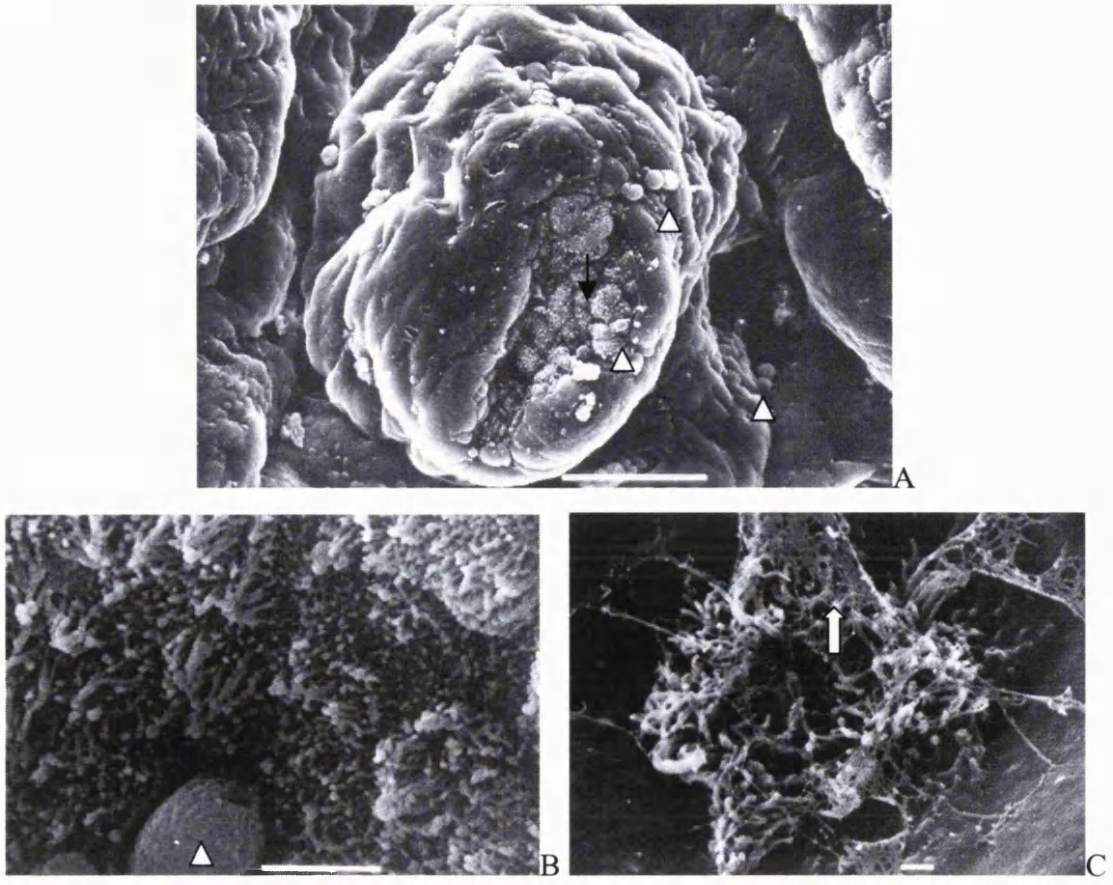


Figure 3.4A bar = 50μm. The black arrow represents an area of A/E lesion formation on the tip of a duodenal villous. The white triangles represent cell rounding. Figure 3.4B shows a region of the intestine without glycocalx and with elongated microvilli and cell rounding (white triangle), bar = 5μm. Figure 3.4C is a micrograph of a region of the intestine with elongated microvilli and glycocalyx (white arrow), bar = 1μm.

The EHEC O157:H7 strain 85/170 produced an A/E lesion on the follicular associated epithelium of an isolated lymphoid follicle in the duodenum. This result could not be repeated in triplicate as lymphoid follicles in the duodenum are not PP and could not be targeted for biopsy during the endoscopic procedures, and thus the presence of lymphoid follicles for analysis was a random event. The A/E lesion produced on the FAE of the duodenum was similar to that produced on the FAE of the Peyer's Patches of the terminal ileum. 85/170 did not adhere to the villi of the duodenum and no adhesion by 85-170 was noted on terminal ileal explants devoid of lymphoid follicles. PP explants also contain villi and one out of the eleven PP explants used in the IVOC assay showed A/E lesion formation on both the villi and the FAE of the PP. 85/170 produced A/E lesions on all PP explants from the terminal ileum. Each follicle within a Peyer's patch varied in size and shape and Peyer's patches were present across a broad patient age range. 85-170 produced A/E lesions that were flat in appearance which was consistent with the epithelial surface, but the bacteria were not all in one plane and due to their adherence to one another some three dimensional aspect could be seen.

Figure 3.5 85-170 A/E lesion formation on explants during IVOC.

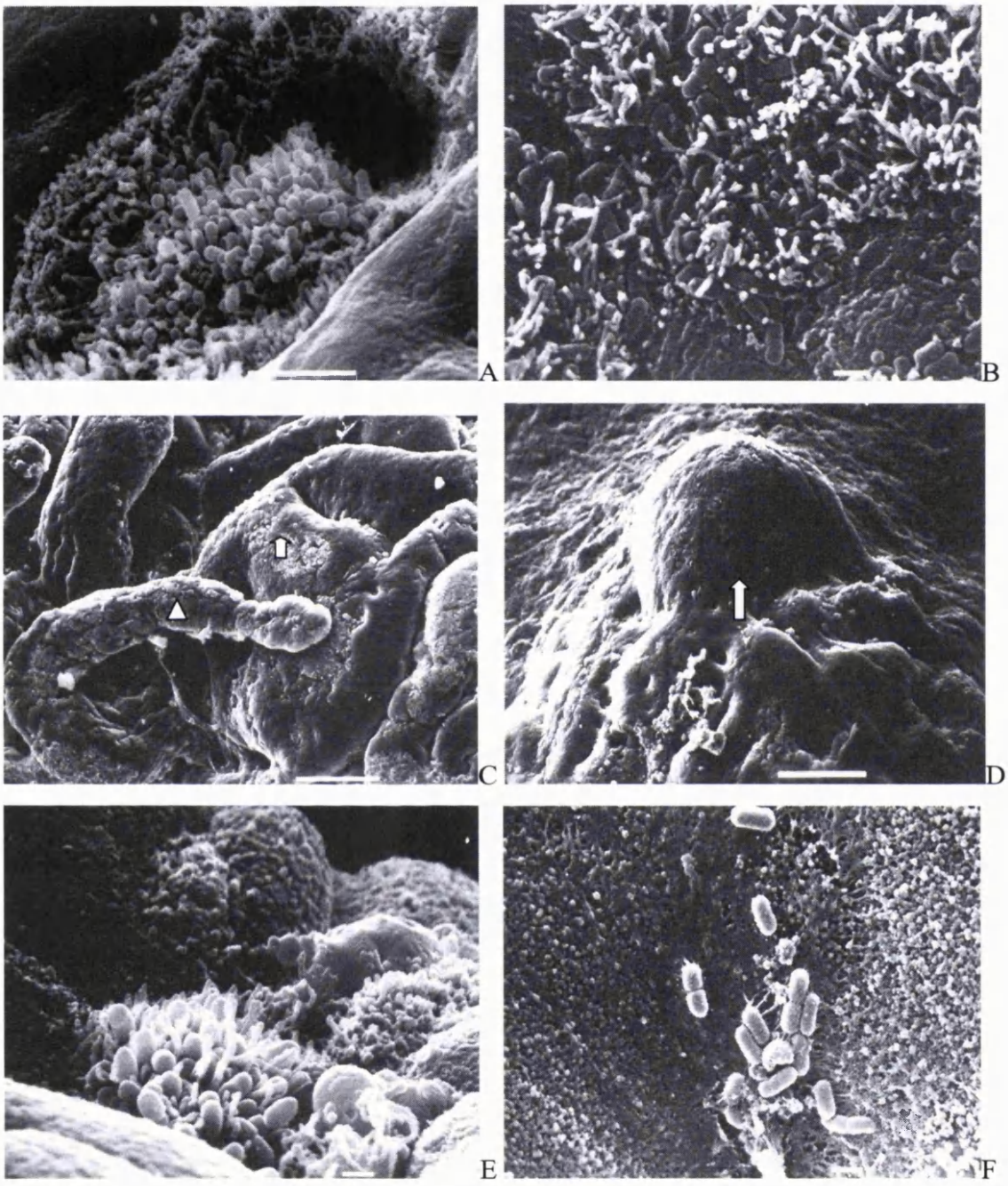


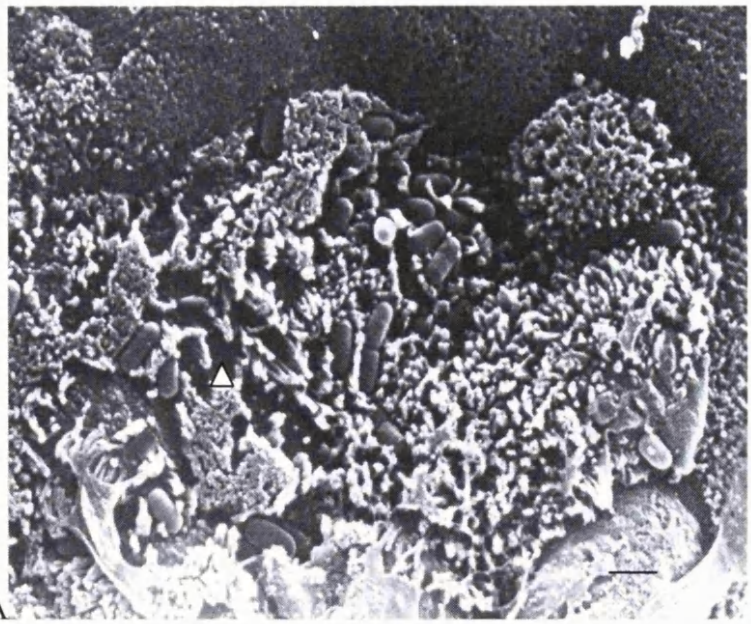
Figure 3.5A shows 85-170 A/E lesions on PP explant, bar = 5μm. Figure 3.5B shows 85-170 A/E lesion formation on the villous epithelium of a terminal ileal villus next to a PP, bar = 1μm. Figure 3.5C shows the terminal ileal villous referred to in figure 3.5B (white triangle) and the PP region next to the villous (white arrow), bar = 100μm. Figure 3.5D is a micrograph of an isolated lymphoid follicle forming part of a duodenal explant, bar =

100μm. Figure 3.5E shows 85-170 A/E lesions on the isolated lymphoid follicle of fourth part of the duodenum, bar = 1μm.

Like all other strains used in this study, 85-170 was tested for adherence to the epithelium of the large intestine. The large intestine was divided into six regions and comprised the caecum, the ascending colon, the transverse colon, the descending colon, the sigmoid colon and the rectum. 85-170 produced a single A/E lesion on the sigmoid colon but did not adhere to any other region of the large intestine. This EHEC O157:H7 strain was therefore positive for A/E lesion formation on only 1/3 sigmoid colon incubations and 1/36 large intestinal incubations.

AGT300 did not adhere to any region of the large intestine during the eight hour IVOC assay. It produced A/E lesions on 2/4 PP explants. In general the A/E lesions produced by this O157:H7 strain were similar to those produced by 85-170. However, A/E lesions were observed without the presence of bacteria and these were termed footprints (see figure 3.6).

Figure 3.6 AGT300 A/E lesion on PP explants.

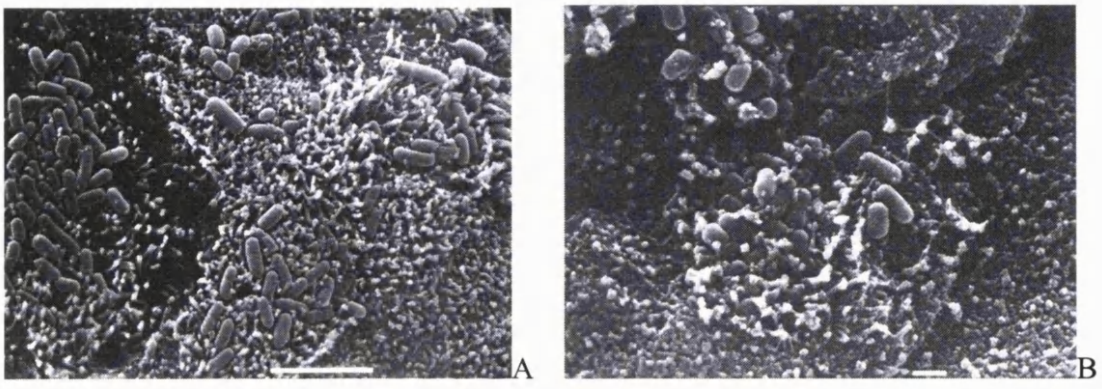


B

Figures 3.6A and 3.6B are micrographs of A/E lesion formation by AGT300 on PP explants, bar = 1um. Footprint structures (white triangles) were observed which consisted of regions of effaced microvilli identical in size and shape to the other bacteria present in the region of A/E lesion formation.

The O157:H7 strain TT12B adhered to the large and small intestine and showed a preferential adhesion to PP explants. TT12B produced A/E lesions on 7/8 PP explants and on 1/1 isolated lymphoid follicles from the duodenum during the eight hour IVOC assay. TT12B produced A/E lesions on the villous epithelium of 2/10 duodenal explants but did not adhere to the terminal ileum villous epithelium. TT12B produced A/E lesions on 1/5 transverse colon explants but adhered to only 1/24 large intestinal explants during IVOC.

Figure 3.7 TT12B A/E lesion formation on intestinal explants during IVOC.



A B

Figure 3.7A is a micrograph of A/E lesions produced by TT12B on duodenum, bar = 5μm. Figure 3.7B shows A/E lesion formation by TT12B on transverse colon explants, bar = 1μm.

The O157:H7 strain H11 did not adhere to any area of the intestine using the eight hour IVOC adhesion assay. Apart from A/E lesions the explants inoculated with H11 showed similar epithelial features to those inoculated with other O157:H7 strains. Viable bacteria could be cultured on LB and MacConkey agar plates after the eight hour IVOC assay. The plates showed colonies with morphology similar to that of *E. coli* and the MacConkey plates showed pink to red colonies lactose fermenting colonies. Another isolate of H11 named 8624JK was provided by Jim Kaper. This isolate also did not adhere to duodenum and did not adhere to PP and acted like H11 in the HEp-2 assay (see section 3.2.3).

The EHEC O26:H11 strain 3801 adhered to the villi and PP of the terminal ileum. In the case of terminal ileal explants without PP present, 3801 produced A/E lesions on 1/3 explants and adherence without A/E lesion formation on 1/3 explants. Adhesion to the epithelium was noted as adhesion to the surface without microvillous effacement or elongation. This occurred with or without the loss of glycocalyx. Strain 3801 produced A/E lesions on 1/3 PP explants and adherence without A/E lesion formation on 1/3 PP explants. The adherence without A/E lesion formation on PP was similar to that seen on terminal ileum explants without PP. 3801 adhered to 2/20 large intestine explants tested with one of these positive results showing A/E lesion formation, in the rectum, the other just showing adhesion on sigmoid colon.

Figure 3.8 Adhesion of 3801 to intestinal explants.

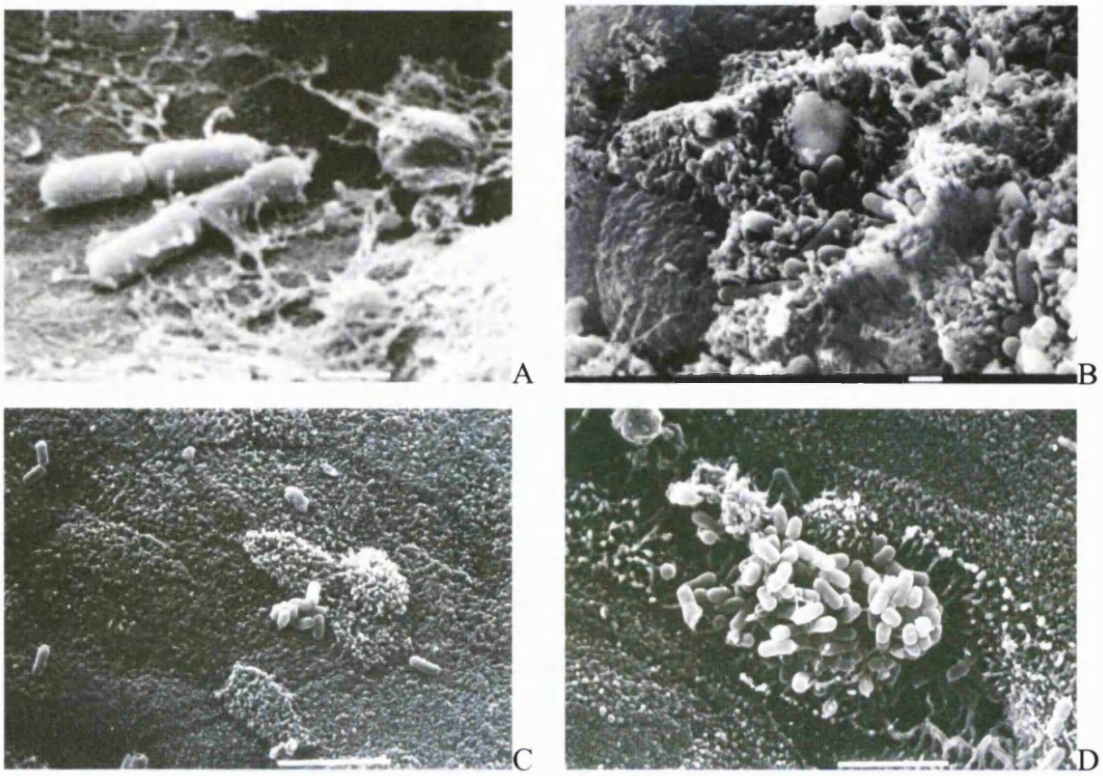


Figure 3.8A shows adhesion of 3801 to the terminal ileal villi without A/E lesion formation, bar = 1μm. Figure 3.8B shows A/E lesion formation by 3801 on terminal ileal villi, bar = 1μm. Figure 3.8C shows the adhesion without A/E lesion formation of 3801 to the sigmoid colon, bar = 5μm. Figure 3.8D shows A/E lesion by 3801 on a rectal explant, bar = 5μm.

The atypical EPEC O103:H- strain E77804 adhered and produced A/E lesions on small intestinal explants. It produced A/E lesions on 7/22 small intestine explants used. It produced A/E lesions on the villi and FAE of the duodenum. This consisted of A/E lesions on 3/9 duodenum explants without lymphoid follicles, and A/E lesions on 2/2 isolated lymphoid follicles found in separate duodenal explants. E77804 did not adhere to terminal ileum explants lacking PP but A/E lesions were noted on 2/3 PP explants. Thus, E77804 adhered to 4/5 explants containing FAE, when combining PP and duodenal results but did not adhere to large intestinal explants during IVOC.

Figure 3.9 E77804 A/E lesion formation on intestinal explants during IVOC.

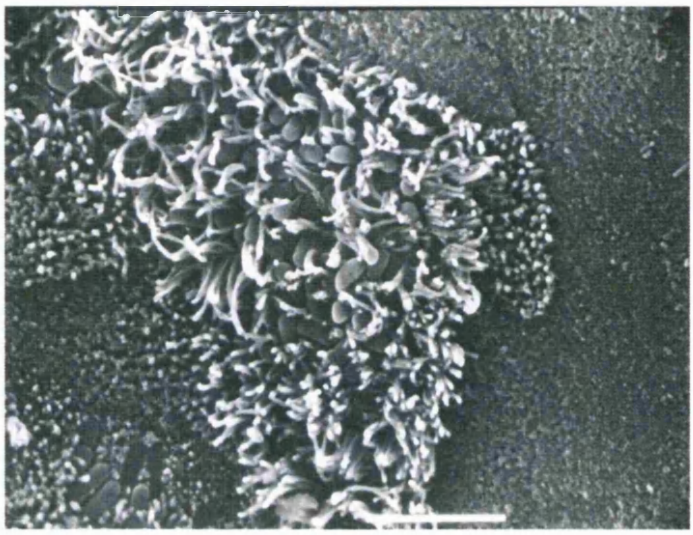


Figure 3.9 is a micrograph of A/E lesion formation by E77804 on duodenum, bar = 5μm.

The EHEC O103:H2 strain PMK5 produced A/E lesions on 1/23 colonic explants and 5/6 PP explants used during IVOC. It adhered to the large intestine with and without A/E lesion formation in 3/23 explants used. This was observed as adhesion without A/E lesion formation to 1/4 ascending colon explants and to 1/5 transverse colon explants, and A/E lesion formation in 1/5 descending colon explants. PMK5 adhered, with A/E lesion formation, to 7/24 small intestine explants but did not adhere to terminal ileal explants lacking PP. A/E lesion formation was observed on the villi of 2/11 duodenal explants. No duodenal lymphoid follicles were obtained for this study.

Figure 3.10 Adhesion of PMK5 to intestinal explants during IVOC.

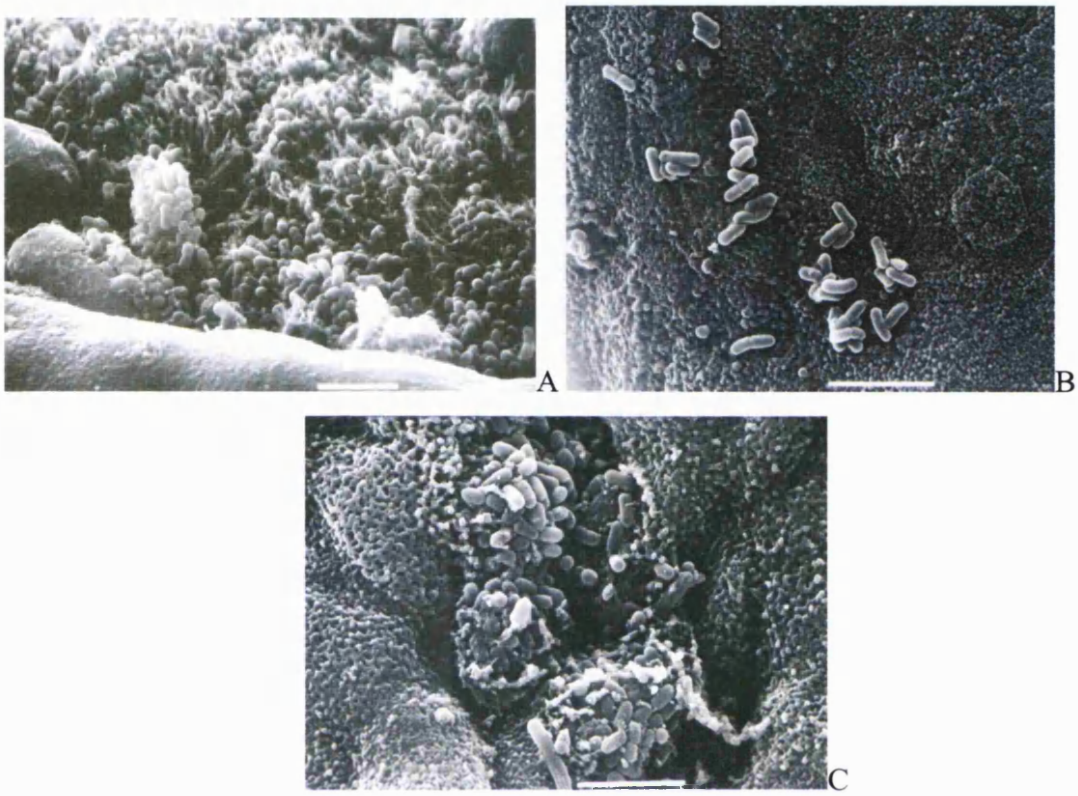


Figure 3.10A shows PMK5 producing A/E lesions on duodenal explants during IVOC, bar = 5μm. Figure 3.10B shows adhesion without A/E lesion formation of PMK5 to ascending colon, bar = 5μm. Figure 3.10C is a micrograph of A/E lesion formation by PMK5 on descending colon, bar = 5μm.

The duodenal explants showing A/E lesions after incubation with EHEC strains and the EPEC strain E77804 did not contain any isolated lymphoid follicles as assessed by SEM of the explants after IVOC and by light microscopy of haemotoxylin and eosin stained biopsy sections provided by the Royal Free Histopathology Department. In addition, no lymphoid follicles were observed in the colonic explants positive for A/E lesion formation and in the associated biopsy sections.

Figure 3.11 Haemotoxylin and eosin stained duodenal biopsy section.

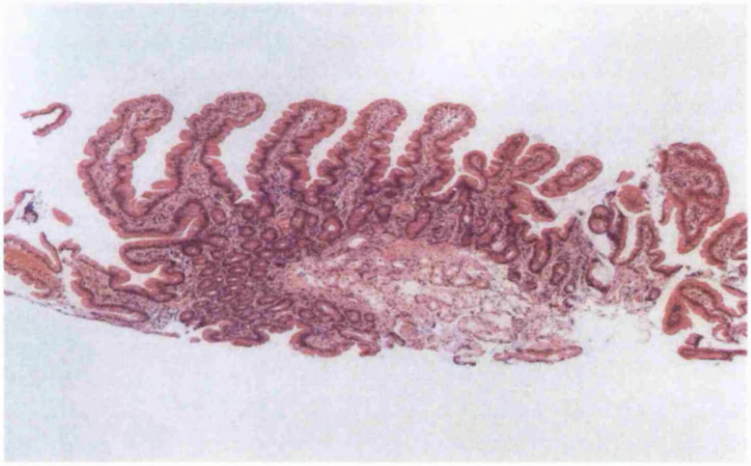


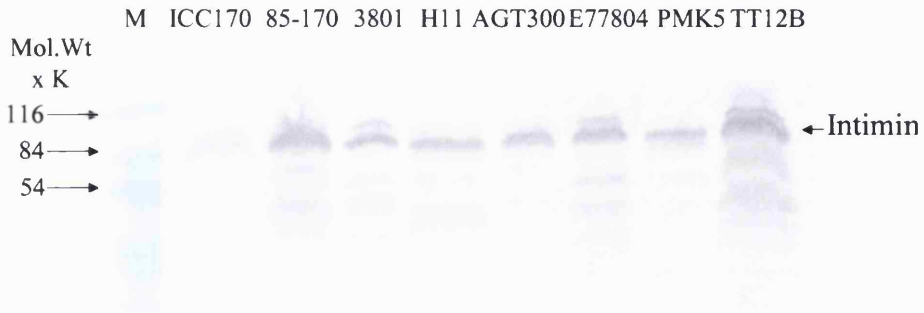
Figure 3.11 shows a haemotoxylin and eosin stained 5 μm biopsy section from the fourth part of the duodenum, magnification x 40. The biopsy was taken from the same area as an explant used for inoculation by an EHEC strain that adhered to duodenum.

All the bacterial strains tested, apart from H11 and 8624JK, showed A/E lesion formation during the eight hour IVOC adhesion assay but adhesion to the villous epithelium of small intestinal explants was limited when compared with E2348/69. EHEC O157:H7 and O103:H2 strains and the atypical EPEC strain E77804 showed preferential adhesion to PP explants with little A/E lesion formation on colonic explants. There was no colonic adhesion in the caecal and ascending regions, only in transverse, descending, sigmoid and rectal, i.e. distal, large intestinal regions. The intimin β expressing EHEC strain 3801 showed similar adhesion to the villi and the PP of the terminal ileum.

3.2.2 Western blot of intimin expression of EPEC and EHEC strains.

Intimin expression of all the bacterial strains listed in table 3.2 was assessed by Western blot using the universal intimin antiserum as described in section 2.5.

Figure 3.12 Western blot of intimin expression of the EPEC and EHEC strains.



Intimin = intimin bands as detected by antiserum raised against the conserved domain of intimin as described in section 2.5.
M = protein ladder marker.
ICC170 = intimin negative EHEC O157:H7 85-170 strain, used a negative control.

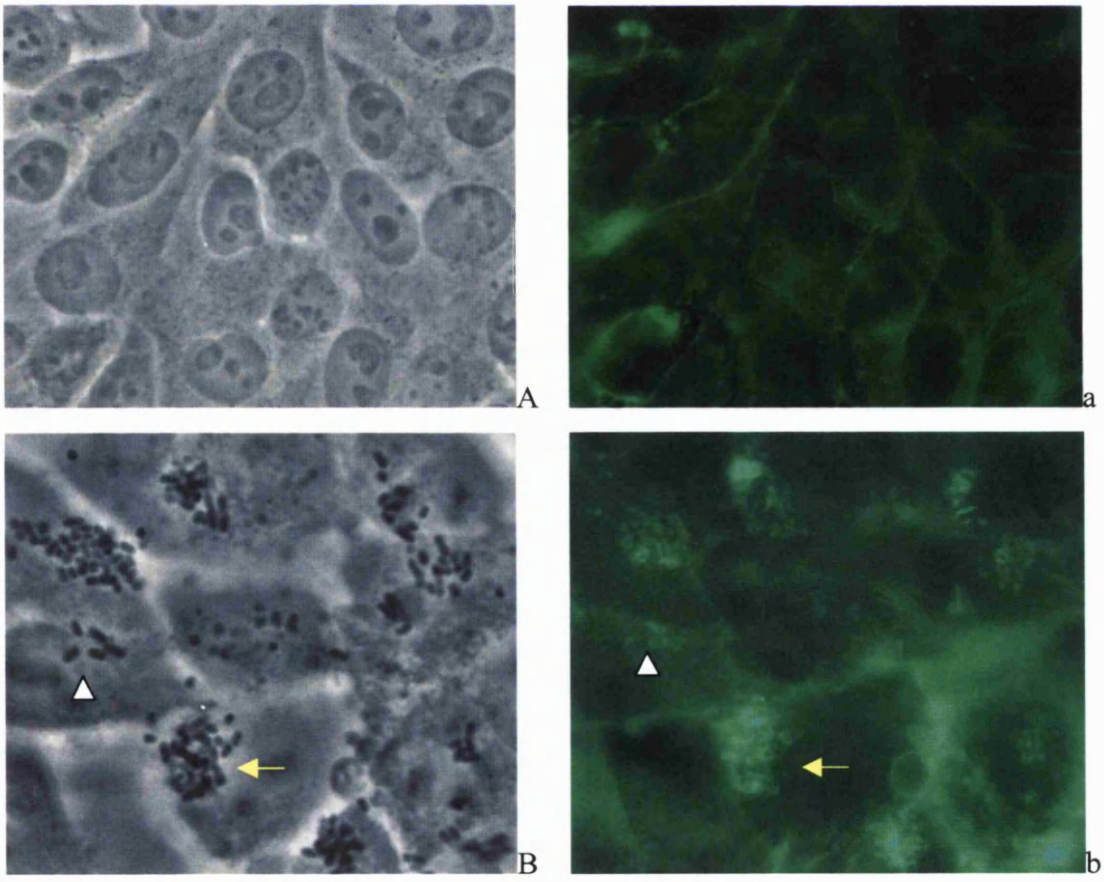
This Western blot showed that all the EHEC strains and the EPEC strain E77804 express intimin after activation in DMEM. The multiple bands show intimin degradation products. The negative control (ICC170) showed some diffuse background staining which was distinct from the intimin bands recorded by the other strains.

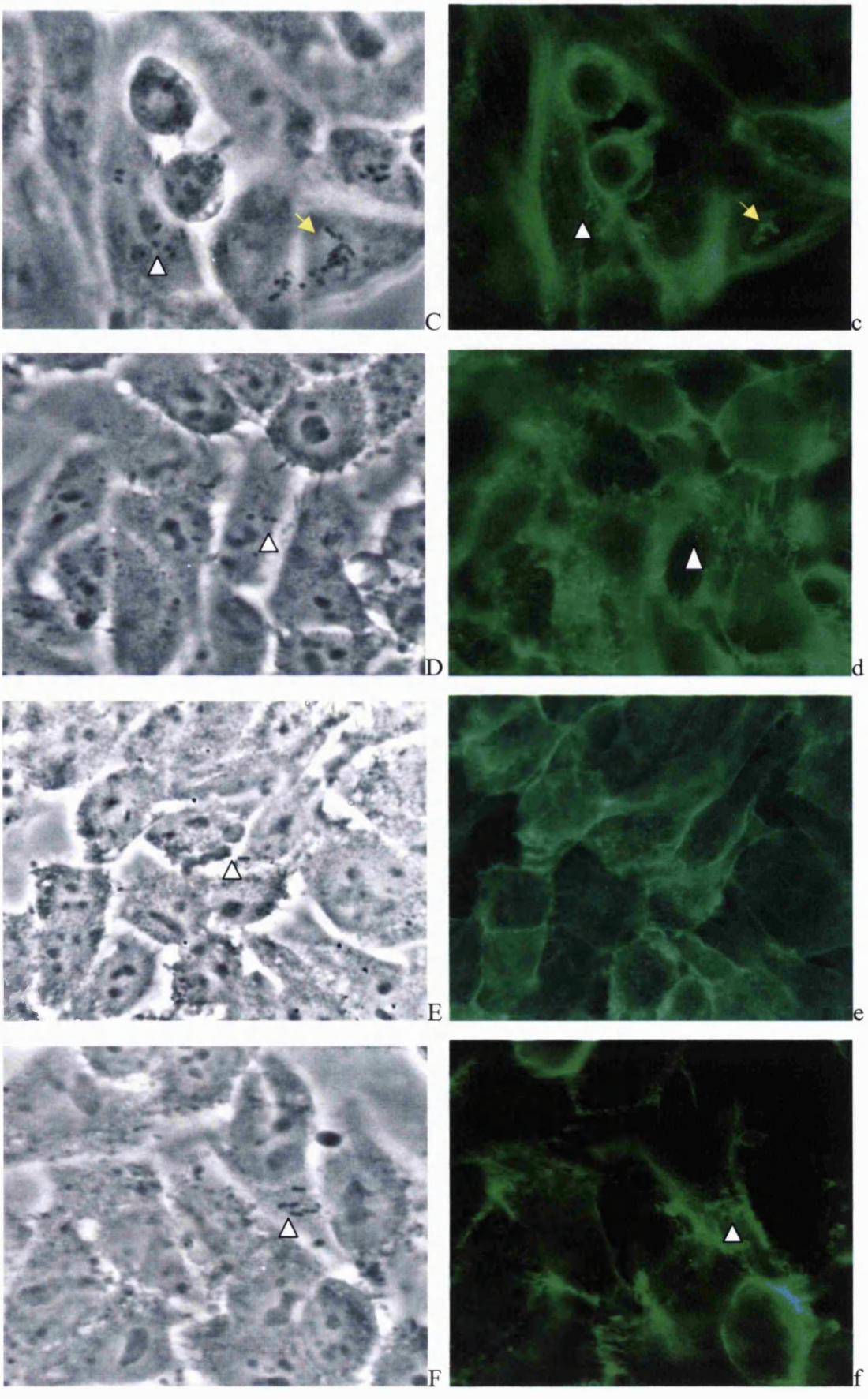
3.2.3 HEp-2 assay of the EPEC and EHEC strains.

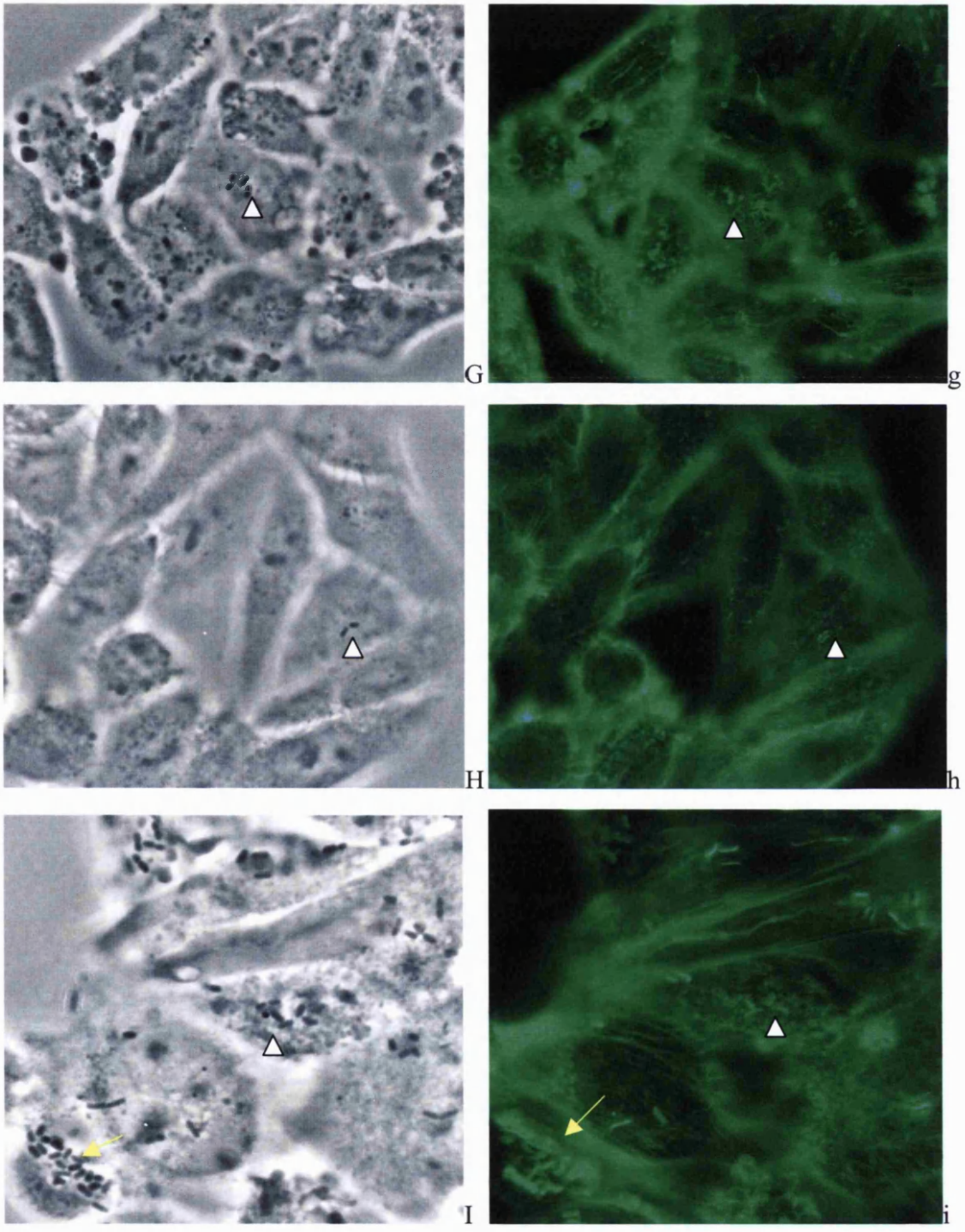
The EPEC E77804 strain and the EHEC strains (except the EHEC strain 3801) used in IVOC in section 3.3.1 showed preferential adherence to PP explants with some strains also adhering to other regions of the intestine. In order to further characterise and compare the strains used in the study, they were assayed for their adhesion to HEp-2 cells

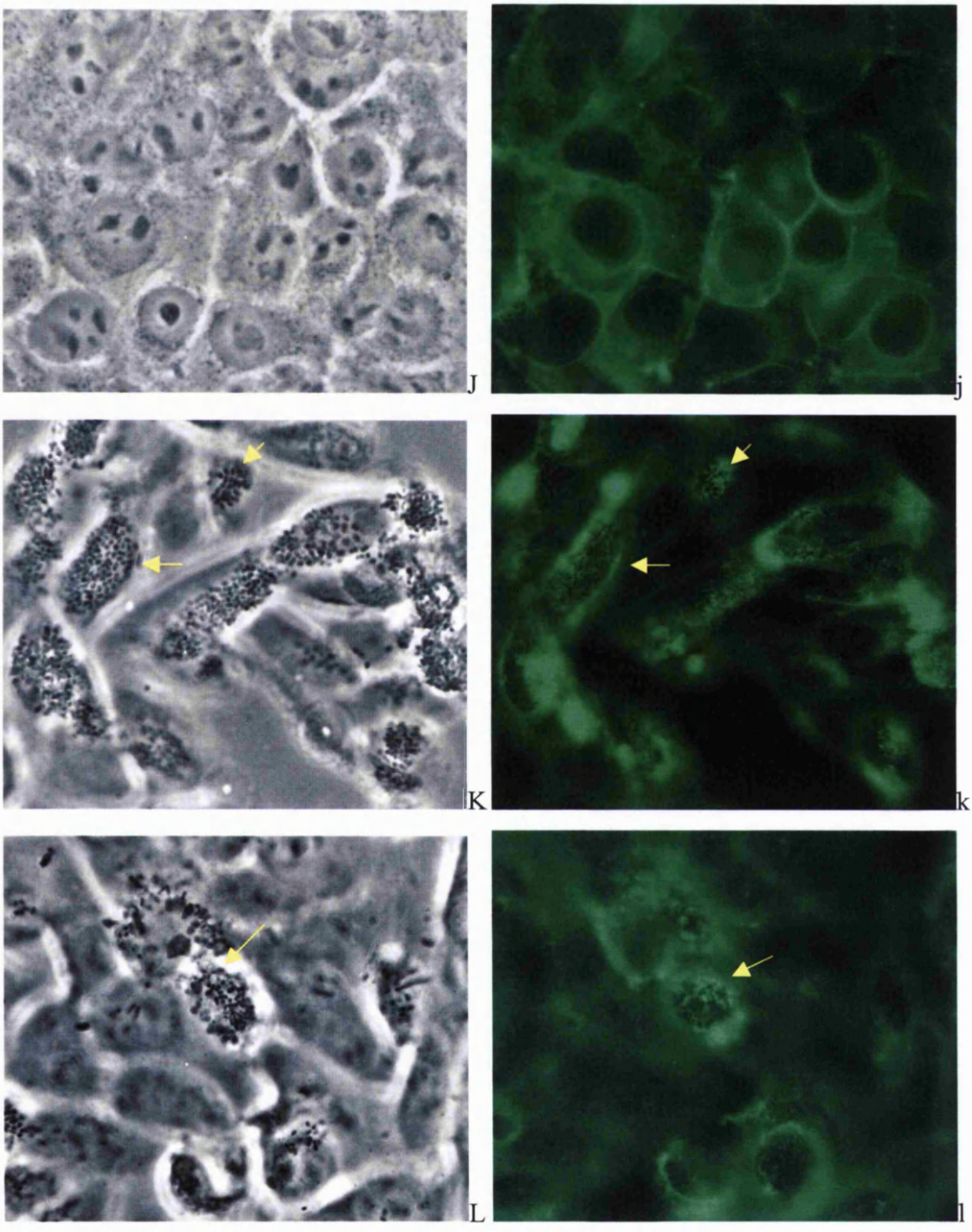
The Hep-2 assay was carried out and the cells were stained using FITC - Phalloidin as described in section 2.4.2. Phase contrast and fluorescent images were obtained for each bacterial strain.

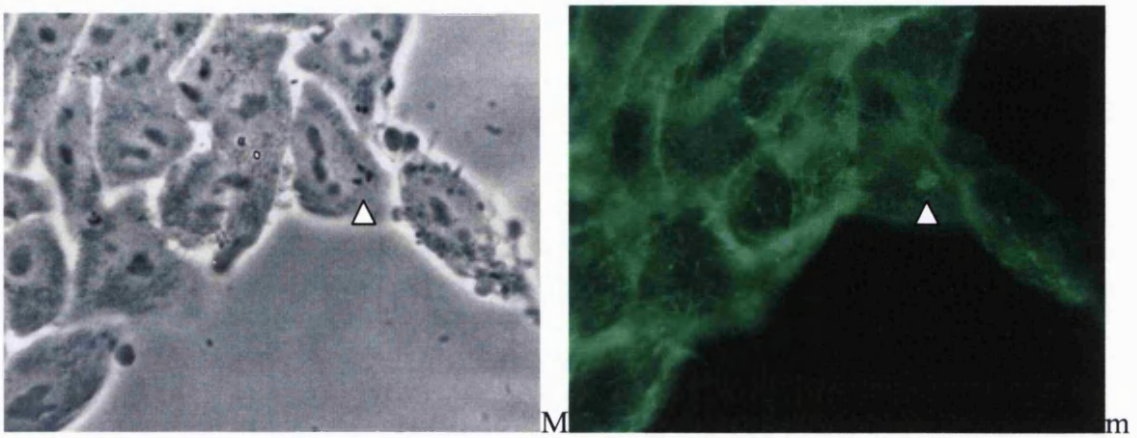
Figure 3.13 Micrographs of the HEp-2 adhesion assay of EPEC and EHEC.



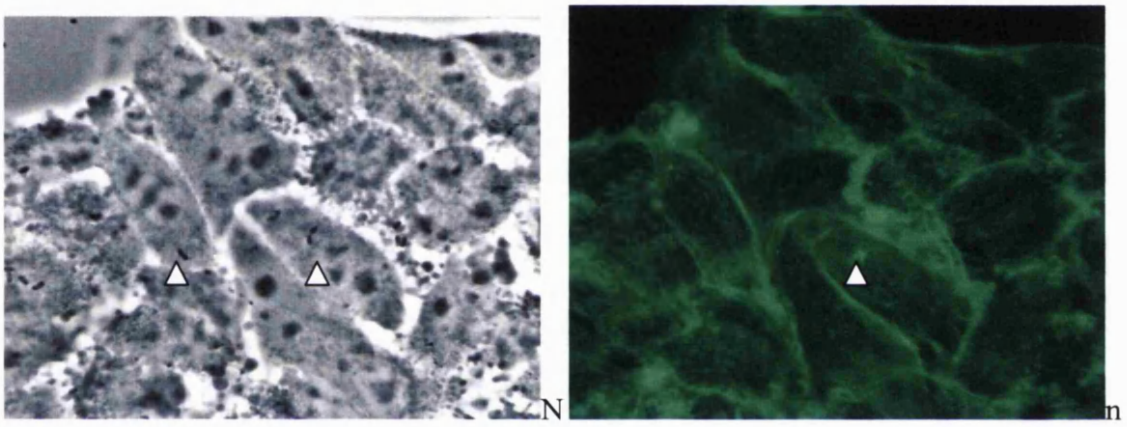

C
c
D
d
E
e
F
f


G
g
H
h
I
i

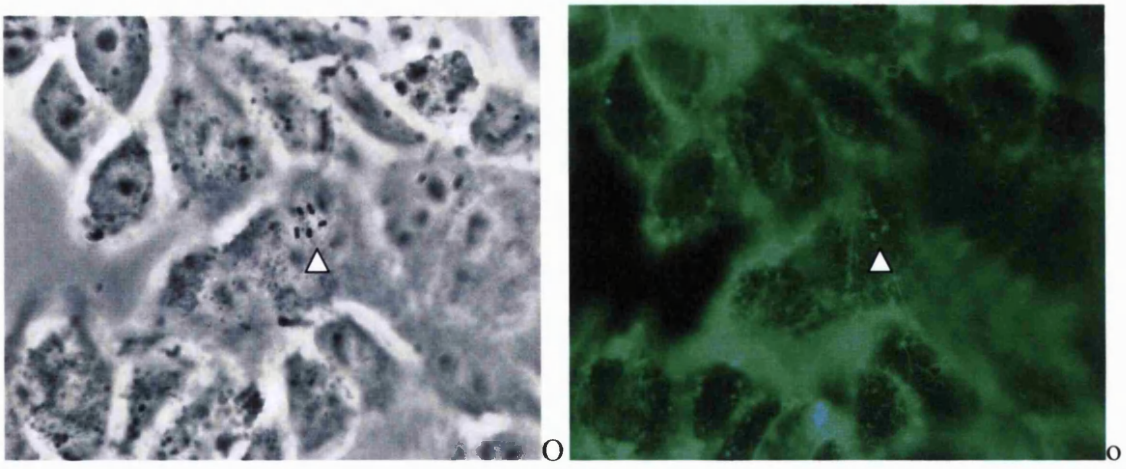

J
j
K
k
L
l



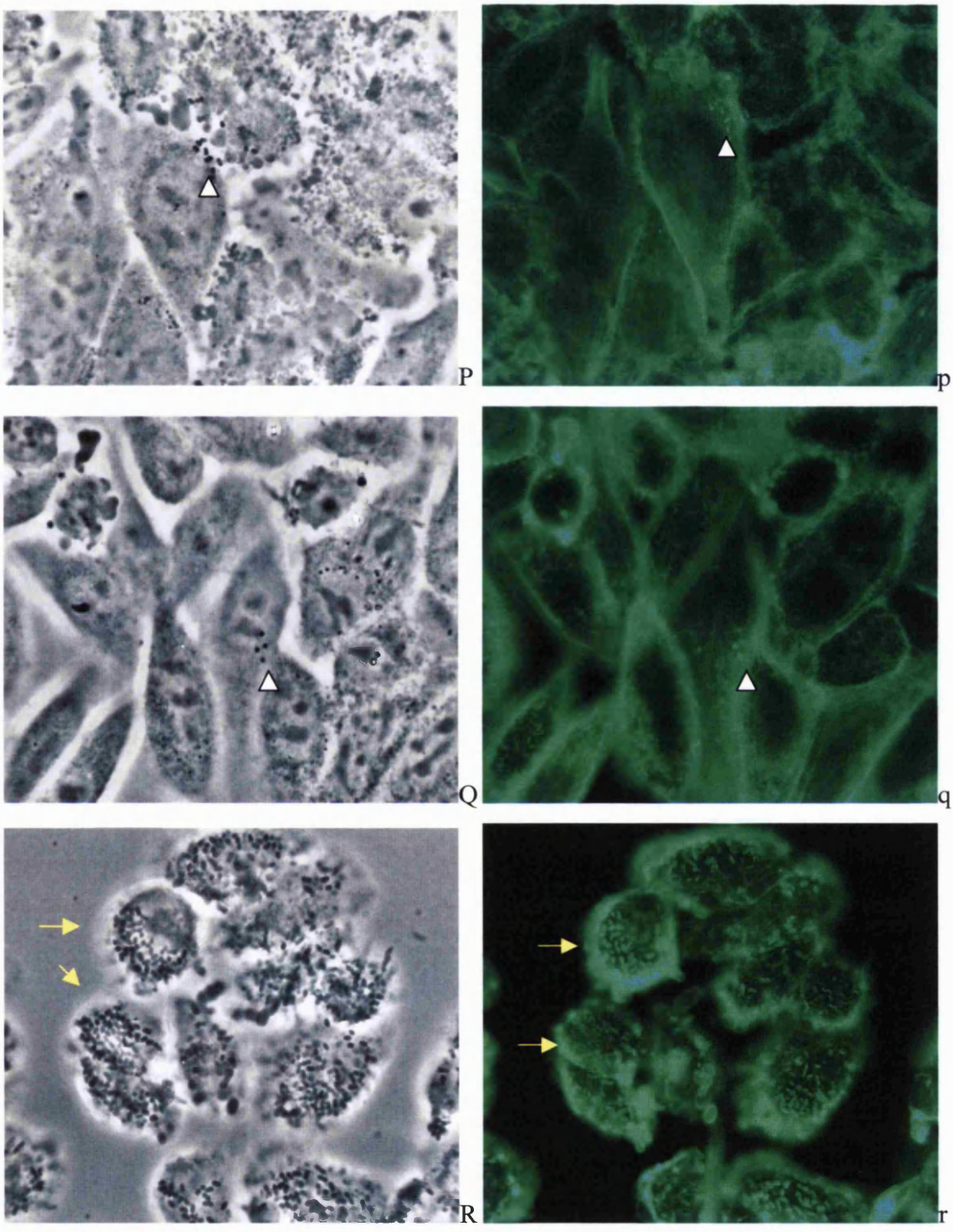
M m



N n



O o



These micrographs are duplicates with the phase contrast images represented by the upper case letters and the fluorescent images by the lower case letters. The HEp-2 assay is divided into 3hr and 6hr incubations periods with letters A to I representing the 3hr HEp-2 assay and letters J to R the 6hr Hep-2 assay. The yellow arrows indicate examples of

bacterial microcolonies where more than five bacteria are present. The white arrow heads indicate bacteria that are not part of a microcolony. Magnification is x 80. The letters indicate the following micrographs: A and J negative control, B and K E2348/69, C and L 85-170, D and M AGT300, E and N H11, F and O TT12B, G and P 3801, H and Q E77804, and I and R PMK5.

The images included in figure 3.13 are representative of the adhesion to HEp-2 cells exhibited by the bacterial strains used in this chapter. After the HEp-2 cells were washed no bacteria were observed adhering to the glass coverslips. After the 6hr incubation period microcolonies were found to be on a different focal plane to the HEp-2 cells. This can be seen with strains E2348/69, 85-170 and PMK5. In addition, the HEp-2 cells incubated with these strains for six hours showed increased cytolysis such as cell swelling and budding when compared to the uninoculated controls and the poorly adherent strains. Cell loss during washing was noted after the six hour incubation period and was also associated with strongly adherent strains.

The typical EPEC strain E2348/69 produced an LA pattern during the 3hr HEp-2 assay. The majority of bacteria showed the ability to nucleate actin. The microcolonies produced during the 6hr incubation contained a greater number of bacteria. The majority of the bacteria were FAS positive. At both the 3 and 6 hour time points some bacteria adhered independently of the microcolonies with a greater proportion of these producing actin nucleation at the site of bacterial adherence after 6 hours incubation (see figure 3.13 B and K).

EHEC O157:H7 strain 85-170 adhered to HEp-2 cells at 3 and 6 hour incubation time points. At 3 hours few microcolonies were observed and the majority of the bacteria present adhered without associating into microcolonies. Not all the bacteria were FAS positive. At 6 hours there was a greater number of microcolonies with some bacteria still adhering independent of microcolonies and more bacteria were FAS positive. 85-170 was designated as being LAL and was used as a standard to compare other strains (see figure 3.13 C and L).

The HEp-2 assay was carried out in triplicate and on at least three occasions. Actin pseudopod or pedestal formation by 85-170 was observed after one of the 6 hour incubation assays (see figure 3.14).

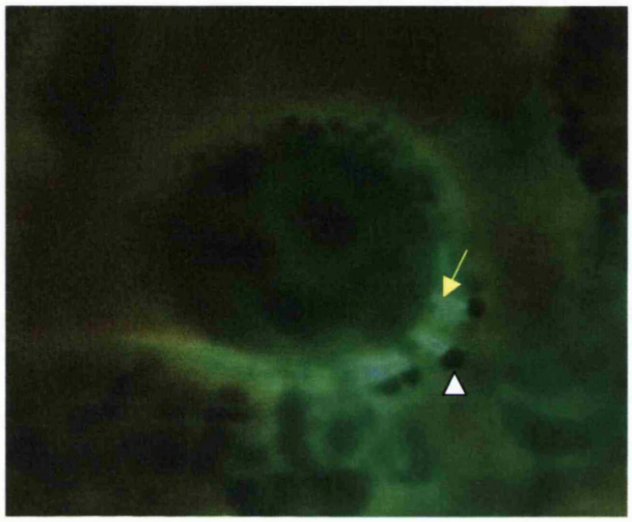


Figure 3.14 Strain 85-170 adhering to actin pseudopods after a 6hr HEp-2 cell assay. The image was taken using the phase contrast setting with fluorescent bleed through used to highlight the actin structures. The yellow arrow indicates the actin pseudopod and the white arrow head the adherent 85-170 bacterium, magnification x 160

The EHEC O157:H7 strain AGT300 adhered to HEp-2 cells after 3 and 6 hour incubation periods. After 3 hours incubation, AGT300 showed few bacteria adhering to HEp-2 cells and few bacteria were FAS positive. After 6 hours incubation there was a greater number of adherent bacteria and a greater proportion of them were FAS positive. After six hours incubation occasional bacterial groups were observed but they did not contain five or more bacteria and were not classified as microcolonies. AGT300 was termed PA (see figure 3.13 D and M).

The EHEC O157:H7 strain H11 adhered in small numbers to HEp-2 cells after 3 hour and 6 hour incubation periods. Actin nucleation by H11 was observed after 6 hours incubation but not all the bacteria showed this ability. Microcolonies were rarely observed at either time point. H11 was termed PA. The 8624JK isolate was tested for adherence to HEp-2

cells and this strain showed a similar number of FAS positive bacteria as H11 (see figures 3.13 E and N).

The EHEC O157:H7 strain TT12B adhered to HEp-2 cells at 3 and 6 hour incubation time points. After 3 hours incubation the bacteria adhered without forming microcolonies and some of the bacteria were FAS positive. After 6 hours incubation the number of bacteria adhering was greater and the majority of the bacteria were FAS positive. TT12B was termed PA and on occasion groups of associated bacteria numbering less than five bacteria were observed after six hours incubation (see figures 3.13 F and O).

The EHEC O26:H11 strain 3801 adhered to HEp-2 cells after 3 and 6 hour incubations. After 3 hours, 3801 adhered in small numbers to the Hep-2 cells and not all the bacteria were FAS positive. After 6 hours, a greater number of bacteria adhered to the HEp-2 cells and the majority of the bacteria were FAS positive, with some bacteria forming groups number less then five bacteria. 3801 was termed PA (see figure 3.13 G and P).

The atypical EPEC O103:H- strain E77804, adhered in small numbers to the HEp-2 cells after 3 and 6 hour incubation periods. The number of FAS positive bacteria was greater after 6 hours incubation but not all the bacteria present at this time point were FAS positive. E77804 was termed PA (see figure 3.13 H and Q)

The EHEC O103:H2 strain PMK5 adhered to the HEp-2 cells after 3 and 6 hour incubation periods. After 3 hours bacteria were seen adhering to HEp-2 cells with the majority of them independent of microcolonies. Microcolonies were observed and they had a less compact appearance than microcolonies formed by E2348/69. The majority of the bacteria were FAS positive. After 6 hours incubation PMK5 adhered in greater numbers to the HEp-2 cells with the majority of the bacteria forming FAS positive microcolonies. PMK5 was termed LA/LAL (see figure 3.13 I and R).

The HEp-2 adhesion assay was repeated using the same strains but with incubation medium lacking D-Mannose. The strains adhered in the same manner to HEp-2 cells with or without D-Mannose present in the incubation medium. Therefore, type-1 fimbriae mediated adherence or catabolic repression of the bacterial strains does not appear to be

involved in adherence by the bacterial strains during the 3 and 6 hour Hep-2 adhesion assay.

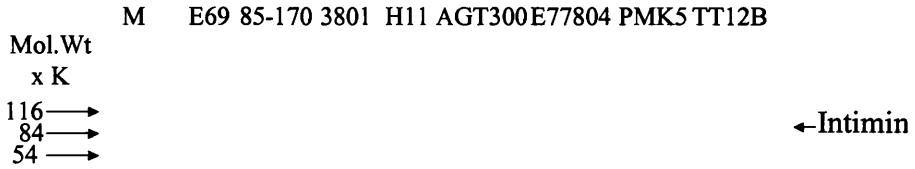
3.2.4 Intimin expression by bacteria during IVOC assay.

Intimin is required for A/E lesion formation during IVOC and is involved in the tissue tropism of EPEC during IVOC (Hicks *et al.*, 1998;Phillips and Frankel, 2000)and of EPEC and EHEC during colonisation of the intestinal tract of gnotobiotic piglets (Tzipori *et al.*, 1995). The EHEC strains and the atypical EPEC strain studied in this chapter express intimin as detected by Western blot of whole cell lysates activated by DMEM *in vitro* (see figure 3.12). Although it was not possible to test for intimin production during an *in* vivo infection, some of the bacterial strains were tested for intimin expression before and during the IVOC assay. This would assess whether intimin can be detected by Western blot of the bacteria present in the medium during IVOC and whether the bacteria expressed intimin during specific time points during IVOC.

The Western blot was carried out as described in 2.5.1. Due to the limited availability of intestinal explant tissue intimin expression, during the IVOC assay, was verified using the EPEC O127:H6 strain E2348/69 and the EHEC O157:H7 strain 85-170. These two strains were tested for intimin expression during their incubation with duodenal, PP and transverse colon explants.

Intimin expression was determined by Western blot in order to compare the intimin expression during IVOC with the intimin expressed by the bacteria when inoculated onto explants after overnight growth in BHI, without activation in DMEM.

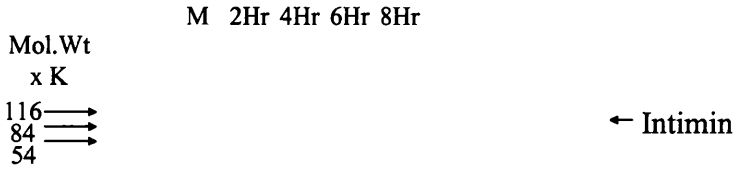
Figure 3.15 Western blot of bacterial strains applied to acrylamide gel after overnight culture in BHI broth at 37°C.



This Western blot showed that E2349/69 expressed intimin before it was added to the IVOC assay (figure 3.15 lane 2). In contrast EHEC strains and the EPEC O103:H- strain did not express after being incubated overnight in BHI broth at 37°C (figure 3.15 lanes 3 – 9).

In order to assess the binding of the intimin antibody to factors within the IVOC assay a Western blot was carried out using the medium from a duodenal explant IVOC assay that was not inoculated with bacteria. This corresponds to the negative control used in the IVOC assays.

Figure 3.16 Western blot of uninoculated IVOC medium used during explant IVOC.



As can be seen from figure 3.16 no bands were detected at any of the time points. Therefore the antiserum raised against the conserved domain of intimin did not bind to the tissue extracts present in the IVOC medium and did not bind to any of the constituents used to make up the IVOC medium, at a level detectable by Western blotting.

A duodenal explant IVOC assay was carried out using the EPEC strain E2348/69 and the EHEC strain 85-170. The medium was removed every two hours during the eight hour incubation period and the bacteria contained in the medium were probed for intimin expression by Western blot (figure 3.17).

Figure 3.17 Western blots of E2348/69 and 85-170 incubated with duodenum, PP and transverse colon explants during an eight hour IVOC.

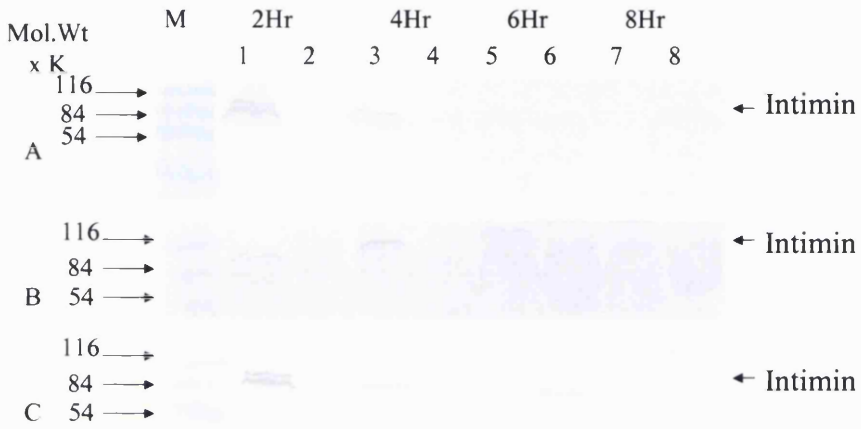


Figure 3.17 shows Western blots used to probe for intimin expression during IVOC of duodenum (A), PP (B) and transverse colon (C) explants. Lanes 1, 3, 5 and 7 correspond to E2348/69 incubations with each lane corresponding to 2, 4, 6 and 8 hour incubation times points, respectively. Lanes 2, 4, 6 and 8 correspond to 85-170 incubations with each lane corresponding to 2, 4, 6 and 8 hour incubation times points, respectively.

The Western blot in figure 3.17A shows that the IVOC medium, removed from the E2348/69 incubation after the two hour and four time points, contains bacteria that produce a band when reacted with the conserved intimin antiserum. Therefore E2348/69 did express intimin during the first four hours of the duodenal IVOC assay as detected by Western blot. The medium removed from the last four hours of the duodenal IVOC assay contains bacteria that give a weak signal. The bands appear to decrease in their signal intensity during the course of the IVOC assay. Therefore E238/69 may not express intimin during the last four hours of a duodenal IVOC assay or the bacteria expressing intimin are not in the IVOC medium.

Intimin expression by 85-170 could not be detected by Western blot throughout the duodenal IVOC assay.

The PP IVOC assay (figure3.17B) was inoculated with E2348/69 and 85-170. E2348/69 expressed intimin during the first six hours of the PP IVOC assay but only a faint band could be detected from the whole cell lysates produced from the bacteria contained in the medium removed from the assay at the eight hour time point. No band corresponding to intimin expression by 85-170 during the PP IVOC assay was detected by Western blot.

The transverse colon IVOC assay (figure 3.17C) was used to detect intimin expression by E2348/69 and 85-170 strains during inoculation with explants from the large intestine. Distinct bands corresponding to intimin expression could be detected during the first four hours of the E2348/69 colonic IVOC assay but the bacteria present in the medium removed from the last four hours of the assay produced a faint band. Therefore the bands decreased in signal intensity as the IVOC assay progressed. Intimin expression by 85-170 could not be detected by western blot throughout the colonic IVOC assay.

The explants used to test for intimin expression by bacteria in the IVOC medium during the eight hour IVOC assay were processed for SEM as described 2.2.10, and analysed. E2348/69 produced A/E lesions on the duodenal and PP explants but did not adhere to the transverse colon explant. 85-170 did not adhere to the duodenal, or transverse colon explants but produced A/E lesions on the PP explant. These results were similar to those described in section 3.2.1. Thus despite the absence of evidence of intimin expression by 85-170 using Western blotting, A/E lesions were present on PP. In addition, viable bacteria were recovered from the duodenal, ileal and colonic IVOC media, used for Western blots, when plated out.

3.2.5 Adhesion of EHEC O157:H7 to the large intestine and the duodenum.

Although the O157:H7 serotype is associated with pathology of the colon during haemorrhagic colitis, no study has yet provided evidence of A/E lesion formation on human colonic epithelium *in vivo*. EPEC have been both shown to adhere to the large intestine and cause colitis in animal models of infection (Mansfield *et al.*, 2001;Dean-

Nystrom *et al.*, 1998;Tzipori *et al.*, 1995). However, A/E lesions have been found in infants infected with EPEC (Rothbaum *et al.*, 1982;Lewis, Walker-Smith, and Phillips, 1987) but no similar phenomenon has been seen with EHEC.

In section 3.2.1 four O157:H7 strains were assessed for their adhesion to a range of areas within the large intestine. Using the standard eight hour IVOC assay, described in section 2.2, the four O157:H7 strains when grouped together produced A/E lesion formation on only 2/115 explants taken from the large intestine. The EHEC and EPEC strain as a whole adhered to 7/173 large intestinal explants and of those only 5/173 explants contained A/E lesions. This implies that colonic infection is infrequent *in vivo* and/or that the IVOC conditions are not conducive to EHEC infection. Alternatively, it reflects the *in vivo* events and colonic infection is a subsequent step following the initial step of infection of the follicular associated epithelium as reported for REPEC (Cantey and Inman, 1981;Heczko, Abe, and Finlay, 2000;Heczko *et al.*, 2001).

The aim of this section was to modify the existing IVOC protocol and determine whether EPEC and/or EHEC could produce A/E lesions on colonic explants with a higher frequency than reported earlier in section 3.2.1, and to see whether the adhesion of certain strains to duodenal explants could be influenced.

The transverse colon region of the large intestine was used to provide explants during the majority of the experiments as it was the most reliable source of large intestinal explants.

3.2.5.1 Effect of D-Mannose on the IVOC of O157:H7 and transverse colon explants.

Mannose is a constituent of the IVOC medium and can be used to prevent non specific binding of EPEC via type-1 fimbriae(Elliott and Kaper, 1997). In addition to binding to type-1 fimbriae, D-Mannose may have some catabolic effects on bacteria(Evans *et al.*, 1991;Nishikawa *et al.*, 1995). However, although type 1 fimbriae are not expressed by all EHEC strains and EHEC O157:H7 strains may contain a 16bp deletion in the genes encoding fimbriae expression (Li, Koch, and Cebula, 1997), some EHEC strains do

express type 1 fimbriae and their role in adhesion to intestinal mucosae has yet to be elucidated (Durno, Soni, and Sherman, 1989;Roe *et al.*, 2001).

The D-mannose was removed from the IVOC medium and the EHEC O26:H11 strain 3801 and the O157:H7 strains 85-170 and H11 were assayed for adherence to transverse colon explants using the IVOC system as described in section 2.2.3. The EAEC strain O42 was included as it adheres to transverse colon in the presence of D-mannose. The strains were tested on three separate occasions using explants from different patients.

Table 3.3 Adherence of EHEC to transverse colon explants using IVOC medium lacking D-mannose.

Strain	Transverse colon
O42	3/3
85-170	0/3
H11	0/3
3801	0/3
Patient age (months)	39, 129, 140

The EAEC O44:H18 strain O42 adhered with an aggregative pattern to all three explants tested. Removal of D-mannose from the IVOC medium had no effect on the ability of O42 to adhere to transverse colon explants using the IVOC assay. No change in the level of adherence was observed due to the removal of D-mannose from the IVOC medium, when compared with the IVOC assay of O42 in the presence of D-mannose.

The EHEC strain 85-170, H11 and 3801 did not adhere or produce A/E lesions on transverse colon when assayed using the IVOC system with medium lacking D-mannose. The epithelium of the explants used in this study showed similar surface integrity when compared with the explants used to test for adherence with medium containing D-mannose. Thus mannose does not influence adhesion of the EHEC strains to colon.

3.2.5.2 The effect of incubation time and frequency of medium change on adherence of bacterial strains during IVOC.

The eight hour IVOC assay used in previous experiments involved a medium change every two hours and washing of the explant before fixing in glutaraldhyde. Changing the medium every two hours during the assay prevented an accumulation of metabolic products and a change in pH. However removal of the medium and its replacement with new medium after two hours may have had a negative effect on the expression of virulence factors by the bacteria that were not removed during the medium change. Although certain strains of EPEC and EHEC can produce A/E lesions on specific regions of the intestinal tract in the standard IVOC assay, it was not known whether the frequency of the medium change had an effect on adhesion to large intestinal explants. In addition, increasing the overall incubation time may have an effect on adhesion to large intestinal explants by the bacterial strains tested in this chapter.

The EHEC O157:H7 strain 85-170 was used to test for adherence to transverse colon after one medium change and without any medium change during an eight hour IVOC assay as described in section 2.2.4.

Table 3.4 Effect of medium change after four hours of incubation and no medium change on adhesion to transverse colon during an eight hour IVOC assay.

	Transverse colon	
Strain	4hr medium change	No medium change
O42	3/3	0/3
85-170	0/3	0/3
Patient age (months)	39, 158, 176	74, 158, 176

Each assay was performed on three separate occasions. The inoculated explants showed some increase in cell rounding and variation in crypt opening size when compared to the uninoculated control explants. This was not consistent and no surface changes in the transverse colon epithelium could be correlated with variation in length between medium

changes or no medium change during the eight hour IVOC assay, when compared with explants cultured according to the standard IVOC protocol described in 2.2.3.

As stated previously in section 3.2.1, the EAEC strain O42 adhered to the transverse colon during the normal eight hour IVOC assay. With a four hour medium change, instead of the medium being changed every two hours, O42 still showed adherence to the transverse colon explants during IVOC. This adherence displayed an aggregative pattern. However, O42 did not adhere to transverse colon explants during the IVOC assay in which the medium was not changed throughout the eight hour incubation period when the medium became acidic, as indicated by phenol red. A similar, but less pronounced, change in pH was observed in the IVOC medium after four hours when the medium was changed. Phenol red covers a pH range of 6.8 – 8.2 and no precise pH measurements were made.

EHEC strain 85-170 did not adhere to transverse colon explants during the eight hour IVOC assay either performed with a medium change after four hours or after no medium change.

The next set of assays involved incubating the explants in IVOC medium overnight at 37°C as described in section 2.2.4.2.

Duodenal and transverse colon explants were used during the overnight IVOC assays. These explants were incubated with the EPEC strain E2348/69 and the EHEC strain 85-170.

Table 3.5 Overnight IVOC assay of EPEC and EHEC.

	Intestinal region	
Strain	Duodenum	Transverse colon
E2348/69	3/5	1/3
85-170	1/4	0/3
Patient age (months) (median (range))	42 (16-134)	64, 112, 186

The age is shown in months and when more than four explants were tested the age is shown as a median, with a range indicated.

After incubation overnight, for ten hours without medium change, the inoculated IVOC medium was becoming acidic as indicated by an orange/yellow colour in appearance. The medium from the uninoculated IVOC assay was red/orange in colour. The explants when washed were more fragile than explants after an eight hour IVOC assay and had to be handled with great care.

The EPEC strain E2348/69 adhered and produced A/E lesions on both duodenal and transverse colon explants after overnight IVOC assay (see figure 3.18). E2348/69 produced A/E lesions on 3/5 duodenal explants. The two explants which showed no A/E lesion formation showed epithelia removal and lack of adhesion to intact epithelial regions. The explants which contained A/E lesions contained a marked increase in cell rounding and glycocalyx removal when compared to explants inoculated during an eight hour IVOC assay. There was a reduction in villous height but this was not greater than that observed in some of the eight hour duodenal IVOC assays. The A/E lesions formed by E2348/69 after overnight incubation were similar to those produced by the eight hour IVOC assay. However some structures were noticed on 2/3 duodenal explants. It was not determined whether these structures were prokaryotic or eukaryotic in origin and they appeared to be rod like (see figure 3.18B). E2348/69 adhered and produced A/E lesions on 1/3 transverse colon explants after overnight IVOC assay. Structures similar to those seen with the prolonged incubation of E2348/69 and duodenum were also observed (see figure 3.18C). The explants showed increased cell rounding, a variation in crypt size and loss of glycocalyx.

Figure 3.18 The interaction of E2348/69 with explants after overnight IVOC.

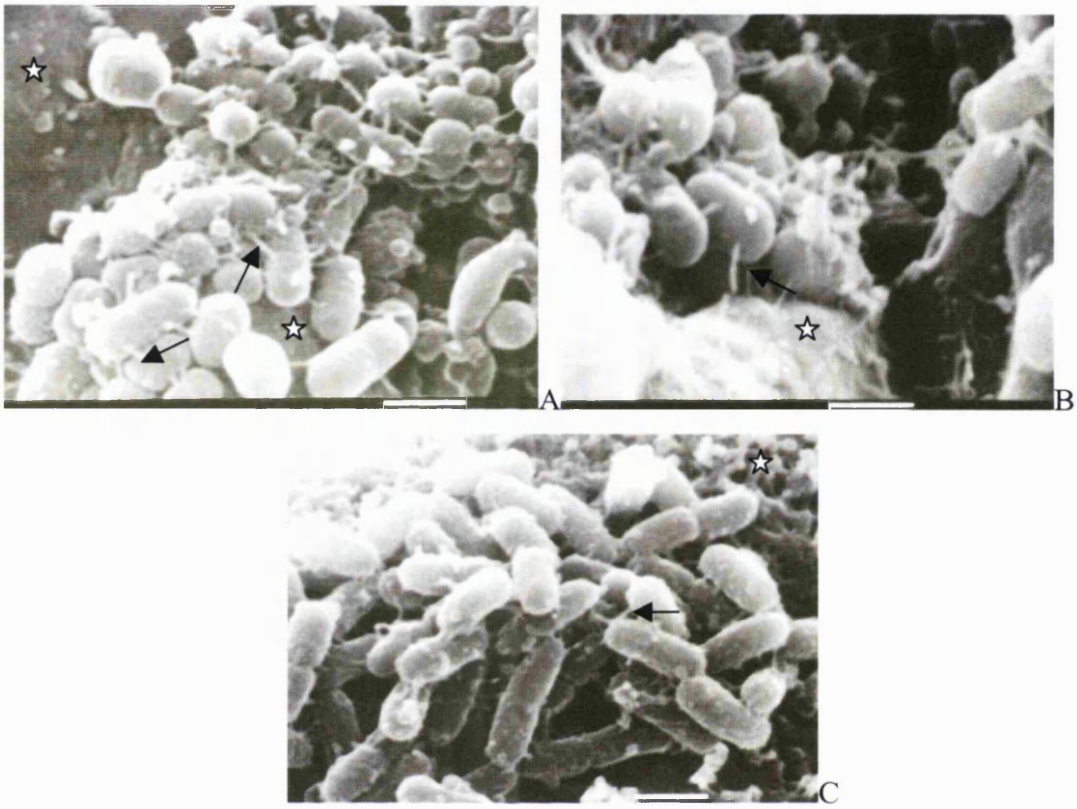
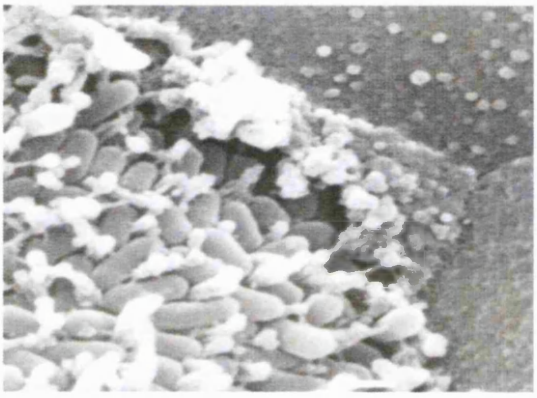


Figure 3.18 A shows an E2348/69 microcolony on duodenum with the presence of surface structures that are approximately 0.5μm in length (bar = 1μm, x15K magnification) and B shows a close up of the structures seen after overnight IVOC assay (bar = 1μm, x20K magnification). Figure 3.18 C shows E2348/69 adhering to transverse colon explants after overnight IVOC assay and the presence of similar surface structures to those observed when E238/69 is incubated with duodenal explants (bar = 1μm, x15K magnification). The black arrows indicate a rod like surface structure and the white star indicates the eukaryotic epithelial surface.

The EHEC strain 85-170 produced A/E lesions on 1/4 duodenal explants after a ten hour IVOC assay followed by incubation at 37^{o}C for ten hours. Under these conditions 85-170 did not adhere to transverse colon. Duodenal and transverse colon samples showed increased cell rounding, some epithelia disruption and glycoclayx removal.

Figure 3.19 A/E lesion after overnight incubation of 85-170 with duodenum.



The bar = 1μm.

Both E2348/69 and 85-170 produced A/E lesions on PP explants after a 10hr IVOC assay followed by ten hours incubation at 37°C. This PP assay was only carried out once.

Uninoculated explants showed cell rounding and glycocalyx removal.

3.2.5.3 The effect of centrifugation on adherence of bacterial strains during IVOC.

During the eight hour IVOC assay the bacterial strains were inoculated into the IVOC medium as close as possible to the intestinal explant. Prior to inoculation the explant was placed on a foam support which allows diffusion of medium and bacteria. When the bacteria were inoculated into medium they formed a suspension with the IVOC medium. Thus it was thought that some of the bacteria adhered to the mucus on the explant surface, leaving the rest to dissipate throughout the IVOC medium. The medium circulated across the explant due to the rocking motion of the platform on which the IVOC dishes sat. This motion allowed the bacteria to come into contact with the explant. The high concentration of the inoculum suggested that there were enough bacteria in solution to adhere to the explant. However the number of bacteria coming into contact with the explant may not have been great enough to produce A/E lesion formation on large intestinal explants. A method was developed to concentrate the bacterial inoculum onto the explant surface by centrifugation and limit the time for the bacterial culture to come into contact with the explant.

The EAEC strain O42 and the EHEC O157:H7 strain 85-170 were used to inoculate transverse colon explants using the assays described in section 2.2.5. The microfuge tubes were inoculated separately with 50µl and 100µl of inoculum for each bacterial strain tested. The microtitre plate was inoculated with 50µl of bacterial culture.

Table 3.6 IVOC of EHEC centrifuged onto the surface of intestinal explants.

	Transverse colon					
Strain	NC	CT50	NC	CT100	NC	CP50
O42	5/5	5/5	5/5	5/5	3/3	3/3
85-170	0/5	1(1adh)/5	0/5	1(1adh)/5	0/3	0(3adh)/3
Patient age (months) (median(range))	76(58-143)		139(63-155)		61, 87, 122	

Note: Values for 85-170 correspond to A/E lesion formation as a proportion of biopsies inoculated. Adh refers to adhesion without A/E lesion. Values for O42 refer to adhesion. NC = not centrifuged CT = centrifuged microtube CP = centrifuged microtitre plate
The age is shown in months and when more than four explants were tested the age is shown as a median, with a range indicated.

The transverse colon explants used in the assays described in this section showed similar surface epithelium to those used during the eight hour IVOC assay without centrifugation.

The EAEC strain O42 adhered to the transverse colon explants that had been centrifuged using both microfuge tube assays and the microtitre plate assay. The control explants that were not centrifuged also had adherent bacteria on their surfaces. O42 adhered to the explants in an aggregative manner.

The EHEC strain 85-170 adhered to the transverse colon explants using both centrifugation techniques. Using the microfuge tube assay, 85-170 adhered to 2/5 explants used, with

both the 50µl and 100µl inoculum. In addition, using both inoculum concentrations, 85-170 produced A/E lesions in 1/5 explants centrifuged in the microfuge tube. The A/E lesions were similar to those produced by 85-170 on other areas of the intestine after the standard eight hour IVOC assay. When 85-170 and transverse colon explants were centrifuged in microtitre plates, 85-170 adhered to 3/3 explants tested in this assay but no A/E lesions were produced. 85-170 did not adhere to or produce A/E lesions on transverse colon when assayed using the IVOC assay without prior centrifugation of explant and bacterial incoculum.

The explants on which 85-170 adhered or produced A/E lesions contained no follicular associated material. This was determined by examination of the morphology of the explants by SEM. In addition, routine histological biopsies taken from adjacent areas and examined by light microscope showed no follicular regions.

Figure 3.20 A/E lesion formation and adhesion of 85-170 to transverse colon explants after centrifugation onto explants.

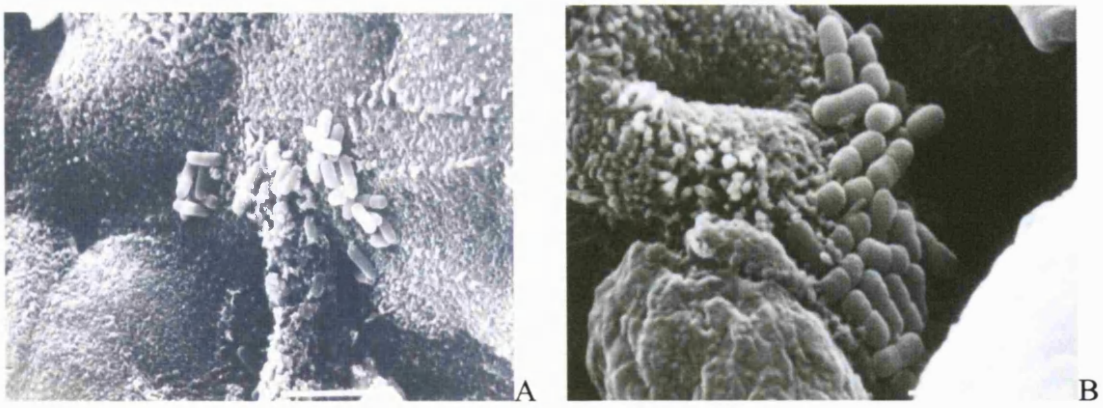


Figure 3.20A is a micrograph of 85-170 adhering to the surface of a transverse colon explant after centrifugation onto the surface of the explant and IVOC, bar = 5µm. Figure 3.20B is a micrograph of 85-170 producing A/E lesions on transverse colon explants after centrifugation onto the surface of the explant, bar = 1µm.

3.2.5.4 Activation of bacteria before IVOC.

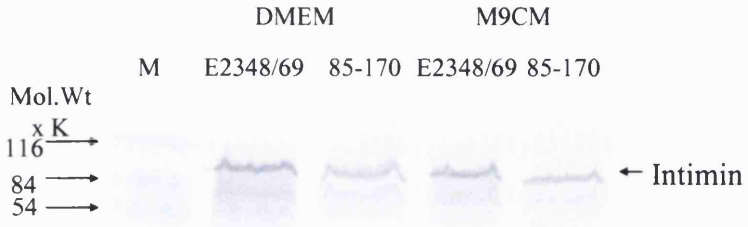
The expression of proteins via the type III secretion system is increased by growth in DMEM at 37°C(Haigh *et al.*, 1995;Jarvis *et al.*, 1995). This is true for both EPEC and EHEC(Jarvis and Kaper, 1996;Sperandio *et al.*, 1999). DMEM is used as a constituent of the IVOC medium in order to promote secretion of virulence factors encoded by the LEE. Intimin a product of the LEE region, is essential for A/E lesion formation by EPEC during IVOC(Hicks *et al.*, 1998). As can be seen from the Western blot in figure 3.15 expression of intimin by EHEC after overnight growth in BHI at 37°C is not possible to detect by Western blot of whole cell lysates. However, intimin is produced by EPEC and EHEC after activation in DMEM (see section 3.2.2). Although variation in intimin type appears to be involved in tissue tropism(Phillips *et al.*, 2000b;Tzipori *et al.*, 1995), the level or timing of intimin expression during the infection process has not been addressed during IVOC. However, alteration of intimin expression has been shown during adhesion of E2348/69 A/E lesion formation on HEp-2 cells. Intimin expression has been shown during E2348/69 A/E lesion formation on HEp-2 cells. Intimin expression was evident during the initial stages but was downregulated following A/E lesion formation (Knutton *et al.*, 1997).

The aim of this section was to induce or activate the bacterial strains prior to inoculation of the bacteria onto intestinal explants in order to see if this would influence the adhesion of bacteria to intestinal explants. The bacterial strains were activated by growing them in DMEM or M9 complemented medium (M9CM) as described in section 2.2.6. M9CM has been shown to produce high levels of protein secretion by EHEC (DeVinney *et al.*, 1999) and to induce the expression of LEE encoded proteins by EHEC and EPEC(Abe and Nagano, 2000).

The EPEC strain E2348/69 and the EHEC strain 85-170 were grown in DMEM and M9CM in order to determine the effect of growth in these media before inoculation of explants.

The expression of intimin by E2348/69 and 85-70 after growth in DMEM and M9CM was determined.

Figure 3.21 Western blot of intimin expression by EPEC and EHEC grown in DMEM and M9CM.



This blot shows the expression of intimin by E2348/69 and 85-170 after growth in DMEM and M9CM. The conserved intimin antiserum binds to the intimin expressed by both strains. The multiple bands shown are intimin degradation products. Therefore intimin expression is activated when E2348/69 and 85-170 are grown in DMEM or M9CM.

IVOC assays were performed to determine whether activation of intimin by DMEM or M9CM would influence the adherence of E2348/69 and 85-170 to duodenal or transverse colon explants during the eight hour IVOC assay.

Table 3.7 Adherence of activated EPEC and EHEC to duodenum and transverse colon.

	Intestinal region			
	Duodenum		Transverse colon	
	Activation medium		Activation medium	
Strain	DMEM	M9CM	DMEM	M9CM
O42	ND	ND	2/3	2/3
E2348/69	3/3	3/3	0/3	0/3
85-170	0/3	0/3	0/3	0/3
Patient age (months) (median(range))	94 (18-171)		172 (24-181)	

ND = not done.

DMEM = Dulbecco's modified Eagle's medium

M9CM = M9 complemented medium

The age is shown in months and when more than four explants were tested the age is shown as a median, with a range indicated.

The growth of E2348/69 and 85-170 in DMEM or M9CM did not induce adhesion to colon (nor to the duodenum in the case of 85-170), and does not appear to alter their tissue tropism. E2348/69 produced A/E lesions on duodenal explants, after growth in DMEM and M9CM, that were similar to those observed after the standard IVOC assay. The activation of 85-170 in DMEM or M9CM did not produce A/E lesions after an eight hour IVOC assay.

The explants' epithelium was similar to that observed when inoculated with bacteria from an overnight BHI culture.

3.2.5.5 The effect of bacterial passage on bacterial tissue tropism in IVOC.

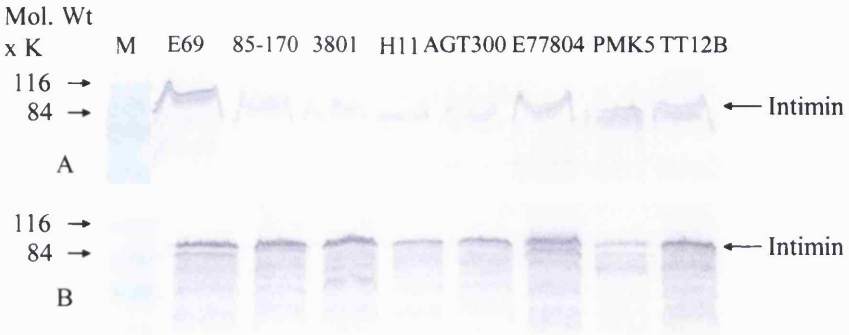
Section 3.2.5.4 showed that short term growth of bacteria in DMEM and M9CM media before inoculation has no significant effect on tissue tropism during the eight hour IVOC assay. The use of centrifugation to concentrate bacteria onto the surface of intestinal

explants and limit bacterial diffusion in IVOC medium, improved the adherence of EHEC strain 85-170 to transverse colon but did not induce A/E lesion formation during an eight hour IVOC assay. The growth of EHEC overnight in BHI at 37°C was used to inoculate explants according to the IVOC protocol. The EHEC cultured under these conditions showed no intimin expression when probed for intimin by Western blot figure 3.15, and in order to promote expression of intimin by EPEC and EHEC the strains were grown in DMEM. However growth of bacteria in rich media such as LB has been shown to promote fimbrial expression by non-pathogenic *E. coli* (Biebricher and Duker, 1984) and diarrhoeagenic *E. coli* (Iida *et al.*, 2001). This can also be achieved by serially passage of bacteria in static, non aerated BHI broth at 37°C for ten days in small test tubes (Old and Duguid, 1970). Type 1 fimbriae have been implicated in the binding of EHEC O157:H7 strain Cl-49 (Durno, Soni, and Sherman, 1989) to isolated human and rabbit ileal brush borders and it is possible that other fimbriae or adhesins activated by passaging bacteria could influence adhesion during IVOC.

In an attempt to promote adhesion of EHEC to the large intestine, bacteria were serially passaged in BHI broth. Transverse colon explants were used to test for adhesion to the large intestine by serially passaged EHEC strains.

The EHEC strains described in table 3.1 and the EPEC strain E77804 were passaged in BHI broth as described in section 2.5.3 and the strains were tested for intimin expression by Western blot after passage for 10, 12, and 14 days in BHI broth in bijoux. The western blots were carried out as described in section 2.5 and the passaged cultures were tested for intimin expression before and after activation in DMEM.

Figure 3.22 Western blot of EPEC and EHEC after 10 day passage in BHI broth.



Mol.Wt = molecular weight

M = protein ladder marker

E69 = E2348/69

A = Western blot of strains after 10 day passage in BHI broth in small test tubes

B = Western blot of strains used in A but activated in DMEM at 37°C

The Western blots of the EHEC and EPEC strains after 10 and 12 day passage in BHI broth in small test tubes were similar.

The EPEC strain E2348/69 expressed intimin after 10 days of passage in BHI broth in small test tubes. The EPEC strain E7804 and the EHEC strains PMK5 and TT12B also expressed intimin after passage in BHI broth (without culture in DMEM). The EHEC strains 85-170, 3801, H11 and AGT300 did not express intimin after 10 days of passage in BHI broth in bijoux. The bands detected for the EHEC strains (TT12B and PMK5) and the EPEC strain E77804, appeared to be of slightly lower molecular weight than the bands detected in the E2348/68 lane.

The EPEC strain E2348/69 expressed intimin after 14 days passage in BHI broth but the EHEC strains and the EPEC strain E77804 did not. However all the strains expressed intimin after growth in DMEM.

All the bacterial strains were positive for intimin expression when grown in DMEM after passage in bijoux containing BHI broth for 10, 12 and 14 days as seen in figure 3.21B

3.2.5.5.1 HEp-2 cell adhesion of bacterial strains after passage for 10, 12 and 14 days in BHI broth in bijoux.

The bacterial strains used in this chapter were passaged for 10, 12 and 14 days in BHI broth bijoux at 37°C and assessed for changes in HEp-2 cell adhesion phenotype in comparison to their phenotype after overnight growth in BHI. There was no change in CFU between 10, 12 and 14 days of passage in BHI broth in bijoux. The average 25µl inoculum contained approximately 5 x 10^7 bacteria/ml.

Table 3.8 Adhesion of bacterial strains to HEp-2 cells after passage in BHI broth in bijoux.

	Growth conditions							
	Standard assay		10DayPBHIB		12DayPBHIB		14DayPBHIB	
Strain	Adh	FAS	Adh	FAS	Adh	FAS	Adh	FAS
ICC170	NA	-ve	NA	-ve	NA	-ve	NA	-ve
E2348/69	LA	+ve	LA	+ve	LA	+ve	LA	+ve
O42	AA	-ve	AA	-ve	AA	-ve	AA	-ve
85-170	LAL	+ve	LAL	+ve	LAL	+ve	LAL	+ve
AGT300	PA	+ve	PA/LAL	+ve	PA/LAL	+ve	PA/LAL	+ve
H11	PA	+ve	PA/LAL	+ve	PA/LAL	+ve	PA/LAL	+ve
TT12B	PA	+ve	LAL	+ve	LAL	+ve	LAL	+ve
3801	PA	+ve	PA	+ve	PA/LAL	+ve	PA/LAL	+ve
E77804	PA	+ve	PA	+ve	PA	+ve	PA	+ve
PMK5	LAL/LA	+ve	LA/LAL	+ve	LAL/LA	+ve	LAL/LA	+ve

10, 12 and 14 DayPBHIB = number of days passage in BHI broth in bijoux at 37°C
Standard assay = Standard Hep-2 assay described in section 2.4.2.
CVD206 = *eae* negative mutant of EPEC O127:H6 strain E2348/69.
NA = No adhesion
Adh = Adhesion pattern on Hep-2 cells viewed under phase a contrast light microscope
FAS = Flourescent actin staining test

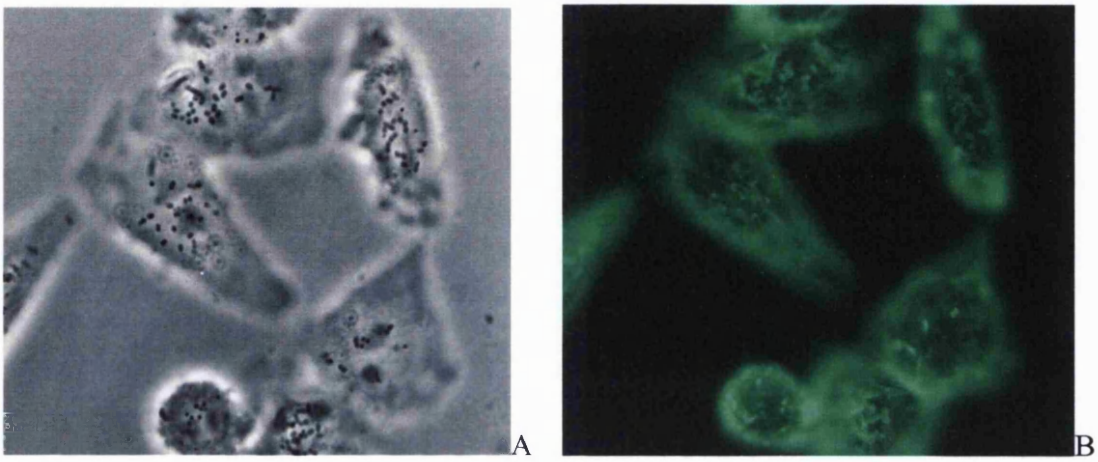
The intimin negative EHEC strain ICC170 did not adhere to HEp-2 cells after passage in BHI broth in bijoux. It was FAS negative.

The passage of E2348/69 in BHI broth in bijoux for 10, 12 or 14 days did not alter its adhesion pattern when compared with the results from the standard HEp-2 adhesion assay. It was FAS positive at all time points.

The EHEC strain 85-170 adhered to HEp-2 cells with an LAL pattern, similar to the result after a standard HEp-2 adhesion assay, after passage in BHI broth in bijoux for 10, 12 or 14 days. It was FAS positive.

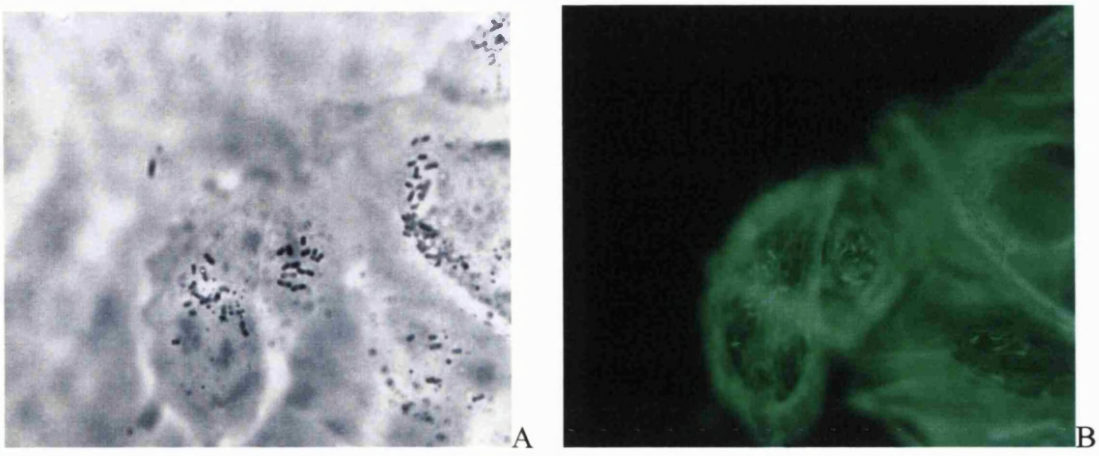
After passage of AGT300 for 10, 12 or 14 days in BHI broth in Bijoux it adhered to HEp-2 cells in a PA/LAL pattern. Therefore the passage of the bacteria in BHI broth in bijoux increased the number of adherent bacteria and resulted in the formation of microcolonies by AGT300 when compared to the result of the standard HEp-2 cell assay. It was FAS positive at each time point.

Figure 3.23 HEp-2 cell adhesion of AGT300 after passage in BHI broth in bijoux.



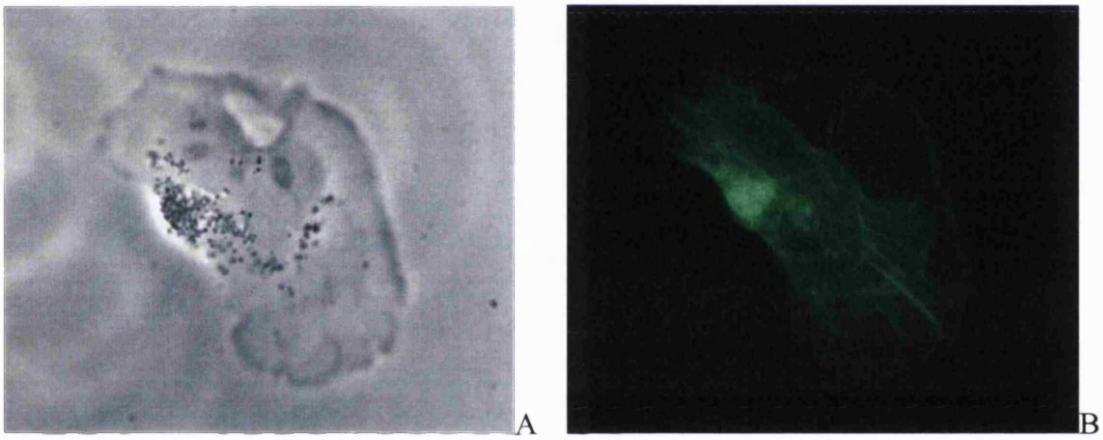
The EHEC strains H11 and 8624JK adhered to HEp-2 cells after passage for 10,12 and 14 days in BHI broth in Bijoux. They showed a PA/LAL pattern of adhesion. During the standard HEp-2 adhesion assay H11 adhered to HEp-2 cells in small numbers. After passage in BHI broth for 10, 12 or 14 days in bijoux it showed increased adherence to the HEp-2 cells and microcolony formation at the 6hr incubation time point. It was FAS positive as in the standard HEp-2 assay. The result was similar for the EHEC strain 8624JK and H11.

Figure 3.24 HEp-2 cell adhesion of H11 after passage in BHI broth in bijoux.



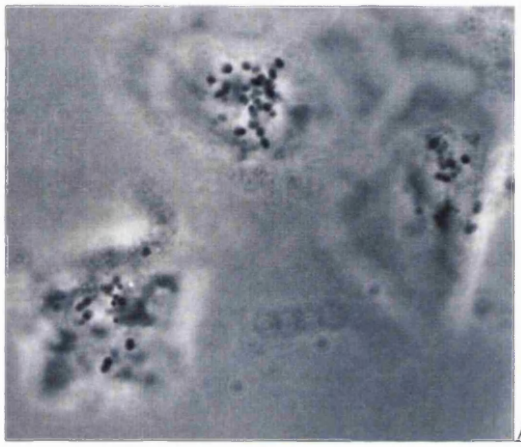
The EHEC strain TT12B adhered to HEp-2 cells after passage in BHI broth in Bijoux for 10, 12 and 14 days. As a result of repeated passage TT12B adhered to HEp-2 cells with an LAL pattern. An increase in adherence to HEp-2 cells at three and six hour time points and microcolony formation after six hours of incubation was observed when compared to the adhesion of TT12B after the standard HEp-2 assay. TT12B was FAS positive.

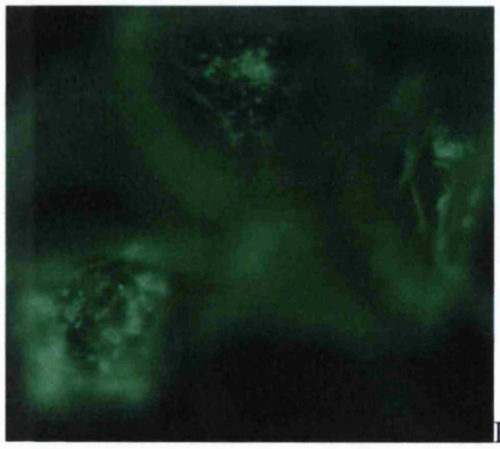
Figure 3.25 HEp-2 cell adhesion of TT12B after passage in BHI broth in bijoux.



The EHEC O26:H11 strain 3801 adhered to HEp-2 cells after passage in BHI broth in bijoux for 10, 12 and 14 days. A change in adherence pattern, when compared with the assay for 3801 using the standard HEp-2 adhesion assay, was observed after 12 and 14 days of passage in BHI broth in bijoux. After 12 and 14 days passage 3801 adhered to HEp-2 cells with a PA/LAL pattern showing an increase in adhesion to HEp-2 cells with the bacteria forming microcolonies after six hours of incubation with HEp-2 cells.

Figure 3.26 HEp-2 cell adhesion of 3801 after passage in BHI broth in bijoux.


A


B

The atypical EPEC strain E77804 adhered to HEp-2 cells after passage for 10, 12 and 14 days in BHI broth in bijoux. It did not show a change in adherence pattern when compared to the result of the standard HEp-2 cell adhesion assay. Magnification is x100.

The EHEC strain PMK5 adhered to HEp-2 cells after passage for 10,12 and 14 days in BHI broth in bijoux. It did not show a change in adherence patter when compared to the result of the standard HEp-2 cell adhesion assay. After passage the number of microcolonies formed after 3 hours incubation with HEp-2 cells did not change and therefore its adhesion pattern was LA/LAL.

The passage of bacteria for 10, 12 and 14 days in BHI broth in Bijoux altered the adhesion pattern, to HEp-2 cells, of some of the bacteria studied in this chapter. This passage altered the adhesion patterns of the EHEC strains AGT300, H11/8624JK, TT12B and 3801. No strains showed reduced adherence due to passage and the EPEC strains showed no change in adhesion phenotype as well as the EHEC strain 85-170 and PMK5. Therefore change in adhesion phenotype on HEp-2 cells does not appear to be related to intimin type.

3.2.5.5.2 IVOC of bacterial after passage for 10, 12 and 14 days in BHI broth in bijoux.

The HEp-2 cell adhesion assay showed that the passage of bacteria could alter the adherence phenotype of certain bacterial strains. Based on these findings IVOC assays were carried to determine the effect of bacterial passage *in vitro* on the adhesion of bacteria to intestinal explants *in vitro*.

The EHEC strains and the EPEC strain E77804 were passaged for 10, 12 and 24 days in bijoux containing BHI broth. At each time point a 25 µl aliquot of each bacterial strain was used to inoculate a transverse colon explant according to the eight hour IVOC protocol.

Table 3.9 Adherence to transverse colon of bacterial strains after passage in BHI broth in bijoux.

	Growth conditions			
Strains	No passage	10DayPBHIB	12DayPBHIB	14DayPBHIB
85-170	NA	0/3	0/3	0/3
AGT300	NA	0/3	0/3	0/3
H11	NA	0/3	0/3	0/3
TT12B	1/5	0/3	0/3	0/3
3801	NA	0/3	0/3	0/3
E77804	NA	0/3	0/3	0/3
PMK5	0(1adh)/5	0/3	0/3	0/3
Patient age (months)	115(38 – 198)	78, 151, 159	71, 84, 131	24, 130, 140

Note: Values correspond to A/E lesion formation as a proportion of biopsies inoculated. Adh refers to adhesion without A/E lesion formation.
10, 12 and 14 DayPBHIB = number of days passage in BHI broth in bijoux at 37°C
NA = No adhesion
The age is shown in months and when more than four explants were tested the age is shown as a median, with a range indicated.

As can be seen from table 3.9 the passage of bacteria in BHI broth in bijoux did not alter the tissue tropism of the EHEC strains and the EPEC strain E77804. The transverse colon explants used during these IVOC assays showed similar surface morphology to the explants used during the IVOC of bacterial strains that had not been passsaged.

In addition to the assay described above, PP explants were used to test for adhesion by bacteria after passage in BHI broth in bijoux. After 10 and 17 days passage, E2348/69 and 85-170 produced A/E lesions on PP explants during an eight hour IVOC assay. After 12 days passage in BHI broth in bijoux the O157:H7 strain H11 produced A/E lesions on 2/2 PP explants, during an eight hour IVOC assay.

Figure 3.27 A/E lesion formation by H11 on PP after 12 days passage in BHI broth in bijoux.

Figure 3.26 bar = 5μm.

The passage of the EHEC and EPEC used in this study did not influence adhesion to transverse colon explants. The passage of the EPEC strain E2348/69 and the EHEC strain 85-170 did not alter their ability to adhere to PP explants. However the passage of H11 produced an isolate of H11 that produced A/E lesions on PP during an eight hour IVOC, thus altering its IVOC adhesion phenotype.

3.2.5.6 The effect of tissue co-incubation on bacterial adherence.

The EPEC and EHEC strains tested in this section adhered to HEp-2 cells and at certain time points displayed different adhesion patterns. However the EPEC and EHEC strains did not adhere to all areas of the intestine during the eight hour IVOC assay. They showed tissue tropism for certain areas within the intestinal tract. Few strains showed the ability to adhere to large intestinal explants and those that did, did so for only a small percentage of the explants tested.

Peyer's patches have been implicated as initial sites of adhesion for REPEC during infection of rabbits and for *S. typhimurium*, *Listeria moncyotgenes* and *S. flexneri* during the infection of mice (Heczko, Abe, and Finlay, 2000;Jensen, Harty, and Jones, 1998). The results outlined in table 3.2 indicate that EHEC strains and the atypical EPEC strain E77804 may adhere initially to PP during *in vivo* infection due to their higher frequency of colonisation of this area during IVOC when compared to other regions of the intestine. Thus it is possible that adhesion to one region may facilitate adhesion to another region.

In order to attempt to promote the adhesion of the bacterial strains to large intestinal explants the HEp-2 adhesion assay was combined with the IVOC assay to determine whether HEp-2 adhesion and A/E lesion formation might produce subsequent adhesion to the transverse colon.

The EAEC strain O42, the EPEC strain E2348/69 and the EHEC strain 85-170 were used during the assays described in section 2.2.8. Each assay was carried out in triplicate using different explants for each assay.

Table 3.10 The effect of co-incubation on adhesion during the IVOC assay.

	Assay conditions		
Strain	T_0 T. colon + HEp-2	3hr HEp-2 + T. colon	6hr HEp-2 + T. colon
O42	3/3	2/3	2/3
E2348/69	1/3	0/3	0/3
85-170	0(1adh)/3	0/3	0/3
Patient age (months)	126, 171, 181	94, 100, 125	

Note: Values correspond to A/E lesion formation as a proportion of biopsies inoculated. Adh refers to adhesion without A/E lesion formation.

T_0 T. colon + HEp-2 = inoculation of T. colon and Hep-2 with bacteria at the start of the assay.

3hr Hep-2 + T. colon = 3hr HEp-2 assay followed by 8hr IVOC assay.

6hr Hep-2 + T. colon = 6hr HEp-2 assay followed by 8hr IVOC assay.

The EAEC strain O42 adhered to transverse colon during each assay. Incubation of O42 with HEp-2 cells prior to IVOC resulted in lack of adhesion by O42 to one of three explants. The HEp-2 assays carried out at each time point in the T_0 T. colon + Hep-2 assay showed adhesion to HEp-2 cells with an aggregative pattern. The HEp-2 cells without explant that were incubated for eight hours with IVOC medium change every two hours and the HEp-2 cells incubated according to the standard HEp-2 assay protocol showed aggregative adhering O42 bacteria when viewed under phase contrast light microscopy. O42 adhered to all explants during the T_0 T. colon + HEp-2 assay.

The EPEC strain E2348/69 produced A/E lesions on 1/3 transverse colon explants when inoculated onto HEp-2 and transverse colon explants at the same time (T_0 T. colon + HEp-2). E2348/69 did not adhere to transverse colon explants after incubation with HEp-2 cells for 3 and 6 hour incubation periods before an eight hour IVOC assay. All the HEp-2 cells viewed under phase contrast microscopy and stained for the FAS test showed the typical LA adhesion phenotype for E2348/69. The majority of the bacteria were grouped into tight microcolonies and E2348/60 was FAS positive.

Figure 3.28 A/E lesion produced by E2348/69 on a transverse colon explant after the T_0 T. colon + HEp-2 assay.

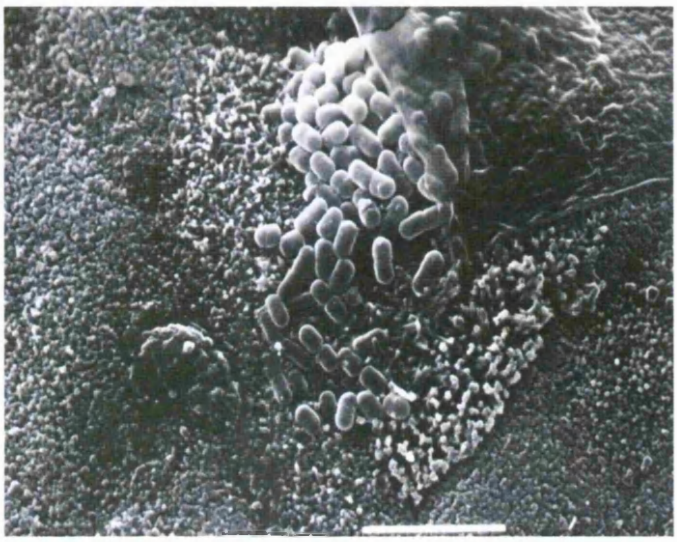


Figure 3.27 bar = 5µm.

The incubation of 85-170 with HEp-2 cells for 3 and 6 hour incubation periods before an eight hour IVOC assay produced no adherence to transverse colon explants. The control HEp-2 assay carried out in parallel produced the same LAL adhesion phenotype as the standard HEp-2 assay.

The inoculation of EHEC strain 85-170 onto Hep-2 cells and a transverse colon explant at the same time, T_0 T. colon + HEp-2, resulted in 1/3 explants showing bacterial adhesion without A/E lesion formation.

Figure 3.29 Adhesion of 85-170 to a transverse colon explant after the T_0 T. colon + HEp-2 assay.

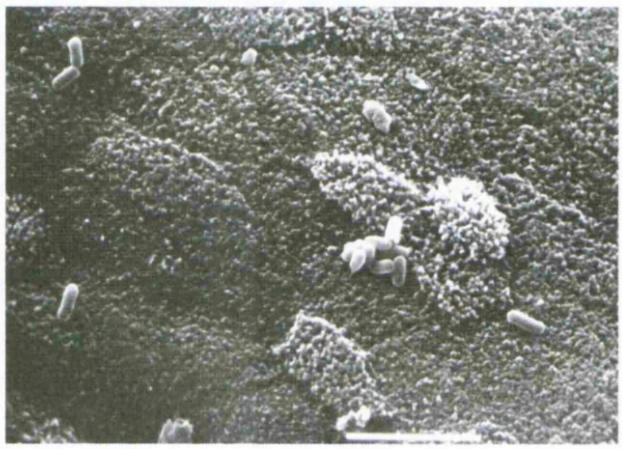
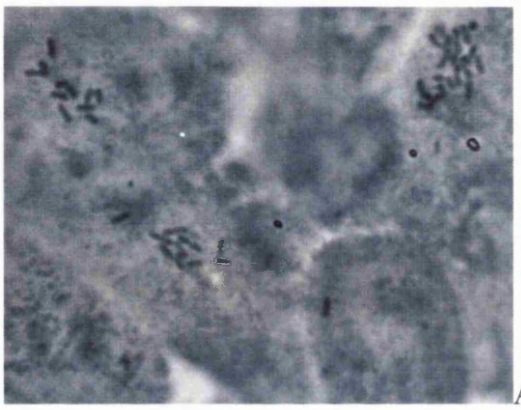
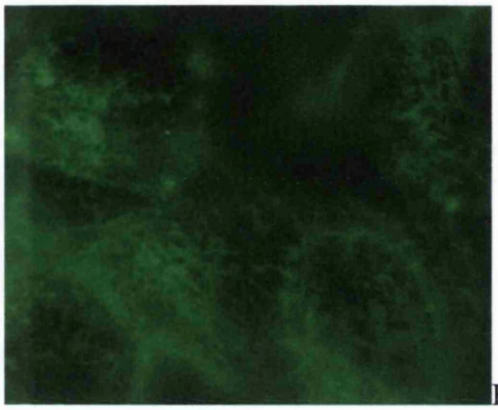


Figure 3.28 bar = 5μm.

The HEp-2 assays carried out at each two hour time point during the T_0 T. colon + HEp-2 assay showed an altered HEp-2 adhesion phenotype when compared with the standard HEp-2 adhesion assay. There was an increase in bacterial adhesion to HEp-2 cells after three hours incubation and six hours incubation periods from bacteria removed at all T_0 T. colon + HEp-2 two hour time points. However this increase in adhesion was similar at each two hour time point and no increase in adhesion proportional to IVOC assay time was observed. The increased adhesion to HEp-2 cells suggested a change in HEp-2 adhesion phenotype from the LAL phenotype to an LA phenotype due to the presence of the transverse colon explant. However the bacterial adhesion was not accompanied by a FAS positive result similar to the prototypical LA forming bacteria E2348/69. The actin nucleation was more diffuse than that seen in for 85-170 incubated in the standard HEp-2 adhesion assay and not similar to E2348/69. This is shown in figure 3.30

Figure 3.30 HEp-2 assay adhesion of 85-170 during T_0 T. colon + HEp-2 IVOC assay.


A


B

Magnification is x 100.

The HEp-2 cells inoculated without explant showed the same adhesion phenotype as in the standard assay and were FAS positive. The HEp-2 cells analysed after eight hours of incubation in IVOC medium contained more adherent microcolonies than after six hours incubation during the standard HEp-2 assay. They were FAS positive. 85-170 did not adhere to the transverse colon explant inoculated in the 24 well plate without the presence of HEp-2 cells.

The explants analysed by SEM showed similar surface morphology when compared with explants incubated during a standard eight hour IVOC assay. The wells containing no inoculum showed no adhesion to HEp-2 cells or SEM and the HEp-2 cells and transverse colon explants appeared similar to previous controls.

3.3 Discussion.

In 1980 Ulshen and Rollo used TEM and intestinal biopsies to show that a diarrhoeagenic *E. coli* strain could produce attaching and effacing lesions on intestinal mucosae *in vivo*(Ulshen and Rollo 99-101). This led to investigators developing models of infection to study the A/E lesions produced by EPEC and EHEC. The use of explants in bacterial adhesion assays has benefited from the study of diseases of the intestine such as ulcerative colitis and coeliac disease involving the culture *in vitro* of colonic and duodenal explants respectively. In addition explants have been used to study the enzyme activity and protein turnover of the intestinal epithelium during explant culture. Browning and Trier in 1969 showed that small intestinal explants could be cultured for 24 hours and that during this period cell proliferation occurred along with fat absorption by the epithelium (Browning and Trier 1423-32). Further studies showed that during the 24 hour culture period protein synthesis by the explant was maintained(Mitchell, Mitchell, and Peters 805-11). In addition, it is possible to culture colonic explants for up to 20 days *in vitro,* although by day 20 the cultured explants consisted of a single layer of cuboidal epithelial cells (Autrup et al. 1248-57).

Intestinal explants as well as primary intestinal epithelial cell lines have been used to study host pathogen interactions (Meng et al. 150-56). EHEC O157:H7 produces A/E lesions on bovine intestinal explants(Phillips et al. 377-81;Baehler and Moxley 239-42) and a mouse-human intestinal xenograft model (Zhang et al. 3240-47) has been used to study host-pathogen interactions. Other investigators have used human gastric explants to study *Helicobacter pylori* adhesion *in vitro* (Smoot et al. 46-54).

The culture of adult human intestine has been used to study the interaction of the intestinal mucosae with pathogenic *E. coli* (Knutton, Lloyd, and McNeish 69-77;Knutton et al. 3364-71;Knutton et al. 2083-91). Phillips et al modified the method used in these studies to incorporate paediatric human intestinal explants. This IVOC system was applied to the study of EHEC O157:H7 and showed that EHEC could produce A/E lesions on human intestinal explants (Phillips et al. 377-81).

Animal models and IVOC have been used to study the colonisation of the intestinal mucosae by A/E lesion forming pathogens. EPEC and EHEC as well as other A/E lesion forming pathogens, such as *Citrobacter rodentium*, exhibit species specificity and tissue tropism (Donnenberg, Kaper, and Finlay, 1997). In EPEC and EHEC the outer membrane protein intimin has been shown to be involved in colonisation and tissue tropism (Tzipori *et al.*, 1995;Phillips and Frankel, 2000). These studies showed that intimin exchange could alter the site of bacterial adherence and that EPEC and EHEC may adhere to specific sites within the intestinal tract. IVOC has also demonstrated that intimin is essential for A/E lesion formation on explants *in vitro* during an eight hour IVOC assay (Hicks *et al.*, 1998).

3.3.1 IVOC of EPEC and EHEC.

The bacterial strains described in table 3.1 were tested for their adherence to explants from representative regions of the intestinal tract. They expressed a range of intimin types including intimins α, β, γ and ϵ. It has been shown previously that EPEC strain E2348/69 and EHEC strain 85-170 can produce A/E lesions on intestinal explants. It was not known whether other EPEC and EHEC strains could produce A/E lesions during the IVOC assay. Both E2348/69 and 85-170 adhered to specific areas of the intestine and one of the aims of this chapter was to test the hypothesis that intimin type is involved in tissue tropism in man by defining the tissue tropism of other EHEC and EPEC strains. It might then be possible to associate adherence to areas of the intestine with defined intimin types and bacterial serotypes and the study of tissue tropism could be used to highlight the possible mechanisms by which an infection is established *in vitro*.

3.3.1.1 Changes in the explant epithelium associated with IVOC.

The explants used during the IVOC assays outlined in table 3.2 showed normal histopathological characteristics prior to IVOC, when viewed under light microscopy. Culturing of the explants during an eight hour IVOC assay did produce some changes in the explant morphology. During some IVOC assays, but not all, shortening of the villi of duodenal explants was observed. This could be assessed qualitatively and has been reported before. In the early experiments by Browning and Trier (Browning and Trier, 1969) they described villi to be shorter and wider after 24 hours culture when compared

with controls. Their method was similar but involved gassing their explants with 95%O_2/5%CO_2 twice during the 24 hour assay whereas the IVOC assay described in section 2.2.3 involves gassing every two hours over an eight hour incubation period. However it has not been determined whether gassing frequency is responsible for morphological changes in villi structure. It is possible that the change from the *in vivo* environment to the *in vitro* environment of the IVOC assay may have caused contraction of the smooth muscle contained in the villi thus producing a shortening of the villi (Womack *et al.*, 1987). In addition, although the microvasculature is maintained during biopsy the blood supply to the mucosae is lost which may result in a loss of turgur of the villi and produce some shortening of the villi (Kelly *et al.*, 1998).

The inoculation of bacteria onto explants produced an increase in cell rounding when compared to uninoculated controls, although the mechanism behind this cell rounding has yet to be defined. The cell rounding was interpreted as cell loss (Phillips, 1981) and was observed as cells rising above the epithelial surface as can be seen in figure 3.4. *In vitro* and *in vivo* models of infection have been used to characterised the mucosal response to EPEC and EHEC infection, and although no mechanism has been suggested to account for the EPEC and EHEC induced cell rounding during IVOC, such studes may provide insights into possible mechanisms. In a rabbit model of infection REPEC have been shown to not promote apoptosis and REPEC may decrease apoptosis during adhesion to PP and the terminal ileum (Heczko *et al.*, 2001). However EPEC factors such as Tir (Malish *et al.*, 2003) and EspF (McNamara *et al.*, 2001) have been implicated in apoptotic events. Cell turnover rates have been shown to be increased during *in vivo* infection by EPEC (Savidge *et al.*, 1996), demonstrating increased cell death, and a severe enteropathy can be found (Rothbaum *et al.*, 1982). The cell rounding seen in IVOC, may therefore have been a direct effect of EPEC. Another factor may have been local changes in pH due to the presence of excess bacteria or the presence of excess metabolic products excreted by the bacterial strains, as IVOC is a closed system. Changes in pH were noted as being adhesion dependent when *E. coli* were incubated with HeLa cells (McCabe, Mann, and Bowie, 1994). Although no link was observed during the IVOC assays performed in this chapter the presence of bacterial virulence factors may have produced an increase in cell rounding during IVOC. The presence of cell rounding in duodenal and terminal ileum explants was noted along the length of the villi and not just at the tip of the villi. This suggests that cell

rounding occurred in epithelia cells regardless of their position during the migration from the crypt to the apical region of the villous. Although the mechanism responsible for cell rounding during IVOC was not elucidated it has been shown that apoptosis augments bacterial adhesion to HEp-2 cells (Barnett *et al.*, 2000).

EPEC and EHEC express fimbriae and fimbriae may be involved in adhesion to host cells and in host inflammatory responses (Elliott *et al.*, 2000;Hedlund *et al.*, 2001b;Torres *et al.*, 2002). The expression of fimbriae by EPEC and EHEC may have had an effect on the epithelium of the explants. EHEC O157:H7 produce a proinflammatory response independent of either Stx or intimin (Berin *et al.*,).. The expression of fimbriae by EHEC may be found in genes located on the pO157 (Brunder *et al.*, 2001) or the EHEC chromosome (Torres *et al.*, 2002) and the EAF plasmids of typical EPEC strains contain genes for BFP which can induce cell death including apoptosis (Abul-Milh *et al.*, 2001). However, volunteer studies have shown that an EAF negative E2348/669 strain can produce diarrhoea suggesting that genes on the EPEC chromosome may be sufficient to cuase diarrhoea (Levine *et al.*, 1985). In addition to fimbriae, flagella have been implicated in EPEC adherence and EPEC and EHEC flagellin increases the production of IL-8 by the host cell (Berin *et al.*, ;Zhou *et al.*, 2003). The expression of fimbriae by *E. coli* can activate epithelial cells through toll receptors by delivering host activating molecules such as LPS to the toll – like receptor 4, this inducing an inflammatory response (Hedlund *et al.*, 2001a)

Intimin, Tir and the Stx of the wildtype Stx expressing strains PKM5 and 84-289 (Stx positive 85-170), did not influence the enteropathogenic response of bovine ligated ileal loops to these strains, suggesting other factors are involved in intestinal inflammation in calves(Stevens *et al.*, 2002). For example, the Esp proteins may have an effect of the host cells and the EspF protein of E2348/69 can induce cell death in cells without involvement in A/E lesion formation (McNamara *et al.*, 2001) but requires the presence of the type-III secretion system to deliver it inside host cells. EspC which does not require the TTSS for elaboration is related to the plasmid encoded toxin of EAEC. The plasmid encoded toxin of EAEC strain O42 has been shown to be involved in cell rounding during the adhesion to transverse colon explants during IVOC (Henderson *et al.*, 1999b). In addition, EspC was shown to act like an enterotoxin on rat jejunal tissue (Mellies *et al.*, 2001). An ORF

homologous to EspF exists in EHEC but has not yet been implicated in pathogenesis and an EspC homologue exists in EHEC and is termed EspP and may be involved in causing HC as it is able to cleave human coagulation factor V but is not a cytotoxin (Brunder, Schmidt, and Karch, 1997;Dutta *et al.*, 2002;Perna *et al.*, 1998). Thus the complete mechanisms involved EPEC and EHEC host cell death remain to be elucidated.

In addition to fimbriae and Esps, EPEC and EHEC express cytotoxins. EPEC are LT and Stx negative and some EPEC strains produce a low molecular weight ST called EAST1 (Enteroaggregative *E. coli* heat-stable enterotoxin 1) which is common in EAEC. E248/69 has two copies of *astA* gene which encodes for EAST1. One is located on the chromosome and one on the EAF plasmid. In addition, EAST1 is expressed by EHEC O157:H7 (Savarino *et al.*, 1996) and *Yersinia pestis* (Yamamoto and Taneike, 2000). However there are no reported cytotoxic effects associated with EAST1 and human intestinal damage produced by EAEC in colonic IVOC assays has been linked to its plasmid encoded toxin (Pet) (Henderson *et al.*, 1999b).

Enterohaemolysin is found in most EHEC O157:H7 strains and is widely distributed in non-O157 Stx producing strains. The genes that encode haemolysin in O157:H7 are found on the 60-MDa pO157(Beutin *et al.*, 1994). Although patients with HUS develop antibodies to haemolysin there have been no reports on its pathological significance of the haemolysin (Schmidt, Beutin, and Karch, 1995). The bacterial strains used in this study were not tested for haemolysin expression.

Therefore it is possible that the increased cell rounding observed on culture of explants and bacteria may have been influences by an uncharacterised virulence factor such as a toxin or fimbriae acting independently of intimate attachment of the bacteria to the host cell. In addition, induction of apoptosis by EHEC, via Stx and non-Stx induced pathways, has been suggested as being advantageous to bacterial adhesion (Barnett *et al.*, 2000).

HEp-2 cells have been used to show that EPEC can produce microvillus – like processes (MLPs) which appear to be similar to the microvillus elongation associated with A/E lesion formation during IVOC. MLPs were noted when HEp-2 cells were infected with E2348/69. Deletion of EspA or EspB increased the length of the MLPs suggesting that

MLP formation is not TTSS dependent and that intimin can activate cell signalling pathways involved in remodelling of the host cell surface. This was highlighted by the induction of MLPs by intimin coated latex beads (Phillips *et al.*, 2000a). All the A/E lesions produced by the EPEC and EHEC strains used in this chapter showed microvillus elongation suggesting that this activity is independent of intimin type. If intimin was responsible for microvillus elongation during A/E lesion formation then it also suggests that the residues involved in this biological function are located in a conserved region of the intimin molecule or that different combinations of amino acid residues, such as those present in the carboxy terminus of intimin, can induce intimin-mediated microvillus elongation.

A mechanism for the loss of glycoclayx during A/E lesion formation has not yet been proposed. The solution of the genome of O157:H7 strain EDL933 may provide opportunities to study the factors involved in the interaction of EHEC with the mucus and glycolcayx layers covering the villi of the intestinal tract (Perna *et al.*, 2001). EspP is homologous to the Pic (protein involved in intestinal colonisation) protein of EAEC and *S. flexneri* and like Pic may have mucinase activity (Al Hasani *et al.*, 2001;Henderson *et al.*, 1999a). However, not all of the glycoclayx loss was present at the site of A/E lesion formation. Glycocalyx thinning was noted on areas of intact epithelium suggesting that environmental conditions or soluble virulence factors not implicated in intimate attachment of the bacterium to the host cell may play a part in the removal or degradation of the glycoclayx. Glycocalyx produced by epithelial cells has a high turnover (Ito, 1969;Weiser, 1973;Leblond and Bennett, 1979) and thus factors which interfere with cell metabolism may disrupt glycocalyx formation.

3.3.1.2 The adhesion and tissue tropism of EPEC and EHEC strains during IVOC.

The IVOC assay uses explants from different patients to perform bacterial adhesion studies. Therefore unlike using HEp-2 cells and inbred animal models there may be some variability between the explants used. In order to counteract this effect each bacterial strain was tested on at least three occasions and on each occasion explants from different patients were used. Therefore this mimics to some degree the susceptibility of the intestine to A/E lesion formation as seen in natural infections. However, a comparison of

uninoculated explants from different patients shows minimal variation in morphological appearance (Autrup *et al.*, 1978).

The use of explant tissue was limited to the studies under investigation at a given time. This restricted the use of IVOC to specific experiments. During the IVOC assays performed in this chapter E2348/69 was used when appropriate as a positive control for small intestinal adherence. It has been shown that E2348/69 adheres to the small intestine with a high degree of reliability (Phillips *et al.*, 2000b). Therefore it was used as a reference point. As outlined in section 3.2.1, E2348/69 adhered to small intestinal explants with a degree of reliability similar to published data. E2348/69 adhered to a small percentage of transverse colon explants. However the EAEC O44:H18 strain O42 did adhere to colon, in these and previous studies, with a high degree of reliability and it was used as an adherence control when appropriate (Hicks, Candy, and Phillips, 1996).

The adhesion of EHEC O157:H7 strain 85-170 during IVOC has been reported previously (Phillips *et al.*, 2000b). In this chapter this work was repeated and extended to include regions of the intestine other than the transverse colon. The proximal and distal regions of the large intestine may show differences in glycoconjugate expression (Caldero *et al.*, 1989), which could influence bacterial adhesion. 85-170 adhered to intestinal epithelium with a restricted tissue tropism. Its adhesion to the small intestine during the eight hour IVOC assay was limited to PP explants and on one occasion to the FAE of an isolated lymphoid follicle in the duodenum. The latter could not be repeated as isolated lymphoid follicles in the duodenum were not observed during endoscopy and therefore selection of this area for biopsy was not possible. Although 85-170 did not adhere to transverse colon explants it did produce A/E lesions on 1/3 sigmoid colon explants. Thus intimin γ expressing 85-170 can produce A/E lesions in the large intestine. However the frequency of A/E lesion formation was low at 3% when compared with E2348/69 at 17% (this study) and adhesion to PP by 85-170 at 100%.. The number of explants tested for E2348/69 adhesion to the large intestine was much lower than that for 85-170 (6 compared to 36). Therefore further studies would have to be carried out to facilitate a comparison of the two strains.

EHEC 85-170 has a restricted tissue tropism during eight hour IVOC when compared to the typical EPEC strain E2348/69. The factors that determine this difference in tissue tropism during IVOC have not yet been fully elucidated. Both strains express different intimin types and this may play a part in tissue tropism. In the gnotobiotic piglet model intimin γ expressing EHEC adheres to the large intestine with intimin α expressing EPEC adhering to the small intestine and large intestine. Exchanging EHEC intimin with the intimin α from EPEC allowed the EHEC strain to adhere to the small intestine. Therefore in the gnotobiotic piglet model intimin is involved in EHEC tissue tropism (Tzipori *et al.*, 1995). During IVOC the adhesion of EPEC to small intestinal explants was abrogated by the exchange of EPEC intimin α with intimin γ from EHEC, allowing the EPEC strain to mimic the restricted tissue tropism of EHEC. Thus intimin is involved in EPEC tissue tropism during IVOC (Phillips and Frankel, 2000). It was shown using both gnotobiotic piglets and IVOC that intimin is necessary for A/E lesion formation. (Donnenberg *et al.*, 1993;Hicks *et al.*, 1998). Unlike E2348/69, 85-170 did not adhere to the epithelium of duodenal villi during the eight hour IVOC assay. The difference in tissue tropism of the two strains could be attributed to their intimin type. The cell-binding domain of intimin resembles, in topology, a C-type lectin and intimin types exhibit antigenic and amino acid variation within this domain (Adu-Bobie *et al.*, 1998). Therefore this region of the intimin molecule may play a part in the variation seen in tissue tropism between E2348/69 and 85-170. Although intimin γ appears to restrict adhesion of EPEC and EHEC to PP explants other factors may be involved. For example, RDEC-1 expresses intimin β and also targets PP during its infection of rabbits. Adhesion to areas within the intestine may not be restricted to one intimin type and may involve other virulence factors and events prior to A/E lesion formation. This may explain why the EHEC O157:H7 strain TT12B adheres to the duodenum, although with a lower frequency than E2348/69. TT12B also produces A/E lesions on the transverse colon underlining that although EHEC O157:H7 strains produce A/E lesions on large intestinal explants with a low frequency, intimin γ expressing EHEC strains are able to produce A/E lesions on the large intestine and the villi of the small intestine. It is possible that the low frequency of adhesion of EHEC O157:H7 strains is a reflection of the ability of these strains to colonise the large intestine in humans or that the IVOC assay needs to be modified.

The formation of A/E lesions by all the strains used in this chapter (including H11 after passage) suggests that the factors involved in the initial adherence of bacteria to the intestinal mucosae may be involved in mediating tissue tropism. The involvement of Tir or the EspA/B/D translocon in tissue tropism during IVOC has not been determined. Intimin α and intimin γ bind the Tir from E2348/69 suggesting that intimin type - Tir interactions were not critical to tissue tropism during IVOC. All the strains used in this chapter expressed EspA. In a study of EspA from different EPEC strains no antigenic variation in EspA was noted between strains and they shared at least 65% homology in their *espA* genes. Strains within an EPEC clone group showed a greater degree of homology (Neves *et al.*, 1998). This study included EPEC expressing intimin β and an intimin γ expressing O55:H7 strain related to O157:H7 but no assessment was made of the variation in EspA belonging to EHEC. Therefore it would be important to assess the events involved in EspA/B/D mediated interactions with the host cell and how they may act during initial adhesion to explants during IVOC.

The adhesion of 85-170 to sigmoid colon and the adhesion of TT12B to the transverse colon suggested that intimin γ expressing strains can produce A/E lesions on large intestinal explants. This is the first report of A/E lesion formation by an O157:H7 on paediatric large intestine cultured *in vitro*. Adhesion of intimin γ and intimin α expressing bacterial strains to large intestinal explants during IVOC occurs at a low frequency when compared to adhesion to the PP of the small intestine. The inability of both strains to produce A/E lesions on large intestinal explants during an eight hour IVOC assay highlights the complexity of the issues surrounding adherence and A/E lesion formation. The inability of α and γ intimin types to adhere to the large intestine with a high frequency may be regulated by factors within the biological activity of intimin or by other factors that act with and independently of intimin. The IVOC assay may have been too short in duration to allow the bacteria to express the factors that are required for A/E lesion formation on the large intestine. The fact that both EPEC and EHEC can adhere to other areas of the intestine during the eight hour IVOC assay implied that the eight hour assay was long enough for A/E lesion formation to occur and that the IVOC medium did not prevent A/E lesion formation. Whether the length of time or the IVOC medium was responsible for the low frequency of adherence of 85-170, TT12B and E2348/69 to the large intestine was not known. Attempts were made during this chapter to counteract such

possible limitations within the IVOC assay system. An increase in the IVOC assay time, activation of the bacteria before inoculation onto explants or stimulation of virulence factors that may be involved in adhesion to large intestine, may all influence the adhesion of EHEC O157:H7 strains to the large intestine. However, unlike strains 85-170 and TT12B the EHEC O157:H7 strains AGT300 and H11 did not adhere to the large intestine suggesting that adhesion during IVOC may exhibit some strain variation amongst EHEC O157:H7 strains. The adherence of 85-170 and TT12B to the FAE of an isolated lymphoid follicle from the duodenum provided further evidence that EHEC O157:H7 strains preferentially adhere to FAE. On 1/11 of the PP explants used to test for adherence by 85-170, A/E lesion formation was noted on the villi of the terminal ileum as well as the FAE of the PP(see figure 3.5B). Therefore adherence to the PP epithelium may be a first stage in the colonisation of the intestinal tract. The expression of virulence factors may be triggered by adhesion to PP explants that would then allow the bacteria to adhere to other areas of the intestine. This strategy has parallels in the infection by pathogenic bacteria of other species. RDEC-1 initially colonises the FAE of rabbit PP, in the first twenty four hours, and then after three days post inoculation adheres to the ileal or colonic mucosae. *Shigella* shows preferential adhesion to the FAE of PP (Sansonetti *et al.*, 1996). Some adhesins have been shown to promote adhesion to PP. This is true for the long polar fimbriae of *Salmonella* (Baumler, Tsolis, and Heffron, 1996), the AF/R1 pilus of RDEC-1 (Von Moll and Cantey, 1997) and β-1 integrin binding by *Yersinia* (Clark, Hirst, and Jepson, 1998). *Shigella* has a low infective dose as does EHEC O157:H7 and transit towards the terminal ileum would allow these acid resistant bacteria to increase their numbers intraluminally prior to colonisation of the PP in the T.ileum. The dose of the inoculum used to initiate the IVOC assay is much greater than that suggested as the infective does of EHEC which suggests that the number of bacteria present in the terminal ileum during O157:H7 infection could be high. It is probable that not all bacteria adhere and the non-adherent bacteria may be regulated by the adhering bacteria allowing them to colonise other regions of the intestinal tract.

Large intestinal lymphoid follicles were not used to test for bacterial adherence during the assays described in Table 3.2. Attempts were made to obtain large intestinal lymphoid follicles but when examined under SEM no follicular morphology was observed.

Apart from 85-170, three other O157:H7 strains were tested for their adhesion to PP explants. AGT300 and TT12B adhered to PP explants but H11 did not during the standard eight hour IVOC assay. Another isolate of H11 was provided by J. B. Kaper of the University of Maryland and this was named 8624JK and this strain did not adhere to PP or duodenal explants thus duplicating the H11 result. AGT300 adhered to 50% of the PP explants tested which was the lowest percentage adherence for any strain that adhered to PP. In addition, AGT300 produced footprints on PP explants which suggests that detachment may occur after A/E lesions formation possibly in order to colonise other regions of the intestinal tract or as a result of partially formed A/E lesions where bacteria are loosely, rather than intimately, attached. However, it was not possible to assess the viability of the bacteria involved in footprint formation as the bacteria were no longer *in situ* and it is also possible that footprints are a result of misformed A/E lesions or are produced if bacteria are no longer viable. Honeycomb structures have been reported during the infection of rabbits by REPEC which are similar in appearance to the footprints observed with AGT300. The honeycomb structures were assumed to be as a result of bacterial detachment before possible migration to and colonisation of other intestinal regions (Heczko *et al.*, 2001). The O157:H7 strain TT12B showed the greatest range of tissue tropism of the O157:H7 strains tested to date using IVOC. It adhered preferentially to PP explants like 85-170 and AGT300 but also adhered to the transverse colon and the villous epithelium of the duodenum. Adhesion to the duodenum and transverse colon was present in 20% of the explants tested from each region. However, like 85-170, TT12B produced A/E lesions on only one of the 24 large intestinal explants used during the eight hour IVOC assay. TT12B was the first O157:H7 strain to produce A/E lesions on the villous epithelium of the duodenum during the eight hour IVOC assay. These explants did not contain lymphoid follicles and suggest that intimin γ expressing strains can adhere to areas devoid of lymphoid follicles during eight hour IVOC thus implying a role for other factors in mediating tissue tropism. However, TT12B adhered to the FAE of an isolated lymphoid follicle in the duodenum and taken together with the PP results suggests that adhesion to FAE is preferential for O157:H7 strains. The colonic pathology associated with EHEC O157:H7 has been described in the ascending and transverse colon (Griffin *et al.*, 1988). The results listed in table 3.1 show that adhesion by EHEC strains to the colon occurs from the ascending colon to the rectum thus mirroring the *in vivo* reports. In a bovine model of infection rectal adhesion was suggested to be due to the increased

presence of follicular associated epithelium (Naylor *et al.*, 2003), but of the few large intestinal explants showing adhesion of EHEC, none contained lymphoid follicles. The reason that so few duodenum and large intestinal explants show adherence has yet to be explained and more work needs to be carried out to elucidate the regulation of O157:H7 virulence factors during *in vivo* infections. In addition, more O157:H7 strains should be tested using the eight hour IVOC assay to improve the tissue tropism description of this serotype.

The viability of H11, and the other bacterial strains used in this chapter, after eight hour IVOC was tested by growth on LB and MacConkey agar plates. MacConkey agar selects for gram negative cultures over gram positive cultures by inhibiting the growth of gram positive strains. This is due to the presence of bile salts and crystal violet (Kehl, 2002). The addition of lactose to the agar allows lactose fermentors to be differentiated from non lactose fermentors. *E. coli* ferment lactose and can be seen as pink/red colonies on MacConket agar. After IVOC culture of H11, colonies were observed on LB and MacConkey agar indicating a viable H11 culture post IVOC. Sorbitol MacConkey agar (sMAC) has been used to detect O157:H7 strains due to the inability of O157:H7 strains to ferment sorbitol. However, this detection method has a low sensitivity and in addition, some O157:H7 strains ferment sorbitol (Kehl *et al.*, 1997). It was not known whether the strains used in this chapter displayed this activity and no sMAC plates were available within department.

The intimin β expressing O26:H11 strain 3801 adhered and produced A/E lesions on the villous epithelium and the FAE of the terminal ileum. It did not adhere to duodenum and no duodenal isolated lymphoid follicles were available for IVOC assay of 3801. 3801 was the only EHEC strain to show adhesion to terminal ileal villous epithelium in explants lacking PP, without adhesion to the villous epithelium of the duodenum. E2348/69 showed adhesion to all areas of the small intestine and the intimin γ expressing strain TT12B and the intimin ϵ expressing strains E77804 and PMK5 showed adhesion to the duodenum without adhesion to the villous epithelium of the terminal ileum. This suggests that not all adhesion during an *in vivo* infection would require a step involving prior adhesion to PP or isolated lymphoid follicles. This may be the case for intimin α, β, γ and ϵ expressing strains, although adhesion without prior adhesion to PP or isolated lymphoid

follicles occurred at a lower frequency than adhesion to PP or isolated lymphoid follicles for all strains except the non-adherent H11 strain and E2348/69. 3801 adhered to the sigmoid colon suggesting that intimin β expressing strains, such as the O26:H11 strains implicated in HUS and HC, may adhere to the large intestine. 3801 adhered to the large intestine in 2/20 explants and produced A/E lesions on a rectum explant. In addition, 3801 adhered without A/E lesion formation in 3/6 adherence positive explants. The lack of A/E lesion formation by 3801 and its adherence to the villous epithelium of the terminal ileum but not the duodenum may be related to its intimin type or factors acting on or independently of intimin. The sequence of the whole intimin β shares the greatest overall sequence identity with intimin ϵ (Oswald et al. 64-71). However, none of the intimin ϵ expressing strains adhered to the villi of the terminal ileum, the sigmoid colon or the rectum suggesting that the tissue tropism of 3801 may not involve the same intimin residues as the intimin ϵ expressing strains E77804 and PMK5. The IVOC assay of further intimin β expressing strains would help to highlight the relationship between these strains and other EHEC and EPEC strains. The O26:H11 strain 3801 has its LEE inserted at a different site than EHEC O157:H7 strains and the typing of the tissue tropism of further strains during IVOC, may help to define the tissue tropism of strains according to their intimin type and their evolution (Sperandio et al. 133-39). 3801 belongs to EHEC group 2 which is related to the typical EPEC group 1 strains suggesting that like the typical EPEC strain E2348/69, 3801 and other EHEC group 2 strains may adhere to the villi of the terminal ileum, but possibly with different frequencies of adherence.

The intimin ε expressing strains E77804 and PMK5 showed a preferential adhesion to PP explants. Both strains showed a limited adhesion to the villous epithelium of duodenum and did not adhere to terminal ileum explants lacking PP. The atypical EPEC E77804 produced A/E lesions on the FAE of isolated lymphoid follicles of the duodenum. E77804 did not adhere to the large intestine but the EHEC strain PMK5 did and is further evidence of the ability of EHEC strains to adhere to the large intestine during IVOC, although with a low frequency when compared to EAEC adhesion and adhesion to the PP explants. It adhered to three regions of the large intestine showing the broadest range of large intestinal adherence of all the strains tested. Intimin ϵ strains have not been assigned to an EPEC or EHEC clonal group. However, intimin ϵ is one of the largest intimins

cloned and it shares overall sequence similarity to intimin β, but the last 280 C terminal amino acids which contain the Tir and cell binding region show a greater similarity with intimins α and γ (Oswald *et al.*, 2000). The IVOC of these strains seems to indicate that in terms of tissue tropism intimin ϵ expressing strains are more closely related to intimin γ than intimin α expressing strains. This is due to adhesion to PP explants with a higher frequency than adhesion to the duodenum or terminal ileum villous epithelium.

It is clear that intimin is necessary for adhesion during IVOC assay and that the FAE of lymphoid follicles is more receptive to EPEC and EHEC adhesion when compared to other areas of the intestine. The initial adherence of other bacterial species to PP in different animal models suggests that either bacteria possess specific mechanisms for PP adherence and/or that the PP environment is suited to bacterial interaction. Adhesion to lymphoid follicles may be an important means of initiating infection as lymphoid follicles are spread along the length of the human intestine (Cornes, 1965;Dukes and Bussey, 1926). Lymphoid follicles such as those contained in PP express different receptors to other areas of the intestine and have structural differences such as a thin glycocalyx layer which allows particle access (Brandtzaeg and Bjerke, 1990;Brown *et al.*, 1990;Chae, 1997;Foster *et al.*, 1998;Frey *et al.*, 1996;Hamzaoui and Pringault, 1998). The inability of the bacterial strains tested in this chapter to adhere to the large intestine or parts of the small intestine with a higher frequency may be linked to factors produced by the intestine. Non-pathogenic bacteria elicit a different cytokine response during epithelia leucocyte co cultures when compared with pathogenic bacteria (Haller *et al.*, 2000). Matrilysin, a matrix metalloproteinase is not expressed by germ-free mice but induced after colonisation with *Bacteriodes thetaiotamicron*. However in epithelia cells bacteria induced matrilysin production which correlated with a defensin-like bacteriocidal activity. The bacterial factor involved was a soluble non-LPS factor (Lopez-Boado *et al.*, 2000). Therefore cells exposed to bacteria can produce an innate immune reaction and thus prevent EPEC and EHEC adhering to certain areas of the intestine. However, *Shigella* has been shown to downregulate the antibacterial peptides LL-37 and human β-defensin-1 during infection and both of these peptides are present in the paediatric rectal biopsies (Islam *et al.*, 2001). The assessment of antibacterial effectors in different regions of the intestine or immune

antagonists expressed by EPEC and EHEC would help in understanding the ability of bacteria to adhere preferentially to one area of the intestine.

The IVOC assay is a system that has allowed the assessment of factors that may be involved in intestinal adhesion by EPEC and EHEC. The strains used in this chapter have shown that intimin may play a part in tissue tropism as suggested by animal models and further work using intimin exchange studies in EHEC during IVOC may allow the role of intimin during tissue tropism to be characterised. However these IVOC assays have shown that all intimin types can adhere to the small and large intestine, with varying frequencies. Therefore other factors may regulate tissue tropism or the spread of colonisation within the intestinal tract. All strains appear to be able to adhere to PP explants and E2348/69 is the only strain that showed a high frequency of adhesion to the duodenum and terminal ileum. In comparison to E2348/69, EPEC strain E77804 show a low frequency of adhesion to the villi of the small intestine with preferential adhesion to the PP. In order to assess the involvement of intimin in tissue tropism other intimin α expressing strains will have to be tested for adherence during IVOC. This work would be enhanced by the testing of a serogroup expressing different intimin types in order to assess the effect of a common genetic background on the tissue tropism of bacteria expressing varying intimin types. It would also provide further information on the adhesion of bacteria to the FAE of PP explants.

Intimin is necessary for EPEC adhesion to explants during IVOC and plays a part in tissue tropism during IVOC. As was demonstrated in figure 3.12, intimin is expressed by all the strains used in this chapter after incubating in DMEM. The bands corresponding to intimin were similar to those observed in previous studies (Batchelor *et al.*, 1999;Hartland *et al.*, 2000). The multiple bands present in some lanes are due to intimin degradation products and this has also been observed in western blots of invasin (Frankel *et al.*, 1998;Isberg, Yang, and Voorhis, 1993). DMEM is a constituent of the IVOC medium but it was not known whether intimin could be detected during the eight hour IVOC assay. If intimin could be detected, western blotting might show whether intimin is expressed or not expressed at certain times points during the IVOC assay or that intimin expression may vary according to tissue type.

3.3.1.3 Intimin expression during the IVOC assay.

In section 3.3.4 the expression of intimin after overnight growth in BHI broth at 37°C was assessed for each strain. After overnight growth it was possible only to detect intimin expression by E2348/69. The other bacterial strains either expressed no intimin or low levels of intimin that cannot be detected with certainty by western blot. The expression of intimin by E2348/69 in the stationary phase after growth in LB has been reported previously (Knutton *et al.*, 1997). It is interesting that intimin is being expressed by a greater number of E2348/69 bacteria after overnight incubation in BHI broth when compared to the other strains. E2348/69 was the only strain that contained the EAF plasmid. The other EPEC, strain E77804, did not. As well as modulating the adhesion of EPEC to Hep-2 cells the EAF plasmid contains gene sequences that make up the *per* or plasmid encoded regulator (Gomez-Duarte and Kaper, 1995). This set of genes can increase the expression of intimin and it may be its presence that allowed intimin to be detected in E2348/69 after growth overnight in BHI broth. However, the EAF plasmid has also been implicated in the downregulation of intimin expression during A/E lesion formation and an EAF negative strain of E2348/69 expressed more intimin after overnight growth in LB, without activation in DMEM, when compared to E2348/69. Thus the EAF plasmid present in E2348/69 regulates intimin expression and may account for the difference in intimin expression between E2348/69 and the EHEC strains and the atypical EPEC strain E77804, after overnight growth in BHI. In addition, the regulation of intimin by environmental factors or quorum sensing, via *per*, may account for the expression of intimin by E2348/69 as detected by Western blot after overnight growth in BHI. Whether this level of intimin expression allows E2348/69 to adhere to the small intestine with a higher frequency than the EHEC strains and the atypical EPEC strain E77804 was not known. However, the adhesion of CVD206 expressing intimin γ, rather than the intimin α of its wildtype E2348/69, to PP explants and not the small intestine suggests that factors expressed by the EAF plasmid do not determine tissue tropism during the eight hour IVOC assay (Phillips and Frankel, 2000). However in this study intimin γ was expressed on a plasmid and it is not known whether the interaction of EAF and plasmid expressed intimin affects tissue tropism during IVOC when compared with EAF acting on chromosomal expressed intimin. The tissue tropism of other EAF containing strains, during the eight hour IVOC assay, may help address this question.

The presence of intimin as detected by western blot during IVOC was assessed for strains E2348/69 and 85-170 after incubation on duodenal, PP and transverse colon explants. These strains and regions of the intestine have produced the most IVOC data to date and as such were chosen to aid in the comparison of EPEC and EHEC during IVOC. Using duodenal explants, intimin was detected in the first four hours of IVOC culture with a decrease in intimin expression with time. E2348/69 can produce A/E lesions after two hours of IVOC and intimin expression in E2348/69 is down regulated after A/E lesion formation (Hicks, Candy, and Phillips, 1996;Knutton *et al.*, 1997). This may have led to lack of intimin detection for E238/69 during the last four hours of IVOC assay. 85-170 did not produce A/E lesions on the villous epithelium of the duodenum and intimin was not detectable during IVOC. The regulation of the LEE in EHEC O157:H7 is different to E2348/69 and the lack of adhesion of 85/170 to duodenal explants may be related to these mechanisms. An EAF negative strain of E2348/69, JPN15, adhered to duodenum but only after 4 hours IVOC and JPN15 does not downregulate intimin expression during adhesion to HEp-2 cells suggesting that the delay in A/E lesion formation during IVOC is not due to intimin expression but may be due to another factor expressed or regulated by the EAF plasmid. However whether the lack of adhesion of 85-170 is due to low levels or delayed intimin expression would have to be addressed by analysing the regulation of 85-170 during PP, duodenal and large intestinal IVOC.

85-170 and E2348/69 both adhered to PP explants. However no intimin could be detected by western blot of 85-170 in the IVOC medium, during the eight hour IVOC assay. This suggested that a low number or none of the bacteria in solution expressed intimin during the eight hour IVOC assay. 85-170 may have only expressed intimin close to the surface of the PP explant and intimin may only be up regulated in the proximity of the PP explant or after other initial events prior to intimate attachment have occurred. E2348/69 expressed intimin during the first six hours of the eight hour IVOC assay. This suggests that like duodenal A/E lesion formation, intimin is expressed by the bacteria in the IVOC medium before A/E lesion formation and then may be down regulated once A/E lesions are formed. Therefore E2348/69 and 85-170 may express intimin at different stages of the adhesion process and the inoculum corresponding to each bacterial strain does not contain the same percentage of bacteria that are intimin positive during the eight hour IVOC assay.

E2348/69 and 85-170 did not produce A/E lesions on transverse colon when they were probed for intimin expression by Western blot during the eight hour IVOC assay. Although the signal intensity decreased with time, E2348/69 was positive for intimin expression during the first four hours of the eight hour IVOC assay suggesting that intimin expression by E2348/69 may be downregulated in the absence of A/E lesion formation. It appeared that intimin expression was maintained by the E2348/69 bacteria in the IVOC medium without subsequent A/E lesion formation. This may mean that events leading up to A/E lesion formation by E2348/69 are required to down regulate intimin expression. Transverse colon explants do not appear to inhibit intimin expression by E2348/69 and other factors may therefore be involved in the low frequency of adhesion of this strain to transverse colon explants during the eight hour IVOC assay. Intimin expression during IVOC appears to be different for E2348/69 and 85-170. The environmental factors involved in A/E lesion formation during IVOC have yet to be determined and interestingly the 85-170 bacteria present in the DMEM of the IVOC medium did not express intimin detectable by Western blot suggesting a local expression of intimin by 85-170 close to the explant surface or inhibited intimin expression by bacteria in the DMEM constituted IVOC medium during inoculation onto explants.

An assessment of intimin expression and the expression of other virulence factors in the IVOC medium and at the bacteria/explant interface would provide useful information on the sequence of regulation of virulence factors by EPEC and EHEC.

3.3.2 Hep-2 cell adhesion of EPEC and EHEC.

The adhesion of bacteria to the intestinal mucosae may play a role in the maintenance of bacteria in a nutrient rich host environment. The development of *in vitro* assays to test for bacterial adherence allows us to postulate on a the ability of a given bacteria's to reside *in vivo* and act a pathogen. In defining the characteristics of EPEC Kaper J.B. (Kaper, 1996) suggested that adherence to cultured HEp-2 cells was could be used to assess the A/E ability of a new bacterial isolate. Sectioning of the infected cells would allow A/E lesions to be observed by TEM or using the FAS test A/E lesions could be observed by actin nucleation under the site of bacterial adherence.

The HEp-2 assay used in this chapter was based on the Hep-2 assay of Cravioto et al (Cravioto *et al.*, 1979). A 25 µl (approximately 4 x 10^7) inoculum was used throughout and was the same size of inoculum used to inoculate the IVOC assay (Knutton, Lloyd, and McNeish, 1987a). Variations in HEp-2 cell adhesion assay protocols exist and depend on the emphasis of each study. Preincubation of the bacteria in various media, selected for their ability to activate virulence factors or prevent adhesin binding, have been used as well as variable incubation time points (Gansheroff, Wachtel, and O'Brien, 1999;Liu *et al.*, 1999;McKee and O'Brien, 1995;Sinclair and O'Brien, 2002). In order, to assess the adhesion pattern of the bacterial strains in described in Table 3.1, three and six hour incubation time points were used. The formation of bacterial clusters numbering five or more bacteria after three hours incubation was termed LA adherence (DeVinney *et al.*, 2001a;Nataro *et al.*, 1985). In order to standardise the assay the adhesion of E2348/69 was used as a reference point when determining whether bacteria adhered to HEp-2 cells with an LA pattern. The clusters or microcolonies produced by E2348/69 are tightly packed in appearance and are not diffuse. The term LAL was used to describe strains that did not produce the LA pattern of adherence exhibited by E2348/69 after three hours of incubation but that did produce microcolonies after six hours incubation (Scaletsky *et al.*, 1999;McKee and O'Brien, 1995). Some microcolonies may be observed after three hours incubation but unless the adhesion pattern was similar to E2348/69 it was referred to as LAL. Poorly adherent bacteria denoted by PA, did not form microcolonies of five or more bacteria after six hours incubation and showed less adherence to the HEp-2 cells after six hours when compared to the LAL pattern of adhesion. Quantitative assessment of bacterial adherence to cell lines has been carried out (Tatsuno *et al.*, 2000) but it has not been used to set the limits of the accepted HEp-2 cell adhesion phenotypes. This is due to the presence of certain virulence factors and serotypes being associated with defined adhesion patterns. This is highlighted by the presence of the EAF plasmid and expression of BFP being linked with the LA pattern of adhesion (Nataro *et al.*, 1985). However due to the difference in assays performed the HEp-2 adhesion assay is a flexible method and some variability may occur between studies (Vial *et al.*, 1990). In order to determine the adhesion patterns of the bacterial strains used in this chapter, each strain was assessed relative to the adhesion pattern of E2348/69.

The typical EPEC strain E2348/69 produced a LA pattern of adhesion on Hep-2 cells after three hours of incubation. The size of these microcolonies appeared to grow with time. After six hours incubation the E2348/69 microcolonies were larger than at the three hour time point and were still tightly packed. E2348/69 was FAS test positive at both incubation time points and the actin staining was defined and localised to the site of bacterial adherence.

The O157:H7 strains did not express the same adhesion phenotype on HEp-2 cells. All the strains tested displayed a PA pattern of adhesion except for 85-170 which had a LAL pattern of adhesion. 85-170 was LAL like because the microcolonies it did form after three hours of incubation were not as tightly clustered or as numerous as E2348/69. In addition, after three hours of incubation 85-170 had fewer FAS positive bacteria then E2348/69. Therefore 85-170 adhered in a different manner to E2348/69 and they did not share the same pattern of adhesion. The other intimin γ expressing strains (AGT300, H11/8624JK, TT12B) showed little adhesion after three hours of incubation and only the occasional grouping of bacteria (less than five bacteria) after six hours incubation with H11 showing the least number of adhering bacteria. However all the intimin γ expressing strains were FAS positive and therefore were A/E lesion positive according to the HEp-2 assay. This suggested that the pattern of adhesion of O157:H7 strains to Hep-2 cells is not determined by intimin type but may depend on factors other than intimin and is highlighted by the fact that the EHEC LEE is necessary but not sufficient for A/E lesion formation on HEp-2 cells (Elliott, Yu, and Kaper, 1999). In addition, although TT12B adheres to the duodenum during IVOC and 85-170, does not, AGT300 does not adhere to HEp-2 cells in greater number than 85-170. Therefore adhesion to IVOC and Hep-2 cells may occur via different mechanisms, and adhesion in one model may not determine adhesion in another model.

The EHEC strain 85-170 formed actin pseudopod structures after six hours incubation with HEp-2 cells. This has been reported previously for the intimin α expressing strain E2348/69 and during the *in vivo* infection of a rabbit model by REPEC O103 (Heczko, Abe, and Finlay, 2000). The mechanism by which EPEC and EHEC form pedestals on cultured epithelia cells appears to be different and yet E2348/69 and 85-170 can both produce similar actin rich pseudopods. It remains to be determined whether other EHEC

strains can produce actin pseudopods and whether such structures play a role during *in vivo* infections and it is interesting to note that EHEC O157:H7 strains do not secrete the same level of Esp proteins under the same growth conditions (Elliott, Yu, and Kaper, 1999;Jarvis and Kaper, 1996). In EPEC, pedestal formation requires the phosphorylation of a tyrosine residue at position 474 in Tir. This phosphorylation is not required for EHEC pedestal formation and it appears that additional EHEC factors need to be delivered into the host cell (DeVinney *et al.*, 2001b). Recently a new Esp, termed EspH, has been characterised and shown to be involved in the elongation of EHEC pedestals (Tu *et al.*, 2003). In addition, the phosphorylation of a serine at position 434 in EPEC Tir has been demonstrated as being required for efficient pedestal formation (Warawa and Kenny, 2001). Although pedestal structures have been observed as being similar in EPEC and EHEC it may be possible that the ability to form pseudopods is not present in all O157:H7 strains and that there may be other factors involved in elongation of pedestals that are not present in all O157:H7 strains. Further studies should assess whether intimin γ expressing strains EPEC and EHEC strains can phosphorylate tyrosine 474 in Tir and whether all EPEC and EHEC strains express EspH. In addition, studies should be carried out to assess the role of genes involved in *in vitro* pedestal elongation for their relevance during *in vivo* colonisation and pathogenicity.

The intimin ϵ expressing strains showed similar tissue tropism during the eight hour IVOC assay, with the exception of the ability of EHEC PMK5 to adhere to the large intestinal explants and the inability of the atypical EPEC strain E77804 to do so. The EAF plasmid was not contained in either strain and they did not share the same adherence pattern to Hep-2 cells. E77804 showed a PA pattern of adhesion to HEp-2 cells whilst PMK5 adhered with a LA/LAL pattern of adhesion. PMK5 did not produce an LA pattern due to the lack of tightly clustered microcolonies formed after three hours incubation. The microcolonies formed by PMK5 appeared to be loose in comparison to E2348/69. The lack of an EAF plasmid may have altered the shape of the microcolonies formed by PMK5 when compared to E2348/69 as the EAF plasmid does not appear to be essential for HEp-2 cell adherence and may be more important in colony type formation. PMK5 showed a greater number of adherent bacteria at three hours when compared with 85-170 and produced more microcolonies. The adhesion pattern of PMK5 does not appear to be determined by the expression of intimin ϵ and it may express other factors, not present in

E77804 or the other bacterial strains used in this chapter, which allows it to adhere with a LA/LAL phenotype. Although E77804 shares the same O103 serogroup, PMK5 expresses a H2 flagellum whilst E77804 could not be typed for a flagellar antigen and is H-. The H6 flagellum of E2348/69 has been shown to be involved in its adherence to epithelia cells (Giron *et al.*, 2002). Flagella expression can be stimulated in motile and non-motile strains by eukaryotic cells in culture. Therefore, it is possible that the H2 flagellum of PMK5 may have been involved in its adhesion to HEp-2 cells and the lack of flagellum in E77804 may have contributed to its lack of adhesion. The involvement of flagella has yet to be formally assessed during IVOC, although a *fliC* mutant of E2348/69 still cuases A/E lesions on duodenal IVOC (Philips, 2003). However the H- status of E77804 did not appear to compromise its adhesion to IVOC when compared with PMK5.

EHEC strain 3801 adhered to Hep-2 cells with a PA pattern of adhesion. Although intimin β shares overall sequence similarity with intimin ϵ it did not adhere in a manner similar to the EHEC PMK5. Some comparison could be made between E77804 and 3801 in that they were both poorly adherent but intimin type did not appear. in general, to determine HEp-2 cell adhesion patterns. Although 3801 was PA O26:H11 EPEC isolates have been typed as having a LAL adhesion pattern to HEp-2 cells(Pelayo *et al.*, 1999;Scaletsky *et al.*, 1999).

The lack of adhesion to HEp-2 cells of strains H11 and 8624JK was mirrored in their lack of adhesion during IVOC. This may be as a result of a lack of environmental signals necessary for the expression of virulence factors. The adhesion of the wildtype strain 8624 to Hep-2 cells has been reported previously as LA after 5 hours of incubation (McKee and O'Brien, 1995). This strain could not be assessed by HEp-2 assay due to the expression of Stx. The deletion of *stxA2* to create strain H11 and 8624JK may have altered the adhesion phenotype of 8624. Carbon source can influence the adhesion of EPEC to HEp-2 cells but growth phase and the number of CFU incubated with the Hep-2 cells does not appear to alter the ability of EPEC during the HEp-2 cell adherence assay (Vanmaele and Armstrong, 1997). Further studies were done to determine whether the tissue tropism or HEp-2 cell adherence of H11 could be altered by growth conditions.

Intimin expression is required for A/E lesion formation on HEp-2 cells and adhesion involves the C-terminal region of intimin (Gansheroff, Wachtel, and O'Brien, 1999). However no adhesion phenotype could be correlated with intimin type and no intimin type showed no adhesion. Therefore factors acting with and independently of intimin may affect adhesion HEp-2 cells and account for the lack of common adhesion phenotype amongst EHEC and EPEC strains and EHEC O157:H7 strains.

The presence of D-mannose in the HEp-2 assay medium did not alter the adhesion patterns produced by the bacterial strains used in this chapter. Therefore mannose sensitive fimbriae or adhesins, such as mannose sensitive type-1 fimbriae (Elliott and Kaper, 1997) do not appear to play a role in the adhesion of the EPEC and EHEC, used in this chapter, during their incubation with HEp-2 cells. Although there may be some redundancy in fimbrial adhesion. O157:H7 strains can contain a 16 bp deletion in the regulatory *fimA* gene resulting in the lack of expression of type 1 fimbriae (Roe *et al.*, 2001). However, type 1 fimbriae have been implicated in the tissue tropism of *Salmonella* (Thankavel *et al.*, 1999) and in the tropism of certain pathogenic *E. coli* for the urinary tract (Hung *et al.*, 2002). Although the majority of the EHEC reported do not express type 1 fimbriae, adhesion of O157:H7 via type 1 fimbriae has been reported using rabbit epithelia cells (Durno, Soni, and Sherman, 1989). An assessment of type 1 fimbriae expression by EHEC and EPEC *in vivo* may provide further information as to the role of type 1 fimbriae expression during pathogenesis and outline why certain strains retain the ability to express type 1 fimbriae.

The growth of bacteria in media containing D-mannose, prior to an adhesion assay, has been shown to alter the adhesion of EHEC and some EHEC strains may therefore be susceptible to catabolic repression (Nishikawa *et al.*, 1995). However the presence of D-mannose during incubation of the EPEC and EHEC strains listed in table 3.1 with HEp-2 cells did not alter their adhesion phenotype and thus catabolic repression as a result of the potential metabolism of D-mannose did not appear to be a factor in determining the adhesion phenotype of the strains used in this study.

The use of the HEp-2 assay as a means of comparison is beneficial when comparing new strains to data on existing strains. Adhesion patterns and tissue tropism during IVOC do

not appear to correlate for the strains tested in this section. However an assessment of this may benefit from an increase in the number of strains tested using both models of infection. HEp-2 cells can be used to study the adhesion and A/E lesion formation in strains that do not adhere during IVOC or when IVOC is not available. Fulfilling Koch's postulates for all pathogenic bacteria (Fredericks and Relman, 1996), such as EHEC, is not possible due to ethical considerations. Therefore the assessment of virulence using genetic and non genetic methods is important. HEp-2 cells have been used to associate certain bacterial adhesion patterns with diarrhoea (Nataro *et al.*, 1987). However genetic analysis of bacterial pathogens may be a better indicator of diarrhoeagenic bacteria (Knutton *et al.*, 2001) and from the study of HEp-2 cell adherence no adhesion pattern appears to be indicative of EHEC O157:H7 or the O103 serogroup. In addition no common pattern of adhesion could be found for EHEC, apart from the fact that the EHEC strains used in this chapter did not resemble E2348/69 adherence. Nonetheless, HEp-2 cells can be used to study the importance of a defined virulence factor in adhesion to eukaryotic cells and/or A/E lesion formation.

3.3.3 Adhesion of EHEC to the large intestine and the duodenum.

The ability of EHEC strains to form A/E lesions in the human large intestine raises pertinent questions about the mechanisms associated with EHEC pathology in general. The failure in detecting A/E lesion formation during *in vivo* infection in man is thought to be due to the collection of biopsies late in the disease. Therefore the assessment to date of the ability of EHEC to cause A/E lesions has been limited to animal studies and assays involving cultured epithelium. However the outcome of EHEC pathogenesis in relation to Stx induced pathology and A/E lesion formation varies according to which animal model is used. In the infant rabbit model diarrhoea was dependent on A/E lesion formation with changes in Na+ and Cl- absorption showing no correlation with Stx production (Li *et al.*, 1993). In a calf model EHEC O103:H2 was shown to induce enteropathogenic effects in ligated ileal loops that were not dependent on intimate attachment of the bacteria or production of Stx (Stevens *et al.*, 2002). Therefore although the ability of EHEC to produce diarrhoea is probably linked to A/E lesion formation the development of bloody diarrhoea and haemorrhagic colitis may require the production of Stx. In addition enteropathogenic effects may involve factors outside the A/E lesion Stx axis.

The mechanisms involved in adhesion of EHEC to the intestine are viewed in relation to EPEC due to the greater amount of knowledge being available about the latter. However the genes required for A/E lesion formation by EPEC E2348/69 are found within the LEE but the EHEC LEE is necessary but not sufficient for A/E lesion formation by O157:H7 on Hep-2 cells (Elliott, Yu, and Kaper, 1999).

The results from the IVOC assays used to define the tissue tropism of the strains listed in table 3.1 questioned whether the limited ability of the strains to adhere to the large intestine was a reflection of the IVOC assay conditions or an inherent characteristic of the strains used. The latter opened up many theoretical possibilities. The bacteria may bind to sites in the small intestine such as PP which have shown the highest degree of adherence per explant used for all strains when compared to other intestinal areas. Subsequent to PP adhesion appropriately activated virulence factors may allow the pathogens to adhere to other areas of the intestine such as the large intestine. However, it is also possible that pathology in the large intestine may result from secretion of Stx by luminal, non-adhering, EHEC, or by secretion of Stx after adhesion to PP of the small intestine with dissemination of the toxin into the large intestine. Such a mechanism may explain the presence of LEE negative STEC strains (Doughty *et al.*, 2002). The low frequency of adhesion to the large intestine may mirror the *in vivo* situation where few subjects may be susceptible to A/E lesion formation. The factors influencing such susceptibility are not known but the immune state of the host and genetic factors may play a role as well as malnutrition (Kurioka, Yunou, and Kita, 1998). No genetic marker has been clearly linked with susceptibility to EHEC infection. For example the exist conflicting reports as to the role played by ABO and P1 blood group antigens in the outcome of O157:H7 infections (Jelacic *et al.*, 2002;Blackwell *et al.*, 2002). The presence of ligands on the surface of the intestinal epithelium that would allow adhesion of EHEC and EPEC to the intestinal mucosae have yet to be identified but a receptor for intimin γ, called nucleolin, has been characterised on HEp-2 cells (Sinclair and O'Brien, 2002). Human intestinal brush borders were shown to contain a 32 to 33 KDa protein that binds to *E.* coli strains associated with the LA phenotype and binding of EHEC to phosphatidylethanolamine may promote adherence and EPEC BFP have been shown to bind to phosphatidylethanolamine (Barnett *et al.*, 1999). In other enteric pathogens such as *Salmonella* binding may be influenced by

cellular glycosylation patterns (Giannasca, Giannasca, and Neutra, 1996). If such ligands to EPEC and EHEC virulence factors were identified on intestinal mucosae it would be possible to establish their presence within a population and this may suggest why certain areas of the intestinal tract are less susceptible to A/E lesions than others. In addition, these ligands could be used to define the differences between animal models and different epithelial cell culture assays.

3.3.3.1 The effect of D-mannose on IVOC.

In section 3.2.5 experiments were designed to try and improve adhesion of bacteria to the large intestine. In the first instance, the IVOC assay medium was made up without the inclusion of D-mannose. As has been outlined previously in the discussion of the Hep-2 assay results the incorporation of D-mannose within assay media has been used to prevent non specific adhesion of bacteria via type 1 fimbriae. Knutton et al have shown that adhesion to the basolateral surfaces may be influenced by type 1 fimbriae and therefore the addition of D-mannose to the HEp-2 and IVOC medium was suggested as means of preventing the influence of basolateral adhesion on the adhesion of bacterial strains to the epithelial surface (Knutton, Lloyd, and McNeish, 1987b). However, the inclusion of D-mannose may affect bacteria through catabolic repression. It has been shown that bacteria grown in medium containing sugars are subject to catabolic repression which manifests itself as reduced adherence in subsequent adhesion to epithelial cells (Nishikawa *et al.*, 1995). It was not certain whether catabolic repression, as a result of D-mannose containing medium, would prevent adhesion during the eight hour IVOC. Adhesion to transverse colon was not altered by using IVOC medium without D-mannose. Therefore it appears that catabolic repression and adhesion via D-mannose sensitive adhesins is not a factor in the adhesion of O42, 85-170, H11 and 3801 to the large intestine during the eight hour IVOC assay.

3.3.3.2 Variation in IVOC incubation time.

The low incidence of A/E lesion formation in the large intestine may have been due to the frequency of the medium changes or the short length of the incubation time. In order to address this, the IVOC assay was performed for a longer period of time (overnight

incubation) and in a separate set of assays with fewer medium change steps. The latter assays involved changing the medium after fours hours and not changing the medium at all during the eight hour IVOC assay. The adhesion of 85-170 to transverse colon was not altered by incubation with fewer medium change events. However, the EAEC strain O42 did not adhere to transverse colon explants during an eight hour IVOC without medium change. This may have been due to environmental factors. A change in pH was noted around the explant and this change in pH was greater in the assays with no medium change than in the assay with one medium change during the eight hour IVOC assay. The metabolic products of the bacteria inoculated close to the explant may have produced this change in pH or it may be due to factors produced by the explant. In addition, CO_2 may have dissolved into the medium thus lowering the pH throughout the medium. The effect of such a change in pH or the presence of an increased level of bacterial and/or explant products may have prevented O42 adhering during the assay.

The extension of the eight hour IVOC assay in order to incubate the explant and bacteria overnight altered the adhesion phenotype of 85-170 to the duodenum but no to the large intestine. Therefore 85-170 can adhere and produce A/E lesions on duodenal explants after prolonged incubation and this showed that adherence of intimin γ expressing strains to duodenum may benefit from an IVOC assay with a longer incubation period. Although adhesion to the large intestine was not observed it is possible that adhesion to the large intestine will occur after prolonged incubation of EHEC and explants and that adhesion to PP is followed by adhesion to other areas with a higher frequency that that observed after the eight hour IVOC assay. Although the percentage of adhesion of E2348/69 may have been altered by overnight incubation the overall phenotype remained the same with adhesion to the small intestine being greater then adhesion to the large intestine. In addition, rod like structures were noted between the host cell and the bacteria and were approximately 0.5μm in length. They did not appear to be BFP and it is possible that they may be EspA as EspA can extend up to 600nm from the bacterial surface when bound to the cell surface (Daniell *et al.*, 2001;Sekiya *et al.*, 2001). The structures viewed after overnight incubation of E2348/69 appeared to occur between bacteria and between bacteria and the host cell suggesting that if they were bacterial in origin they may play a role in colony formation and adherence. They can be seen as rigid structures when connecting bacteria to with the host cell and this has also been noted with EspA filaments (Knutton *et*

al., 1998;Sekiya *et al.*, 2001). In addition, EspA filaments are present on bacteria after intimate adhesion of the bacteria to the host cell and actin accumulation has occurred. These structures were not routinely observed during the eight hour IVOC assay of E2348/69 and they were not present in all overnight incubations. Therefore, further study of these structures was not carried out and their characterisation has yet to be defined.

The presence of A/E lesions after twenty hours of E2348/69 incubation with duodenal, PP and large intestinal explants suggests that the A/E lesion may be long lived interaction with the intestinal mucosae or that E2348/69 forms A/E lesions at different time points during the IVOC assay. E2348/69 forms A/E lesions after two hours of incubation with duodenal explants (Hicks *et al.*, 1998) and no mechanism to disassociate the bacteria from the site of A/E lesion has been characterised during IVOC. No footprint structures were observed after overnight incubation and the A/E lesions were observed only in areas of intact epithelium suggesting that areas in which the epithelium had been removed may have contained adherent bacteria leading to possible epithelial removal.

The development of a prolonged IVOC assay would benefit from the development of a method to continuously change the medium surrounding the explant. This would have to be achieved without disrupting the interaction of the bacteria and the explant and not alter the viability of the explant. In addition, gassing of the IVOC assay would be needed to provide the explant with oxygen.

3.3.3.3 Centrifugation of bacteria onto intestinal explants.

The third set of assays involved trying to focus the bacteria onto the surface of the explant and at the same time limiting the effect of bacterial diffusion in the IVOC medium and the time it takes for the bacteria to come into contact with the mucosal surface of the explant. In order to do this, bacteria were centrifuged onto the surface of the explants. During the centrifugation process the explants were held in a microtube or a microtitre plate well. Centrifugation has been used with EPEC and other pathogenic bacteria to co-ordinate the infection process (Collington, Booth, and Knutton, 1998). Whether having bacteria diffuse in the medium or focused in one area affects bacterial signalling events is not known. The use of centrifugation did not alter the adhesion of O42 to transverse colon and did not

affect the epithelial morphology of the explants used. However, the centrifugation did alter the adhesion phenotype of the EHEC strain 85-170. It adhered to transverse colon explants whether the microtube or microtitre plate was used. The size of the bacterial inoculum did not appear to play a part in this result. The microtitre plate assay showed a higher frequency of adhesion to the microtube assay. However, A/E lesions were observed using the microfuge tube method suggesting that the focusing of bacteria onto the explant surface had allowed 85-170 to produce A/E lesions on transverse colon. This may have been due to a reduction in the time it takes for the bacteria in the inoculum to come into contact with the host cell surface when compared with the standard IVOC assay. In addition the concentrating of the inoculum by centrifugation may have altered the expression of virulence factors, possibly though quorum sensing, and led to a higher frequency of adhesion of 85-170 to the large intestine and the formation of A/E lesions by 85-170 on transverse colon explants. Comparing the expression of virulence factors during a standard IVOC assay with the IVOC assay involving centrifugation of the bacterial inoculum may highlight factors involved in the adhesion of 85-170 to large intestinal explants.

3.3.3.4 Activation of EPEC and EHEC prior to IVOC.

Media have been used in the study of various pathogens to stimulate the production of virulence factors and the presence of dyes like Congo red, in media, activate the expression of virulence factors. This is true for the type III secreted Ipa proteins of *Shigella flexneri* (Bahrani, Sansonetti, and Parsot, 1997). The expression of LEE encoded virulence factors is increased when EPEC and EHEC are grown in DMEM and the expression of EHEC virulence factors such as Tir and EspA were increased by growth in M9CM (Abe and Nagano, 2000). Previously the growth of overnight cultures of EPEC and EHEC in DMEM prior to inoculation onto HEp-2 cells has been used to stimulate the A/E lesion forming process (Rosenshine, Ruschkowski, and Finlay, 1996). Activation of EPEC strain E2348/69 and EHEC strain 85-170 in DMEM and M9CM prior to inoculation of duodenal and transverse colon explants was used to determine whether growth in these media before eight hour IVOC would affect the adhesion of the two strains to intestinal explants. Expression of intimin could be detected in both strains after growth in DMEM and M9CM. The signal was stronger than the signal for both strains after overnight growth in BHI. The

IVOC assay results of these activated strains showed that growth in DMEM and M9CM medium prior to IVOC did not alter the adhesion phenotype of both strains. Therefore growth in DMEM or M9CM did not result in the expression of virulence factors that altered adhesion during the eight hour IVOC assay or the activation of virulence factors by DMEM and M9CM before IVOC did not alter the adhesion phenotype of the strains. However it is possible that virulence factors may have been switched off when the strains were inoculated onto the explants in the IVOC medium and that altering variables that are effective during the IVOC assay may result in a higher frequency of A/E lesions on transverse colon by E2348/69 and 85-170.

3.3.3.5 Passage of the bacterial strains before IVOC.

The serial passage of bacteria in nutrient rich media has been used to promote the expression of fimbriae in bacteria and oxygen limited and anaerobic cultures of *E. coli* strains of serotype O157 have been shown to be more adherent on HEp-2 cells (James and Keevil, 1999). Therefore growth conditions may stimulate the production of different adhesins. Strains grown under oxygen limiting conditions lower their metabolism and this may trigger the expression of virulence factors. In addition, *ler* EPEC and EHEC mutants express novel fimbriae and the *ler* has been shown to be regulated by environmental signals (Umanski, Rosenshine, and Friedberg, 2002;Sperandio, Li, and Kaper, 2002;Elliott *et al.*, 2000;Sperandio *et al.*, 1999).

The strains described in table 3.1 were passaged in BHI broth in bijoux and assayed, using Hep-2 cells and IVOC, for a change in their adhesion phenotype. After passage in static BHI broth for 10, 12 and 14 days the strains were probed for intimin by western blot. After 10 and 12 days of passage E2348/69, E77804, PMK5 and TT12B were positive for intimin expression without activation in DMEM. Therefore the serial passage of these bacterial strains had resulted in the stimulation of intimin expression without activation in DMEM. All the strains were intimin positive after growth in DMEM suggesting that serial passage had not negatively affected the mechanisms involved in regulation of the LEE. However intimin expression was only detected for E2348/69 after 14 days passage in BHI broth without activation in DMEM. The altered expression of intimin after serial passage in nutrient rich medium was observed in both intimin ϵ expressing strains and it may be

that strains with the O103 serotype respond differently to growth conditions than other EPEC and EHEC strains. TT12B showed the broadest range of tissue tropism of the O157:H7 strains tested in this chapter and this may be correlated with its ability to express intimin after serial passage in nutrient broth. Both E77804 and PMK5 showed a similar ability to adhere to duodenal explants as TT12B, which was different from the other strains tested.

A Hep-2 cell adhesion assay was used to detect any changes in adhesion patterns due to serial passage of the bacterial strains. The serial passage of the bacteria produced a change in adhesion phenotype in all the strains showing a PA phenotype except EPEC strain E77804. Therefore it is possible that the detection of intimin expression after serial passage in BHI broth may not correlate with improved adhesion to HEp-2 cells. It may also have been easier to detect changes in the PA phenotype than in the LA and LAL phenotypes. Strain 3801 showed a delayed change in adhesion pattern when compared to the other PA strains. Therefore the effect of serial passage on each strain may be translated by different mechanisms in each strain. This highlights the fact that optimal growth conditions can vary from strain to strain and that assay results may be limited by such variables. The expression of fimbriae and other virulence factors appear to be regulated by complex pathways that may not respond to all growth conditions.

The passaged strains were tested for their adhesion to transverse colon explants to see if any variation in expression of virulence factors would mediate a higher frequency of adhesion to the large intestine. The eight hour IVOC assay was used to test whether changes in HEp-2 adhesion could be correlated with a variation in adhesion to the large intestine. After passage none of the strains tested showed adhesion to transverse colon explants. However, strain H11 which showed a change in adhesion to HEp-2 cells after serial passage in BHI broth, adhered to PP explants. Thus the serial passage of H11 had converted it from being non adherent during the eight hour IVOC assay to being adherent to PP explants. Therefore factors involved in adhesion to explants during the eight hour IVOC assay may be sensitive to growth conditions and that lack of adhesion to explants may be as a result of inappropriate growth conditions rather than a lack of pertinent virulence factors. In addition, prolonged serial passage up to 17 days did not alter the ability of strains E2348/69 and 85-170 to adhere to PP explants showing that extended

serial passage may be a possible mechanism in preparing bacteria for adhesion assays. However the passage of bacteria cannot be carried our indefinitely without acquiring deleterious mutations (Muller's ratchet) (Andersson and Hughes, 1996).

3.3.3.6 Co-incubation of HEp-2 cells with explants during IVOC.

Bacteria have been shown to respond to elements within their growth medium and in addition it is possible that factors expressed by eukaryotic cells allow bacteria to adhere or prevent adhesion (Giron *et al.*, 2002;Sperandio *et al.*, 1999). Different models of infection show different adhesion phenotypes and this may be related to the cell types involved, the effect of the immune system and the complexity of the barriers in place to prevent bacterial adhesion. These barriers include the glycocalyx which can modulate particle access to the epithelial surface (Frey *et al.*, 1996) and mucus which has been shown to be involved in activating Stx (Melton-Celsa, Darnell, and O'Brien, 1996) inhibiting binding of EPEC to HEp-2 cells (Smith, Kaper, and Mack, 1995). The surface of the mammalian epithelium undergoes marked structural and functional changes during postnatal development (Mahmood and Torres-Pinedo, 1983) and receptors are expressed at different points in the cell cycle (Majoul *et al.*, 2002). In addition, bacteria may stimulate the expression of ligands and immune effectors. LPS promotes the expression of soluble CD14 on intestinal epithelial cells (Funda *et al.*, 2001) and the epithelial secretion of the complement component C3 promotes the colonisation of the upper respiratory tract by *E. coli* (Springall *et al.*, 2001). Receptors specific to certain pathogens such as the nucleolin receptor present on the surface of HEp-2 cells during O157:H7 adhesion exist (Sinclair and O'Brien, 2002). *Shigella* have been shown to downregulate the expression of bactericidal peptides during infection and this process may be mediated by plasmid DNA (Islam *et al.*, 2001). In addition, conjugation has been shown to be possible between bacteria and mammalian cells (Waters, 2001).

Bacteria isolated from a host or bacteria that have been incubated with eukaryotic cell lines have been shown to be more virulent that those grown in culture (Garduno *et al.*, 2002). The EPEC and EHEC strains used in this chapter did not adhere to all areas of the intestine, and it is possible that adhesion in certain areas such as the large intestine may have been limited by host cell factors. Assays were performed to determine whether

coincubation or prior incubation of EPEC strain E2348/69 and EHEC strain 85-170 with HEp-2 cells would promote adhesion to transverse colon explants, and in the former whether incubation of transverse colon with HEp-2 cells can prevent adhesion to HEp-2 cells. Prior incubation of the bacterial strains with HEp-2 cells for three and six hours had no effect on the adhesion of the bacterial strains in a subsequent eight hour IVOC assay. This was possibly due to the downregulation of adhesion factors after A/E lesion formation or the presence of factors on the transverse colon explant that inhibited binding of the bacteria regardless of whether they had adhered to HEp-2 cells or not. The extended incubation of bacteria in the presence of epithelial cells did not alter the ability to adhere to transverse colon suggesting that virulence factors may be downregulated, such as intimin, during the three and six hour HEp-2 assays or that factors involved in adhesion to HEp-2 cells do not determine adhesion to the transverse colon. The initial adhesion to HEp-2 cells did not promote adhesion to the transverse colon. However, adhesion to other regions of the mucosal tract may promote adhesion to the transverse colon.

During the control HEp-2 assays E2348/69 produced an LA adhesion pattern showing no variation in adhesion to HEp-2 cells regardless of the growth conditions or presence of an explant. Coincubation of HEp-2 cells and transverse colon during the E2348/69 T_0 T. colon + HEp-2 assay produced A/E lesion in 1/3 explants used. Although this is a greater percentage of adhesion than during the standard eight hour IVOC assay it was not significant enough to suggest that the presence of HEp-2 cells had promoted A/E lesion formation on the transverse colon. However the presence of HEp-2 cells and transverse colon explants did not inhibit binding in the respective assays suggesting no negative effect of one to another. During the T_0 T. colon + HEp-2 assay 85-170 adhered to the transverse colon suggesting a possible effect on adhesion to transverse colon explants due to the presence of the HEp-2 cells. 85-170 adhered to the sigmoid colon during the standard IVOC assay but did not adhere to the transverse colon and it was not known whether factors involved in sigmoid colon adhesion were the same as those expressed during adhesion to transverse colon during the T_0 T. colon + HEp-2 assay. However some differences were detected. The HEp-2 assay of bacteria at each time point revealed and increase in the number of adherent bacteria and a change in the actin nucleation of the bacteria. The actin nucleation was more diffuse than during the standard assay and not comparable to the actin nucleation of any other strain. Therefore it is possible that the

adhesion to the transverse colon by 85-170 during the T_0 T. colon + HEp-2 assay produced different cell signalling events or involved a different adherence mechanism. The increased adhesion of 85-170 at three and six hour time points showed that adhesion as well as cell signalling events may have been altered by the presence of the transverse colon and HEp-2 cells in the same assay. Removal of the bacteria from the environment of the T_0 T. colon + HEp-2 assay and incorporation into a standard HEp-2 assay did not abrogate this changed phenotype suggesting that a novel bacterial adhesion mechanism had been selected for that is not inhibited by any factors involved in the standard HEp-2 assay.

3.3.4 Conclusions.

The experiments in this section suggest that intimin is involved in tissue tropism but that factors acting with or independently of intimin may influence tissue tropism during the eight hour IVOC assay. All the EHEC and EPEC strains listed in table 3.1 can produce A/E lesions on explants during IVOC. The variation in frequency of adhesion to regions of the intestine suggest that EHEC and EPEC may represent heterogenous groups of pathogens and that adhesion mechanisms can vary or be regulated in different ways. A/E lesion formation on the large intestine and the villi of the small intestine during IVOC can occur with EHEC strains and the atypical EPEC strain E77804 but this adhesion is lower in frequency that adhesion to PP explants.

Experiments to influence the adhesion of EPEC and EHEC strains to the large intestine and the duodenum showed that prolonged IVOC incubations, centrifugation of the bacteria onto the explant surface and coincubation of HEp-2 cells and explants had an effect on adhesion during IVOC. However, activation of the bacteria prior to IVOC did not have a similar effect and further work is required to determine the growth conditions necessary to influence the adhesion of EPEC and EHEC to explants during IVOC. Passage of the bacteria in BHI appears to have influenced the intimin expression of certain EHEC strains and passage in BHI produced a H11 isolate that adheres to PP explants during IVOC. Further work will be carried out to determine the effect of bacterial passage and growth conditions on the expression of virulence factors by EPEC and EHEC.

In order to define the role of intimin in tissue tropism the IVOC assay will be used to determine whether the tissue tropism of the EHEC O157:H7 strain 85-170 during IVOC can be altered by intimin exchange. In addition, IVOC will be used to define the tissue tropism of the O55 serogroup which contains strains that express intimin α and intimin γ and are both typical and atypical EPEC. This will provide further information about the tissue tropism of intimin α strains and the tissue tropism of EPEC strains, such as O55:H7, that are related to EHEC O157:H7.

4.0 Intimin and tissue tropism in man.

4.1 Introduction.

The importance of intimin in human disease was supported by the presence of a high titre of serum intimin antibodies in individuals infected with EHEC (Jenkins *et al.*, 2000) and in the colostrum of mothers in Brazil where EPEC infection is endemic (Loureiro *et al.*, 1998). In addition, an *eae* negative strain of EPEC was significantly attenuated when administered to human volunteers (Donnenberg *et al.*, 1993a) and did not adhere to intestinal explants during IVOC (Hicks *et al.*, 1998).

In the previous chapter, we have shown that, although EPEC and EHEC expressing different intimin types can adhere to the small and large intestine, intimin type was associated with preferential adhesion to areas of the intestine. The involvement of intimin in tissue tropism has been suggested previously using a gnotobiotic piglet model of infection (Tzipori *et al.*, 1995).

In addition, to mapping out the intestinal IVOC tissue tropism of the strains in table 3.1, the previous chapter sought to modify the IVOC protocol to determine whether tissue tropism could be altered by a change in IVOC methodology and/or activation of the bacterial strains. Although some positive results were obtained during these experiments, this chapter describes the results of experiments using the standard IVOC protocol to answer questions about the involvement of intimin in the intestinal tissue tropism of EPEC and EHEC.

Although the results in section 3.0 suggest that different intimin types can adhere to both the small and large intestine, preferential adhesion to PP explants appears to be common amongst EHEC strains and in atypical EPEC strains such as E77804. In addition to the gnotobiotic piglet model, IVOC has shown that intimin is involved in the tissue tropism of EPEC during IVOC (Phillips and Frankel, 2000a). Therefore initially this section sought to characterise the role of intimin in the tissue tropism of EHEC during IVOC. Intimin exchange studies were used to determine whether intimin type could influence the tissue tropism of O157:H7 during IVOC. Secondly strains belonging to the O55 serogroup,

which contains both typical and atypical EPEC strains, and strains linked to O157:H7, were used to determine the tissue tropism of bacterial strains from one serogroup expressing different intimin types. In addition, site directed mutagenesis of intimin α were performed to assess the biological activity of this intimin type during IVOC.

4.2 Results.

4.2.1 IVOC of the O157:H7 strain, 85-170, expressing different intimin types.

The first gene to be associated with A/E lesion formation was the EPEC *eae* gene encoding intimin. The *eae* gene was also detected in EHEC O157 and other serogroups (Huppertz *et al.*, 1996;Yu and Kaper, 1992), and has been shown to be required for the colonisation of O157:H7 in animal models and for A/E lesion formation by EPEC on intestinal explants using the IVOC model (Dean-Nystrom *et al.*, 1998;Donnenberg *et al.*, 1993b;Hicks *et al.*, 1998).

The prototype EPEC strain E2348/69 expresses intimin α and adhered to the small and, to a lesser extent, large intestine during IVOC. However, exchanging its intimin α for intimin γ limited its adhesion to PP explants during IVOC, thus mimicking the tissue tropism of the intimin γ expressing O157:H7 strain 85-170 during IVOC (Phillips and Frankel, 2000b). The intimin α from E2348/69 has been used to study the biological activity of intimin and the properties of amino acid residues within the C-type lectin binding domain (CTLD) have been characterised (Reece *et al.*, 2001). Binding of EPEC to HEp-2 cells but not to Tir is dependent on the presence of a disulphide bridge at cysteine (C) 937 (Hartland *et al.*, 1999). Substituting C937 with alanine (Ala, A) and expressing this intimin derivative in the EPEC CVD206 background resulted in the loss of adhesion of EPEC to intestinal explants during the eight hour IVOC (Hicks *et al.*, 1998). Using a gnotobiotic piglet model in which EHEC only adhered to the large intestine it was shown that EHEC expressing the intimin α from EPEC adhered to the small and large intestine and this result suggested that intimin was involved in tissue tropism in gnotobiotic piglets (Tzipori *et al.*, 1995).

In order to determine whether intimin α could modulate the tissue tropism of EHEC in man, an *eae* negative derivative of the O157:H7 strain 85-170 was created. This strain was complemented with plasmids expressing intimin α and γ, and intimin α with a disrupted disulphide bridge.

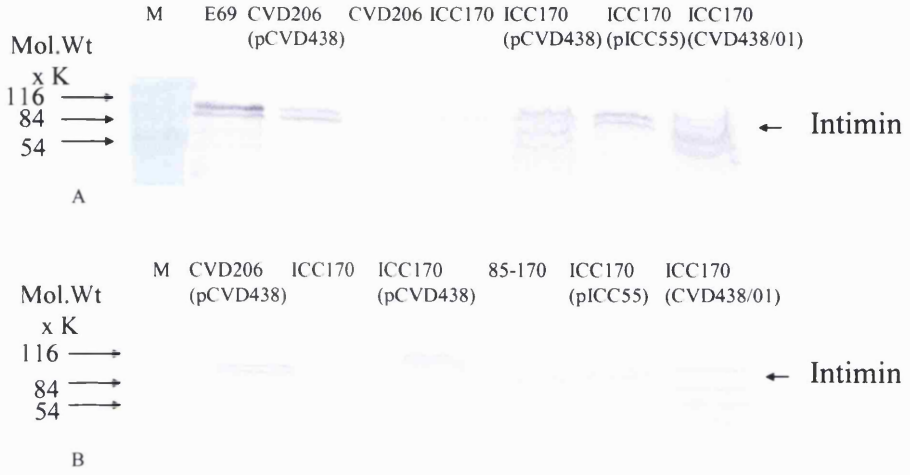
Table 4.1 Strains.

Strain	Description	Reference/Source
85-170	Spontaneous Stx negative derivative of intimin γ expressing EHEC O157:H7 strain 84-289	(Kelly et al. 313-18;Tzipori et al. 3117-25)
ICC170	Intimin negative strain of 85-170	(Fitzhenry et al. 180-85)/ Imperial College
ICC170pCVD438	ICC170 harbouring the intimin α expressing plasmid pCVD438	(Fitzhenry et al. 180-85)/ Imperial College
ICC170pICC55	ICC170 harbouring a pCVD438 derivative plasmid, pICC55, expressing recombinant intimin γ	(Fitzhenry et al. 180-85)/ Imperial College
ICC170pCVD438/01	ICC170 harbouring the intimin α expressing plasmid pCVD438, containing a C937A substitution	(Fitzhenry et al. 180-85)/ Imperial College

The ICC170 strains listed in Table 4.1 were generated in the laboratory of Professor G. Frankel, Centre for Molecular Microbiology and Infection, Imperial College, London, U.K. The construction of these strains is outlined in section 2.6

The strains in Table 4.1 were probed for the intimin production using an antibody against the conserved region of the intimin molecule as described in 2.5.

Figure 4.1 Western blot of intimin expression of 85-170 derivative strains.



Mol.Wt = molecular weight. M is a protein ladder marker.

The western blot in figure 4.1A shows that no intimin was detected after growth of strains ICC170 and CVD206 for four hours in DMEM. Intimin was detected in the strains expressing chromosomal intimin, E2348/69, and intimin on a plasmid, CVD206(pCVD438), ICC170(pICC55), ICC170(pCV438) and ICC170(pCV438/01). Therefore the *eae* negative derivative of 85-170, ICC170, could be complemented with plasmids expressing different intimin types. As has been reported previously (Frankel *et al.*, 1998), strains complemented with a plasmid (pCVD438/01) expressing intimin α with a C937A substitution resulting in a disrupted disulphide bond still expressed intimin that could be detected by western blot.

After overnight growth of the strains in BHI broth at 37°C (figure 4.1B) intimin expression was detected in strain CVD206(pCVD438), ICC170(pCVD438) and ICC170(pCVD438/01). No intimin was detected in the strain harbouring the pICC55 intimin γ expressing plasmid or the intimin negative strains.

Having established that the *eae* negative strain ICC170 could be complemented with plasmids expressing different intimin types, the bacterial strains were tested for their ability to colonise intestinal explants during eight hour IVOC.

Table 4.2 Regional adherence of O157:H7 strain 85-170 derivative strains, expressing no intimin and intimin of different types.

	Intestinal region			
Strain	D4	T. ileum	PP/T. ileum	Transverse colon
85-170	0/3	0/4	3/3	0/4
ICC170	ND	ND	0/3	ND
ICC170pICC55	0/4	ND	2/3	ND
ICC170pCVD438	5(+4 adh)/9	2(+2 adh)/4	3/3	0/3
CVD206pCVD438	6(+3adh)/9	ND	ND	ND
ICC170pCVD438/01	0/4	ND	0/3	ND
Patient age (months) (median(range))	68(34-212)	38 (24-148)	82(42-170)	116(38-198)

Note: Values correspond to A/E lesion formation as a proportion of biopsies inoculated. Adh refers to adhesion without A/E lesion formation. When more than four explants were tested the age is shown as median (range).

D4= fourth part of the duodenum PP= Peyer's patch

T. ileum= terminal ileum ND= not done

As in the IVOC assays outlined in section 3, each bacterial strain was incubated in the IVOC system at least three times per mucosal region. No bacteria were found adhering to the uninoculated control explants after processing for SEM and the uninoculated explants showed similar surface morphology, after IVOC, to uninoculated explants used during previous IVOC assays.

The O157:H7 derivative strain, ICC170, did not express intimin as detected by western blot and did not adhere to PP explants during the eight hour IVOC. In previous

experiments 85-170 adhered preferentially to PP explants and the inability of ICC170 to adhere to PP suggested that ICC170 would not adhere to other areas of the intestine and therefore ICC170 was not tested for adhesion to other areas of the intestine.

Figure 4.2 Lack of adhesion of ICC170 to PP explants.

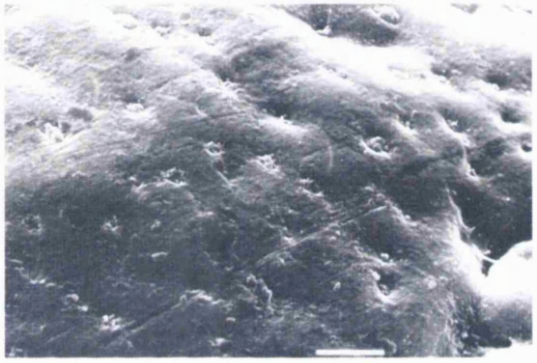


Figure 4.2 is a micrograph of the epithelium of a PP explant incubated with ICC170, showing no adhesion, bar = 10μm.

ICC170 was complemented with a plasmid expressing a recombinant intimin γ molecule. This resultant strain, ICC170pICC55, adhered to 2/3 PP explants during the eight hour IVOC. The A/E lesions were similar to those produced by 85-170 and other EPEC and EHEC. ICC170pICC55 did not adhere to duodenal explants during the eight hour IVOC assay. Lack of adhesion to the villous epithelium of the small intestine and adhesion to PP epithelium indicated that the intimin γ associated adhesion phenotype of the wildtype parent strain 85-170 had been restored by complementing ICC170 with a plasmid expressing recombinant intimin γ, and therefore ICC170pICC55 was not tested for adhesion to other areas.

Figure 4.3 ICC170(pICC55) A/E lesion formation on PP explants.

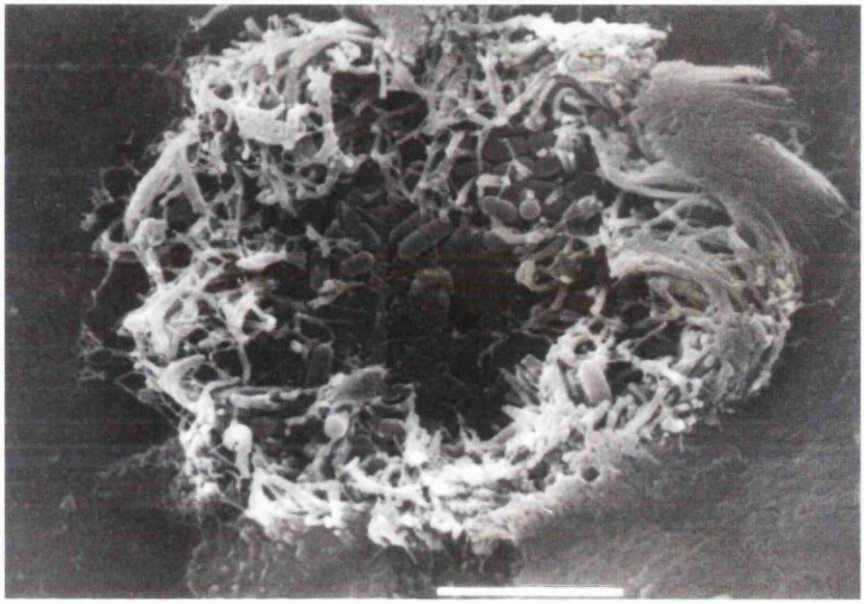


Figure 4.3 is a micrograph of ICC170(pICC55) adhering to PP explants, bar = 5 μm.

The intimin α expressing O157:H7 recombinant strain ICC170pCVD438 adhered to PP explants in a manner similar to the parent strain 85-170 and the intimin γ expressing recombinant strain ICC170pICC55. In addition, it adhered to duodenal and terminal ileum explants during the eight hour IVOC. ICC170pCVD438 did not adhere to transverse colon explants. ICC170pCVD438 adhered to 9/9 duodenal explants but only produced A/E lesions on 5/9 explants used during the eight hour IVOC. It adhered to 4/4 terminal ileum explants but only 2/4 of these explants showed A/E lesion formation. Adhesion without A/E lesion formation was similar to previous examples of adhesion without A/E lesion formation, as seen with the strains E2348/69, 3801 and PMK5 described in table 3.2. Glycocalyx thinning was noted at site of adhesion in some cases but adhesion was typified by contact with the epithelium without loss or elongation of microvilli. No bacterial footprints were noted.

Figure 4.4 Adhesion of ICC170(pCVD438) to intestinal explants.

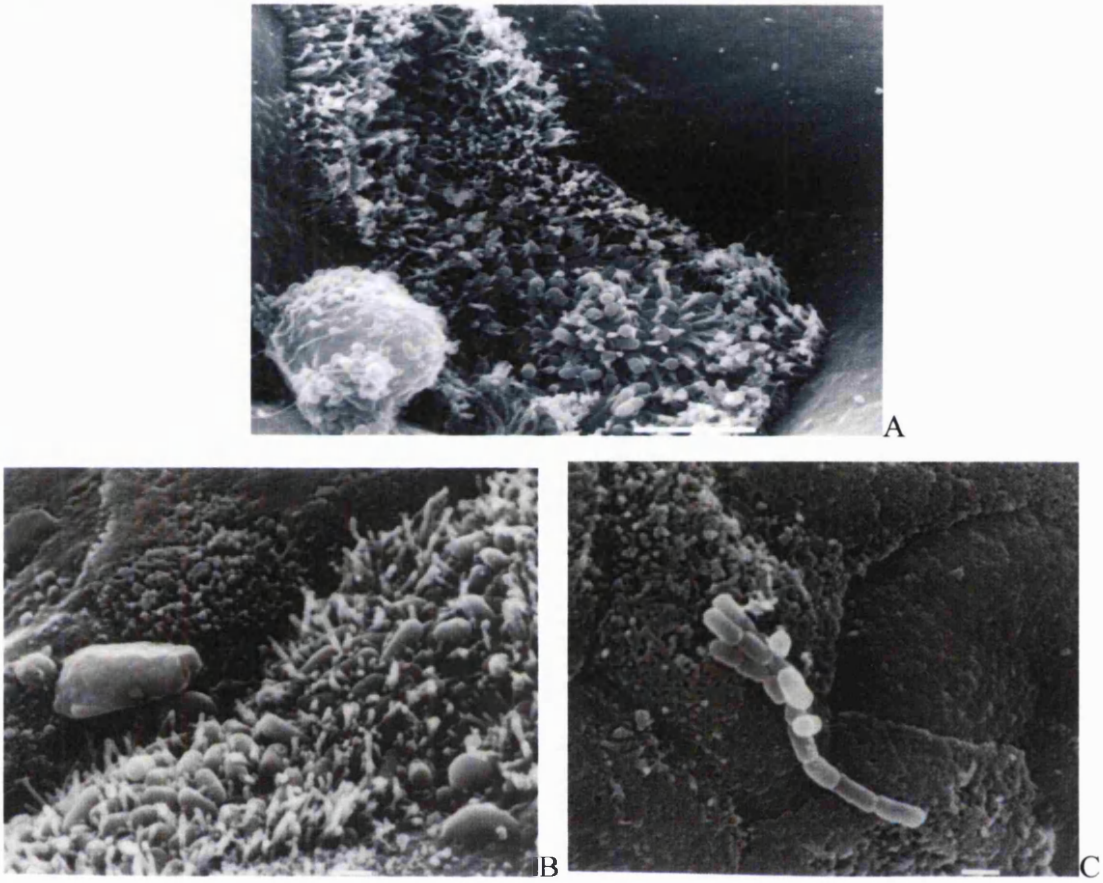


Figure 4.4 A is a micrograph of ICC170(pCVD438) producing A/E lesions on PP explants, bar = 5μm. Figure 4.4B is a micrograph of ICC170 producing A/E lesions on small intestinal explants during IVOC, bar = 1μm. Figure 4.4C is a micrograph of ICC170(pCVD438) adhering to small intestinal explants during IVOC without A/E lesion formation, bar = 1 μm.

The intimin α expressing recombinant derivative of E2348/69, strain CVD206(pCVD438) adhered to duodenal explants during the eight hour IVOC. CVD206(pCVD438) adhered to 9/9 explants during the eight hour IVOC but A/E lesions were present on only 6/9 explants. The adhesion without A/E lesion formation of EPEC strain CVD206(pCVD438) was similar to the adhesion showed by E2348/69, 3801, PMK5 and ICC170(pCVD438). The A/E lesions formed by CVD206(pCVD438) were similar to the A/E lesions formed by its parent strain E2348/69.

Figure 4.5 Adhesion of CVD206(pCVD438) to intestinal explants.

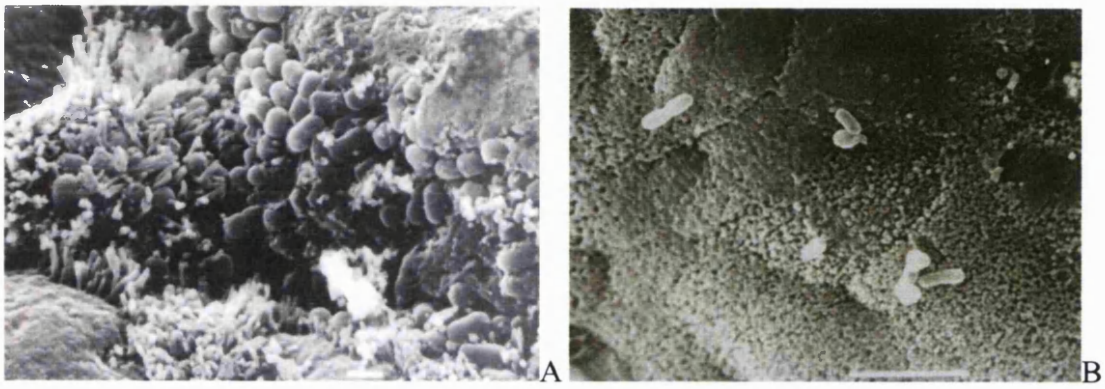


Figure 4.5A is a micrograph of A/E lesion formation by CVD206(pCVD438) on small intestine, bar = 1μm. Figure 4.5B is a micrograph of CVD206(pCVD438) adhering to the small intestine without A/E lesion formation, bar = 5 μm.

The intimin α expressing recombinant O157:H7 strain ICC170(pCVD438/01) expressed an intimin α protein with a C937A mutation, thus disrupting the disulphide bridge in the Tir binding domain of intimin α. ICC170(pCVD438/01) did not adhere to duodenal or PP explants during the eight hour IVOC assay. The surface epithelium of the PP explants used during the IVOC assay of ICC170(pCVD438/01) was similar to the surface epithelium of ICC170 PP explants, after IVOC assay.

4.2.2 HEp-2 cell adhesion of the O157:H7 strain, 85-170, expressing different intimin types.

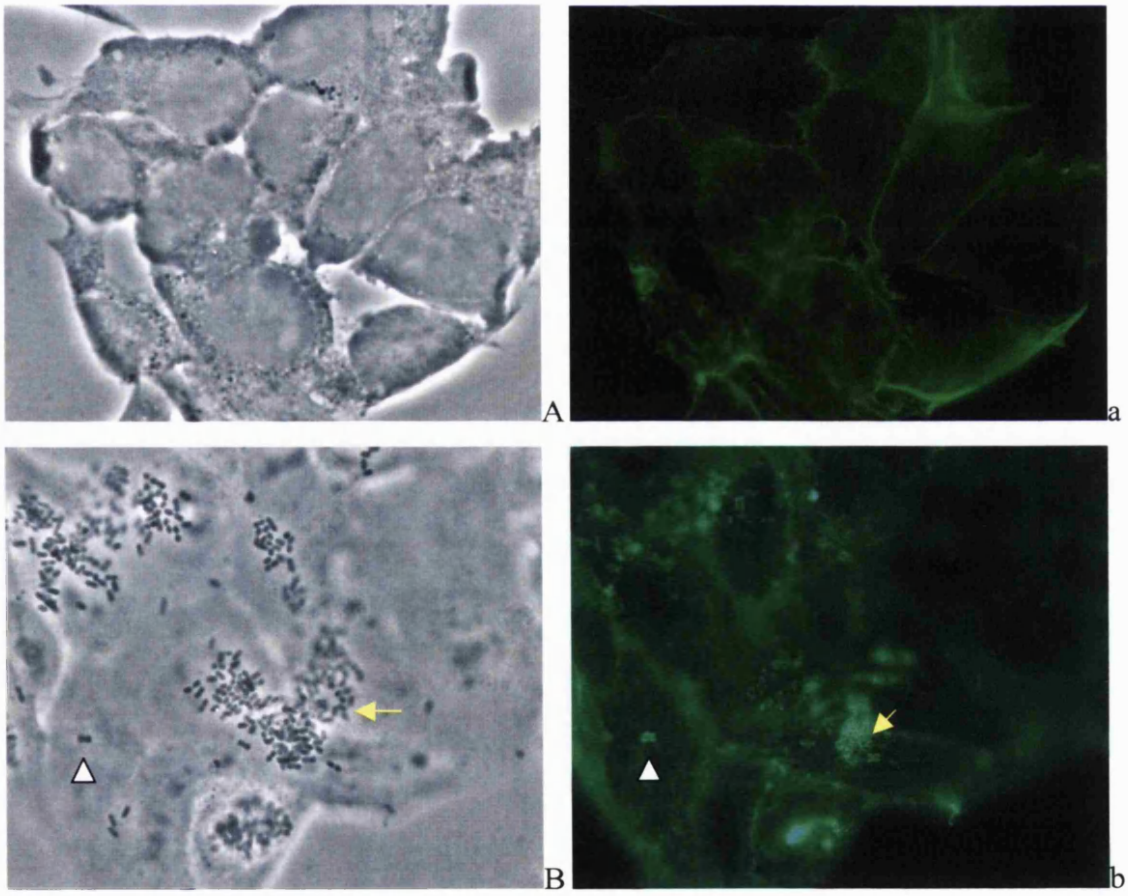
The strains listed in table 4.1 were assayed for their adhesion to HEp-2 cells in order to assess the affect of expressing different recombinant intimin proteins in an 85-170 intimin negative derivative strain ICC170. Included in this assay was the strain CVD206(pCVD438) which is an intimin negative derivative of the prototype intimin α O127:H6 strain E2348/69 complemented with a plasmid expressing the intimin α from E2348/69. CVD206(pCVD438) was used as a recombinant mimic of the wildtype E2348/69 strain in order to assess how the expression of the intimin α on a plasmid affected the adhesion of this strain to HEp-2 cells. CVD206 was also complemented with a plasmid expressing intimin γ (pICC55) in order to compare the expression of

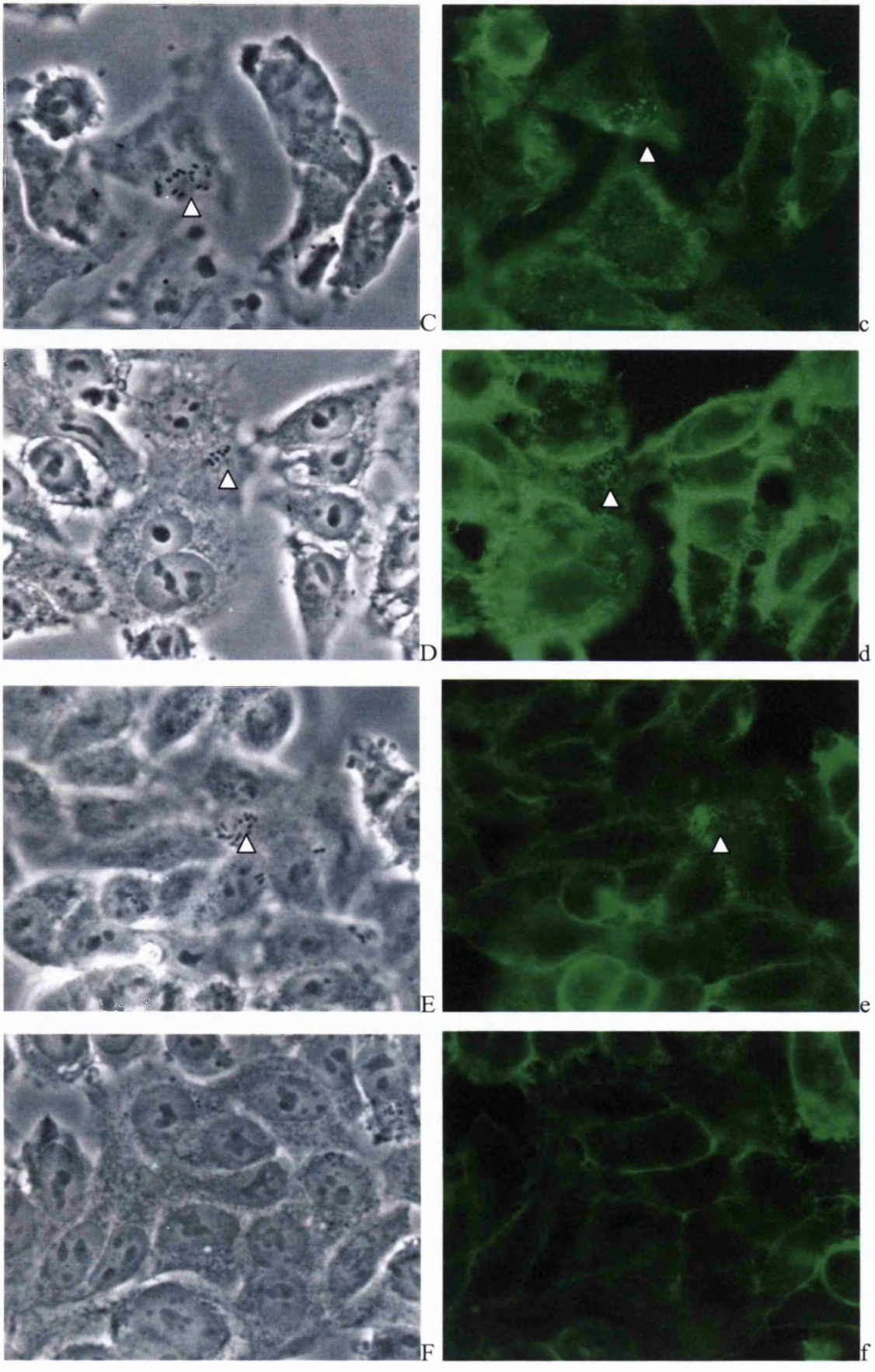
recombinant intimin γ in EPEC (CVD206(pICC55) and EHEC (ICC170(pICC55) backgrounds on HEp-2 cell adhesion.

An intimin negative derivative strain of the O157:H7 strain 85-170, was included in the HEp-2 assay.were included in the HEp-2 assay.

The HEp-2 cell adhesion assay was carried in triplicate and on three separate occasions.

Figure 4.6 Adhesion of 85-170 derivatives expressing different intimin types to HEp-2 cells.




C
c
D
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E
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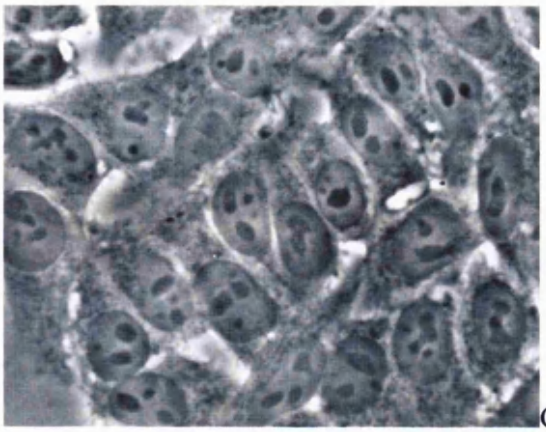
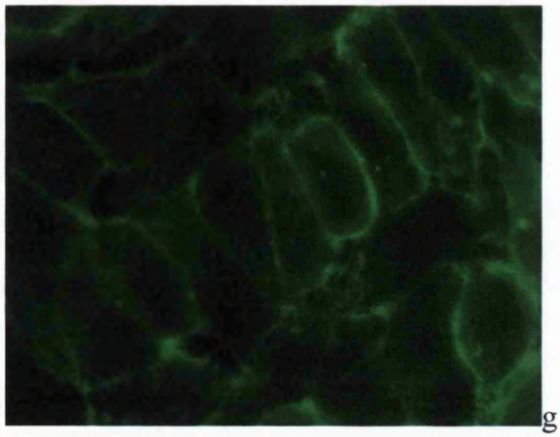

G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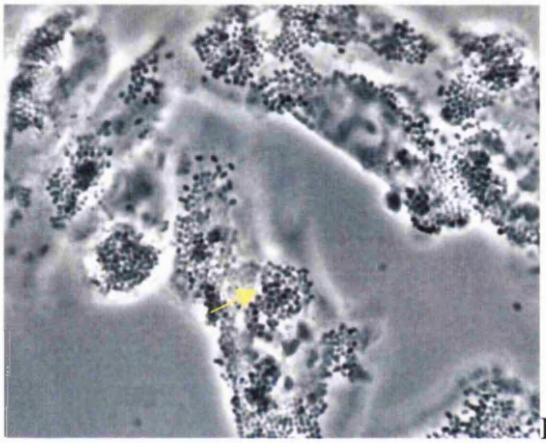
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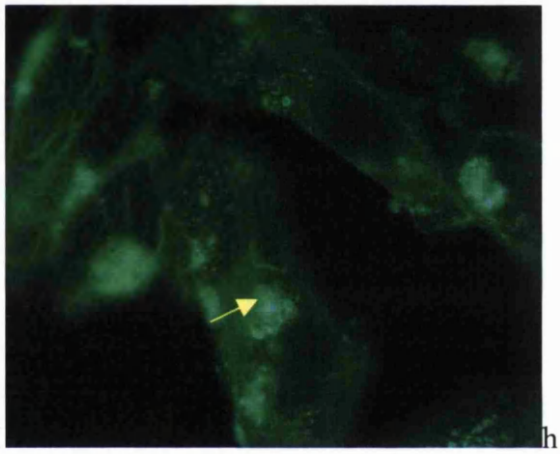
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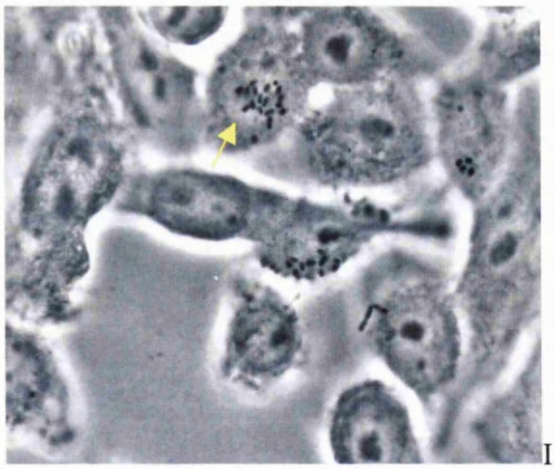
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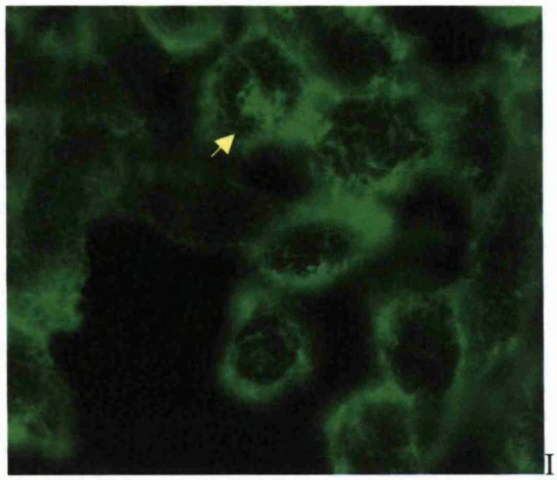
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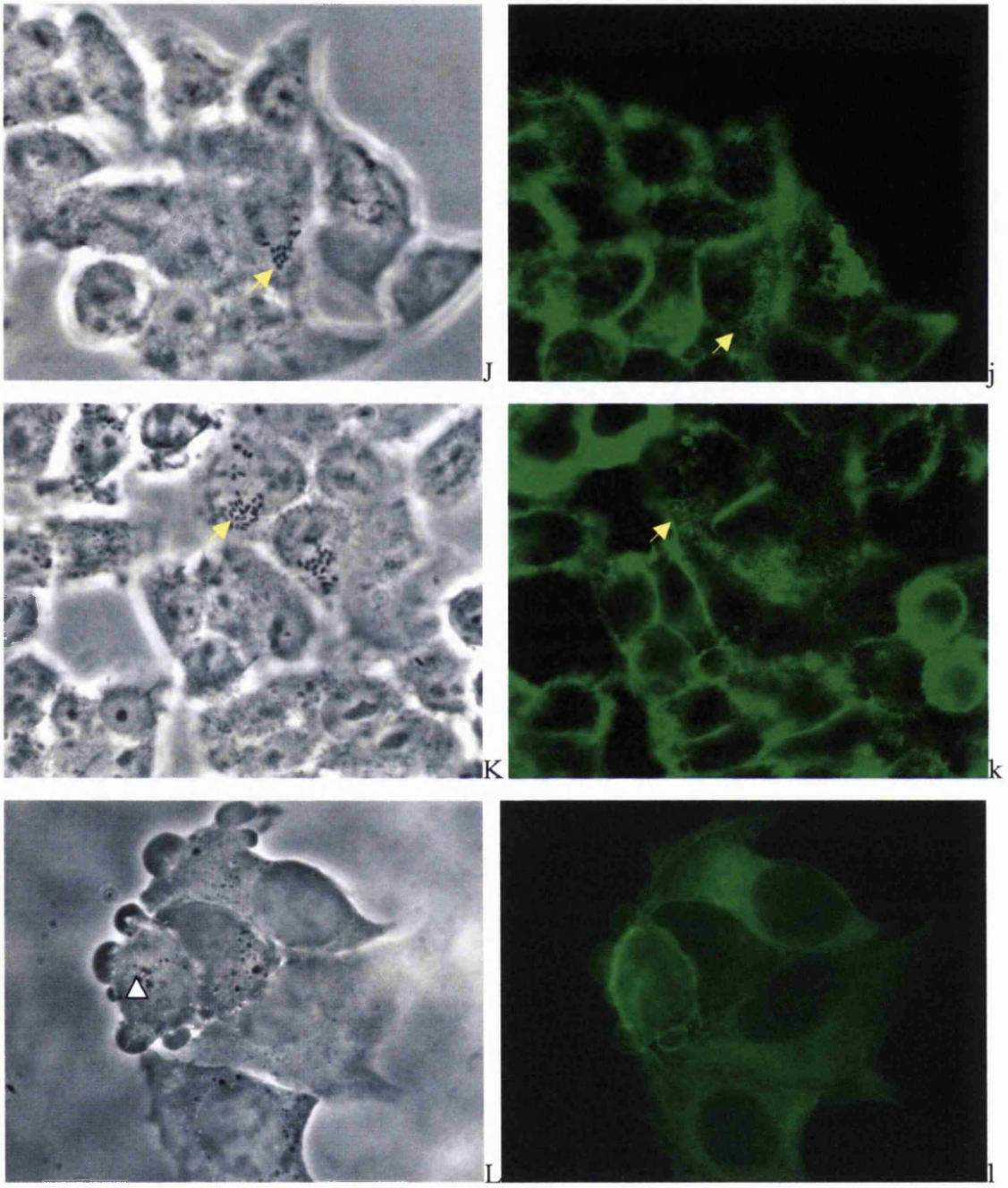


These micrographs are duplicates with the phase contrast image represented by the upper case letters and the fluorescent images represented by the lower case letters. The HEp-2 assay was divided into three and six hour incubation periods with the letters A to F representing the 3Hr Hep-2 assay and the letters G and M representing the 6Hr HEp-2 assay. The yellow arrows indicate examples of bacterial microcolonies where the number of bacteria forming the microcolony is greater than 5. The white triangles indicate adherent bacteria that are not part of a microcolony. Magnification is x 80.

In these micrographs strain ICC170 is represented by A and G, CVD206(pCVD438) by B and H, ICC170(pCVD438) by C and I, ICC170(pICC55) by D and J, CVD206(pICC55) by E and K and ICC170(pCVD438/01) by F and L.

The intimin negative strain ICC170 (see figures 4.6 A and G) did not adhere to HEp-2 cells after three and six hours of incubation. CVD206(pCVD438) adhered to HEp-2 cells after three and six hour incubation periods. Microcolonies were seen at both time points as well as bacteria adhering independently of microcolonies. The number of adherent bacteria whether adhering independently or as part of a microcolony, was greater after six hours of incubation than that observed after three hours of incubation. The microcolonies formed were similar to those formed by E2348/69 but in some cases they did not appear to be as tight as E2348/69 microcolonies. CVD206(pCVD438) was FAS positive and was designated LA (see figures 4.6 B and H)

The recombinant intimin α expressing strain ICC170(pCVD438) adhered to HEp-2 cells after three and six hours of incubation. The majority of the bacteria adhered to the HEp-2 cells independently of microcolonies after three hours of incubation with microcolonies being observed in a greater number after six hours of incubation. Not all the bacteria were FAS positive after three hours of incubation and a greater number of bacteria were FAS positive after six hours of incubation, particularly those forming part of a microcolony. ICC170(pCVD438) was designated LAL (see figures 4.6 C and I).

The recombinant intimin γ expressing strain ICC170(pICC55) adhered to HEp-2 cells and was FAS positive after three and six hours of incubation. The majority of the bacteria adhered to the HEp-2 cells independently of microcolonies with a few microcolonies observed after six hours of incubation. ICC170(pICC55) was designated as being PA/LAL (see figures 4.6 D and J).

The recombinant intimin γ expressing strain CVD206(pICC55) adhered to HEp-2 cells and was FAS positive after three and six hours of incubation. The majority of the bacteria adhered to the HEp-2 cells independently of microcolonies but microcolonies were observed after six hours of incubation. CVD206(pICC55) was designated as being PA/LAL (see figures 4.6 E and K).

Strain ICC170(pCVD438/01) expressing an intimin α with a disrupted disulphide bridge adhered to HEp-2 cells in very few numbers after three hours of incubation and these bacteria were not FAS positive. After six hours incubation, ICC170(pCVD438/01) adhered to HEp-2 cells in few numbers with no FAS positive stain observed. ICC170(pCVD438/01) was designated as being PA (see figure 4.6 F and L).

The expression of intimin α by an 85-170 host, ICC170, did not alter the HEp-2 cell adhesion phenotype of this strain and like 85-170 it produced an LAL pattern of adhesion on HEp-2 cells. Therefore unlike during IVOC the expression of intimin α by an EHEC background, ICC170, did not result in a change in the adhesion phenotype of EHEC to HEp-2 cells. However, the expression of the intimin γ expressing plasmid in both EPEC, CVD206, and EHEC, ICC170, backgrounds did result in a change in the adhesion phenotype of these strains. CVD206(pICC55) showed a reduced adhesion phenotype when compared with CVD206(pCVD438) and E2348/69. This correlates with the IVOC results in which CVD206(pICC55) showed a reduced tissue tropism when compared with E2348/69 and CVD206(pCVD438), by only adhering to PP explants in a manner similar to the intimin γ expressing 85-170 strain. In addition, ICC170(pICC55) showed a change in HEp-2 adhesion phenotype with a reduction in adhesion and a change from a LAL phenotype of the parent 85-170 strain to a PA/LAL phenotype. Therefore expression of intimin γ by strains may result in reduced adhesion to HEp-2 cells and a restricted IVOC tissue tropism.

4.2.3 IVOC of EPEC strains belonging to the O55 serogroup.

The bacterial strains used in this section are listed in table 4.3. The list of strains included typical and atypical EPEC. The strains expressed intimin α or intimin γ, and the list included intimin γ strains with and without the EAF plasmid. In previous sections, the intimin α expressing strains tested for adhesion to intestinal explants during the eight hour IVOC included the prototype typical EPEC O127:H6 strain E2348/69 and recombinant strains bearing an intimin α expressing plasmid. The aim of this section was to obtain more data concerning the tissue tropism of wildtype intimin α expressing strains. In addition, the strains selected from the O55 serogroup, and listed in table 4.3 , provided

information on the tissue tropism of intimin γ expressing EPEC strains, including O55:H7 isolates which have been suggested to be related to EHEC O157:H7. TEM was used to analyse the morphology of the A/E lesions produced by the O55 strains after the eight hour IVOC assay.

Table 4.3 Strains.

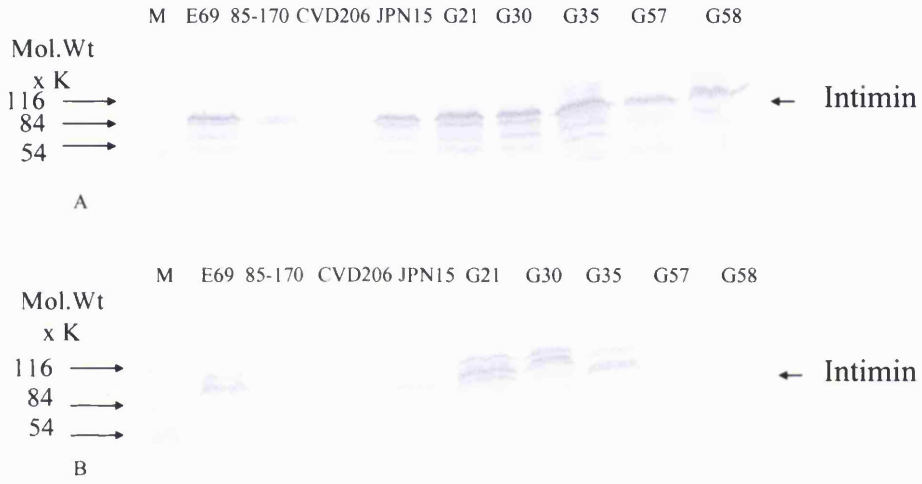
Strain	Description	Source/reference
CVD206	Intimin negative, EAF+, BFP+ O127:H6 E2348/69 derivative strain	(Donnenberg and Kaper 4310-17;Kelly et al. 313-18)
JPN15	Intimin α expressing, EAF-, BFP- O127:H6 E2348/69 derivative strain	(Gomez-Duarte and Kaper 1767-76)
G21	Intimin α expressing, EAF+, BFP+ O55:H6 strain	(Adu-Bobie et al. 662-68;Rodrigues et al. 2680-86)
G30	Intimin α expressing, EAF+, BFP+ O55:H6 strain	(Adu-Bobie et al. 662-68;Rodrigues et al. 2680-86)
G35	Intimin γ expressing, EAF+, BFP+ O55:H- strain	(Adu-Bobie et al. 662-68;Rodrigues et al. 2680-86)
G57	Intimin γ expressing, EAF-, BFP- O55:H7 strain	(Adu-Bobie et al. 662-68;Rodrigues et al. 2680-86)
G58	Intimin γ expressing, EAF-, BFP-, O55:7 strain	(Adu-Bobie et al. 662-68;Rodrigues et al. 2680-86)

The strains listed in table 4.3 contained two derivatives of the prototype EPEC O127:H6 strain E2348/69 that can act as controls for intimin mediated events in EPEC (CVD206) (Donnenberg and Kaper 4310-17;Knutton et al. 1644-52) and as an EAF control in EPEC (JPN15) (Gomez-Duarte and Kaper 1767-76).

The O55 strains were isolated from patients in Brazil who had presented with diarrhoea between 1950 and 1993. Another O55:H6 isolate obtained from a four month old infant by the center for disease control and prevention in the U.S.A. has been shown to produce diarrhoea in human volunteers but not protect against subsequent challenge with E2348/69.

The strains were probed for intimin expression by western blot using a conserved intimin antibody as described in section 2.5. The strains were grown overnight in BHI broth at 37°C and probed for intimin expression before and after growth in DMEM at 37°C.

Figure 4.7 Western blot of strains from the O55 serogroup.



Mol.Wt = molecular weight. M is a protein ladder marker.

The bacterial strains CVD206 and JPN 15 were used as control strains. CVD206 was an intimin negative derivative and JPN15 was an EAF negative derivative, of E2348/69. E2348/69 was abbreviated as E69.

All the strains, except CVD206, expressed intimin after growth in DMEM at 37°C (figure 4.7A). After overnight growth in BHI broth 37°C, and without subsequent growth in DMEM, only the strains expressing intimin α and the G35 intimin γ expressing strain expressed levels of intimin detectable by western blot (figure 4.7B).

An eight hour IVOC assay was carried out on G21, G35, G57 and G58 and the explants sectioned for analysis by TEM as described in 2.10.1. Some of the data concerning the tissue tropism of G21, G30, G35, G58 was kindly provided by Ms. Saghar Navabpour, while gaining work experience within the department. This section included the analysis of the tissue tropism of O55:H7 strain G57.

Table 4.4 Tissue Tropism of O55 strains.

	Intestinal region				
Strain	Small intestine		PP/T. ileum		Transverse colon
G21	0/4		2/3		0/3
G30	0/3		ND		0/3
G35	0/4		4/4		0/3
G58	0/7		3/3		0/3
Patient age (months) (median(range))	115(9-188)		73(30-171)		119(103-167)
Strain	D4	FAE/D4	T. ileum	PP/T. ileum	Transverse colon
G57	0/5	1/1	1/3	4/4	0/3
Patient age (months) (median(range))	109(24-172)	124	85, 131, 176	85, 140, 149, 161	74, 132, 176

Note: Values correspond to A/E lesion formation as a proportion of biopsies inoculated.
When more than four explants were tested the age is shown as a median (range).
D4= fourth part of the duodenum FAE= follicular associated epithelium
T. ileum= terminal ileum PP= Peyer's patch
ND= not done

In the study involving the bacterial strains G21, G30, G35 and G58 the small intestine results were grouped together.

As was shown in figure 4.7 all the strains listed in table 4.1 expressed intimin.

The intimin γ expressing strains G35 and G58, containing and not containing an EAF plasmid respectively, adhered to PP explants during the eight hour IVOC assay. These strains did not adhere to small intestinal explants devoid of lymphoid follicles and did not adhere to transverse colon explants.

Figure 4.8 G35 and G58 A/E lesions on PP explants.

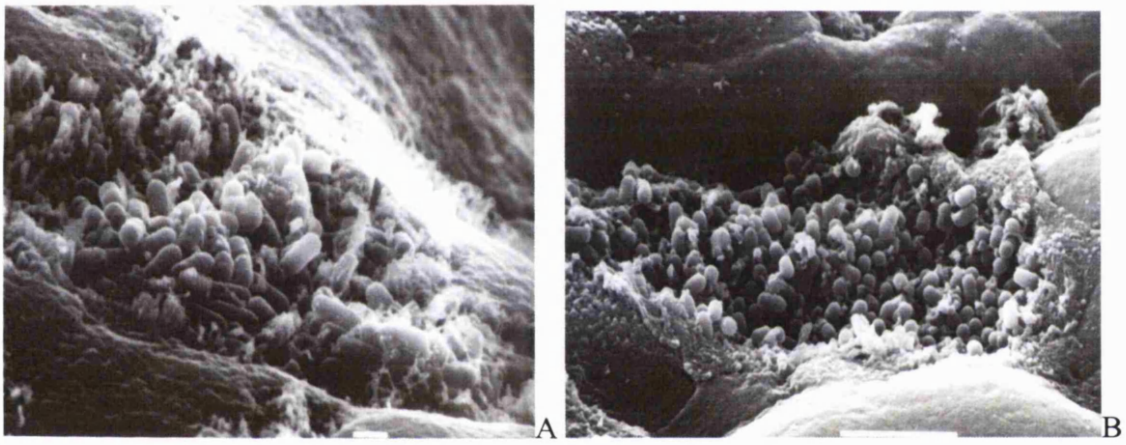


Figure 4.8A is a micrograph of O55:H- strain G35 producing A/E lesions on PP explants, bar = 1μm. Figure 4.8B is a micrograph of O55:H7 strain G58 producing A/E lesions on PP explants, bar = 5μm.

The intimin α expressing strain G21 adhered to 2/3 PP explants and adhered to 0/4 small intestine and 0/3 transverse colon explants during the eight hour IVOC assay. Strain G30 did not adhere to the small intestine or the large intestine and due to the lack of adhesion of strain G21 and the limit on tissue availability G30 was not assessed for adhesion to PP explants.

Figure 4.9 G21 A/E lesions on PP explants.

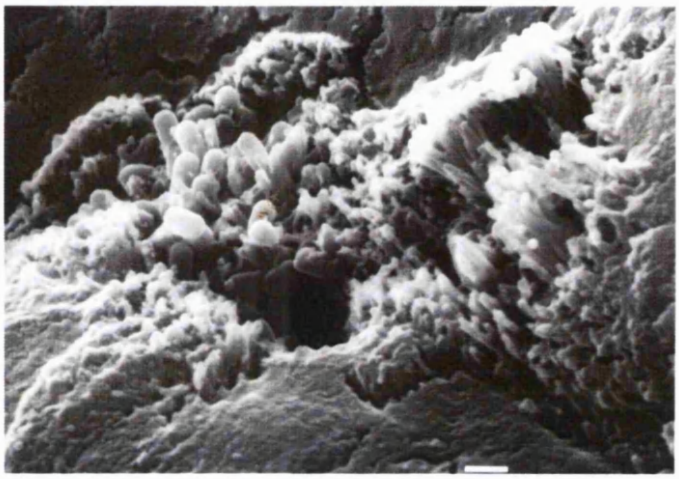


Figure 4.9 is a micrograph of O55:H6 strain G21 producing A/E lesions on PP explants, bar = 1 μm.

The intimin γ expressing strain G57 produced A/E lesions on 4/4 PP explants and 1/3 terminal ileal explants. It did not adhere to duodenal or transverse colon explants.

Figure 4.10 G57 producing A/E lesions on intestinal explants.

Figure 4.10 is a representative micrograph of the A/E lesions produced by O55:H7 strain G57 on the villous epithelium of the terminal ileum, PP explants and the FAE of isolated lymphoid follicles from the duodenum, bar = 1μm.

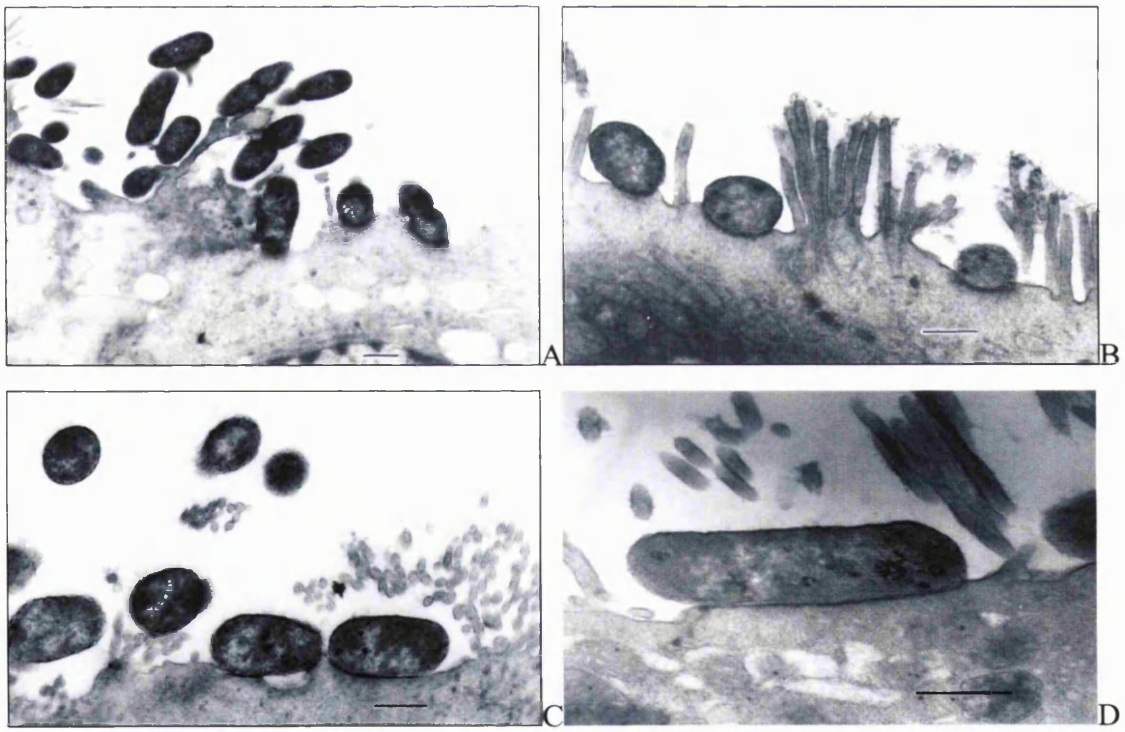
The O55 strains showed a preferential adhesion to PP explants during the eight hour IVOC assay regardless of intimin type or presence of the EAF plasmid.

The uninnoculated control explant and those incubated with bacteria resulting in lack or production of A/E lesions showed similar cell surface morphological traits to this described in previous IVOC experiments.

4.2.3.1 TEM of O55 A/E lesions.

IVOC assays of PP explants explants and strains G21, G35, G57 and G58 were carried out in duplicate and the explants were processed for TEM as described in 2.10.1

Figure 4.11 TEM of O55 PP A/E lesions



A= G21, B= G35, C= G57, D= G58 showing attaching effacing lesion on FAE of ileal Peyer's patch. Bar = 0.5μm

TEM was used to confirm the presence of A/E lesions on intestinal PP explants. The O55 strains G21, G35, G57 and G58 showed intimate adherence of bacteria to the enterocyte

with effacement of microvilli. Actin filaments were observed at the base of intact microvilli and the A/E lesions appeared discrete. A mucus/glycoclayx layer could be observed on intact microvilli next to A/E lesions. In some cases vacuole-like structures were seen close to the surface of the epithelium and on occasion close to the site of A/E lesion formation (see figure 4.11A).

The A/E lesions produced by the O55 strains after the eight hour IVOC assay do not show elongated pedestal structures. The bacterium-enterocyte interface was primarily seen as a depression, with only a few short pedestal-like structures observed. On occasion bacteria were seen adhering to extruding cells where cytoplasmic extrusion gives the appearance of elongated pedestals. This was noted in the G21 micrograph figure 4.11A. No bacteria were observed within enterocytes.

4.2.3.2 Intimin expression during IVOC of EPEC O55 strains.

Due to the different intimin expression profiles of the O55 strains after overnight growth in BHI at 37°C (see figure 4.7), the strains were used to see if it was possible to detect any differences in intimin expression during intestinal IVOC. Although all the O55 strains showed similar tissue tropism patterns after the eight hour IVOC it was not known whether the presence of an EAF plasmid would lead to difference in the detection of intimin during IVOC of the strains.

The strains used to analyse the expression of intimin during IVOC of the O55 serogroup were CVD206, JPN15, G21, G35 and G57. CVD206 was used as an intimin negative control and JPN15 was used to control for the effect of an EAF plasmid on intimin expression in the IVOC medium during IVOC. The O55:H7 strain G57 was selected during these studies because the data concerning its tissue tropism had been elucidated during the studies described in this section. Ideally PP explants would have been used to assess the intimin expression of the O55 strains during IVOC but PP were of limited availability and duodenal explants were used.

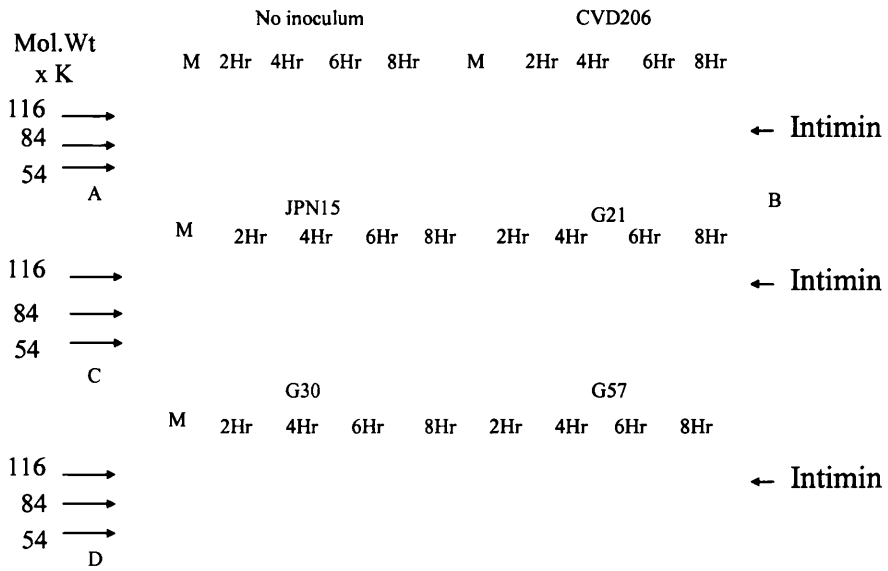
After IVOC culture the explants were processed for SEM. The only strain which showed A/E lesion formation on the duodenal explant was JPN15.

The western blot was carried out as described in 2.5.1.

The uninoculated medium from a negative control duodenal IVOC assay and the medium inoculated with CVD206 during IVOC assay of a duodenal explant showed no intimin expression at each time point (see figure 4.12A and B).

The intimin α expressing strains JPN15 and G21 showed similar results (see figure 4.12C). In both cases intimin was detected in the their respective IVOC medium at each time with a weaker signal being detected in the last two time points, or last four hours of IVOC. The signal detected at the first two time points or the first four hours of IVOC appeared to be slightly stronger in strain G21 when assessed visually. Thus both JPN15 and G21 expressed intimin in the IVOC medium during IVOC with duodenal explants in a manner similar to E2348/69.

Figure 4.12 Western blot of intimin expression by JPN15 and strains from the O55 serogroup during IVOC.



In figure 4.12 hour has been abbreviated to Hr and M indicates a protein ladder marker. Mol. Wt = molecular weight.

The IVOC medium at removed at each time point from the IVOC assays of the intimin γ strains showed weak signals when probed by western blot for intimin (see figure 4.12D). For strains G35 and G57 the medium removed from the last two time points produced a weaker signal than the medium removed from the first two time points. The medium removed after two and four hours IVOC of strain G57 appeared to generate a slightly stronger signal than the corresponding medium from the G35 IVOC assay. However, both strains appeared to express little or no intimin in the IVOC medium during duodenal IVOC assay.

4.2.4 The adhesion of strains from the EPEC O55 serogroup to cultured HEp-2 cells

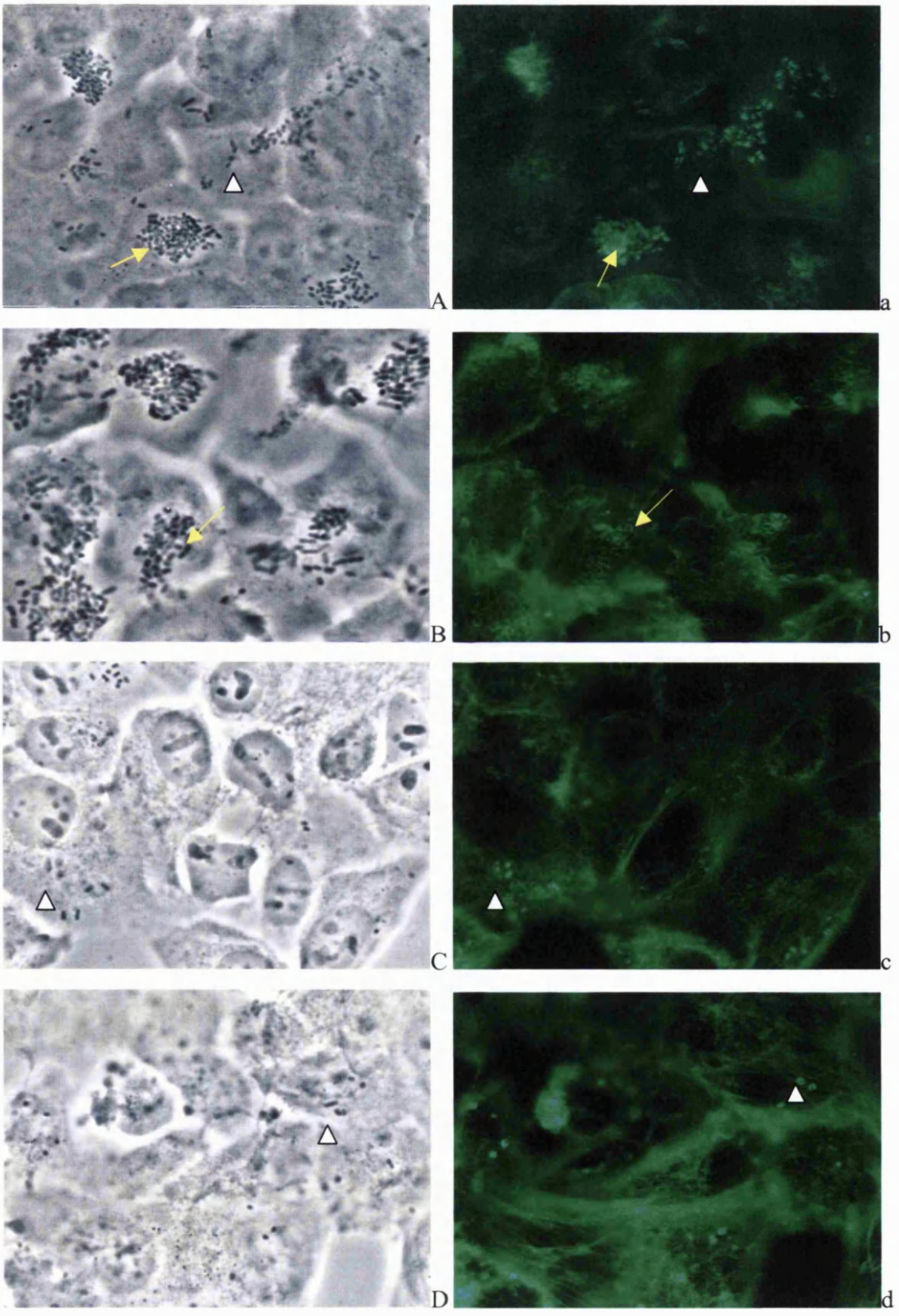
As stated in the previous chapter the HEp-2 adhesion patterns of pathogenic *E. coli* have been used to characterise EPEC and EHEC strains. In this section, the HEp-2 adhesion assay was used to compare the adhesion patterns of the strains from the O55 serogroup to the EPEC and EHEC strains characterised in section 3. In addition, the HEp-2 assay was used to determine whether the Tir proteins expressed by the O55 strains are tyrosine phosphorylated during A/E lesion formation.

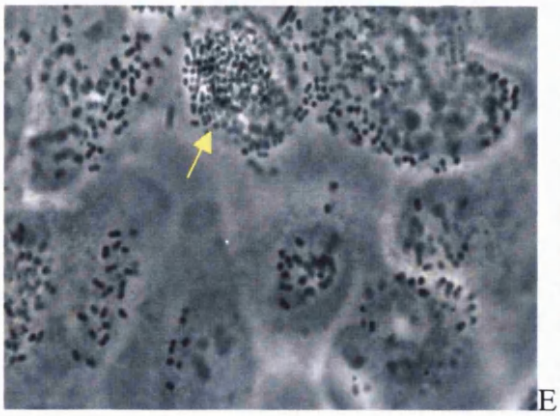
4.2.4.1 HEp-2 cell adhesion assay of EPEC strains from the O55 serogroup.

The O55 strains G21, G35, G57 and G58 were assayed for their adhesion to Hep-2 cells following three and six hour incubation periods at 37°C. The HEp-2 adhesion assay may highlight some differences in adhesion characteristics between the O55 strains whose tissue tropism was similar during the eight hour IVOC assay.

The HEp-2 adhesion assay was carried out as described in section 2.4.2 and FITC Phalloidin was used to highlight actin nucleation at the site of bacterial adhesion, which was representative of A/E lesion formation (Knutton *et al.*, 1989).

Figure 4.13 Micrographs of the Hep-2 adhesion of O55 strains G21, G35, G57 and G58.

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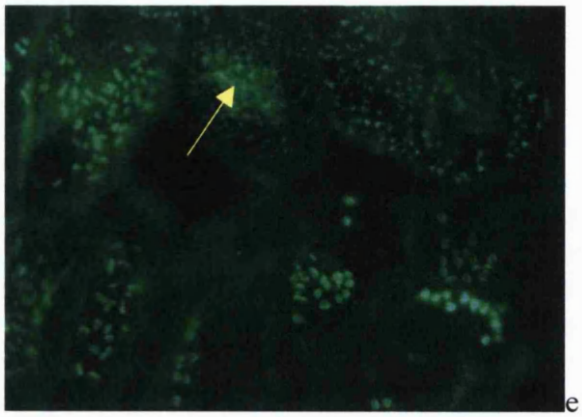
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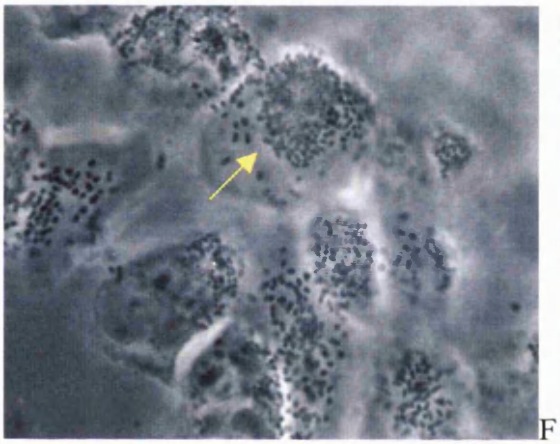
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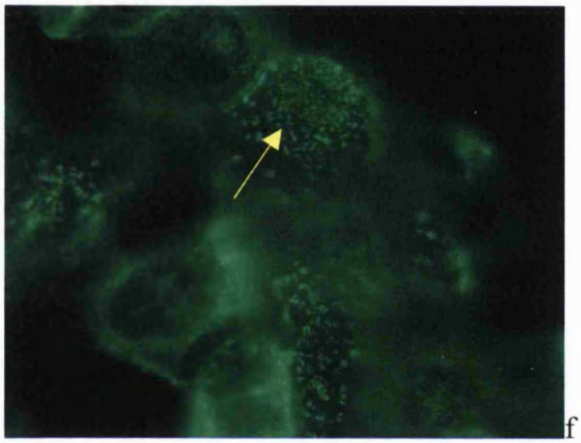
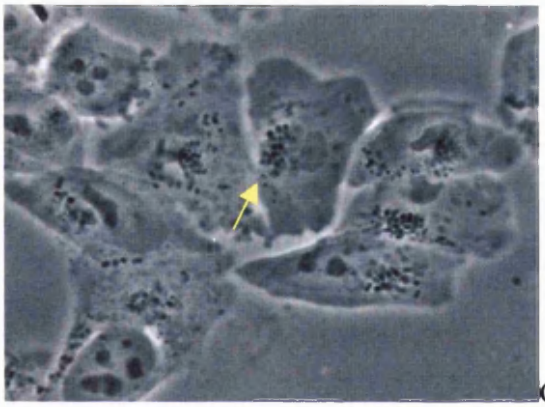
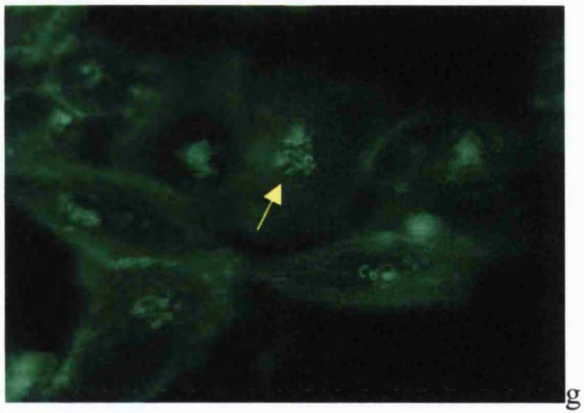
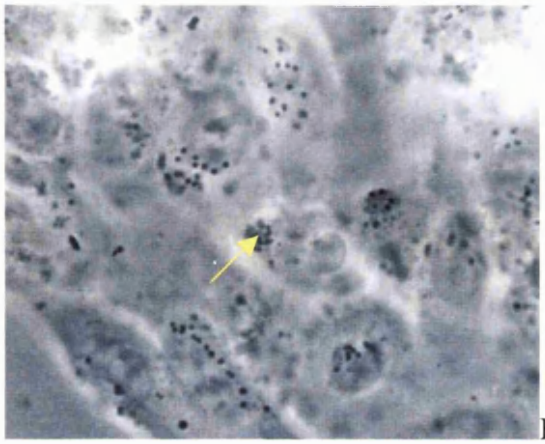
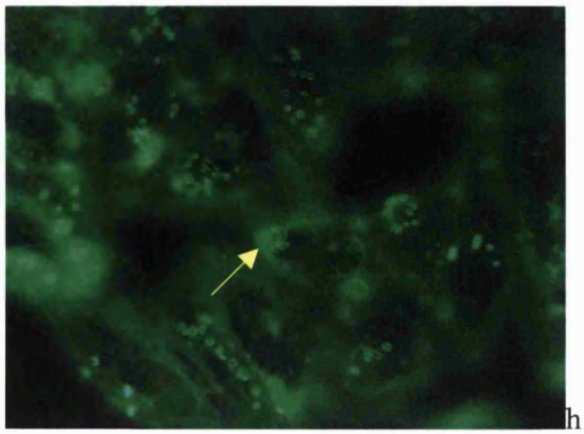

f


G


g


H


h

These micrographs are duplicates with the phase contrast image represented by the upper case letters and the fluorescent images represented by the lower case letters. The Hep-2 assay was divided into three and six hour incubation periods with the letters A to E representing the 3Hr Hep-2 assay and the letters F to J representing the 6Hr Hep-2 assay. The yellow arrows indicate examples of bacterial microcolonies where the number of bacteria forming the microcoloby is greater than 5. The white triangle indicates adherent bacteria that are not part of a microcolony.

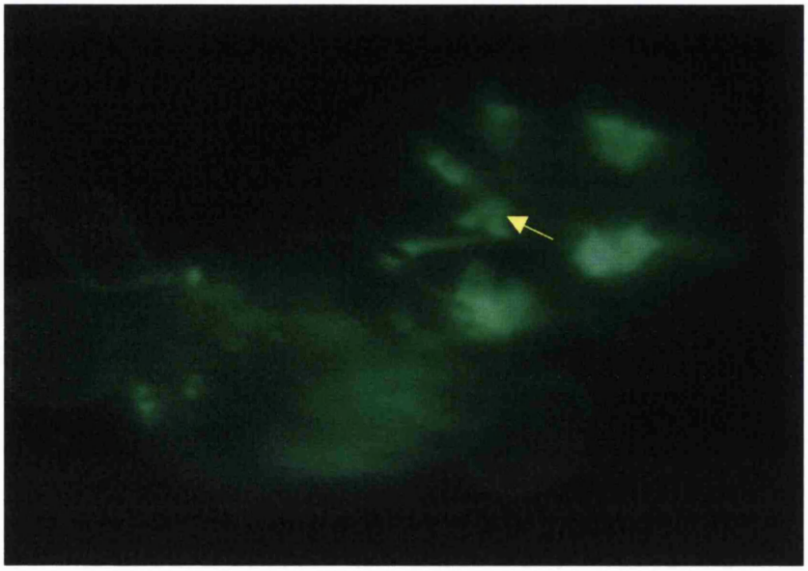
In these micrographs G21 is represented by A and E, G35 by B and F, G57 by C and G, G58 by D and H. Magnification is x 80.

The four O55 strains adhered to Hep-2 cells after three and six hour incubations periods. However, unlike their tissue tropism during IVOC the O55 strains did not share a common adhesion phenotype.

The typical EPEC O55:H6 strain G21 produced an LA pattern during the 3Hr Hep-2 assay. The majority of the bacteria were FAS positive but not all the bacteria adhered to the Hep-2 cells as part of microcolonies. The microcolonies produced during the 6Hr incubation contained a greater number of bacteria and there were a greater number of bacteria adhering independently of microcolonies. G21 was designated as being LA (see figures 4.13 A and E).

In addition, actin pseudopods or elongated pedestals (denoted by yellow arrow) were noted after six hours incubation and were similar to those noted for 85-170 in figure 3.14.

Figure 4.14 O55 strain G21 producing HEp-2 cell actin pseudopods.



Magnification is x120

The intimin γ, EAF plasmid containing O55:H- strain G35 showed an LA pattern of adhesion to HEp-2 cells after three hours of incubation. The majority of the bacteria were FAS positive as this time point. The microcolonies were bigger after six hours of incubation and both three and six hour incubation periods contained FAS positive bacteria adhering independently of microcolonies. G35 was designated as being LA (see figures 4.13 B and F).

The EAF plasmid negative, intimin γ expressing O55:H7 strain G57 adhered to HEp-2 cells after three hours of incubation but the majority of the bacteria adhered independently of microcolonies. Microcolonies were noted after six hours of incubation and the number of FAS positive bacteria was greater after six hours incubation when compared with the three incubation results. G57 was designated as being LAL (see figures 4.13 C and G).

The EAF plasmid negative, intimin γ expressing O55:H7 strain G58 adhered to HEp-2 cells after three hours of incubation with the majority of the bacteria adhering independently of microcolonies. Microcolonies were noted after six hours of incubation. G58 was FAS positive and the bacteria adhering independently of microcolonies adhering

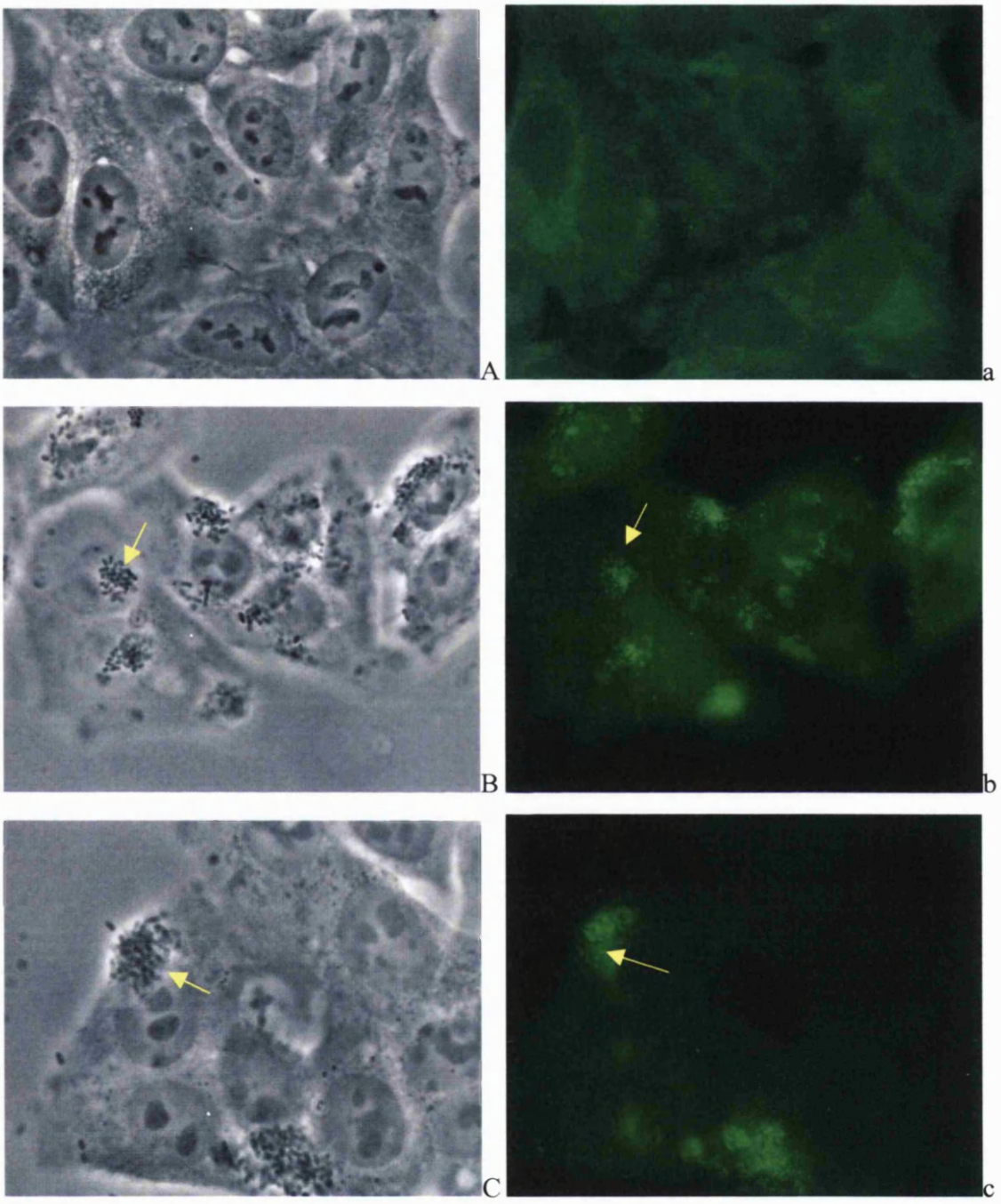
after six hours and adhering without microcolony formation after three hours incubation. G58 was designated as being LAL (see figures 4.13 D and H)

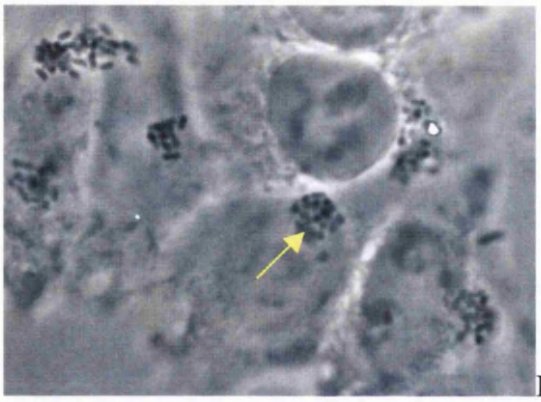
The HEp-2 cell adhesion of the O55 strains was repeated using incubation medium lacking D-mannose. The strains adhered to the HEp-2 cells with the same pattern regardless of the presence or lack of D-mannose in the incubation medium. Thus type-1 fimbriae mediated adhesion of the O55 strains to HEp-2 cells or catabolic repression of the O55 strains does not appear to be involved in adherence by the bacterial strains during the three and six hour HEp-2 adhesion assays.

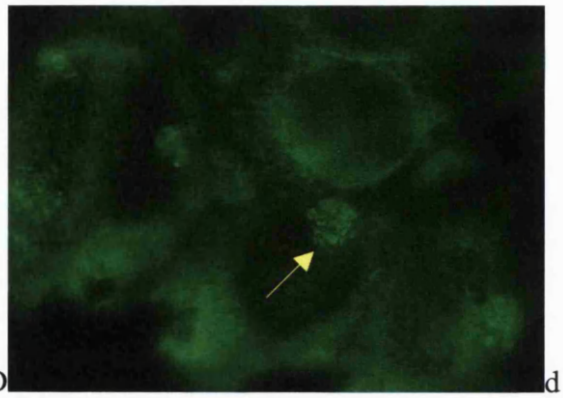
4.2.4.2 The Tir tyrosine phosphorylation of EPEC and EHEC strains.

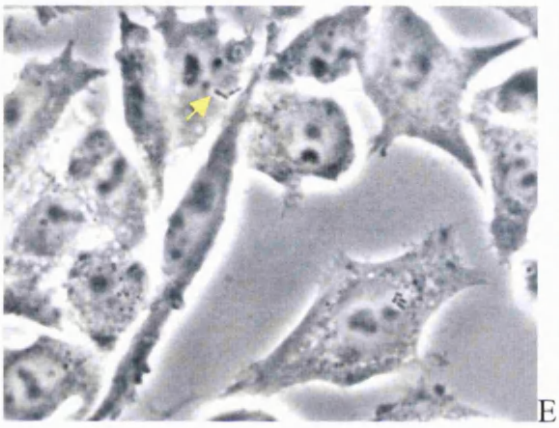
Tir is essential for A/E lesion formation in both EPEC and EHEC (DeVinney *et al.*, 1999). The ability of a tyrosine residue, at position 474 in the Tir molecule, to be phosphorylated has been used to gain an initial insight into the differences in the signal transduction mechanisms that are involved in EPEC and EHEC A/E lesion formation (Kenny, 1999). It is not known whether all EPEC strains express Tir molecules that undergo tyrosine phosphorylation during A/E lesion formation but it has been suggested that the phosphorylation of the tyrosine residue at position 474 is essential for pedestal formation by the prototype EPEC E2348/69 (DeVinney *et al.*, 2001). During this section the O55 strains G21, G35, G57 and G58 were assessed for the ability of their Tir molecules to be tyrosine phosphorylated by staining infected HEp-2 cells with a phosphotyrosine antibody (Rosenshine *et al.*, 1992). In addition the EHEC O157:H7 strain 85-170 was also tested for Tir phosphorylation during its adhesion to HEp-2 cells. The O55 strains covered a range of typical and atypical EPEC strains that in association with 85-170 provided data about the phosphorylation of Tir during EPEC and EHEC adhesion to HEp-2 cells.

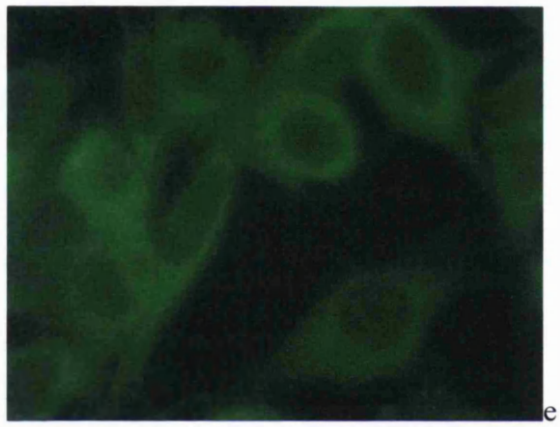
Figure 4.15 EPEC O55 and EHEC 85-170 Tir phosphorylation during adhesion to HEp-2 cells.




D


d


E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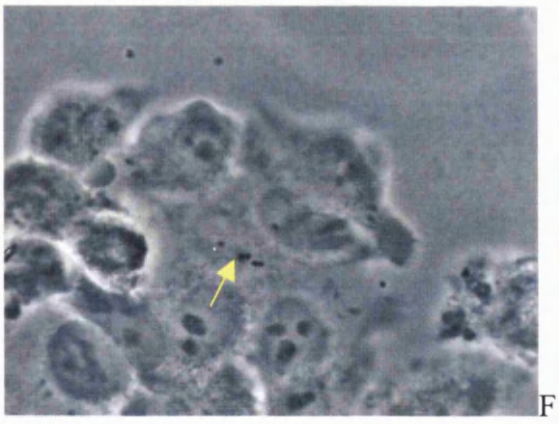
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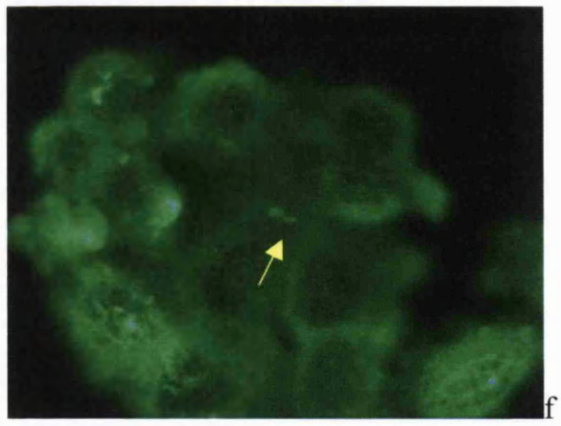
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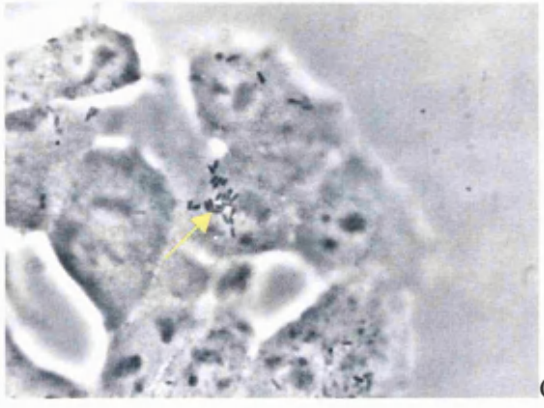
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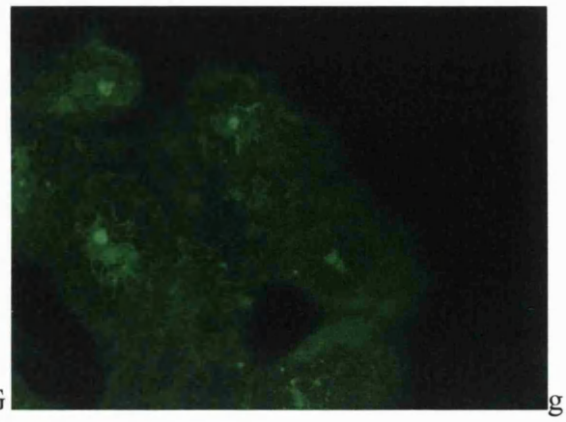
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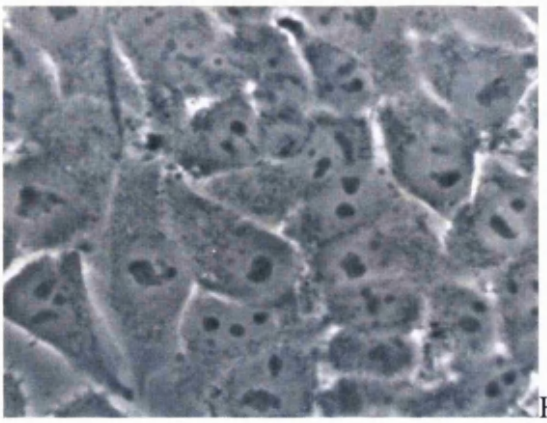
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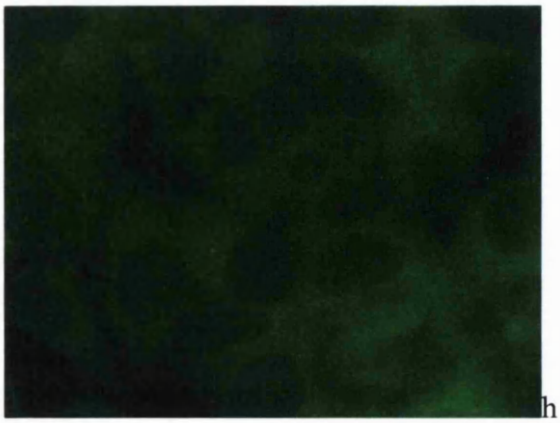
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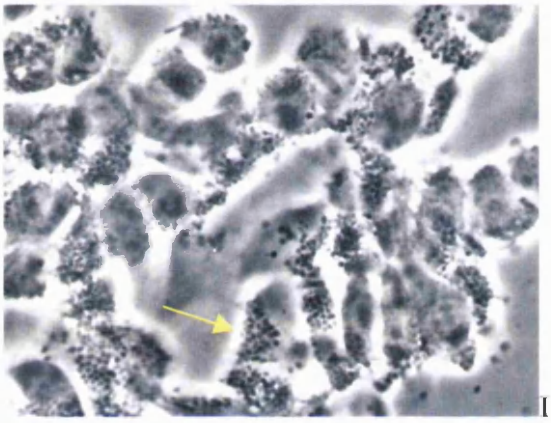
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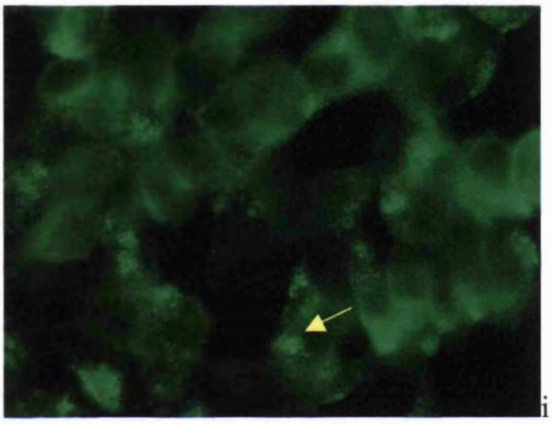
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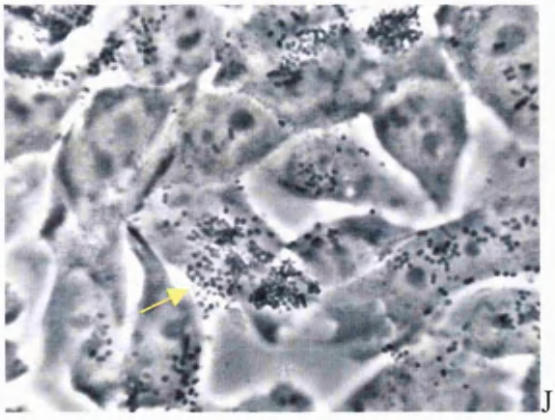
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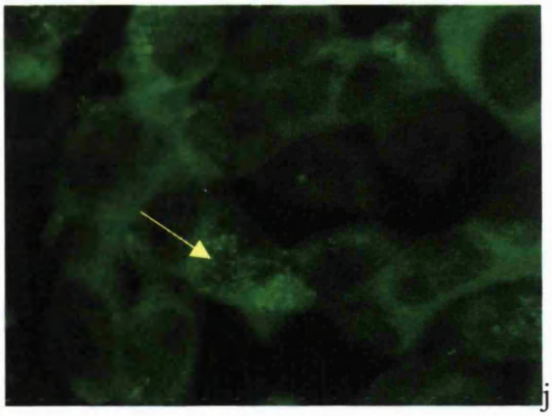
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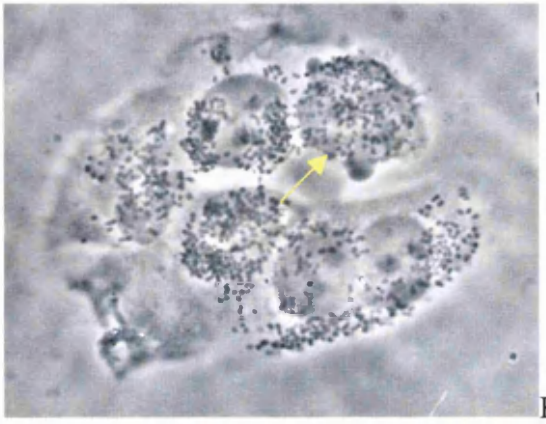
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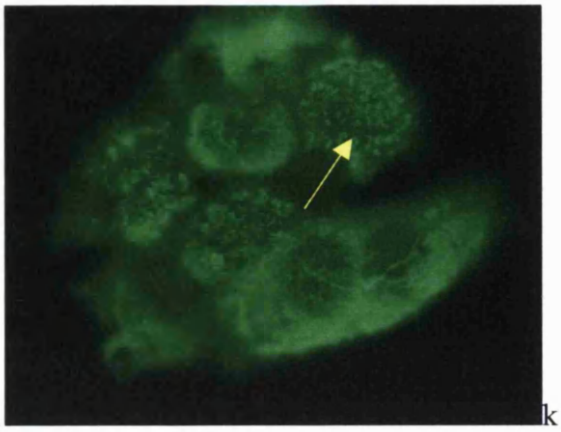
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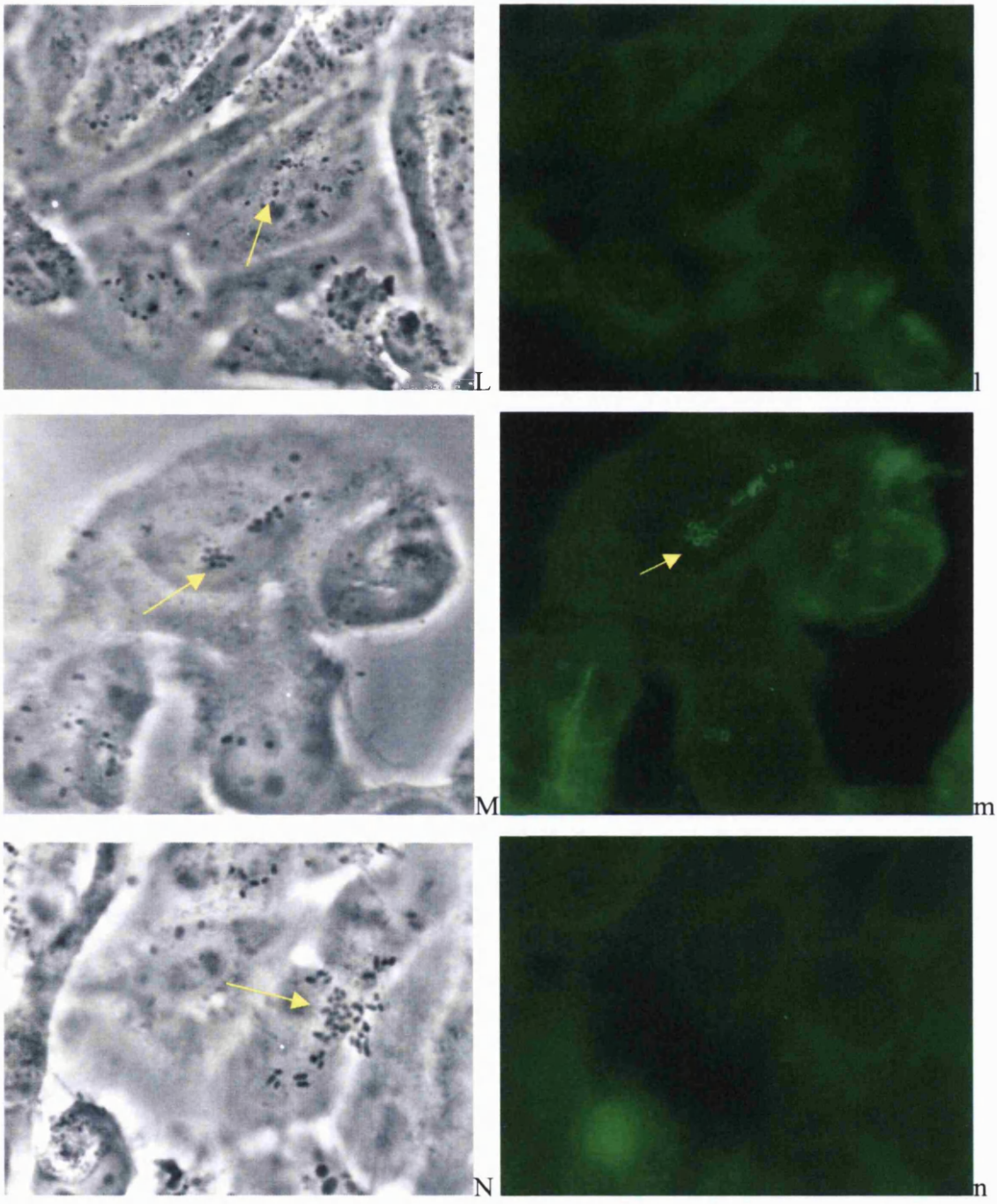


These micrographs are duplicates with the phase contrast image represented by the upper case letters and the fluorescent images represented by the lower case letters. The Hep-2 assay was divided into three and six hour incubation periods with the letters A to G representing the 3Hr Hep-2 assay and the letters H to N representing the 6Hr Hep-2 assay. The yellow arrow indicates examples of bacterial adhesion and Tir associated tyrosine phosphorylation.

The micrographs designated by the following letters A and H are uninfected control HEp-2 cells, B and I are E2348/69 infected HEp-2 cells, C and J are G21 infected HEp-2 cells, D and K are G35 infected HEp-2 cells, E and L are G57 infected HEp-2 cells, F and M are G58 infected HEp-2 cells and G and N are 85-170 infected HEp-2 cells. Magnification is x 80.

The strains used in this study adhered to HEp-2 cells after three and six hour incubation periods. E2348/69 was used as a control for Tir tyrosine phosphorylation (Rosenshine *et al.*, 1992). All the strains, except the O55:H7 strain G57 and the EHEC O157:H7 strain 85-170, showed tyrosine phosphorylation staining at the site of bacterial adherence as described by others (Goosney *et al.*, 2000;Goosney, DeVinney, and Finlay, 2001;Rosenshine *et al.*, 1992). The stain was concentrated around the site of bacterial adhesion and formed a partial or complete outline of the bacterium. In addition to the sites of bacterial adhesion being stained, the anti-phosphotyrosine antibody stained the HEp-2 cells, but this did not prevent visualisation of bacterial Tir tyrosine phosphorylation.

4.2.5 The involvement of intimin α from O55:H6 strain G21 in IVOC tissue tropism

Intimin has been shown to be involved in tissue tropism in a gnotobiotic piglet model of infection (Tzipori *et al.*, 1995) and during IVOC in this section and in previous studies (Phillips and Frankel, 2000b;Phillips *et al.*, 2000). Efforts have been directed towards understanding the basis of this tissue tropism by looking at the importance of amino acid residues within the intimin α of O127:H6 strain E2348/69, highlighting the contribution of residues to the colonisation of the intestinal tract and binding to Tir (Reece *et al.*, 2001). These studies showed that mutating a valine (Val, V) at position 911 in intimin α to an Ala resulted in an intimin α which did not bind to Tir in a gel overlay. When this intimin α V911A protein was expressed in a *C. rodentium* strain it resulted in a reduced level of colonisation of the mouse intestinal tract showing that it may have a role in initiation or maintenance of the colonisation. When intimin α V911A was expressed in CVD206 (intimin negative E2348/69 strain) and tested for adhesion during IVOC no adhesion to the duodenal explants was observed, but there was adhesion to the PP epithelium. This indicated that this residue may be involved in the tissue tropism of E2348/69 during IVOC.

Interestingly, a V906A substitution in intimin γ, equivalent to V911A in intimin α, has been reported and shown to disrupt intimin Tir binding. In addition, a Tir binding pocket has been characterised for EHEC intimin γ (Liu *et al.*, 2002).

In this chapter we have shown that intimin α expressing EPEC strain G21 has a restricted tissue tropism similar to the EHEC strains reported in section 3. Its A/E lesions were shown to be similar to previously reported human A/E lesions using TEM and its adhesion pattern to HEp-2 cells was similar to the prototype intimin α expressing strain E2348/69. The observation that mutating 911 valine to alanine in intimin α of E2348/69 changed tropism to an intimin γ associated phenotype (Reece *et al.*, 2001), raised the possibility that a similar change might be present in the intimin of O55 strain G21. In order to determine the basis of the variation in IVOC tissue tropism amongst intimin α strains, the intimin from G21 was sequenced and the results used to define further IVOC assays to investigate the role of intimin during IVOC tissue tropism.

4.2.5.1 DNA Sequencing of the intimin α from the EPEC O55:H6 strain G21

To determine whether the *eae* gene of strain G21 contained any variation in the CTLD region, the carboxy terminal region was compared with that of the prototype intimin α from EPEC O127:H6 strain E2348/69.

The DNA sequencing of the intimin α from the O55:H6 strain G21 was carried out as described in section 2.7.

Figure 4.16 Comparison of the carboxy terminus DNA sequence of the *eae* gene from E2348/69 (NCBI genbank accession number AF022236.1 region 24849 to 27668) and G21 (AF475062).

```
E69 (1)     TGGTCAGGAT GCTATTACAT ACACTGTTAA AGTGATGAAG GGGGATAAGC
G21         TGGTCAGGAT GCTATTACAT ACACTGTTAA AGTGATGAAG GGGGATAAGC
E69(51)     CTGTATCTAA TCAGGAAGTG ACCTTTACGA CGACCTTAGG TAAGTTAAGT
G21         CTGTATCTAA TCAGGAAGTG ACCTTTACGA CGACCTTAGG TAAGTTAAGT
E69(101)    AATTCCACTG AAAAAACGGA TACGAATGGC TATGCCAAAG TAACATTAAC
G21         AATTCCACTG AAAAAACGGA TACGAATGGC TATGCCAAAG TAACATTAAC
E69(151)    ATCGACAACT CCAGGAAAAT CACTCGTTAG TGCCCGTGTT AGCGATGTCG
G21         ATCGACAACT CCAGGAAAAT CACTCGTTAG TGCCCGTGTT ATCGATGTCG
E69(201)    CCGTTGATGT CAAAGCACCT GAAGTTGAAT TTTTTACAAC GCTTACAATT
G21         CCGTTGATGT CAAAGCACCT GAAGTTGAAT TTTTTACAAC GCTTACAATT
E69(251)    GATGACGGTA ATATTGAAAT TGTTGGAACC GGAGTTAAAG GGAAGTTACC
G21         GATGACGGTA ATATTGAAAT TGTTGGAACC GGAGTTAAAG GGAAGTTACC
E69(301)    CACTGTATGG TTGCAATATG GTCAAGTTAA TCTGAAAGCC AGCGGAGGTA
G21         CACTGTATGG TTGCAATATG GTCAAGTTAA TCTGAAAGCC AGCGGAGGTA
E69(351)    ACGGAAAATA TACATGGCGC TCAGCAAATC CAGCAATTGC TTCGGTGGAT
G21         ACGGAAAATA TACATGGCGC TCAGCAAATC CAGCAATTGC TTCGGTGGAT
E69(401)    GCTTCTTCTG GTCAGGTCAC CTTAAAAGAG AAGGGAACTA CAACTATTTC
G21         GCTTCTTCTG GTCAGGTCAC CTTAAAAGAG AAGGGAACTA CAACTATTTC
E69(451)    CGTTATCTCA AGTGATAATC AAACTGCAAC TTATACTATT GCAACACCTA
G21         CGTTATCTCA AGTGATAATC AAACTGCAAC TTATACTATT GCAACACCTA
E69(501)    ATAGTCTGAT TGTTCCTAAT ATGAGCAAGC GTGTGACCTA TAATGATGCT
G21         ATAGTCTGAT TGTTCCTAAT ATGAGCAAGC GTGTGACCTA TAATGATGCT
E69(551)    GTGAATACAT GTAAGAATTT TGGAGGAAAG TTGCCGTCTT CTCAGAATGA
G21         GTGAATACAT GTAAGAATTT TGGAGGAAAG TTGCCGTCTT CTCAGAATGA
E69(601)    ACTGGAAAAT GTCTTTAAAG CATGGGGGGC TGCAAATAAA TATGAATATT
G21         ACTGGAAAAT GTCTTTAAAG CATGGGGGGC TGCAAATAAA TATGAATATT
E69(651)    ATAAGTCTAG TCAGACTATA ATTTCATGGG TACAACAAAC AGCTCAAGAT
G21         ATAAGTCTAG TCAGACTATA ATTTCATGGG TACAACAAAC AGCTCAAGAT
E69(701)    GCCGAAGAGTG GTGTTGCAAG TACATACGAT TTAGTTAAAC AAAACCCTCT
G21         GTTGAAGAGTG GTGTTGCAAG TACATACGAT TTAGTTAAAC AAAACCCTCT
E69(751)    GAATAATATT AAGGCTAGTG AATCTAATGC TTATGCCACT TGTGTAAAA
G21         GAATAATATT AAGGCTAGTG AATCTAATGC TTATGCCACT TGTGTAAAA
```

For the purpose of alignment E2348/69 was abbreviated to E69.

The DNA sequence was aligned using the BLASTn program from the NCBI.

When the DNA sequence recovered from the sequencing of the intimin α from O55:H6 strain G21 was compared to the DNA sequence of the intimin α from the O127:H6 strain E2348/69, two base changes were noted. The first was at position 192 in the alignment which corresponds to position 2720 in the E2348/69 *eae* DNA sequence, which is 2820 bases long. This position was a guanine in E2348/69 and a thymine in G21. This base difference produced a serine (Ser, S) in E2348/69 and an isoleucine (Ile, I) in G21. The second base change was at position 702 in the alignment which corresponds to 2210 in the

E2348/69 *eae* DNA sequence. This position was a cytosine in E2348/69 and a thymine in G21. This base change produced an alanine (Ala, A) in E2348/69 and a valine (Val, V) in G21.

Figure 4.17 Comparison of the carboxy terminal amino acid sequences of the intimin α from E2348/69 and G21.

E69 (673)	GQDAITYTVKVMKGDK
G21	GQDAITYTVKVMKGDK
E69 (691)	PVSNQEVTFTTTLGKLSNSTEKTDTNGYAKVTLTSTTPGKSLVSARVSDVA
G21	PVSNQEVTFTTTLGKLSNSTEKTDTNGYAKVTLTSTTPGKSLVSARVIDVA
E69 (741)	VDVKAPEVEFFTTLTIDDGNIEIVGTGVKGKLPTVWLQYGQVNLKASGGN
G21	VDVKAPEVEFFTTLTIDDGNIEIVGTGVKGKLPTVWLQYGQVNLKASGGN
E69 (791)	GKYTWRSANPAIASVDASSGQVTLKEKGTTTISVISSDNQTATYTIATPN
G21	GKYTWRSANPAIASVDASSGQVTLKEKGTTTISVISSDNQTATYTIATPN
E69 (841)	SLIVPNMSKRVTYNDAVNTCKNFGGKLPSSQNELENVFKAWGAANKYEYY
G21	SLIVPNMSKRVTYNDAVNTCKNFGGKLPSSQNELENVFKAWGAANKYEYY
E69 (891)	KSSQTIISWVQQTAQDAKSGVASTYDLVKQNPLNNIKASESNAYATCVK **(939)**
G21	KSSQTIISWVQQTAQDVKSGVASTYDLVKQNPLNNIKASESNAYATCVK

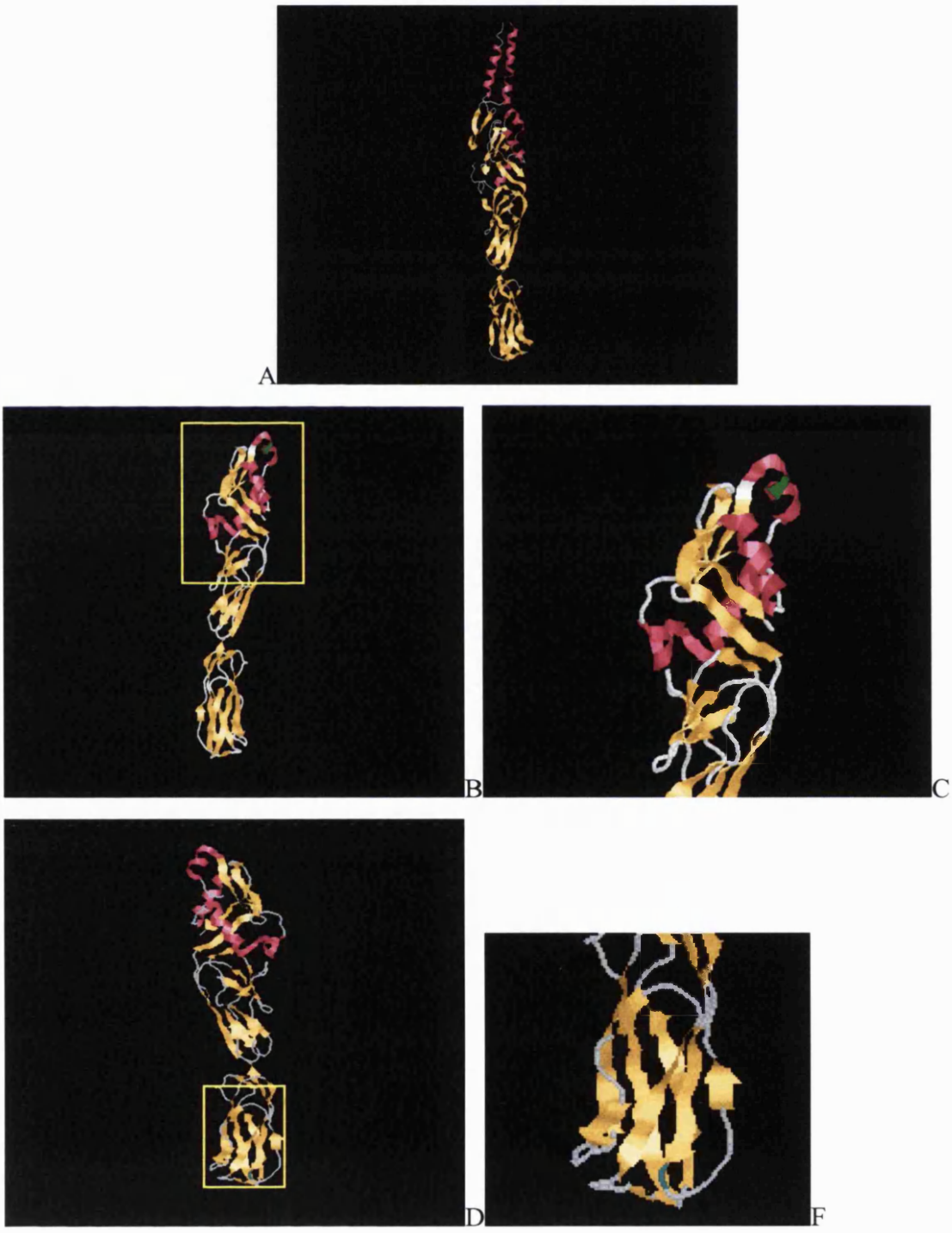
Amino acids numbered according to position in intimin α of E2348/69 (pubmed accession number ACC38392). For the purpose of alignment E2348/69 was abbreviated to E69.

The amino acid sequences were aligned using the SIM program from ExPASy after being translated using the translate tool in the ExPASy menu.

After DNA sequencing of the *eae* gene of O55:H6 strain G21 the gene sequence recovered was translated to give a 266 long amino acid sequence. This was compared to the carboxy terminal amino acid sequence of E2348/69. When numbered according to the position of the amino acid residues in the intimin α of E2348/69, one single variation, in the putative CTLD amino acid sequence of strain G21, was noted. The was noted as a single amino acid change at position 907 which was found to be a valine (Val, V) residue in G21 instead of an alanine (Ala, A) residue in E2348/69. This residue is highlighted in figure 4.17. In addition, outside the putative CTLD of G21 another amino acid change was noted as an

isoleucine (Ile, I) at position 737 in G21 and a serine (Ser, S) residue in E2348/69. This residue is highlighted in figure 4.17

Figure 4.18 Representative images of Int 280 from E2348/69 in complex with Tir and highlighting of residues 907, 911 and 737 the intimin α of E2348/69.



A= int-Tir

B and C (enlarged region designated in B) = white valine residue at 911 in E2348/69 and green alanine residue at 907 in E2348/69 (corresponds to valine 907 in G21 intimin α)

D= cyan serine residue at position 737 in E2348/69 (corresponded to isoleucine737 in G21)

E= enlarged area showing cyan serine residue highlighted in D.

This diagram was made using the RasMol version 2.6 program and the files were saved as BMP files.

It was assumed that the structural information for intimin α280 of E2348/69 could be used to make structural predictions about the carboxy terminal intimin α of G21 since intimin structures were predicted to be similar (Reece *et al.*, 2001;Kelly *et al.*, 1999;Luo *et al.*, 2000). This showed that the residue at position 907 of intimin α in E2348/69 and hence G21 was located on an α-helix and did not have the same orientation as the residue at position 911 with respect to Tir. The residue at position 737 was located on a hairpin loop at the end of a β-sheet at the base of the D1 region.

4.2.5.2 The presence of valine and isoleucine residues, in other strains, that align with V907 and I737 in G21.

The SIM alignment tool for protein sequences (http://us.expasy.org/tools/sim-prot.html) was used to determine whether other EPEC and EHEC strains found in the NCBI database (http://www.ncbi.nlm.nih.gov/) had a valine at position 907 and/or an Isoleucine at position 737 in their intimin molecules.

Table 4.5 The alignment of V907 and I737 of G21 with intimins from other intimin expressing strains.

NCBI protein bank ID number	Strain	Intimin type	Sequence identity %	Residue aligned with V907	Residue aligned with I737
P19809	O127:H6 E2348/69	α	99.2	A	S
AAG58823	O157:H7 EDL933	γ	48.7	Q	S
CAA73559	Human EPEC	β	52.3	Q	S
AAK48432	Caprine O3	β	52.1	Q	S
AAK26724	REPEC O15:H-	β	52,4	Q	S
AAD16298	REPEC O153:H7	β	52.4	Q	S
AAD27847	Human O26:NM	NG	52.4	Q	S
AAB37574	Human EHEC	NG	52.4	Q	S
AAB37572	Human EPEC	NG	52.4	Q	S
AAF21451	Swine O45	NG	52.1	Q	S
CAC81733	Calf STEC	NG	52.4	Q	S
AAL06378	Citrobacter DBS100	β	53	S	S
AAK48434	Ovine O91	γ	46.7	K	Gap
AAF23359	Human O103:H2	ε	49.3	K	S
CAC81744	Calf STEC	ε	50.7	K	S
CAC21552	Bovine O84:NM	ζ	69.3	M	S
CAC81930	Human EPEC O125:H-	η	52	K	S
AAL67423	EHEC O111:H8	θ	48.5	K	Gap
CAC59747	Human STEC O145:H4	ι	51.1	K	S
CAC59748	Human STEC O118:H5	κ	51.3	L	S
AAL32028	Stx negative *E. coli*	λ	56.2	V	S
AAN04017	Stx negative bovine *E. coli*	λ	56.2	V	S

Note: A(alanine), V(Valine), S(Serine), Q(Glutamine), K(Lysine), M(Methionine), L(Leucine) and a Gap signifies that no amino acid residue was aligned between the test intimin and the intimin from G21.

Of the twenty two strains chosen from the NCBI protein database, two had valine residues that aligned with the valine at position 907 in G21, numbered according to the alignment of G21 with E2348/69. No strain contained an Isoleucine residue that would align with the I737 of G21 and the strains either had a serine that aligned with I737 or a gap. The strain which contained the highest sequence similarity to the intimin expressed by G21 was the intimin α of E2348/69. The two intimin γ strains that were aligned showed a sequence similarity of 46.7 and 48.7 %. The intimin β had either a glutamine or a serine aligned with V907 and showed sequence similarity between 52.1 and 53%. Both intimin ϵ strains had a lysine that aligned with V907 and had a sequence similarity of 49.3 and 50.7 %. Although the strains whose intimin type could not be found all had a glutamine residue aligned with V907 the intimin type could not be assigned with any certainty.

4.2.5.3 Site-directed mutagenesis of intimin α from O127:H6 strain E2348/69 and incorporation into EPEC and EHEC backgrounds for use in *in vitro* adhesion studies.

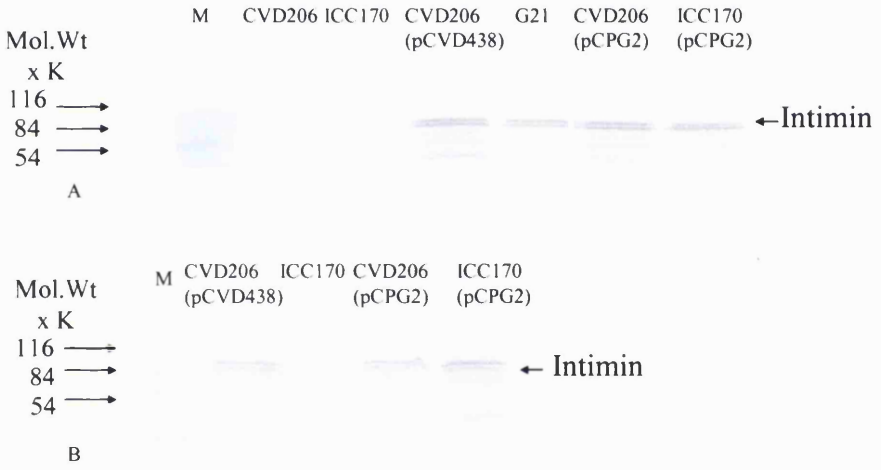
The sequencing of intimin α from O55:H6 strain G21 showed that it differed in one CTLD amino acid residue when compared with the intimin α from E2348/69. Due to the proximity of the valine at position 907 to the valine at position 911, which has been shown to be involved in the tissue tropism of E2348/69 during IVOC, IVOC and HEp-2 cell adhesion assays were developed to study the role of the valine residue at position 907 during intimin α mediated tissue tropism. The amino acids located in the D1 region of the intimin α 280 expressed by E2348/69 (residues 659 – 750) were not necessary for Tir binding and detailed analysis of their structure and function has yet to be resolved (Batchelor *et al.*, 2000). The presence of serine conserved across different intimin types at position 737, and in some cases a gap, (when aligned with the intimin sequences from G21, numbered according to its alignment with E2348/69) in the alignments outlined in table 4.17 indicated that this residue may not be involved in tissue tropism and may play a structural function in the intimin molecule.

An intimin α bearing template plasmid, derived from the intimin α of E2348/69, was used to create an intimin α A907V baring plasmid (pCPG2) which was used to transform intimin negative EPEC (CVD206) and EHEC (ICC170) strains. These strains were

designated as CVD206(pCPG2) and ICC170(pCPG2) and their construction is outlined in section 2.7.2. CVD206(pCPG2) and ICC170(pCPG2) were tested for their adhesion to intestinal epithelium during IVOC and for their adhesion to HEp-2 cells.

A western blot was used to confirm that intimin was expressed by the EPEC and EHEC strains expressing the intimin α A907V protein.

Figure 4.19 Western blot of intimin expression by CVD206(pCPG2) and ICC170(pCPG2)



Mol.Wt = molecular weight. M is a protein ladder marker.

This western blot showed that both CVD206 and ICC170 harbouring the pCPG2 plasmid expressed intimin after growth in DMEM at 37 °C (figure 4.19A). Strains CVD206(pCPG2) and ICC170(pCPG2) expressed intimin after overnight growth in BHI at 37°C (figure 4.19B).

4.2.5.3.1 IVOC of CVD206(pCPG2) and ICC170(pCPG2) strains.

EPEC and EHEC intimin negative strains were transformed with a mutated intimin α bearing plasmid, which has been used in previous studies (Reece *et al.*, 2001), containing a A907V mutation in order to determine the involvement of the amino acids at position 907 during intimin α mediated IVOC intestinal tissue tropism.

The EPEC CVD206(pCPG2) and the EHEC ICC170(pCPG2) strains were tested for their adhesion to intestinal explants during the eight hour IVOC assay. Duodenal explants were used in order to determine whether the A907V mutation would abrogate the adhesion of intimin α expressing EPEC and EHEC strains to duodenal explants, i.e. indicating that they had a FAE restricted phenotype which could be tested on IVOC with PP explants.

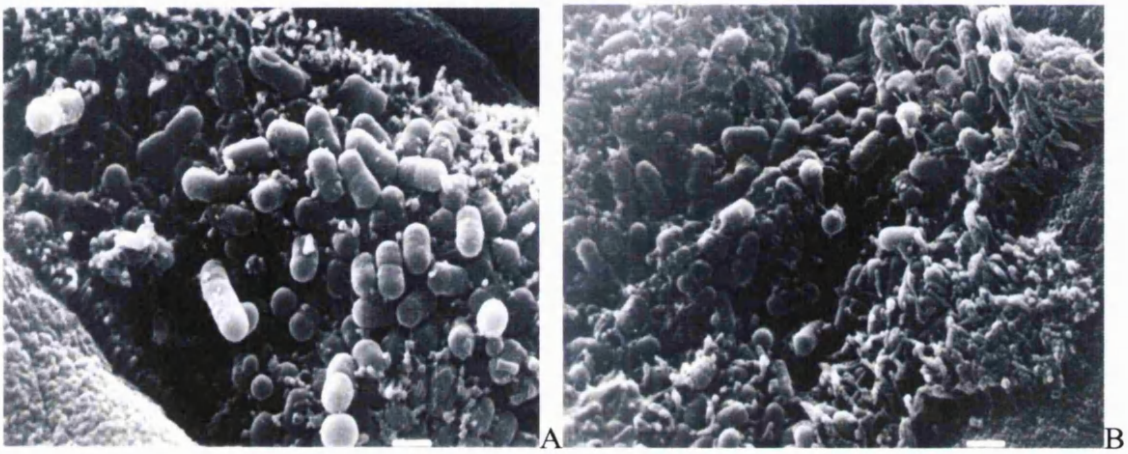
Table 4.6 IVOC of CVD206(pCPG2) and ICC170(pCPG2)

	Intestinal region
Strain	D4
CVD206(pCVD438)	8/9
CVD206(pCPG2)	5/6
ICC170(pCPG2)	3/3
Patient age (months) (median(range))	115(14-170)

Note: Values correspond to A/E lesion formation as a proportion of biopsies inoculated. When more than four explants were tested the age is shown as median (range). D4= fourth part of the duodenum

The EPEC CVD206(pCPG2) and EHEC ICC170(pCPG2) strains produced A/E lesions on duodenal explants. Therefore CVD206(pCPG2) and ICC170(pCPG2) did not show lack of adhesion to duodenal explants in a manner similar to the O55:H6 strain G21. PP explants were limited in availability but CVD206(pCVD438) adhered to 2/2 PP explants.

Figure 4.20 A/E lesion formation of strains CVD206(pCPG2) and ICC170(pCPG2) on duodenum during IVOC.



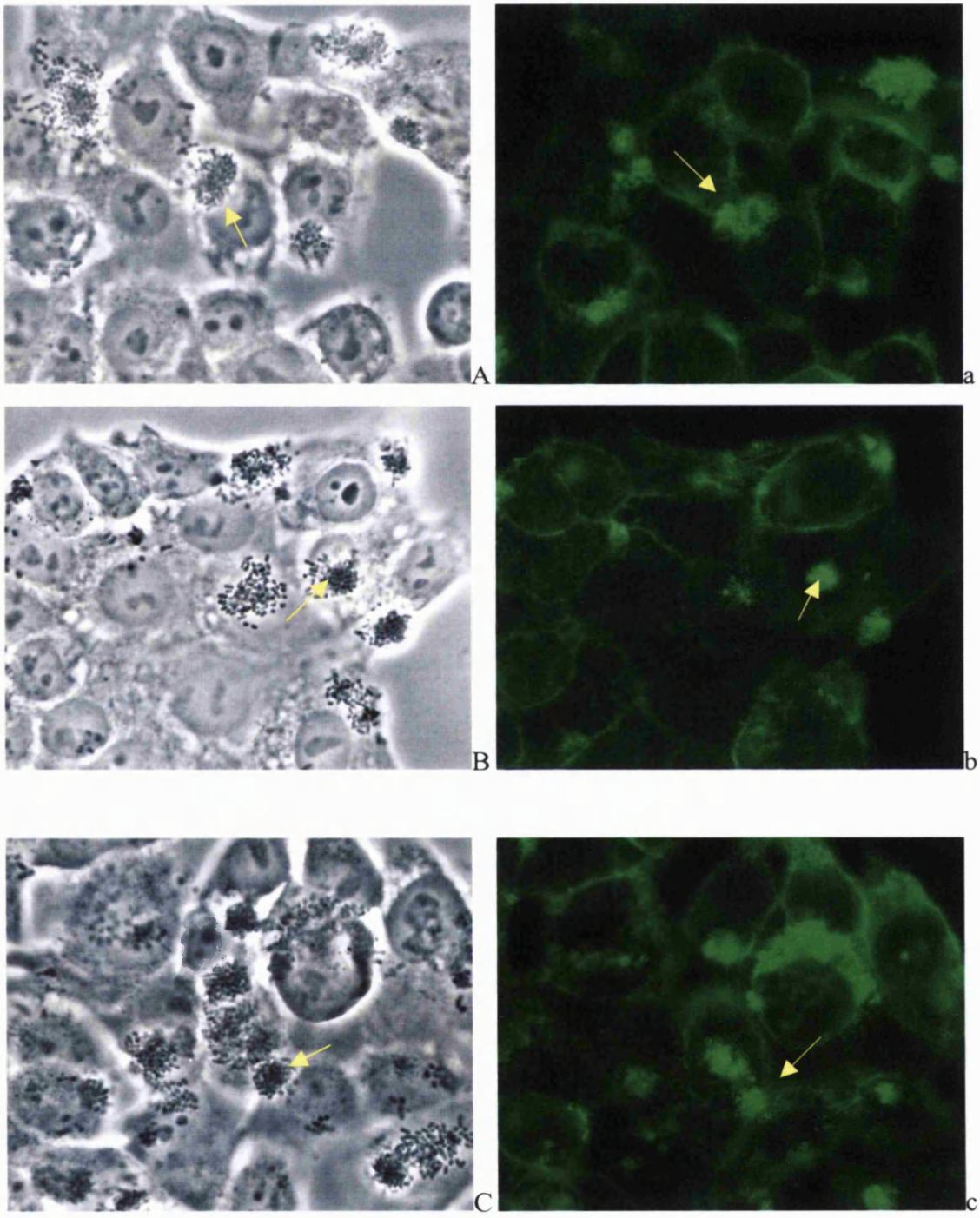
SEM of duodenal IVOC of A. CVD206(pCPG2), B. ICC170(pCPG2). Both strains showed attaching and effacing lesion formation on duodenum, bar = 0.5μm.

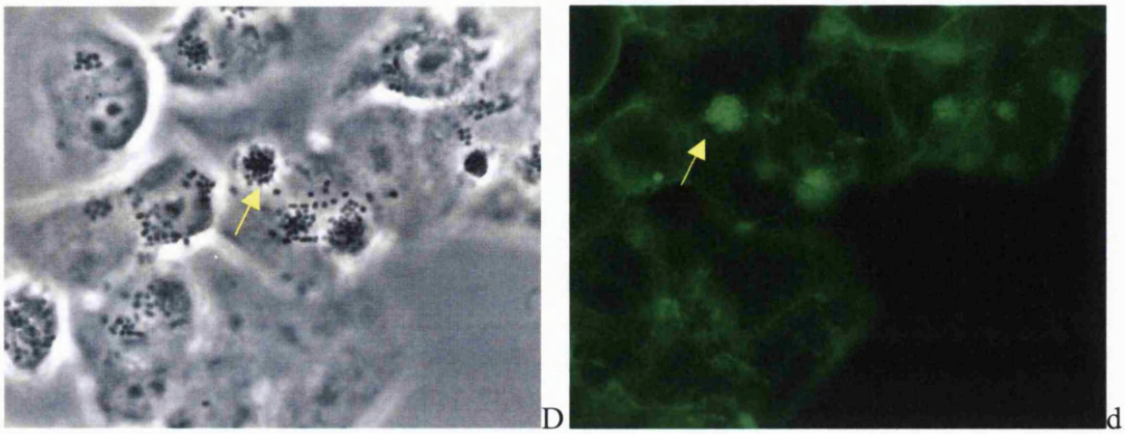
4.2.5.4. HEp-2 cell adhesion of CVD206(pCPG2) and ICC170(pCPG2).

In order to compare CVD206(pCGP2) and ICC170(pCPG2) to other strains, they were assayed for adhesion to HEp-2 cells. In addition, the HEp-2 cell assay was used to provide information on differences between these strains and other intimin α expressing strains.

The HEp-2 cell adhesion assay was carried in triplicate and on three separate occasions.

Figure 4.21 HEp-2 cell adhesion of CVD206(pCPG2) and ICC170(pCPG2)





These micrographs are duplicates with the phase contrast image represented by the upper case letters and the fluorescent images represented by the lower case letters. The HEp-2 assay was divided into three and six hour incubation periods with the letters A and B representing the 3Hr HEp-2 assay and the letters C and D representing the 6Hr Hep-2 assay. The yellow arrows indicate examples of bacterial microcolonies where the number of bacteria forming the microcolony is greater than 5. Magnification is x 80.

The strains CVD206(pCPG2), panels A and C, and ICC170(pCPG2), panels B and D, adhered to HEp-2 cells after three and six hour incubations periods. They shared similar adhesion patterns and which were described as an LA pattern. This was observed as microcolonies being formed, after three hours of incubation, that were similar to those produced by the O127:H6 strain E2348/69. These microcolonies contained more bacteria after six hours incubation and the number of bacteria adhering to HEp-2 cells independently of microcolonies was greater after six hours incubation when compared to the observations made after three hours of incubation. CVD206(pCPG2) and ICC170(pCPG2) were FAS positive after three and six hour incubation periods.

4.3 Discussion.

The experiments outlined in section 4.0 focused on using IVOC to outline the possible role of intimin during intestinal tissue tropism.

4.3.1 IVOC of the EHEC O157:H7 strain 85-170 expressing different intimin types.

The *eae* gene that encodes the outer membrane adhesion protein, intimin, was the first gene to be associated with A/E lesion forming activity and *eae* positive strains of EHEC and EPEC have been recovered from both animals and humans (Zhang *et al.*, 2002;Blanco *et al.*, 2003;Osek, 2001;Jerse and Kaper, 1991). During experimental infections, EPEC intimin was required for full virulence in human volunteer studies and EHEC O157:H7 required intimin to produce enteropathogenic effects in calves (Donnenberg *et al.*, 1993a;Dean-Nystrom *et al.*, 1998). The involvement of intimin in tissue tropism during adhesion to the intestinal tract was suggested by intimin exchange studies using a gnotobiotic piglet model. The *eae* gene is located in the LEE pathogenicity island of EPEC, EHEC and *C. rodentium* and this island was shown to be necessary and sufficient for adhesion to HEp-2 cells by the EPEC strain E2348/69 and was necessary but not sufficient for adhesion of EHEC to HEp-2 cells. Therefore the adhesion of EHEC and EPEC to the intestine may occur via different yet overlapping mechanisms. In order to assess the role of intimin in tissue tropism during IVOC intimin exchange studies were performed in combination with IVOC.

The EHEC intimin negative strain ICC170, like the intimin negative EPEC strain CVD206, did not express intimin as detected by Western blot (figure 4.1). Both strains displayed the ability to be complimented with plasmids expressing intimin α and ICC170 was also complimented with plasmids expressing intimin γ and intimin α with a disrupted disulphide bridge. In accordance with previous studies of intimin, the expression of intimin by bacteria was recorded after activation in DMEM at 37°C. However, without activation no intimin was detected for the strains expressing intimin from the pICC55 intimin γ plasmid. The strains harbouring intimin α plasmids expressed intimin after overnight growth in BHI broth at 37°C. The reasons for the difference in intimin expression are not clear.

The plasmids expressing different intimin types were all constructs of the same parent plasmid (Frankel *et al.*, 1998). This plasmid was used to generate pCVD438 which expressed the intimin α from the O127:H6 strain E2348/69. This intimin α expressing plasmid was used to create pICC55, which expressed an intimin with an amino terminal α tail and a carboxy terminal γ head. Intimin α is expressed by the pCVD438 plasmid in both EPEC and EHEC backgrounds and intimin can be detected as a result by Western blot after activation or no activation. However pICC55 contains changes in the carboxy terminal portion of the pCVD438 intimin α and this change may have disrupted the regulation of intimin expression in the absence of DMEM. However the ability of ICC170(pICC55) to produce intimin after activation in DMEM suggests that there is no error within the plasmid and that certain regulatory factors induced by DMEM may be required for expression of intimin from pICC55. In addition, since the bacteria were lysed prior to probing by the antibody it is not a lack of surface expression that is at fault.

The antibody used during the western blot analysis was raised against the conserved region of the intimin α from E2348/69. The polypeptide used incorporated amino acid residues glycine 388 to lysine 667 and was upstream of the cell binding domain. This region is highly conserved in all intimin proteins sequenced to date and is present in the intimin protein produced by pICC55. The antibody to this conserved region of intimin has been shown to be less reactive to intimin produced by EPEC strains cured of their EAF plasmid (Batchelor *et al.*, 1999) but similar levels were observed between wildtype EPEC and EHEC strains regardless of whether they possessed the EAF plasmid. The surface expression of intimin was also shown to vary according to intimin type and that this may be due to background genetic elements in the strains (Batchelor *et al.*, 1999). The lack of detection of intimin by western blot without DMEM activation of ICC170(pICC55) may have been due to the incorporation of an intimin γ carboxy head onto a amino terminal intimin α tail, but the reasons for this are unclear.

Since all the strains listed in table 4.1 produced intimin during growth in DMEM, a constituent of the IVOC medium, the strains were tested for their adhesion to the intestinal tract by IVOC.

As described in chapter 3, 85-170 showed preferential adhesion to the PP of the terminal ileum. The intimin negative EHEC O157:H7 strain ICC170 did not adhere to PP explants during IVOC suggesting that like the prototype EPEC strain E2348/69, intimin was necessary for A/E lesion formation by EHEC during IVOC (Hicks *et al.*, 1998). Therefore although the LEE is necessary but not sufficient for EHEC O157:H7 to produce A/E lesions on HEp-2 cells, the intimin protein encoded by the LEE is necessary for both EPEC and EHEC to produce A/E lesions on PP explants during IVOC (Elliott, Yu, and Kaper, 1999). This result correlated with results using animal models, in which intimin was shown to be necessary for A/E lesion formation by REPEC using rabbit ligated ileal loops, necessary for A/E lesion formation by EHEC O157:H7 on the large intestine of gnotobiotic piglets and required for A/E lesion formation and disease in newborn calves infected with EHEC O157:H7 (Dean-Nystrom *et al.*, 1998;Krejany *et al.*, 2000;Tzipori *et al.*, 1987). Thus intimin is a crucial part of the A/E lesion forming mechanism for different pathogenic *E. coli* and in different hosts.

In order to determine if different intimin types could be used to alter the tissue tropism of an EHEC O157:H7 strain during IVOC, ICC170 was transformed with plasmids expressing intimin α, intimin γ and intimin α with a disrupted disulphide bond. The strain harbouring the intimin γ plasmid, ICC170(pICC55), was used to verify that the deletion of the *eae* gene from the LEE on the 85-170 chromosome did not inhibit the ability of the strains A/E lesion forming activity on PP explants to be restored. Like its parental strain this intimin γ expressing strain adhered preferentially to PP explants during IVOC thus mimicking previous intimin exchange studies showing that an intimin negative strain of EPEC E2348/69, CVD206, could be complemented with an intimin α expressing plasmid resulting in restored A/E lesion formation activity and tissue tropism characteristics (Phillips and Frankel, 2000a). In addition, the intimin chimera expressed by the pICC55 plasmid showed that the tissue tropism activity of intimin γ, from EHEC O157:H7, was primarily located in the carboxy terminal amino acids.

Using the pCVD438 plasmid a EHEC O157:H7 strain was created that expressed intimin α. This strain, ICC170(pCVD438), adhered to PP explants during IVOC in a manner similar to 85-170 but in addition it adhered to the villous epithelium of the terminal ileum and the fourth part of the duodenum thus showing a similar tissue tropism to E2348/69.

This establishes that intimin is involved in the tissue tropism of EHEC O157:H7 strains during IVOC. However, not all of the small intestine IVOC incubations showed A/E lesions. This observation was also noted for the adhesion of E2348/69 to duodenal explants in chapter 3 suggesting that using IVOC it may be possible to separate adhesion to the intestinal mucosae from A/E lesion formation and underlined the possibility that intimin is involved in initial events during adhesion (Frankel *et al.*, 2001). The intimin negative EPEC E2348/69 strain, CVD206, complemented with intimin α (CVD206(pCVD438)) was used in this study to identify whether the adhesion without A/E lesion formation seen with the ICC170(pCVD438) strain was due to an element unique to the ICC170 genetic background. Such an adhesion phonotype was not limited to ICC170 derivative strains. CVD206(pCVD438) showed adhesion to duodenal explants without A/E lesion formation suggesting that in certain incubations A/E lesion formation may have been delayed and that the eight hour IVOC was not sufficient to allow the lesion to develop fully. The importance of intimin during A/E lesion formation and tissue tropism in EHEC O157:H7 was highlighted by the inability of the ICC170 strain expressing intimin α with a disrupted disulphide bridge in the CTLD, to form A/E lesions on duodenal and PP explants. This result suggested that disrupting the disulphide bridge in the CTLD affected the ability of the strain to adhere to the explants and form A/E lesions. Since the intimin α C937A protein was still expressed, as shown in the western blot (figure 4.1), this is evidence that as in EPEC, amino acids in the CTLD are involved in the initial interaction of EHEC with the intestinal mucosae and in subsequent A/E lesion formation. Thus intimin is a key factor in the A/E lesion forming mechanisms of both EPEC and EHEC during IVOC. In addition, these results combined with previous experiments by Phillips et al (Phillips and Frankel, 2000b) showed that for EPEC strain E2348/69 and for EHEC O157:H7 strain 85-170 tissue tropism is Tir type independent. These studies provided further evidence that IVOC can be used to study the relationship between intimin and tissue tropism and that this can be carried out using strains expressing different intimin types or EPEC and EHEC strains expressing different recombinant intimin types.

4.3.2 HEp-2 cell adhesion of the ICC170 strains expressing different intimin types.

The HEp-2 cell adhesion assay was used to characterise the adhesion of the strains listed in table 4.1, thus facilitating their comparison to other studies. ICC170 did not adhere to

HEp-2 cells after three and six hour incubation periods. In comparison, the intimin negative EPEC strain CVD206 has been reported to adhere to HEp-2 cells with an LA pattern, presumably due to the expression of BFP (Knutton *et al.*, 1997). This strain produces a shadow pattern of actin accumulation after three hours of incubation and demonstrated the possibility of intimin-independent actin accumulation during EPEC infections. However, since ICC170 did not adhere or produce an actin shadow it suggests that cell signalling events may be different between EPEC and EHEC O157:H7 strains. Such differences may be due to different Tir mediated cell signalling mechanisms (DeVinney *et al.*, 2001) or due to differences in virulence factor expression by EPEC and EHEC O157:H7.

The two strains harbouring the intimin α expressing plasmid CVD206(pCVD438) and ICC170(pCVD438), did not share the same adherence pattern. CVD206(pCVD438) adhered to HEp-2 cells with an LA pattern similar to E2348/69 but ICC170(pCVD438) was LAL which was the pattern of adhesion exhibited by the EHEC O157:H7 strain 85-170. The only difference that was observed between CVD206(pCVD438) and E2348/69 was that the microcolonies produced by CVD206(pCVD438) appeared to be less tight than those produced by E2348/69. Both strains express BFP and only differ in their expression of intimin from a plasmid and chromosome respectively, so that the *eae* copy number in the former is higher than in the latter. The observations of differences in adhesion phenotype between IVOC and HEp-2 assays may remain difficult to explain until the mechanism of A/E lesion formation has been fully elucidated in the two situations.

Although ICC170(pCVD438) produced an LAL pattern of adhesion the transformation of ICC170 with pCVD438 did not produce a different HEp-2 cell adhesion phenotype to 85-170. This suggested that although intimin is involved in adhesion to HEp-2 cells, and is necessary for EHEC O157:H7 adhesion, other factors may be involved in microcolony development and the production of an LA pattern on HEp-2 cells. One candidate is the type IV pilus BFP which is required for assigning an EPEC strain to the typical EPEC group (Kaper, 1996). Indeed, HEp-2 data has been used to suggest a three stage model of EPEC A/E lesion formation (Donnenberg and Kaper, 1992) in which BFP is the initial adhesin, however this was contradicted by studies which showed that BFP negative EPEC strains still adhered to the intestinal mucosae (Hicks *et al.*, 1998). The current situation

could be summed up as reported in a recent review article "while it seems evident that BFP mediate interbacterial interactions, it si not clear whether BFP acts as a host cell adhesin" (Nougayrede, Fernandes, and Donnenberg, 2003). Although the role of BFP during adhesion is still being investigated it has been shown to be involved in the production of an LA pattern of adhesion on HEp-2 cells(Donnenberg *et al.*, 1992;Giron, Yue Ho, and Schoolnik, 1991;Giron *et al.*, 1993;Giron, Ho, and Schoolnik, 1993). Therefore a lack of BFP or other virulence factor production by ICC170(pCVD438) may have contributed to its LAL pattern of adhesion. In addition, work carried out on the O157:H7 Sakai strain (Tatsuno *et al.*, 2001) has shown that a *toxB* gene located on its 93-kb plasmid (pO157) is involved in microcolony development and regulation of the secretion of type III secreted proteins suggesting that EPEC and EHEC may have evolved different mechanisms to regulate adhesion and microcolony development.

ICC170pICC55 (PA/LAL) did not show the same adherence pattern as its parent strain 85-170 (LAL) showing that the HEp-2 cell adhesion phenotype was not fully restored by the complementation of ICC170 with pICC55. This difference may have been due to the regulation of intimin expression when expressed on a plasmid and may have also affected the adhesion of CVD206(pICC55) to HEp-2 cells which was PA/LAL instead of the LAL pattern adhesion of intimin γ expressing 85-170 or the LA pattern of the intimin α expressing strains E2348/69 and CVD206(pCVD438). CVD206(pICC55) contains the genes for BFP production but it did not produce an LA pattern of adhesion, indicating that BFP-mediated events do not act in isolation from intimin mediated events.

The ICC170 strain expressing intimin α with a C937A mutation adhered in very few numbers after six hours of incubation and was FAS negative. In contrast the EPEC strain CVD206(pCVD438/01) has been shown to adhere after three hours of incubation producing microcolonies with a FAS negative result, possibly due to the presence of BFP. Interaction with the host cell can occur via Tir independent and BFP independent mechanisms, and such mechanisms are not necessarily identical in EPEC and EHEC. Fimbriae have been suggested as a possible adhesin in LEE negative STEC strains (Doughty *et al.*, 2002) and annexin 2 and lipid rafts components have been shown to be recruited to the site of EPEC adherence independently of Tir suggesting a role for intracellular Ca^{2+} in non Tir mediated cell signalling events (Zobiack *et al.*, 2002).

The HEp-2 adhesion assay of the strains listed in table 4.1 suggests that expression of intimin γ by EPEC and EHEC strains limits adhesion. This also appears to be the case in IVOC. The expression of wildtype intimin α in EPEC and EHEC backgrounds appears to only change adhesion phenotypes during the IVOC assay, producing small intestinal EHEC adhesion. In contrast expression of intimin α with a A907V mutation, as described in section 4.3.3.4.2 shows that a mutated intimin α may confer an LA pattern of adhesion in both EPEC and EHEC backgrounds during the HEp-2 adhesion assay. Thus the expression of different intimin types in CVD206 and ICC170 backgrounds can produce different adhesion phenotypes during HEp-2 cell adhesion but as shown in section 3 and in section 4 no intimin type is associated with a particular adhesion phenotype during the HEp-2 adhesion assay. An LA phenotype produced by an EHEC derivative strain (ICC170(pCPG2)) suggests that intimin and other factors may be involved in adhesion and microcolony formation on HEp-2 cells, and it does not solely rely on the presence of BFP.

4.3.3 The tissue tropism of EPEC strains belonging to the O55 serogroup.

The *E. coli* O55 serogroup is an important cause of infantile diarrhoea and is made up of strains belonging to the classic EPEC genotype and others with an atypical EPEC genotype. The O55:H7, atypical EPEC, serotype is closely related to EHEC O157:H7 as can be seen by their near identical sequences for the H7 flagellin gene and the similarity of their *eae* genes (McGraw *et al.*, 1999;Rodrigues *et al.*, 1996). As the O55 serogroup contains strains expressing intimin α and γ, it was considered as an ideal serogroup to try and bridge the gap between the prototype EPEC E2348/69 and the EHEC O157:H7 strain 85-170. The Western blot of the strains listed in table 4.3 showed that all strains expressed intimin after growth in DMEM (see figure 4.7). The O55:H-, EAF positive, strain G35 expresses intimin γ after overnight growth in BHI without activation in DMEM as does the EAF negative strain JPN15. As shown in figure 4.7 CVD206(pICC55) did not express intimin γ after overnight growth in BHI. Therefore the *per* located on the EAF plasmid may regulate intimin expression after overnight growth in BHI differently in different EPEC strains. Whether intimin type or regulators such as the *ler* are involved in the expression of intimin after overnight growth in BHI remains to be elucidated.

The IVOC of the O55:H7 strain G57 showed that it adhered preferentially to lymphoid follicles in a manner similar to the intimin γ expressing EHEC O157:H7 strains 85-170, AGT300 and TT12B, the related G58 O55:H7 strain, and the intimin ϵ expressing strains E77804 and PMK5. G57 was the first intimin γ expressing strain observed producing A/E lesions on villous epithelium of terminal ileum explants lacking PP regions. The A/E lesions were similar to E2348/69, but G57 adhered to the terminal ileum with a lower frequency than E2348/69.

The IVOC of the strains from the O55 serogroup showed that intimin α strains can display a PP restricted tissue tropism and that the tissue tropism of O55:H7 strains appears to be related to that of EHEC strains. In addition to the tissue tropism of EPEC strain E77804 this showed that typical EPEC strains can have different tissue tropism phenotypes and that typical and atypical EPEC strains can have the same tissue tropism phenotypes. Since intimin is essential for adhesion during IVOC and intimin α has been shown in section 4.3.1 to modulate tissue tropism in EHEC O157:H7 these results suggest that the intimin α expressed by the O55:H6 strains may be different to the intimin α expressed by E2348/69 or that other factors in the O55 genome may act to influence tissue tropism during IVOC. This is mirrored in part by strains expressing other intimin types. The 3801 O26:H11 strain expresses intimin β and adheres to both PP and the villous epithelium of the terminal ileum with the same frequency during the eight hour IVOC assay, however RDEC-1, which expresses intimin β adheres initially to rabbit PP with subsequent adhesion to other areas of the intestinal tract (Cantey and Inman, 1981). Thus strains expressing similar intimin types may have different initial sites of adhesion.

Another aspect of the IVOC results was the presence of the EAF plasmid in an intimin γ expressing strain, G35. Since this strain possessed the EAF plasmid and hence the genes for BFP production it can be considered as a typical EPEC (Giron, Yue Ho, and Schoolnik, 1991;Kaper, 1996;Rodrigues *et al.*, 1996). The restricted adhesion of G21 and G35 to PP explants suggested that possession of BFP or the EAF plasmid are not synonymous with small bowl villous adhesion during the eight hour IVOC assay. This was underlined by the ability of the intimin α expressing O157:H7 derivative, ICC170(pCVD438), to adhere to the villous epithelium of the small bowl. Thus BFP may not be involved in tissue tropism during IVOC. It would appear that the EAF plasmid and BFP may be involved in three

dimensional colony formation (Hicks, Candy, and Phillips, 1996) and such a role would remain compatible with adhesion restricted to PP. BFP may act to form establish colony units within the gut environment, allowing non-intimate bacterium to bacterium adhesion, and once a quorum of bacteria have been established, to permit disaggregation and colonisation of other regions of the gut.

4.3.3.1 TEM of the strains belonging to the O55 serogroup.

In order to confirm and elucidate the characteristics of the O55 serogroup A/E lesions, PP explants were sectioned for TEM after IVOC. The TEM micrographs showed that in general the O55 strains did not produce marked pedestal formation, which is also a characteristic of EHEC O157:H7 (Phillips *et al.*, 2000;Xia *et al.*, 2000). The sites of adhesion were seen as forming depressions in the surface of the enterocyte with microvillus effacement. A similar appearance has been documented in newborn pigs infected with O55:H7(Staley, Jones, and Corley, 1969), and in cell lines using EHEC O157:H7 (Xia *et al.*, 2000), indicating that the observations may not have been due to the relatively short length of incubation in IVOC. On occasion some pedestal-like structures were noted but these were usually seen on extruding cells where cytoplasmic extrusion gives the appearance of elongated pedestals. It is possible that this extrusion is due to the cytopathic effects of EPEC adhesion rather than a directed cell signalling event. However, intimin has been shown to be able to remodel the surface of HEp-2 cells and EspH has been implicated in fillopodia formation, pedestal attenuation and marked pedestal elongation in EHEC (Tu *et al.*, 2003). Further examination of *in vivo* EPEC infections (Hill, Phillips, and Walker-Smith, 1991;Rothbaum *et al.*, 1983;Ulshen and Rollo, 1980) and animal models of human EPEC infection (Moon *et al.*, 1983) showed the A/E lesions presented in these studies to be similar to those produced by IVOC. The lack of elongated pedestal or pseudopod formation (Rosenshine *et al.*, 1996) on gut explants by EPEC O55 strains, highlights the possible differences between IVOC and cell line adhesion studies. Using nonpolarised cells derived from the larynx, lung and cervix allows us to carry out *ex vivo* investigations into bacterial pathogenesis. However, such cells may not possess tight junctions, can lack a terminal web and do not have the complex actin based cytoskeleton of the intestinal brush border. Therefore is it possible that actin polymerisation at the

bacterium-host interface may be a more tightly regulated process in the gut epithelium than in cell lines.

An O55:H7 strain has been shown to invade the enterocytes of newborn pigs, implying that the O55 serogroup may have an invasive potential (Staley, Jones, and Corley, 1969). During the IVOC carried out on the O55 strains listed in table 4.3 no invading bacteria were noted after eight hours of IVOC with PP explants. A case report has shown that an O18 EPEC strain may invade enterocytes during *in vivo* infection. This strain was also shown to be invasive for HeLa cells and rabbit ileal loop enterocytes (Scaletsky, Pedroso, and Fagundes-Neto, 1996). However, the invasive potential of EPEC strains has yet to be fully elucidated.

4.3.3.2 Intimin expression during IVOC of strains belonging to the O55 serogroup.

In order to compare the O55 strains in this study to the EPEC and EHEC strains analysed for adhesion, during IVOC (see section 3.2), intimin expression during IVOC of the O55 strains was tested. In addition, CVD206 and JPN15 were used to control for intimin and the EAF plasmid. As was to be expected CVD206 showed no intimin expression during IVOC. The two intimin α strains tested, JPN15 and G21, showed similar results. Both strains showed intimin expression during duodenal IVOC assay. Testing the intimin γ strains for intimin expression during duodenal IVOC produced a background signal indicating that these strains did not produce enough intimin for a clear positive result. In addition, as in previous results (section 3.2.4) it appears that strains expressing intimin α showed detectable levels of intimin expression during IVOC. The lack of detection in intimin γ strains may be due to the affinity of the antibody for intimin γ and/or the amount of intimin produced by intimin γ strains during IVOC, but this characteristic remains unclear. However, it possible that the EAF plasmid present in strain G35 may have contributed to its intimin expression during IVOC. This may highlight certain regulatory differences between intimin γ and intimin α expressing strains and intimin γ expressing strains may be more sensitive to regulation by environmental factors or intestinal flora via a quorum sensing mechanism.

4.3.3.3 The HEp-2 cell adhesion patterns of strains belonging to the O55 serogroup.

The HEp-2 adhesion assay was used to characterise the O55 strains and to highlight any possible differences in their adhesion characteristics. The four O55 strains tested did not share a common HEp-2 cell adhesion phenotype unlike their IVOC tissue tropism phenotype. Both of the strains that contained an EAF plasmid, G21 and G35, adhered to HEp-2 cells with an LA pattern of adhesion providing further evidence that the EAF plasmid plays a role in determining the LA phenotype, particularly since each strain expressed a different intimin type. This result also showed that intimin γ expressing strains can produce an LA pattern of adhesion and that the expression of intimin γ, *per se*, does not appear to be a limiting factor in microcolony formation after three hours of incubation. In addition, G21 was observed to produce actin pseudopods suggesting that like 85-170 in section 3.2.3, G21 may contain an EspH that was overexpressed or upregulated during HEp-2 cell adhesion resulting in pseudopod formation (Tu *et al.*, 2003). The intimin γ expressing EAF negative O55:H7 strains G57 and G58 both had an LAL pattern of adhesion similar to 85-170. The lack of an EAF plasmid may account for the lack of microcolony formation after three hours of incubation but may be due to factors that influence O55:H7 strains, and not other O55 strains, and that O55:H7 strains share in common with 85-170 but not all O157:H7 strains. However, the differences which appeared when the strains were tested for adhesion during a HEp-2 assay were not present after IVOC suggesting that different adhesion mechanisms may exist for different host cells and/or environments or that factors which are important during HEp-2 cell adhesion do not play a significant role during the eight hour incubation period. Perhaps with longer incubation periods or using different host models, differences in adhesion may become apparent.

4.3.3.3.1 The Tir tyrosine phosphorylation of strains belonging to the O55 serogroup during their adhesion to HEp-2 cells.

EPEC strain E2348/69 contains a tyrosine residue at position 474 in its Tir molecule which has yet to be found in an EHEC O157:H7 isolate. The phosphorylation of this residue during A/E lesion formation on cell lines is said to be a distinguishing feature of EPEC Tir induced cell signalling events (Kenny, 1999). It has been shown for E2348/69 that

phosphorylation of Tyr474 is essential for pedestal formation and that EPEC strain E2348/69 and the EHEC O157:H7 strain 86-24 utilise different Tir based mechanisms for pedestal formation (DeVinney *et al.*, 2001). In section 4.2.4.2, four O55 strains and strain 85-170, were tested for Tir phosphorylation by indirect immunoflourecense during the HEp-2 adhesion assay. One intimin α expressing strain, G21, and two intimin γ expressing strains, G35 and G58, showed Tir phosphorylation but the EPEC O55:H7 strain G57 and the EHEC O157:H7 strain 85-170 did not. Thus Tir phosyphorylation appears to be intimin type independent and may provide information about how strains are related to each other. G57 may be more closely related to O157:H7 strains than G58, and is the first EPEC strain to be associated with a non Tir phosphorylating phenotype. In the study carried out by DeVinney et al (DeVinney *et al.*, 2001) an O55:H7 strain was used but this strain was considered to be an EHEC strain rather than an EPEC O55:H7 strain and this was reflected in its inability to phosphorylate Tir. Other studies have defined O55:H7 as being EPEC or EHEC strains, depending on the absence or presence of Shiga toxin respectively, showing that this serotype lies at the junction between EPEC and EHEC characteristics and pathology (Gansheroff, Wachtel, and O'Brien, 1999;Whittam *et al.*, 1993). Further screening of EHEC and EPEC strains would help to add weight to the assumption that Tir phosphorylation is a defining EPEC characteristic.

4.3.3.4 The involvement of the intimin α from O55:H6 strain G21 in its tissue tropism during IVOC.

The O55 strains listed in table 4.3 shared the same tissue tropism during IVOC. The O55:H6 strain G21, a typical intimin α expressing EPEC, showed a restricted tissue tropism similar to that documented for intimin γ expressing strains (Phillips *et al.*, 1999;Phillips and Frankel, 2000b). Evidence that amino acids within the CTLD of intimin α may contribute to tissue tropism during IVOC was provided by mutational analysis of the intimin α of E2348/69. This showed that a V911A mutation resulted in an intimin α that conferred a restricted tissue tropism phenotype during IVOC, similar to that of intimin γ expressing strains. In order to determine whether a mutation in the intimin α of O55:H6 was responsible for its restricted tissue tropism during IVOC, the intimin α from G21 was sequenced. When the sequence of intimin α from G21 was compared to the intimin α sequence from E2348/69 two base pair changes were observed. These two base pair

changes resulted in the intimin α of G21 being different for two amino acids when compared to the amino acid sequence of E2348/69. These were noted as S737I and A907V with respect to E2348/69. Since the valine residue at position 907 in the intimin α of G21 was located in the CTLD, it was thought that this residue may play a critical role in the interaction with Tir and possibly influence tissue tropism. Although, it was not certain to have the same effect as mutating the valine at 911 (Reece *et al.*, 2001) in the intimin α of E2348/69 since the orientation of V907 with respect to Tir was not identical to V911 (see figure 4.18).

The valine at position 907 in G21 has a longer hydrocarbon side chain than the alanine present at position 907 in E2348/69 and both are considered to be structural amino acids. Whether the presence of a larger side chain will have any effect on the structure of intimin and result in a change in its biological properties was tested using IVOC. Interestingly Liu et al (Liu *et al.*, 2002) have shown that by mapping the Tir binding pocket of intimin γ a V906A substitution disrupted intimin-Tir binding. However this mutation in intimin γ was thought to be equivalent to a V911A mutation in intimin α, but may imply that amino acids in this region are important in the biological activity of intimin α and intimin γ. This is underlined by X-ray crystallography evidence that showed that in the intimin α of E2348/69 there is a positively charged tip that was formed by an α-helix between residues 904-907. This region of the intimin molecule is hydrogen bonded through the main chain carbonyl oxygens of S909 and K908 to the main chain amides of N315 and N316 within Tir (Luo *et al.*, 2000). Therefore the presence of a valine instead of an alanine residue in between both these hydrogen bonded residues may have altered the tissue tropism of G21.

4.3.3.4.1 The adhesion of EPEC and EHEC strains expressing the pCGP2 plasmid during IVOC.

An intimin α A907V expressing plasmid was created (pCPG2) and used to transform CVD206 and ICC170. Both strains expressed intimin after growth in BHI overnight and after subsequent growth in DMEM. This mirrored previous results for intimin α expressing CVD206 and ICC170 strains, and wildtype intimin α expressing strains E2348/69 and G21. Duodenal explants were used to determine whether CVD206(pCPG2) would mimic the tissue tropism of G21 and not adhere to duodenum during IVOC, unlike

the prototype intimin α expressing strain E2348/69 which adhered to duodenum. Unlike G21, CVD206pCPG2 adhered to duodenal explants in a manner similar to E2348/69. This suggested that the mutation at position 907 in G21 when compared to the intimin α of E2348/69 was not critical to the tissue tropism of O55:H6 strain G21. In order to determine whether the EPEC background of CVD206 was a factor in the inability of CVD206pCPG2 to reproduce the restricted tissue tropism associated with G21, pCPG2 was transformed into an EHEC background. The resultant strain ICC170pCPG2 produced A/E lesions on duodenal explants similar to that of CVD206pCPG2. Therefore it was concluded that the restricted tissue tropism of O55:H6 strain G21 was associated with a factor distinct from intimin α, which was not present in the EPEC E2348/69 or the EHEC 85-170 backgrounds. Interestingly a different O55:H6 isolate obtained from a four month old infant by the center for disease control and prevention in the U.S.A. has been shown to produce diarrhoea in human volunteers but not protect against subsequent challenge with E2348/69 (Donnenberg *et al.*, 1998). This suggests that although intimin type specific immune responses do exist different strains harbouring similar intimin types may contain other factors that are important in pathogenic effects in humans (Ghaem-Maghami *et al.*, 2001;Higgins *et al.*, 1999;Sanches *et al.*, 2000).

In the study of the biological activity of intimin α carried our by Reece et al the valine at position 911 was hydrophobic and thought to lie at the top end of the Tir-binding site in intimin α. It was solvent exposed and considered to be ideally placed to interact with Tir (Reece *et al.*, 2001). Its mutation to an alanine residue resulted in a restricted tissue tropism, similar to intimin γ expressing strains, when it was placed in the EPEC CVD206 background. As mentioned earlier an equivalent alanine substitution at position 906 in intimin γ resulted in disrupted intimin γ-Tir binding activity (Liu *et al.*, 1999). Thus the removal of a hydrophobic valine residue, within the Tir binding site of intimin α and intimin γ, and a substitution with an alanine residue led to altered binding activity and in the case of intimin α, restricted A/E lesion formation during IVOC. However a valine at position 907 in intimin α does not appear to alter the tissue tropism associated with the intimin α from E2348/69. This suggested that other factors may be involved in tissue tropism or that residues outside the CTLD may have an effect on tissue tropism. The amino acid residues outside the CTLD show a high degree of homology suggesting that this region may not play an important role in tissue tropism. The I737 present in the

intimin of G21 is part of the D1 280 amino acids of the carboxy terminal of intimin α (Kelly *et al.*, 1998;Kelly *et al.*, 1999). This region was not essential for Tir binding and formed a globular domain comprising two β-sheet sandwiches. Even though this domain lacks a disulphide bond it resembled the type I set of the immunoglobulin superfamily (IgSF). IgSF domains are present in cell-surface adhesion molecules and can act in the recognition of integrins. However, no recognised integrin binding motif is present in D1 and integrins are not required for intimin mediated cell binding (Liu, Magoun, and Leong, 1999). In addition, the D1 domain is very similar to the first IgSF domain of the *E. coli* pili chaperone PapD. PapD was shown to be involved in organelle assembly and acts to control subunit folding during pili biogenesis (Behrens, 2003). The IgSF domains in intimin may act to project the CTLD domain away from the bacterial surface so that it can interact with Tir (Luo *et al.*, 2000). The D1 region has yet to be highlighted as essential in intimin Tir binding or in the interaction of intimin with other receptors involved in the adhesion process. A study of conserved tryptophan residues showed that conserved amino acids within D2, although all located within the region necessary for Tir binding and thought to interact with the CTLD, may have biological activities as well as being important in the structural hydrophobic interactions (Reece *et al.*, 2002a). Indeed, two of the tryptophan residues examined were located in the second IgSF domain of the 280 carboxy terminal amino acids of intimin α and mutation of both showed some change in the biological activity of intimin α suggesting that amino acids in the IgSF domains may play a role in adhesion. Whether this extends to residues within D1 is not known as unlike residues within the D2 IgSF region, no interactions between D1 and the CTLD have been postulated form the structural data. Figure 4.18 shows the putative position of the G21 I737 based on the structural data for E2348/69. This residue appears to be positioned on a hairpin loop next to a β-sheet at the base of the D1 domain and therefore further from Tir than any of the residues implicated in Tir binding examined to date (Batchelor *et al.*, 2000;Luo *et al.*, 2000;Reece *et al.*, 2001;Reece *et al.*, 2002a;Reece *et al.*, 2002b). The region which precedes D1 (558-651) may also be an Ig-like domain and it is possible that residues at position 737 interact with this precedent domain, although how this would alter the biological activity of intimin remains to be resolved.

Therefore the presence of an ethyl (CH_2CH_3) side chain at I737 in the intimin from G21 when compared to the hydroxyl (OH) side chain of the serine at 737 in E2348/69 may have

had an effect on the structure and/or function of the intimin molecule by potentially removing the possibility of hydrogen bond formation via the hydroxyl group. Whether this affected the structure of the D1 domain or its interactions with other domains has yet to be resolved. However none of the mutations noted in the carboxy terminal intimin sequence of G21, when compared to the intimin of E2348/69, inhibited A/E lesion formation during IVOC suggesting that they were not essential for A/E lesion formation and that other factor(s) outside intimin may play a role in tissue tropism.

Since several different infection models have shown that intimin is involved in colonisation and tissue tropism, it appeared that a factor(s) in G21 is operating to prevent colonisation of the small intestinal villous epithelium despite the possession of intimin α. In future, if this factor can be identified it will be interesting to determine whether it is also present in the intimin γ positive O55:H-, O55H7 andO157:H7 strains and the intimin ϵ O103:H- and O103:H2 strains, which would suggest a multifactorial control of the restricted adhesion to the FAE of PP explants.

4.3.3.4.2 The HEp-2 cell adhesion of EPEC and EHEC strains expressing the pCPG plasmid.

The HEp-2 cell adhesion assay was used to determine the effect of pCPG2 in an EPEC and EHEC background. In previous studies the intimin α plasmid pCVD438 had been able to restore the LA adhesion phenotype in the intimin negative EPEC strain CVD206. However, as was shown in figure 4.6 ICC170(pCVD438) did not adhere to HEp-2 cells in a manner similar to CVD206(pCVD438), although CVD206(pCPG2) and ICC170(pCPG2) adhered to HEp-2 cells with an LA pattern of adhesion (figure 4.3.3.4.2). A possible explanation of this was that, as well as intimin, there may be other factors involved in producing an LA phenotype on HEp-2 cells after three hours of incubation. One factor which has been suggested to be involved in producing an LA pattern is the expression of BFP on the EAF plasmid (Vuopio-Varkila and Schoolnik, 1991). However, ICC170(pCPG2) strain does not express BFP and this is the first observation of an intimin molecule conferring an LA phenotype on ICC170 and suggests that O157:H7 strains could produce an LA pattern of adhesion without harbouring an EAF plasmid and producing BFP. It may also be possible that factors other than BFP may promote EPEC and EHEC

microcolony formation. Such factors may include the *efa1* gene in bovine STEC strains and the *toxB* gene in O157:H7 (Stevens *et al.*, 2002;Tatsuno *et al.*, 2001). In addition, these observations suggested that certain mutations in intimin may have a positive effect in terms of adhesion rather than a negative effect. The presence of a valine residue at position 907 in intimin α may have increased the binding affinity of intimin α for Tir or indeed for a human receptor allowing the EHEC ICC170(pCPG2) strain to establish microcolonies at an earlier time point. In addition, the intimin expressed by pCPG2 did not contain an I737 residue suggesting that it was not essential for the binding of G21 to HEp-2 cells nor in the development of microcolonies.

4.3.4 Conclusions.

Thus this section has shown that intimin is necessary for the adhesion of EHEC O157:H7 to PP explants during IVOC and is involved in the tissue tropism of EHEC O157:H7 via a Tir type independent mechanism. However, different strains expressing the same intimin type may not share the same tissue tropism and hence tissue tropism may involve factor(s) that act interpedently of intimin during IVOC. In order, to determine the mechanisms involved in tissue tropism other putative tissue tropism factors need to be examined.

5.0 The expression of fimbriae by EPEC and EHEC and their role in adhesion.

5.1 Introduction.

The previous sections have focused on examining the role played by intimin in tissue tropism. The results of these experiments suggest that other factor(s) may play a role in tissue tropism. Such factors may include molecules that interact directly with the host cell, act as chaperones to adhesions or form part of the regulatory mechanisms associated with adhesion. Some regulatory factors have been found for EPEC and EHEC. In EPEC the genes necessary for EPEC adhesion to HEp-2 cells were found to be present in the LEE (McDaniel and Kaper, 1997). The EAF plasmid of EPEC contains the sequence for a plasmid encoded regulator (*per*) which activates the expression of intimin and BFP in E2348/69 and *per* in turn is regulated by GadX and quorum sensing signals (Shin *et al.*, 2001;Sperandio *et al.*, 1999). For both EPEC and EHEC the LEE encoded regulator (*ler*) regulates the expression of intimin and Tir as well as the Esp proteins. In addition, *ler* regulates the expression of proteins outside the LEE which may be significant for EHEC O157:H7 as its LEE is necessary but not sufficient to generate adhesion to HEp-2 cells (Elliott, Yu, and Kaper, 1999). Such global regulators suggest that adhesion, A/E lesion formation and microcolony development may involve cascade mechanisms with multiple regulators. Studies which focus on characterising adhesins rather than effectors of adhesins have been used to isolate adhesion candidates other than intimin and in some cases included proteins that are expressed by genes outside the LEE. Two surface appendages which have been shown to be expressed by EPEC are BFP and EspA. EspA, expressed by both EPEC and EHEC, forms a filamentous structure which was shown to bind to the putative type III secretion needle complex protein, EscF, and form a tube-like extension to the needle complex. Thus EspA is an extension to the needle complex, which is sometimes called a translocon. The *Shigella* needle complex is similar to the EPEC and EHEC needle complex but EspA is an additional EPEC and EHEC component (Daniell *et al.*, 2001;Daniell *et al.*, 2003;Rosqvist *et al.*, 1995). The TTSS, of which EspA forms a part, has been shown to translocate EspB and other effector proteins into cells and therefore form a bridge between the bacteria and the cell (Knutton *et al.*, 1998). In

addition, the EspA filaments of EPEC and EHEC appear to be polymorphic with E2348/69 having a similar EspA filament to O55:H6 and 85-170 having an antigenically related EspA filament to the O157:H7 related EPEC O55:H7. The other surface appendage that has been extensively studied in EPEC is BFP, which is a type IV pilus whose major structural unit is bundlin encoded by *bfpA* (Donnenberg *et al.*, 1992). Other intestinal pathogens express type IV pili such as the toxin co-regulated pilus (Tcp) of *V. cholerae*. Although bundlin shares low sequence homology with other type IV pilin proteins, *V. cholerae* and EPEC regulate the expression of their pili by transcriptional regulators that are part of the same AraC family (Winther-Larsen and Koomey, 2002). BFP was shown to bind to phosphatidylethanolamine (PE) and this phospholipid was present in both bacterial and eukaryotic membranes but adherence of E2348/69 was only partially inhibited by anti-PE antibodies (Khursigara *et al.*, 2001;Barnett *et al.*, 1999). In addition, BFP has been implicated in determining the cell type that EPEC bind to by studies using mouse and human derived cell lines. However, the role of BFP in aggregation correlates more readily with the data provided by IVOC studies (Knutton *et al.*, 1999;Hicks *et al.*, 1998). In addition to BFP flagella have been implicated in the adhesion of EPEC to eukaryotic host cells. The expression of flagella appears to be stimulated by host cell signals and the host cell can stimulate the expression of flagella by non motile EPEC strains. Antibodies to flagella were shown to inhibit binding of EPEC to the host cell but no adhesin has yet been described for flagella and the role of flagella in EHEC infections has yet to be determined (Giron *et al.*, 2002). BFP negative (Hicks *et al.*, 1998) and *fliC* negative mutants (Philips, 2003) of EPEC E2348/69 still cause A/E lesions on IVOC indicating that BFP and flagella are not necessary for intestinal colonisation and that there may be some redundancy in host cell adhesins in EPEC.

The investigation of the expression of fimbriae by EPEC and EHEC has benefited from the study of fimbriae in other pathogenic bacteria. Uropathogenic *E. coli* (UPEC) have been shown to produce type 1 fimbriae that are important in the adhesion to the urinary tract and may act as initial adhesions (Connell *et al.*, 1996). The FimH subunit of the type 1 fimbria acts as an adhesin and may also be involved in invasion of human bladder epithelial cells. The size of type 1 fimbriae has been estimate in isolates from urinary tract infections and

shown to be approximately 1-2μm long and 7 nm wide along their length with a tip structure of approximately 3 nm (Martinez *et al.*, 2000). A study by Giron et al (Giron, Ho, and Schoolnik, 1993) showed that apart from BFP EPEC expressed three other potential adhesions, showing homology with F9 and F7(2) fimbriae of UPEC and F1845 of diffuse adhering *E. coli*, respectively. A possible role for type 1 fimbriae in EPEC pathogenesis was suggested by an immune response to type 1 fimbriae in human volunteers challenged with E2348/69 (Karch *et al.*, 1987). However, type 1 fimbriae were later shown to not be involved in the adhesion to HEp-2 and Caco-2 cells (Elliott and Kaper, 1997). The expression of type 1 fimbriae by EHEC strain has been examined in several studies with the majority of EHEC strains being negative for type 1 fimbrial expression (Enami *et al.*, 1999). This is due in certain O157:H7 strain to the presence of a 16 base pair deletion within the regulatory region of *fimA*, which is part of the *fim* switch involved in the control of type 1 fimbrial expression (Roe *et al.*, 2001). However, O26 strains do not possess the 16bp deletion and therefore have the potential to express type 1 fimbriae. This suggested that this 16bp deletion could be a marker for O157:H7 strains (Li, Koch, and Cebula, 1997). However, an EHEC O157:H7 strain CL-49 has been shown to produce type 1 fimbriae after serial passage in static non aerated broth suggesting that growth conditions and strain selection may be important in EHEC fimbrial expression (Durno, Soni, and Sherman, 1989). The adhesion of Cl-49 to rabbit ileal brush borders and to a human colonocyte cell line *in vitro* was mannose sensitive. This suggested a role for type 1 fimbriae expressed by this O157:H7 isolate during the adhesion process. Both pathogenic bacteria and commensal bacteria express type 1 fimbriae. The *E. coli* strain HB101 is frequently used as a host for plasmids expressing bacterial fimbriae. However, it has been shown that under the appropriate growth conditions, passage in standing broth culture, this strain produced mannose sensitive type 1 fimbriae (Elliott, Nandapalan, and Chang, 1991). Thus an assessment of the fimbrial expression of EPEC and EHEC under different growth conditions may highlight the presence of fimbriae, which may after further study be implicated in adhesion.

Recently two other fimbriae have been associated with EHEC strains. A sorbitol fermenting EHEC O157:H- strain has been shown to express a novel fimbriae which is

encoded by its pO157 plasmid (Brunder *et al.*, 2001). The fimbriae were expressed after cloning of the appropriate genes into HB101. The expressed recombinant fimbriae were up to 0.4μm in length and had a diameter of 3 to 5 nm. A fimbrial operon has been identified in the chromosome of EHEC O157:H7. This operon contained six putative open reading frames that were found to be closely related to the long polar fimbriae (LPF) of *Salmonella enterica* serovar *typhimurium* (Torres *et al.*, 2002). Exact measurements for the fimbriae observed were not given but they were noted as being similar to those expressed by recombinant *Salmonella* strains, 2 – 10 μm long. The presence of LPF in EHEC is an important consideration as they have been shown to mediate the adhesion of *S. typhimurium* to murine Peyer's patches (Baumler, Tsolis, and Heffron, 1996). Clinical isolates of EHEC may express curli which are surface expressed fibrous proteins made up of a 15 kDa subunit protein, CsgA (Olsen *et al.*, 1993;Arnqvist, Olsen, and Normark, 1994). Curli have broad binding capacity and have been shown to bind human plasma proteins (Sjobring, Pohl, and Olsen, 1994). Curli are regulated by temperature (growth at 26°C and not 37°C) and stress conditions such as low osmolarity and starvation (Arnqvist *et al.*, 1992). Curli have yet to be implicated in the adhesion of EHEC to the intestinal mucosae but they have they appear to be involved during inflammation and may play a role in the pathogenesis of EHEC (Olsen *et al.*, 2002).

The Efa1 of EHEC an O111:H- strain has been shown to be necessary for the adherence of this strain to CHO cells and its gene sequence is virtually identical to the gene encoding the LifA of EPEC (Badea *et al.*, 2003;Nicholls, Grant, and Robins-Browne, 2000). In addition, a homologue of Efa1 exists on the pO157 of EHEC O157:H7 strains and is termed *toxB* and is involved in adhesion of EHEC to Caco-2 cells (Tatsuno *et al.*, 2001). Thus EPEC and EHEC express additional adhesins to intimin and these adhesins may be shared between the two pathogenic types or be unique to one category.

Therefore in this section EHEC and EPEC strains were grown under a variety of growth conditions and viewed using TEM in order to determine the presence of fimbriae or other surface appendages. In addition to TEM, EHEC strains defective in LPF production were

used to assess the role of the putative *lpf* operon in adhesion during IVOC. Attempts were made at characterising the fimbriae observed.

5.2 Results.

5.2.1 The analysis of EPEC and EHEC by TEM after overnight growth in different media.

The assessment of fimbrial and flagella expression was carried out as described in section 2.10.2. The expression of surface structures was correlated with previous morphological studies of fimbrial expression. However in brief, type 1 fimbriae, whose haemagglutination is prevented by coincubation with D-mannose, are 7 to 8 nm wide and are approximately 0.5 to 2 μm long (Old, 1972). They can appear like hollow rod like structures due to the arrangement of their subunits around a hollow core. Type 2 fimbriae are similar in morphology to type 1 fimbriae but do not haemagglutinate erythrocytes. Type three fimbriae are 4 to 5nm in diameter and only haemagglutinate erythrocytes after they have been treated with tannic acid. Type 4 fimbriae can measure between 10 – 20 μm. Type 1, 2 and 3 fimbriae are peritrichous whereas type 4 (IV) fimbriae are polar (Giron *et al.*, 1997).

Long polar fimbriae (LPF) expressed in *S. typhimurium* are 7-8nm wide, are polar and are between 10 and 20 μm long (Baumler and Heffron, 1995). When the genes of an *lpf* – like operon, found in EHEC, are expressed in a non-fimbriated *e. coli* strains, peritrichous fimbriae similar to type 1 fimbriae are observed. They are 7-8 nm wide and 2 – 10μm long (Torres *et al.*, 2002).

The EPEC and EHEC strains listed in table 3.1 were grown overnight at 37°C to assess whether they produced fimbriae or flagella after overnight growth. The strains examined and the growth conditions they were subjected to prior to examination by negative staining TEM are listed in table 5.1.

Table 5.1 The expression of surface structures by EPEC and EHEC after overnight growth.

	Growth conditions					
Strain	BHI broth bijoux	BHI broth uni	LB broth	LB plate	LB slope	DMEM
E2348/69	Fim, FL	Fim, FL	Fim, FL	Fim, FL	Fim, FL	Fim, FL
85-170	Fim, FL	Fim, FL	Fim, FL	Fim	Fim, FL	Fim
AGT300	Fim, FL	Fim, FL	FL	FL	FL	FL
TT12B	Fim, FL	Fim, FL	Fim, FL	FL	FL	Fim
H11	-ve	-ve	-ve	-ve	-ve	-ve
3801	Fim, FL	Fim	FL	-ve	Fim	Fim
E77804	Fim	Fim	Fim	Fim	Fim	Fim
PMK5	Fim, FL	Fim, FL	Fim, FL	Fim, FL	Fim, FL	Fim

Note: Fim = surface structure which appeared to be fimbriae like

FL = Flagella

-ve = no surface structures seen

BHI = brain heart infusion (Oxoid)

LB = L-broth (Sigma)

DMEM = Dulbecco's modified Eagle's medium (Sigma)

Uni = plastic universal container

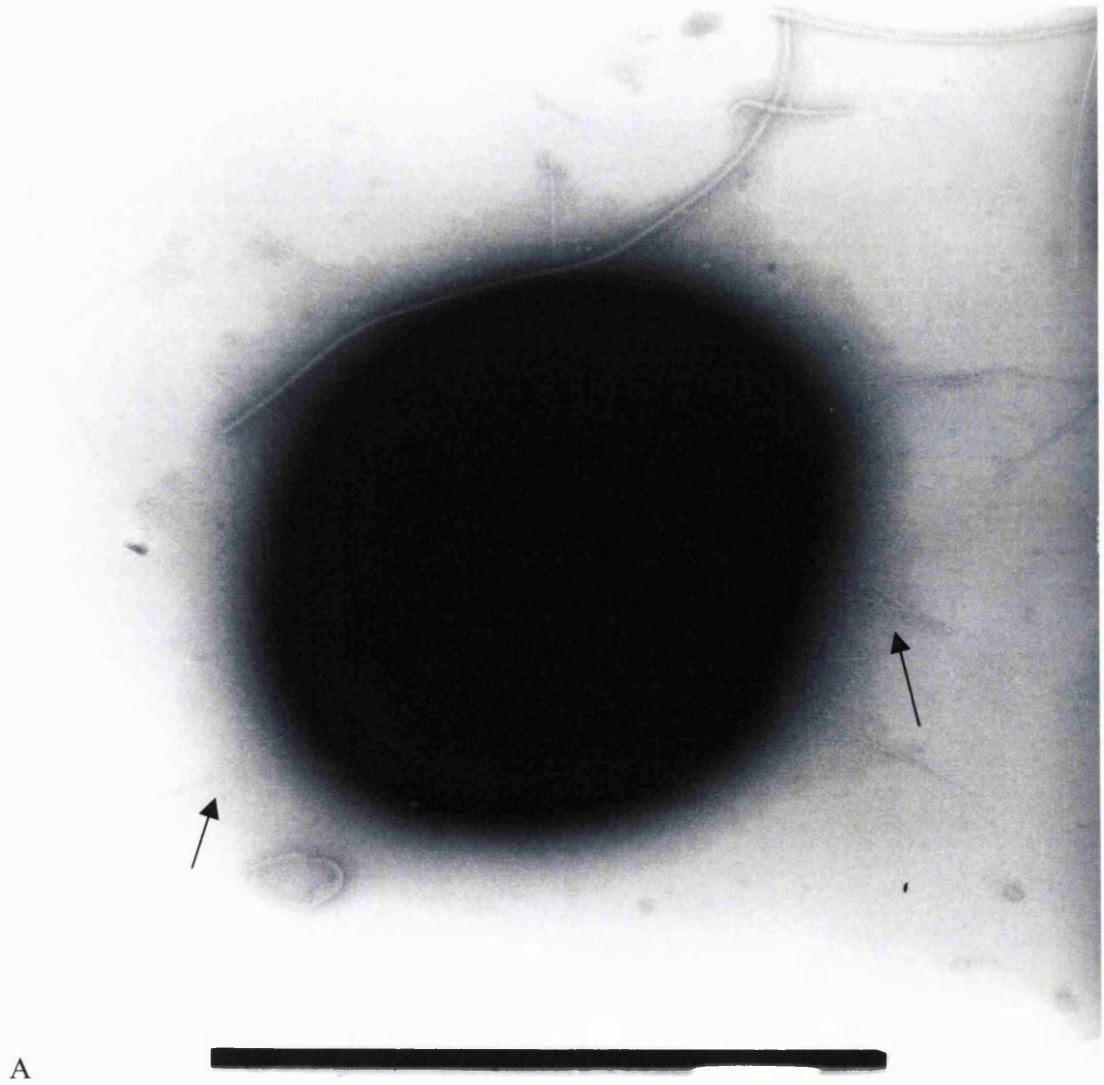
As described in section 2.10.2 each strain was examined twice and on two separate occasions. The number of bacteria observed on each formvar grid varied. In addition, the structures seen were not observed on every bacterium present. Thus the observations are statements about what each strain may potentially express under a given growth condition. Approximations of length and width of the structures seen were assessed as described in section 2.10.2 and further characterisation was carried out, described in subsequent sections, to determine if the surface structures were similar to any known fimbriae.

The growth conditions used in this section are described in section 2.10.1 but briefly the BHI broth cultures were made up in glass bijoux which contained 5ml of broth and up to

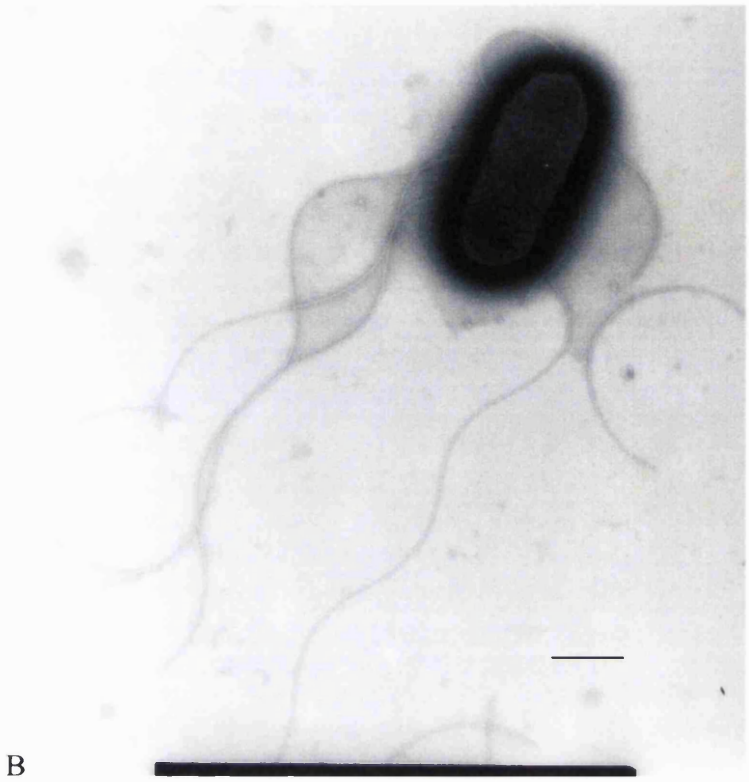
1ml of air and plastic universals which contained 5ml of broth and up to 20ml of air. The DMEM cultures were grown in universals.

The EPEC strain E2348/69 produced structures resembling fimbriae and flagella after overnight growth in all the media. The fimbriae like structures were up to 550nm in length and approximately 7nm in width. The LB plate, LB slope and DMEM cultures were observed to produce the least expression of surface structures.

Figure 5.1 Fimbrial – like structures and flagella expressed by E2348/69.



A

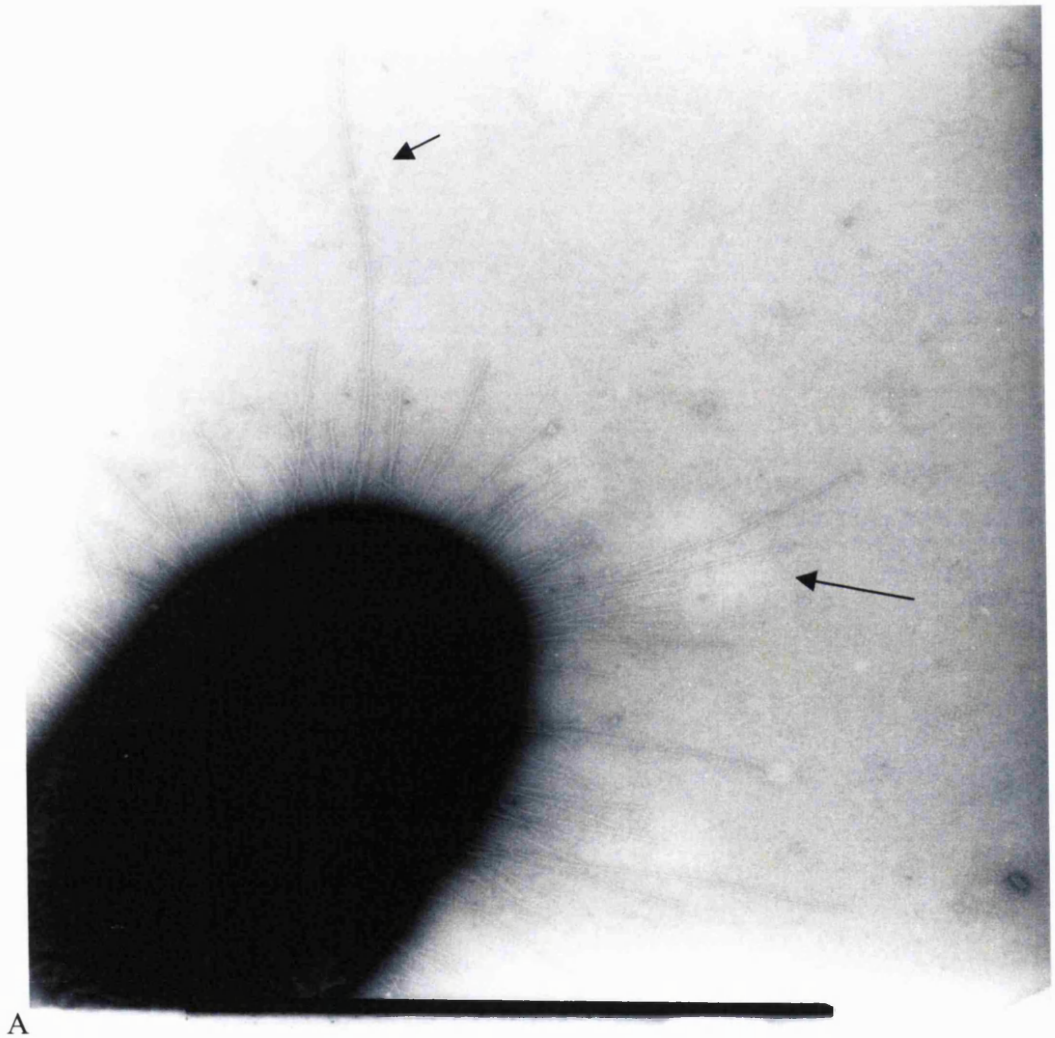


B

Figure 5.1A is a micrograph of the fimbrial – like structures produced by E2348/69 after overnight growth in BHI, bar = 200nm. Figure 5.1B is a micrograph of the flagella produced by E2348/69 after overnight growth in BHI, bar = 500nm.

The EHEC 85-170 produced both fimbrial - like structures and flagella after overnight growth in BHI and LB broth as well as LB slopes but only produced fimbriae on LB plates and in DMEM culture. The fimbriae were up to 900nm in length and approximately 7nm in width.

Figure 5.2 Fimbrial – like structures and flagella expressed by 85-170.



A

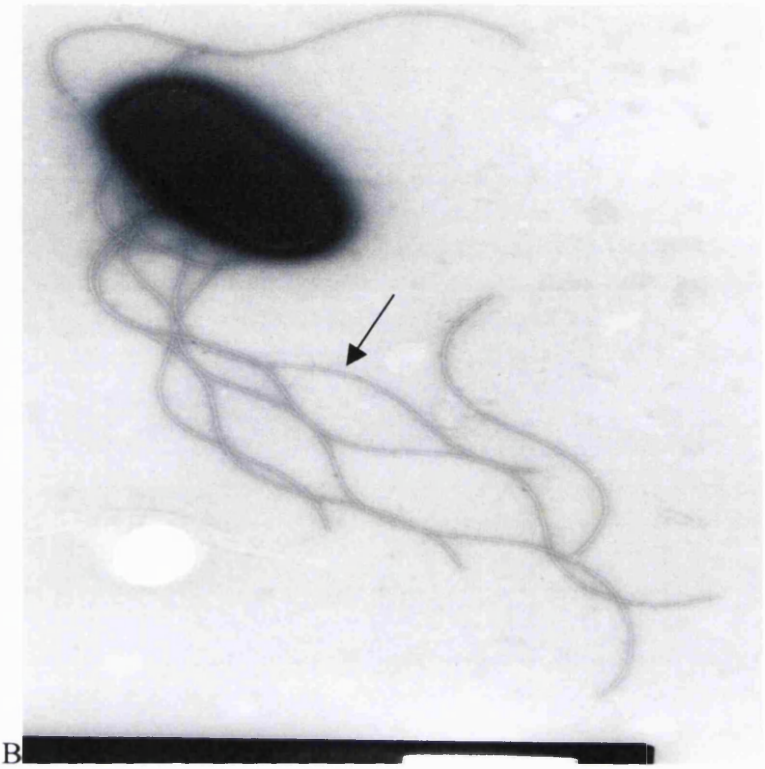


Figure 5.2A is a micrograph of 85-170 producing fimbrial – like structures (black arrow) after overnight growth in BHI, bar = 200nm. Figure 5.2B is a micrograph of 85-170 producing flagella (black arrow) after overnight growth in BHI, bar = 1μm.

The O157:H7 strain AGT300 produced fimbrial - like structures and flagella after overnight growth in BHI broth but only flagella were seen after growth in the other media. The fimbriae were up to 300nm in length and approximately 7nm in width.

Figure 5.3 Fimbrial – like structures expressed by AGT300.

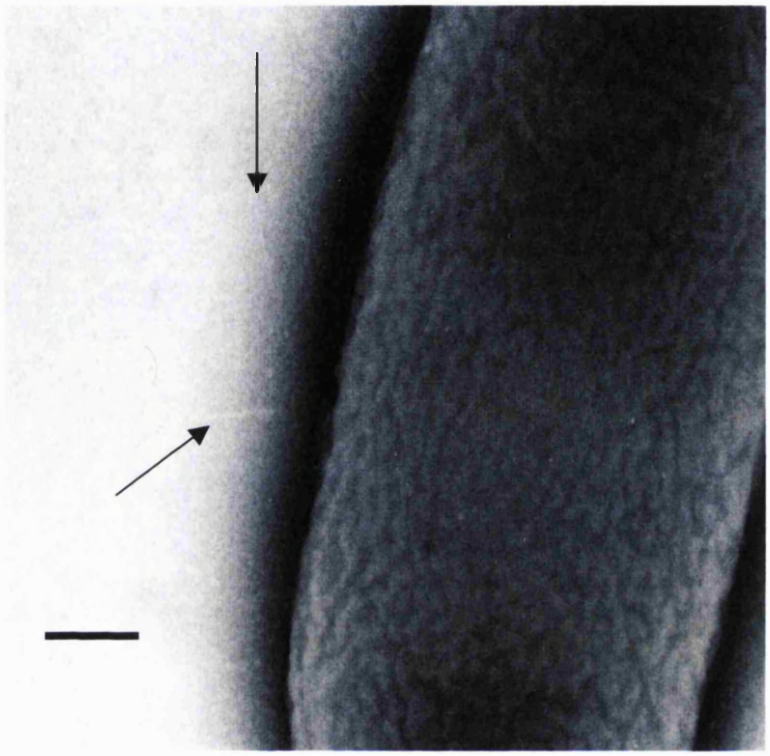


Figure 5.3 is a micrograph of AGT300 producing fimbrial – like structures (black arrow) after overnight growth in BHI, bar = 200nm.

The O157:H7 strain TT12B produced fimbrial - like structures after overnight growth in each of the liquid cultures (BHI, LB, DMEM) and produced flagella on LB plates, LB slopes, BHI broth and LB broth. The fimbriae were up to 1.7 μm in length and 8nm in width.

Figure 5.4 Fimbrial – like structures expressed by TT12B.

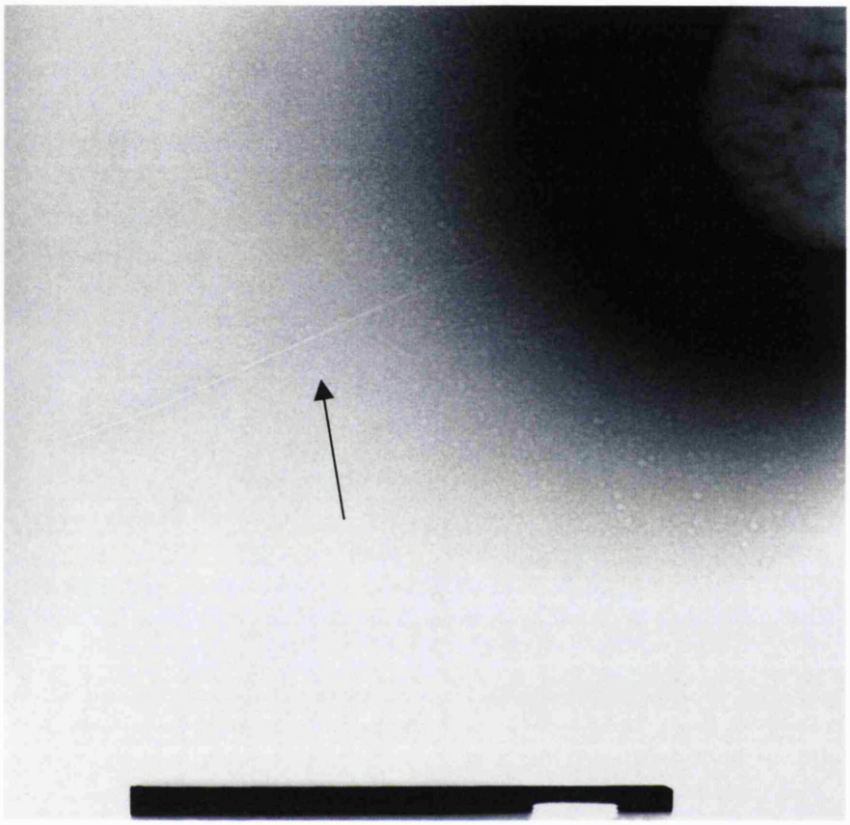


Figure 5.4 is a micrograph of TT12B producing fimbrial – like structures (black arrow) after overnight growth in BHI, bar = 200nm.

No surface structures were seen after overnight growth of O157:H7 strain H11.

The O26:H11 strain 3801 produced surface structures resembling fimbriae after overnight growth in BHI broth, LB slope and DMEM. Growth in LB broth resulted in the production of flagella but not fimbrial - like structures and no structures were observed after growth on LB plates. The analysis of the strains by TEM after overnight growth in universals containing BHI broth showed similar results to those for growth in BHI bijoux. However, one difference was observed in that the O26:H11 strain 3801 expressed flagella and fimbriae after growth in a universal whereas it only expresses fimbrial - like structures after growth in BHI broth in bijoux. The fimbriae were up to 950 nm long and approximately 8nm in width.

Figure 5.5 Fimbrial – like structures and flagella expressed by 3801.

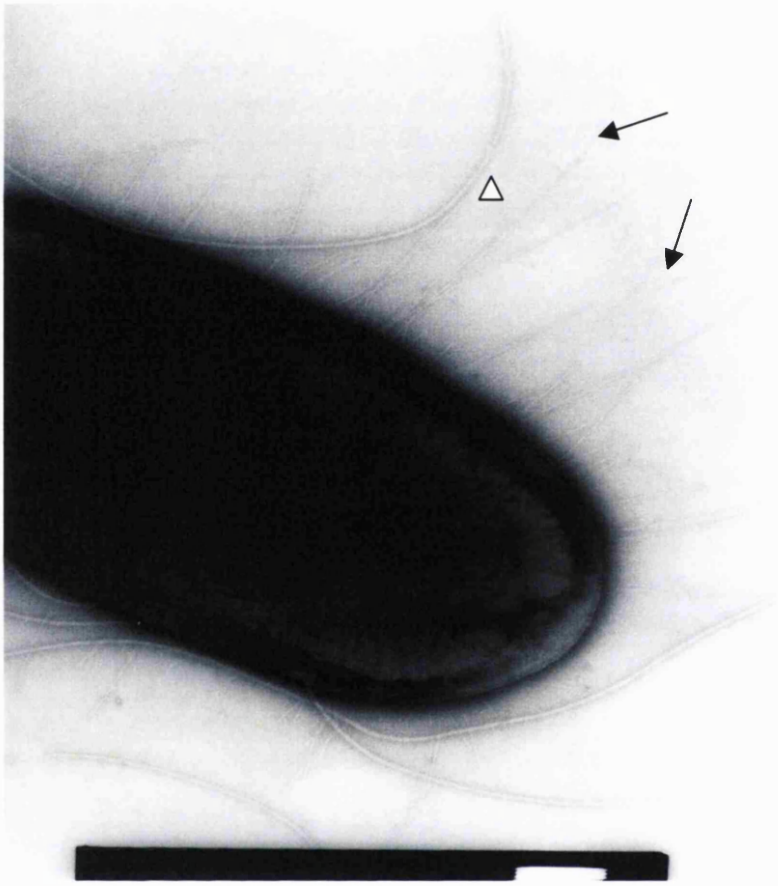


Figure 5.5 is a micrograph of the fimbrial – like structures (black arrow) and flagella (white triangle) produced by 3801 after overnight growth in BHI, bar = 200nm.

The O103:H- strain E77804 produced fimbrial - like structures after growth in all media and no flagella were observed. The fimbrial were up to 1 μm in length and approximately 8nm wide.

Figure 5.6 Fimbrial – like structures expressed by E77804.

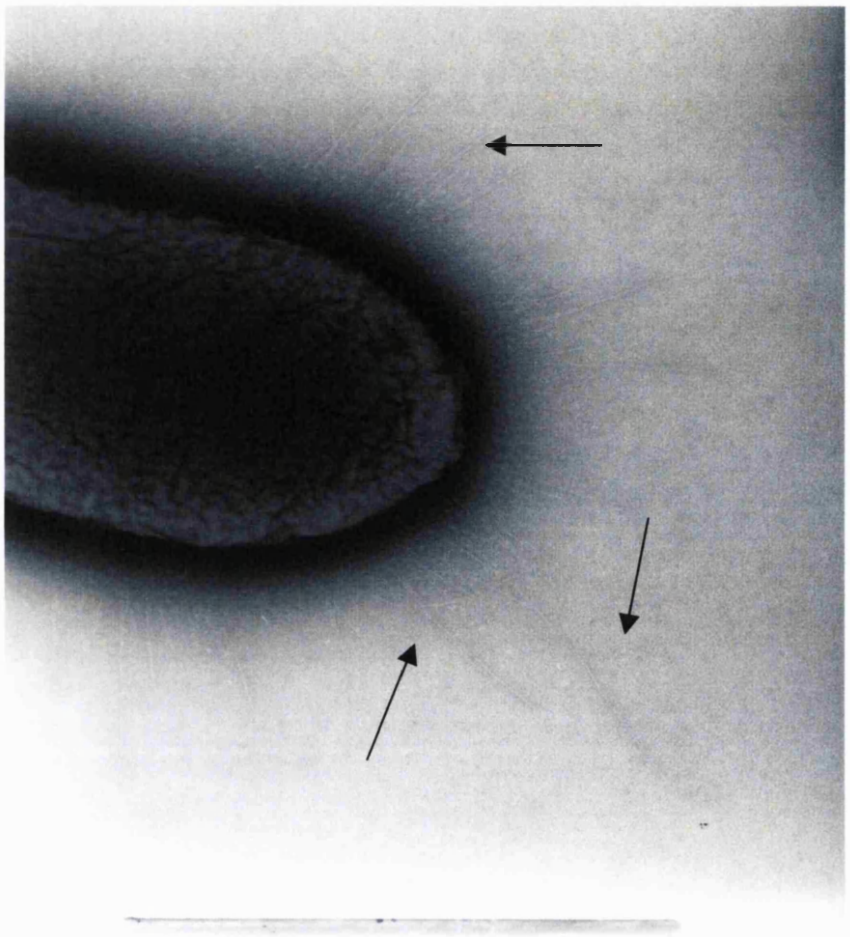


Figure 5.6 is a micrograph of the fimbrial – like structures (black arrow) produced by E77804 after overnight growth in BHI, bar = 200nm.

The O103:H2 strain PMK5 produced fimbriae like structures and flagella in all growth media except DMEM were only fimbriae like structures were observed. The fimbriae were up to 900nm in length and approximately 7nm wide.

Figure 5.7 Fimbrial – like structures and flagella expressed by PMK5.

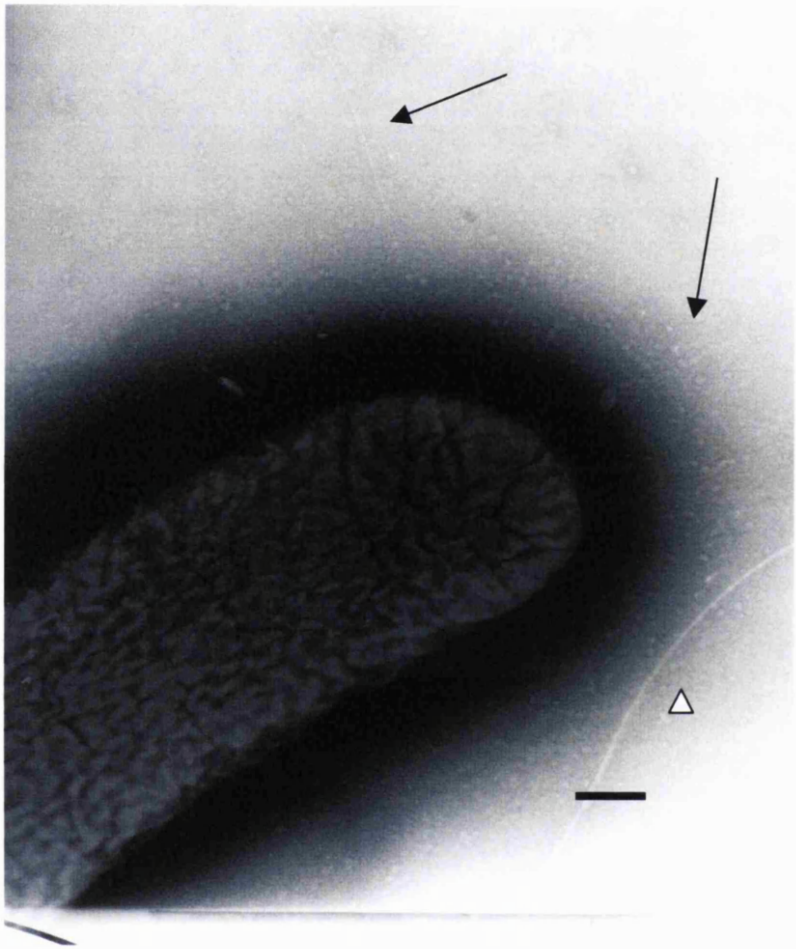


Figure 5.7 is a micrograph of the fimbrial – like structures (black arrow) and flagella (white triangle) produced by PMK5 after overnight growth in BHI, bar = 200nm.

The flagella structures observed varied significantly in length and were up to 15μm in length and approximately 10-12 nm wide.

The fimbrial – like structures observed resembled type 1 or 2 fimbriae in size and morphology as they were between 0.5 and 2 μm in length and were approximately 7nm wide. AGT300 produced fimbrial – like structures which were shorter than the fimbrial – like structures produced by the other EPEC and EHEC strains but they were similar in width to the other fimbrial – like structures. This difference in length may have been due

to sample preparation resulting in shearing of the fimbriae. The flagella observed were distinct from the fimbrial – like structures and were wider and longer than the fimbriae. The fimbriae and flagella were peritrichous.

After overnight growth in DMEM pH changes were observed, with all the strains decreasing the pH of the medium, indicated by the orange colour of the medium. Overnight growth in BHI broth appeared to be the most appropriate medium for the production of surface structures. All of the strains, except H11, produced fimbrial - like structures after overnight growth and this included thee O157:H7 strains and three non O157:H7 strains.

5.2.2 The use of yeast and guinea pig red blood cells to determine mannose sensitive agglutination using bacterial strains grown overnight at 37°C.

The yeast and guinea pig red blood cell assay is described in section 2.11.

The assays were used to test for the presence of type 1 fimbriae after overnight growth of the bacterial strains analysed by TEM and listed in table 5.1. The strains were either grown in bijoux or universals in order to see if the difference in the amount of air present would influence the assays. BHI broth was used as it was the most consistent media in terms of expression of fimbrial – like structures. The use of rich broths to produce fimbriae has been documented (Biebricher & Duker 1984;Schwan *et al* 1992).

Table 5.2 Effect of overnight growth in BHI broth in bijoux or universals on the yeast and red blood cell assay for mannose sensitive adhesion.

	Growth conditions					
	BHI broth bijoux			BHI broth universal		
Strain	Yeast agglutination	GPRBC agglutination	Mannose sensitive	Yeast agglutination	GPRBC agglutination	Mannose sensitive
E2348/69	+ve	+ve	+ve	+ve	+ve	+ve
85-170	-ve	-ve	ND	+ve	+ve	+ve
AGT300	-ve	-ve	ND	-ve	-ve	ND
TT12B	-ve	-ve	ND	-ve	-ve	ND
H11	-ve	-ve	ND	-ve	-ve	ND
3801	-ve	-ve	ND	-ve	-ve	ND
E77804	+ve	+ve	+ve	+ve	+ve	+ve
PMK5	+ve	+ve	+ve	+ve	+ve	+ve

Note: +ve = agglutination of either yeast or guinea pig red blood cells and/or mannose sensitive agglutination.

-ve = no agglutination of yeast or guinea pig red blood cells and/or not mannose sensitive.

ND = not done

GPRBC = guinea pig red blood cells.

After one to two minutes of gently rocking the slides, as described in section 2.11, the suspension of yeast/GPRBC and bacteria either maintained its appearance or agglutination of the suspension was observed. In the case of a positive result the assay was repeated with the inclusion of 1%D-mannose. Inhibition of the agglutination of the yeast/GPRBC was noted as a mannose sensitive result. All the strains which were positive for Yeast/GPRBC agglutination were also mannose sensitive. Thus the presence of mannose binding surface adhesins was detected after overnight growth in BHI and these may be type 1 fimbriae.

After overnight growth in bijoux E2348/69 and the intimin ϵ expressing strains E77804 and PMK5 produced mannose sensitive agglutination in both assays. None of the EHEC O157:H7 strains or the O26:H11 strain 3801 showed this characteristic after growth in BHI bijoux. However, after growth in universals the O157:H7 strain 85-170 showed mannose sensitive agglutination of the Yeast/GPRBC. Thus it may also have expressed mannose sensitive type 1 fimbriae. Those strains expressing fimbriae with type 1 fimbriae morphology, AGT300, TT12B and 3801, but do not agglutinate yeast or GPRBC may express type 2 fimbriae. These results suggest that the amount of air present in the culturing vial during bacterial growth had an effect on the expression of fimbriae in strain 85-170.

5.2.3 The passage of bacterial cultures in BHI broth.

In order to attempt to induce fimbriae in the EPEC and EHEC strains listed in table 5.1 the strains were passaged as described in section 2.10.2.1 for10, 12 and 14 days in BHI broth in bijoux or universals. In addition, bacterial strains were grown as static cultures for 10, 12 and days without passage (NP).

Table 5.3 The effect of passage and NP of the bacterial strains on the yeast and GPRBC agglutination assays.

	Growth conditions			
	BHI bijoux passage	BHI bijoux NP	BHI uni passage	BHI uni NP
	Days 10, 12, 14	Days 10, 12, 14	Days 10, 12, 14	Days 10, 12, 14
Strain	Yeast/GPRBC	Yeast/GPRBC	Yeast/GPRBC	Yeast/GPRBC
E2348/69	+ve/+ve	+ve/+ve	+ve/+ve	+ve/+ve
85-170	-ve/-ve	-ve/-ve	+ve/+ve	+ve/+ve
AGT300	-ve/-ve	-ve/-ve	-ve/-ve	-ve/-ve
TT12B	-ve/-ve	-ve/-ve	-ve/-ve	-ve/-ve
H11	-ve/-ve	-ve/-ve	-ve/-ve	-ve/-ve
E77804	+ve/+ve	+ve/+ve	+ve/+ve	+ve/+ve
PMK5	+ve/+ve	+ve/+ve	+ve/+ve	+ve/+ve

Note: +ve = agglutination of yeast or GPRBC.

-ve = no agglutination of yeast or GPRBC

NP = no passage which indicates no subculturing of the bacteria into fresh medium throughout growth period

uni = universal container

The results in table 5.3 indicate that the passage or no passage (NP) of the EHEC and EPEC strains in BHI broth gave the same agglutination phenotype. The passage or NP of bacteria did not produce the expression of mannose sensitive agglutination positive adhesins in strains which were agglutination negative after overnight growth in BHI.

The number of days that the strains are subcultured did not produce a change in the agglutination phenotype of the strains. Strains E2348/69, E77804 and PMK5 are all positive for agglutination of yeast and GPRBC at all time points and growth conditions. All agglutination was mannose sensitive.

The number of bacteria/ml was assessed for each strain after a 10 day growth period in BHI bijoux and universals and compared with the overnight growth of the bacterial strains.

Table 5.4 The effect of passage and NP on the growth of the bacterial strains.

	Growth conditions					
	BHI bijoux	BHI uni	10 day BHI bijoux		10 day BHI uni	
Strain	Overnight		Passage	NP	Passage	NP
E238/69	6×10^8	1×10^9	7×10^8	1×10^8	2×10^9	5×10^8
85-170	5×10^9	1×10^9	9×10^8	9×10^7	2×10^9	4×10^8
AGT300	8×10^8	5×10^8	9×10^8	2×10^7	5×10^8	7×10^8
TT12B	1×10^9	1×10^9	8×10^8	6×10^7	5×10^8	7×10^8
H11	9×10^8	2×10^8	6×10^8	2×10^7	8×10^8	4×10^8
3801	1×10^9	2×10^9	4×10^8	1×10^8	6×10^8	7×10^8
E77804	5×10^8	1×10^9	8×10^8	1×10^8	7×10^8	9×10^8
PMK5	7×10^8	9×10^8	5×10^9	8×10^7	5×10^9	3×10^8

Note: values are given as CFU/ml

NP = no passage

Uni = universal container

The data outlined in table 5.4 suggests that the growth of the EPEC and EHEC strains in BHI broth overnight, with passage and without passage in bijoux or universal containers did not have a marked effort on the growth of the bacteria. The one growth condition that shows a possible negative effect on growth is growth after 10 days in BHI broth in bijoux without passage in which the bacteria were subjected to subculture into fresh medium. This growth condition shows a decrease in the amount of bacteria present, for each strain, when compared to the other growth conditions.

5.2.4 The effect of passage and NP on intimin expression.

In section 3., the western blot of the EPEC and EHEC strains shows that after 10 days of passage in BHI broth in bijoux, all the strains produce intimin after activation in DMEM and E2348/69, E77804, PMK5 and TT12 B produce intimin prior to activation in DMEM. After 12 days the same result was observed but after 14 days only strain E2348/69 was positive for intimin expression. In addition, figure 3.15 shows that after overnight growth in BHI prior to IVOC inoculation, only E2348/69 expresses intimin, as detected by western blot.

The EPEC and EHEC strains were probed for intimin expression by western blot after passage and no passage in BHI broth in bijoux and universal containers, as described in 2.5.3.

Table 5.5 The expression of intimin by the bacterial strains after passage and NP in bijoux and universals as assessed by Western blot.

	Growth conditions											
	10 days BHI bijoux				10 days BHI uni				12 days BHI uni		14 days BHI uni	
	NP		Passage		NP		Passage		Passage		Passage	
Strain	NAct	Act	NAct	Act	NAct	Act	NAct	Act	NAct	Act	NAct	Act
EPEC												
E2348/69	+	+	+	+	+	+	+	+	+	+	+	+
E77804	+	+	+	+	+	+	+	+	+	+	+	+
EHEC O157:H7												
85170	-	+	-	+	-	+	-	+	-	+	-	+
AGT300	-	+	-	+	-	+	-	+	-	+	-	+
TT12B	-	+	+	+	-	+	-	+	-	+	-	+
H11	-	-	-	+	-	+	-	+	-	+	-	+
EHEC others												
3801	-	+	-	+	-	+	-	+	-	+	-	+
PMK5	-	+	+	+	-	+	-	+	-	+	-	+

Note: - = no band corresponding to intimin after probing with conserved intimin antiserum.

+ = band present corresponding to intimin after probing with conserved intimin antiserum.

NP = no passage

Act = growth/activation in DMEM after growth in BHI.

NAct = no growth in DMEM, bacterial samples from BHI cultures.

No EHEC strains produce intimin, as detected by western blot (see figure 3.22), after overnight growth in BHI, unlike E2348/69 which does. The passage of bacteria in bijoux or universals does not affect the EPEC or EHEC in their ability to express intimin after growth in DMEM (i.e. activation). As described in section 3.2.5.5, the passage of bacteria for 10 and 12 days does affect the expression of intimin in the EPEC and EHEC strains resulting in the E77804, PMK5 and TT12B being positive for intimin expression without activation in DMEM (included in table 5.5 for comparison under the 10 days BHI bijoux column). They are negative after overnight growth in BHI without passage. In comparison, as shown in table 5.5, passage in universals produces a different result with EPEC strain E77804 and EHEC strains PMK5 and TT12B remaining intimin negative after 10, 12 and 14 days passage unlike after passage in bijoux.

As a result of not subculturing the EPEC and EHEC strains (NP), EHEC strain H11 was not intimin positive after growth in DMEM. The EPEC strains E2348/69 and E77804 were positive for intimin expression after NP in bijoux and universals after 10 days static culture and without activation in DMEM.

5.2.5 The analysis of bacterial strains by negative staining TEM after passage for 10 days.

In Table 5.1 the effect of different growth media on surface structure expression by the EPEC and EHECS strains is listed. The yeast/GPRBC agglutination assay and the analysis of intimin expression shows that passage after 10 days in bijoux or universals alters the intimin expression by EPEC and EHEC strains. In addition overnight growth of 85-170 and passage of 85-170 for 10, 12 and 14 days in BHI broth in universals results in agglutination of yeast and GPRBC, which does not occur when 85-170 is grown in bijoux.

Thus the EPEC and EHEC strains were analysed for surface structure expression after 10 days of passage in bijoux and universals.

Table 5.6 The effect of 10 days of passage in BHI in bijoux and universals on the expression of surface structures by EPEC and EHEC.

	Growth conditions	
Strain	Bijoux	Universal
E2348/69	Fim, FL	Fim, FL
85-170	Fim, FL	Fim, FL
AGT300	Fim, FL	FL
TT12B	FL	FL
H11	-ve	FL
3801	Fim, FL	Fim, FL
E77804	Fim	Fim
PMK5	Fim, FL	Fim, FL

Note: Fim = fimbrial surface structures

FL = flagella

-ve = no surface structures observed

uni = universal container

The morphology of the fimbriae and flagella expressed in this section were similar to that observed in section 5.2.1.

Passaging the bacteria into fresh medium everyday for 10 days changed the expression of surface structures, in some of the EHEC O157:H7 strains, when compared with overnight growth in BHI. Strain AGT300 does not produce fimbrial - like structures after passage for 10 days in universals and the TT12B strain does not produce fimbrial - like structures after 10 days of passage in bijoux and universals. Both strains produce flagella after 10 days of passage in bijoux and universals and the O157:H7 strain H11 produces flagella after 10 days of growth in universals, unlike after growth in all other growth conditions studied in section 5. Passage of the bacterial strains did not change the flagella - positive phenotype of EPEC and EHEC strains that expressed flagella after overnight growth in BHI.

Figure 5.8 The expression of flagella by H11 after passage in BHI broth..

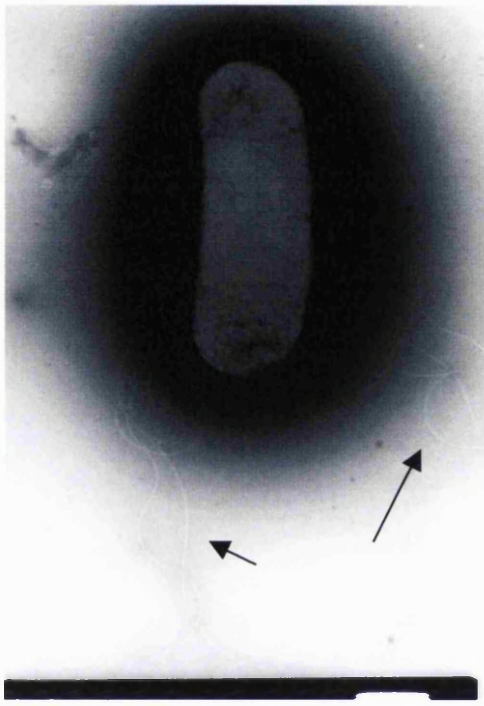


Figure 5.8 is a micrograph of the flagella produced by H11 after passage in BHI, bar = 500nm.

5.2.6 The HEp-2 cell adhesion assay of bacterial strains after passage for 10, 12, and 14 days in BHI broth in universals and after NP for 10 days in bijoux and universals.

As was shown in section 3.2.5.5.1 the passage of bacteria in bijoux for 10, 12 and 14 days altered the HEp-2 cell adhesion phenotype of the EHEC strains AGT300, H11, TT12B and 3801. All the strains showed an increase in their adhesion to HEp-2 cells when compared to the standard HEp-2 assay. In this section, growth in universals changed the agglutination phenotype of EHEC strain 85-170 and passage in universals altered the expression of fimbriae in EHEC strain AGT300. Therefore passage in universals and NP in universals and bijoux was used to test whether the HEp-2 adhesion phenotype could be altered by these growth conditions when compared to the standard HEp-2 assay and the results of the HEp-2 assay after passage in bijoux.

The EPEC and EHEC strains were assessed for their adhesion phenotype when passaged in universals instead of bijoux, and when left in standing culture (NP) for 10 days in bijoux and universals.

Table 5.7 The effect of passage in universals for 10, 12 and 14 days, and NP for 10 days in bijoux and universals on adhesion to HEp-2 cells.

	Growth conditions							
	Standard assay		10-14 days P uni		10 days NP uni		10 days NP bijoux	
Strain	Adh	FAS	Adh	FAS	Adh	FAS	Adh	FAS
Intimin negative strains								
ICC170	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
EPEC								
E2348/69	LA	+ve	LA	+ve	LA	+ve	LA	+ve
E77804	PA	+ve	PA	+ve	PA	+ve	PA	+ve
EHEC O157:H7								
85-170	LAL	+ve	LAL	+ve	LAL	+ve	LAL	+ve
AGT300	PA	+ve	PA	+ve	PA/LAL	+ve	PA/LAL	+ve
TT12B	PA	+ve	PA	+ve	PA	+ve	PA	+ve
H11	PA	+ve	LAL	+ve	PA	+ve	PA	+ve
EHEC others								
3801	PA	+ve	PA/LAL	+ve	PA	+ve	PA	+ve
PMK5	LA/LAL	+ve	PA	+ve	PA	+ve	PA	+ve

Note: 10-14 P uni = 10, 12 and 14 days of passage in BHI universals.
10 days NP uni = 10 days standing culture in BHI universals.
10 days NP bijoux = 10 days standing culture in BHI in bijoux.
Adh = adhesion pattern of bacteria to HEp-2 cells
FAS = fluorescent actin staining test

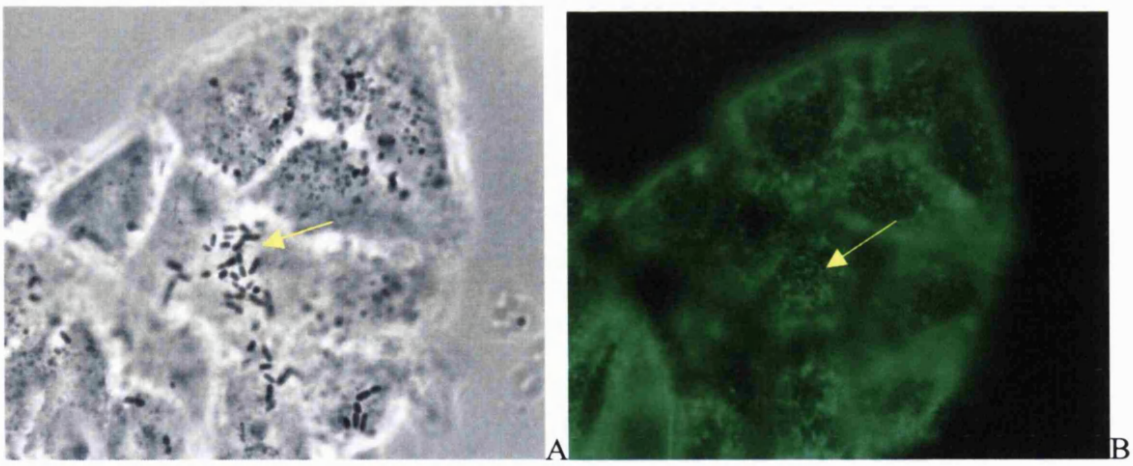
The passage or NP of the bacterial strains in bijoux and universals did not alter the FAS phenotype of the bacterial strains.

The passage for 10, 12 and 14 days of the EHEC strains H11, 3801 and PMK5 in BHI in universals causes a change in their adhesion phenotype. Strains H11 and 3801 have an increase in their adherence as a result of passage in BHI in universals whilst PMK5 shows

a decrease in adhesion when compared to the standard assay and passage for 10, 12 and 14 days in BHI bijoux. It no longer forms microcolonies as a result of passage in BHI universals for 10, 12 and 14 days.

The EHEC O26:H11 strain 3801 has a PA/LAL pattern of adhesion to HEp-2 cells after passage in BHI broth in universals for 10, 12 and 14 days. This is a similar level of adhesion when compared to passage in BHI broth in bijoux and an increase on the standard assay. However growth in standing culture (NP) did not alter its phenotype in relation to the standard assay. Strain 3801 produces microcolonies after six hours of incubation that are lower in number than 85-170. An example of the 3801 microcolonies (yellow arrow) is shown in figure 5.9. Figure 5.9A is the phase contrast image and figure 5.9B is the actin stained image. The magnification is x 80.

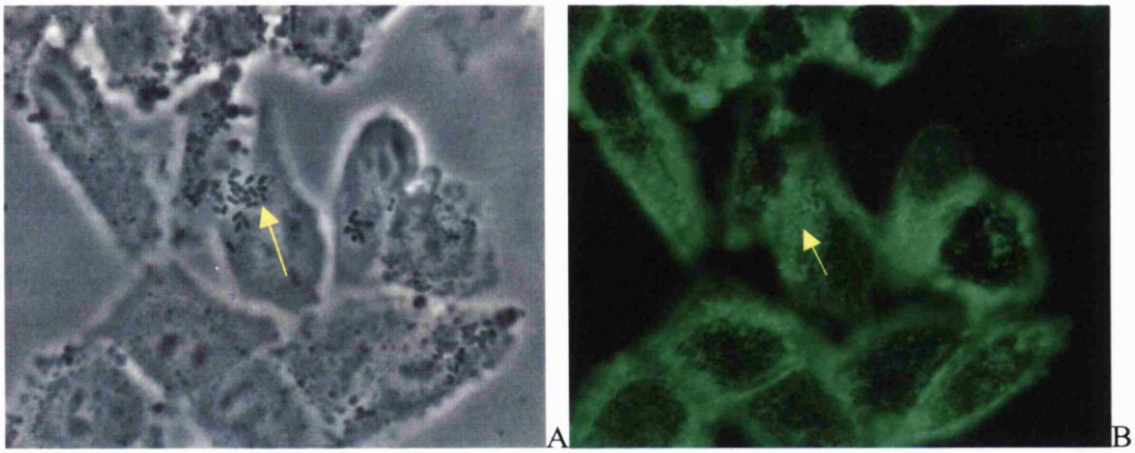
Figure 5.9 HEp-2 cell adhesion of 3801 after passage in BHI in universals for 10 days.



The growth of the bacterial strains in standing culture (NP) for 10 days in BHI universals and bijoux results in a change in the HEp-2 adhesion phenotype of the EHEC strains AGT300 and PMK5 when compared to the standard assay and adhesion after passage of the bacteria.

The PA/LAL adherence pattern of EHEC strain AGT300 can be seen in figure 5.10. AGT300 produced a PA/LAL pattern of adhesion when grown in standing culture and after passage in bijoux but not when passaged in universals. This is an example of a microcolony formed after six hours of incubation with HEp-2 cells after growth of AGT300 in standing culture for 10 days in BHI broth in bijoux and universals. It is not LAL, as it does not produce as many microcolonies as 85-170. Figure 5.10A is the phase contrast image and figure 5.10B is the actin staining image showing actin accumulation at the site of bacterial adhesion.

Figure 5.10 The HEp-2 cell adhesion of AGT300 grown in standing culture (NP) for 10 days in BHI in bijoux and universals.



Passage in universals and NP in bijoux and universals does not change the adhesion phenotype of E2348/69, 85-170, TT12B, E77804, PMK5, and ICC170 when compared to the standard assay.

Thus passage and NP does not alter the adhesion phenotype of all of the bacterial strains and no general correlation with intimin production, without activation in DMEM, or agglutination of yeast or GPRBC can be made.

5.2.7 The use of immuno-EM to type the putative fimbriae of the EPEC and EHEC strains.

All of the EPEC and EHEC strain, except H11, produce fimbrial – like structures after overnight growth in BHI in bijoux and universals. The strains E2348/69, E77804 and PMK5 agglutinate yeast and GPRBC after growth in bijoux and universals whereas this is true of 85-170 only after growth in universals. This agglutination is mannose sensitive in all cases.

Therefore as described in 2.10.3 anti-type 1 fimbriae (T1F) antibody and anti-LPF antibody were used to characterise the fimbriae produced by these EPEC and EHEC strains.

Table 5.8 The binding of anti-fimbrial antibodies to EPEC and EHEC strains.

	Growth conditions							
	Overnight BHI bijoux				Overnight BHI uni			
Strains	α-T1FG	α–T1FT	α–LPF2	α–LPF5	α-T1FG	α–T1FT	α–LPF2	α–LPF5
E2348/69	-ve	-ve	-ve	-ve	-ve	+ve	-ve	-ve
85-170	-ve	-ve	-ve	-ve	-ve	+ve	-ve	-ve
E77804	-ve	+ve	-ve	-ve	+ve	+ve	-ve	-ve
PMK5	-ve	+ve	-ve	-ve	+ve	+ve	-ve	-ve

Note: α-T1FG = anti – type 1 fimbriae antibody raised in rabbits against the type 1 fimbriae of *E. coli* K12 and kindly provided by Giron J.A., CVD, University of Maryland, U.S.A.

α–T1FT = anti - type 1 fimbriae antibody raised in rabbits against the type 1 fimbriae of the UTEC strain LE17 and kindly provided by Toma C., Department of Bacteriology, University of the Ryukyus, Japan.

α–LPF2 = anti – LPF antibody from rabbit number 2, kindly provided by A.G. Torres, CVD, University of Maryland, U.S.A.

α–LPF5 = anti – LPF antibody from rabbit number 5, kindly provided by A.G. Torres, CVD, University of Maryland, U.S.A.

+ve = indicates specific binding of gold particles to fimbrial - like projections

- ve = lack of specific binding

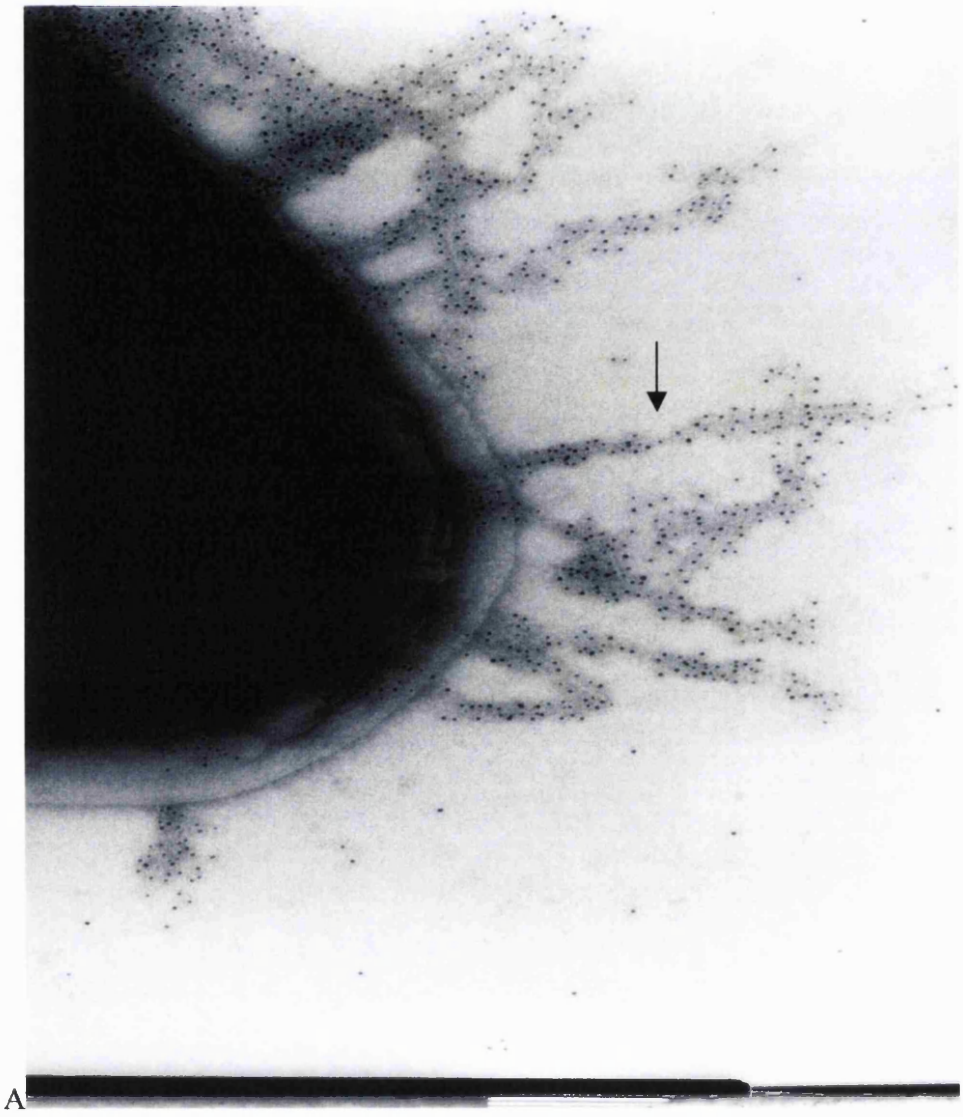
The presence or lack of BSA during the staining procedure did not influence the results.

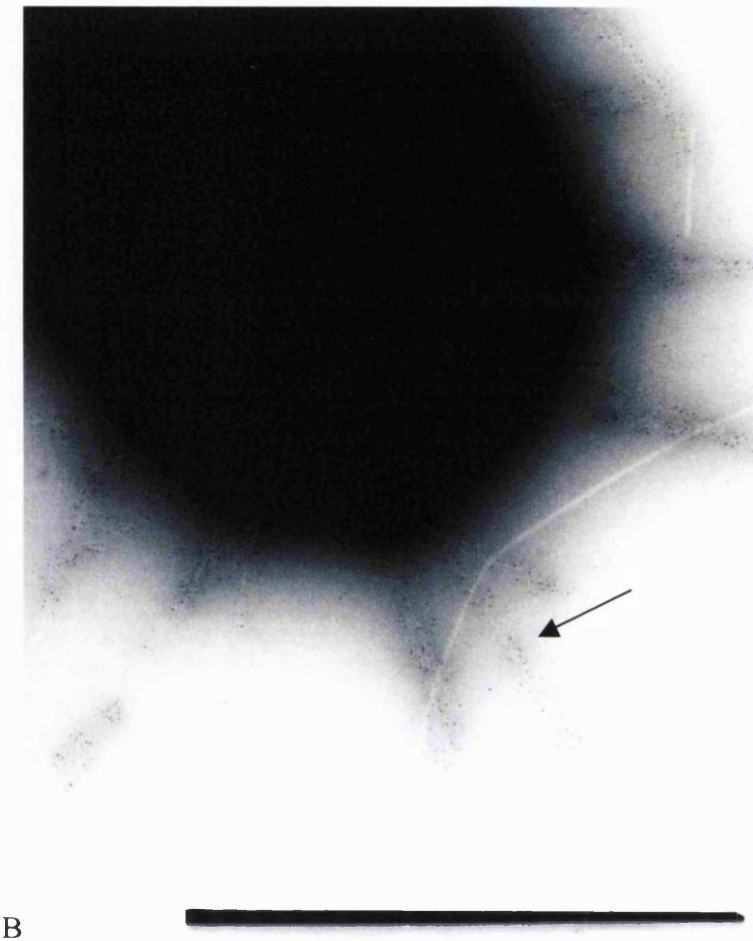
Binding is seen as binding of the 10nm size gold particles to the fimbrial - like structures in a manner that was distinct from any binding of the 10nm gold particles to the formvar grid.

The anti – LPF antibody isolated from two different immunised rabbits did not bind to any of the fimbrial - like structures seen using the TEM.

The EPEC and EHEC intimin ϵ expressing O103:H- and O103:H2 strains, E77804 and PMK5, produce fimbriae that bind both of the anti – T1F antibodies. As can be seen in figure 5.11 A and B the binding of the anti – T1F antibody occurs along the length of the fimbriae structure.

Figure 5.11 Binding of anti – type 1 fimbrial antibody to E77804 and PMK5.





B

Figure 5.11A is a micrograph of the binding of anti – TIFT antibody to the fimbrial – structures (black arrow) produced by E77804 after overnight growth in BHI, bar = 200nm. Figure 5.11B is a micrograph of the binding of anti – TIFT antibody to the fimbrial –like structures (black arrow) produced by PMK5 after overnight growth in BHI, bar = 200nm.

E2348/69 produces fimbrial - like structures that are recognised by the anti – T1FT antibody after growth in overnight in BHI in universals, but not recognised by the anti – T1FG antibody. The fimbriae produced by E2348/69 do not bind to any of the antibodies after overnight growth in BHI in broth. As can be seen in figure 5.12 the fimbrial staining is less marked than with the intimin ϵ strains E77804 and PMK5.

Figure 5.12 Binding of anti – type 1 fimbriae antibody to E2348/69.

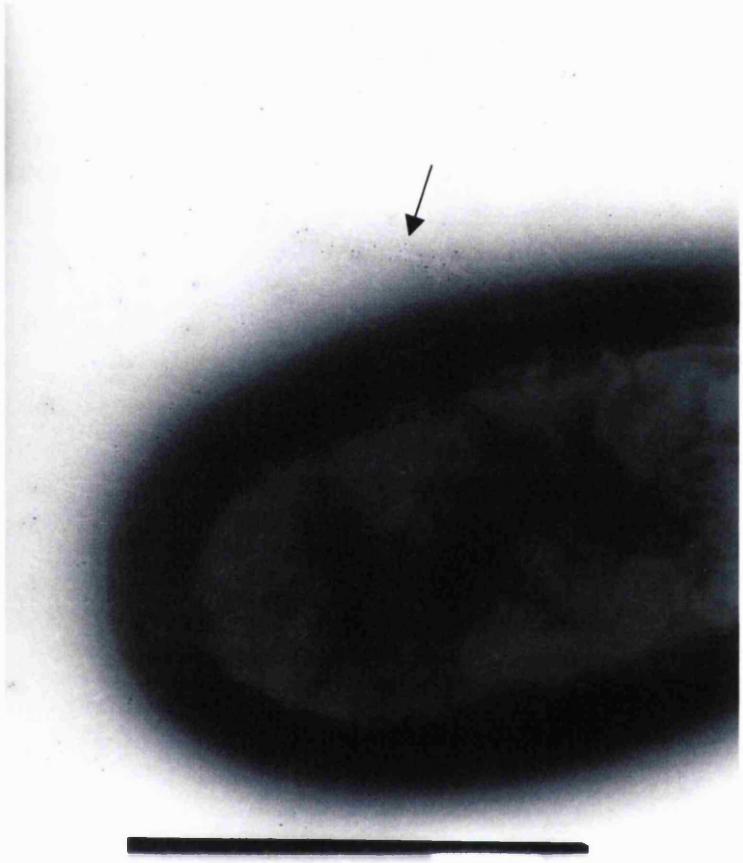


Figure 5.12 is a micrograph of the binding of the anti – TIFT antibody to the fimbrial – like structures (black arrow) produced by E2348/69 after overnight growth in BHI, bar = 200nm.

The EHEC 85-170 strain produces fimbrial - like structures that bind to the anti – T1FT antibody after overnight growth in BHI in universals, but not BHI broth, and does not bind the anti – T1FG antibody or the anti – LPF antibodies. 85-170 binds under the same conditions and to the same antibody that E2348/69 binds to.

Figure 5.13 Binding of anti – type 1 fimbriae antibody to 85-170.

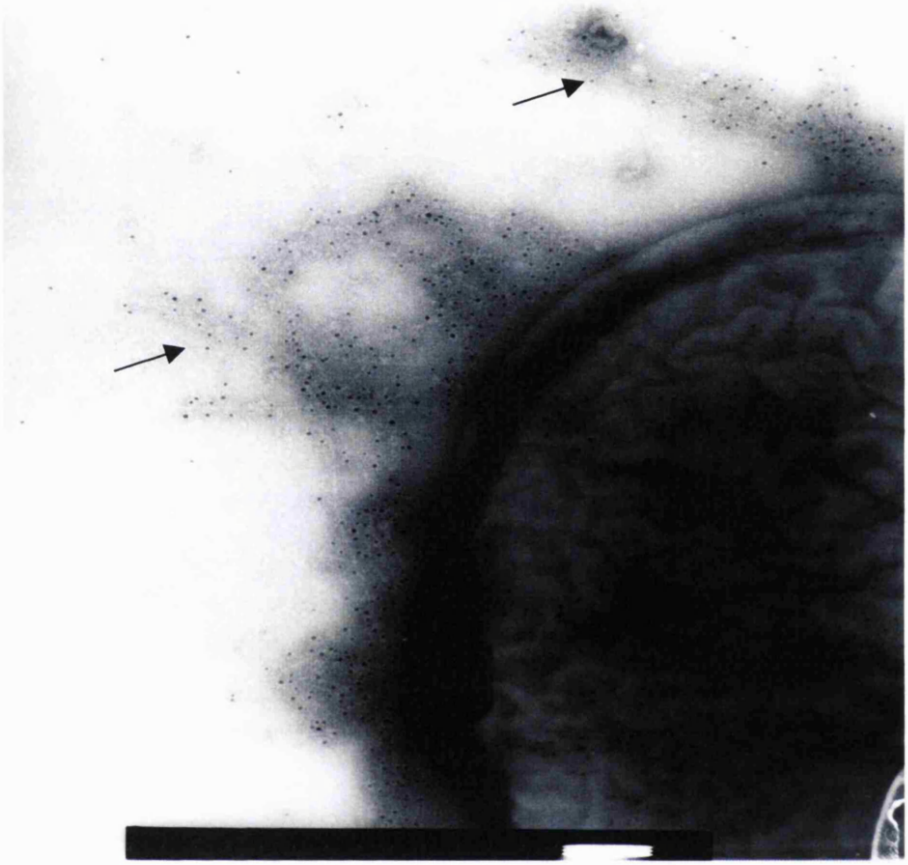


Figure 5.13 is a micrograph of the binding of the anti – TIFT antibody to the fimbrial – like structures (black arrow) produced by 85-170 after overnight growth in BHI broth, bar = 200nm.

The EPEC strains E2348/69 and E77804 and the EHEC strains 85-170 and PMK5 produce fimbriae that are recognised by anti - type 1 fimbriae antibodies.

5.2.8 The use of western blot to detect type 1 fimbrial expression and long polar fimbrial expression in EPEC and EHEC.

As described in section 2.5 the EPEC and EHEC strains used in section 5 were probed for type 1 fimbriae expression and long polar fimbriae expression by western blot using the anti – T1FG and anti – LPF2 antibodies. This was carried out in order to check if a Western blot might detect fimbrial production by the EPEC and EHEC strains with antibodies that had not yielded positive results using immuno-EM.

The detection of long polar fimbriae and type 1 fimbriae by western blot did not work using the antibodies mentioned. The western blots were repeated without BSA blocking and with a longer incubation time for the antibodies but this also gave a negative result.

5.2.9 The expression of surface structures by pathogenic *E. coli* during IVOC.

The EPEC and EHEC strains used in section 5 bind to explants during IVOC (see section 3). TEM was used, as described section 2.10.2.3, to determine whether any fimbrial - like structures or flagella are produced during IVOC. Duodenal and transverse colon explants were used.

Table 5.9 Expression of surface structures by pathogenic *E. coli* during IVOC.

	Intestinal region							
	Duodenum				Transverse colon			
	Time point							
Strain	2Hr	4Hr	6Hr	8Hr	2Hr	4Hr	6Hr	8Hr
E2348/69	-ve	Fim	-ve	-ve	Fim, FL	Fim, FL	Fim	Fim
85-170	-ve	-ve	-ve	-ve	Fim, FL	-ve	Fim	Fim, FL
O42	ND	ND	ND	ND	-ve	-ve	Fim	-ve

Note Time point = refers to the time at which the medium is changed during the eight hour IVOC assay.

-ve = no surface structure observed.

Fim = surface structure which appeared to be fimbrial like.

FL = flagella

ND = not done

Duodenum = fourth part of the duodenum.

The IVOC explants samples were processed for SEM after the eight hour period and E2348/69 produced A/E lesions on duodenum and O42 adhered to the transverse colon with an aggregative pattern. 85-170 and E2348/69 did not adhere to transverse colon explants.

Bacteria with the size and appearance of *E. coli* were analysed for expression of flagella and fimbrial – like structures by TEM.

Taking the four time points together for each region and strain the least number of surface structures are seen when strains adhere to the explants.

The EPEC strain E2348/69 only produced flagella during IVOC with transverse colon during the first four hours of the eight hour IVOC assay. It produces fimbrial - like structures at each transverse colon time point. The fimbrial - like structures are similar to those produced during IVOC with duodenal explants and after growth in BHI. It produces fimbrial - like structures after four hours incubation with duodenum and these fimbriae are up to 600nm long and approximately 7nm wide. This makes the fimbriae approximately the same width as type 1 fimbriae and within the length range exhibited by type 1 fimbriae (0.5 – 2 μm).

Figure 5.14 Fimbrial – like structures produced by E2348/69 during IVOC.

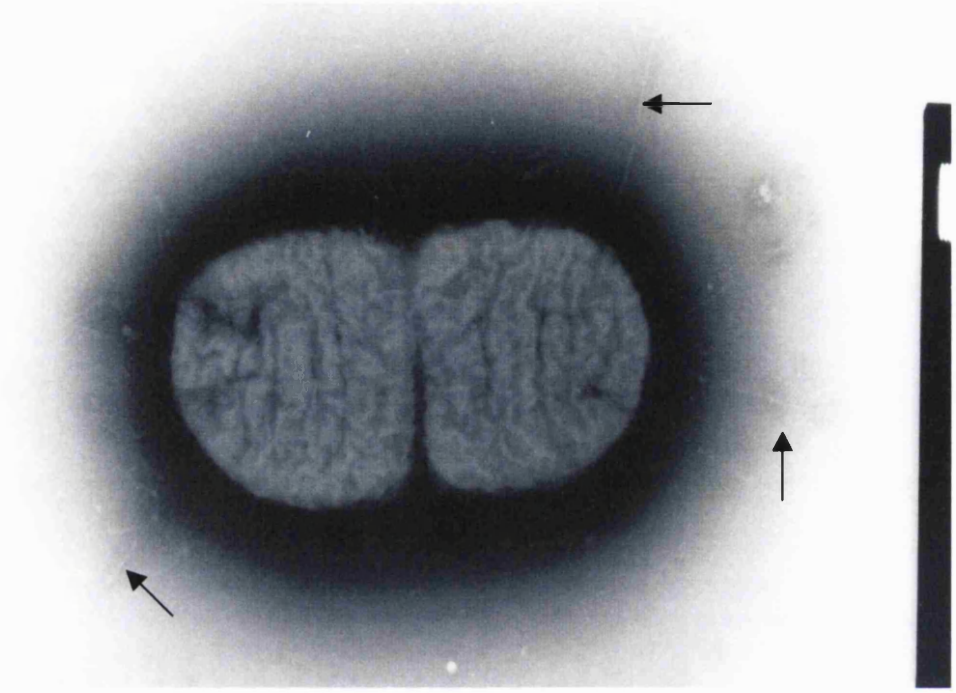


Figure 5.14 is a micrograph of E2348/69 producing fimbrial – like structures (black arrow) during IVOC with transverse colon and duodenal explants, bar = 200 μm.

The EHEC strain 85-170 did not adhere to duodenum or transverse colon explants. It does not produce fimbrial –like structures or flagella during incubation with duodenum 85-170 produces fimbrial – like structures at each time point during incubation with transverse colon, except the four hour time point. It produces flagella at the start and the end of the IVOC assay with transverse colon. The fimbrial - like structures that 85-170 produces during transverse colon IOVC can be seen in figures 5.15. The fimbriae were up to 850nm long and were approximately 7nm wide and were morphologically similar to type 1 fimbriae.

Figure 5.15 Fimbrial – like structures produced by 85-170 during IVOC.

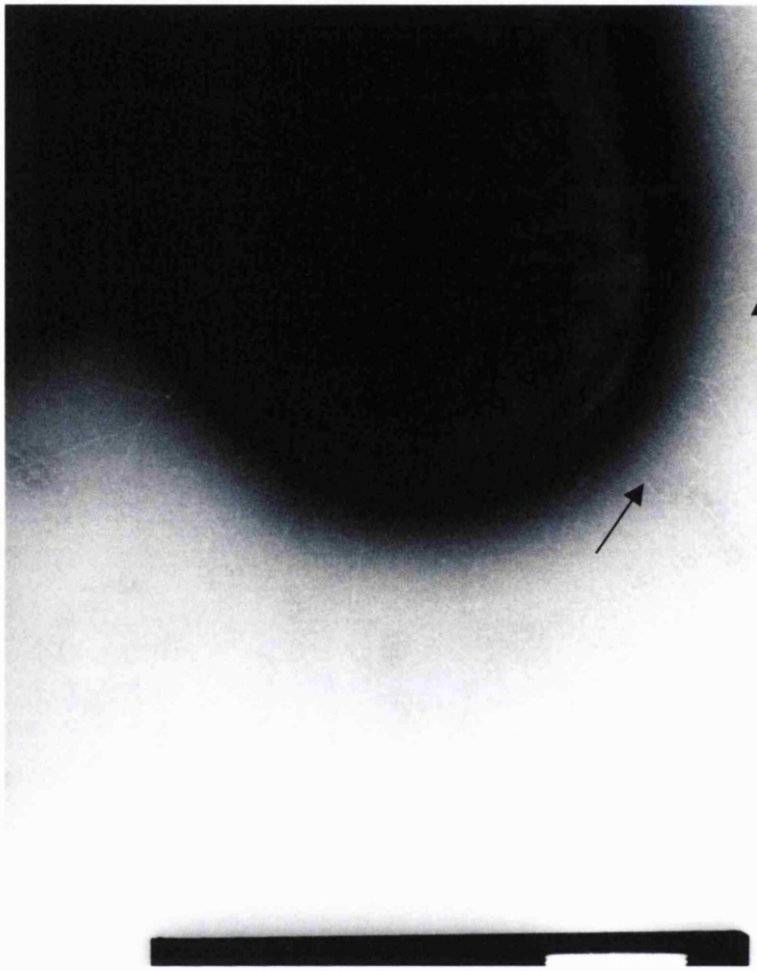


Figure 5.15 is a micrograph of the fimbrial – like structures (black arrow) produced by 85-170 during IVOC with transverse colon explants, bar = 200nm.

The EAEC strain adheres to transverse colon and a fimbrial like structure was seen after four hours of incubation with transverse colon.

Thus it is possible to observe bacterial surface structures during IVOC and this may be used in the future to assess the role of fimbriae and flagella during IVOC. This would be improved by a method involving analysing the bacteria close to the explant surface rather than the bacteria in the IVOC medium.

5.2.10 IVOC of EHEC strains with mutations in their *lpf* - like operons.

The long polar fimbriae of *S. typhimurium* mediate adherence to PP in mice (Baumler, Tsolis, and Heffron, 1996). In an effort to characterise the role of putative adhesive factors during IVOC, strains with deletions in operons similar to the *lpf* operon of *S. typhimurium* strains, kindly provided by Dr. A.G. Torres and Professor J.B. Kaper of the Center for Vaccine Development, University of Maryland, U.S.A, were tested for adhesion during IVOC.

Table 5.10 List of strains with mutations in their *lpf* - like operons.

Strain	Description	Source/reference
85-170lpf1-	The EHEC O157:H7 strain 85-170 with a deletion in the *lpfA* of the first *lpf* - like operon. Cm^r	Dr A.G. Torres CVD, University of Maryland, U.S.A.
85-170lpf1/c	Strain 85-170lpf1- complemented with pBR322*lpfABCC'DE*. Ap^r	Dr A.G. Torres CVD, University of Maryland, U.S.A.
85-170lpf2-	The EHEC O157:H7 strain 85-170 with a deletion in the *lpfA* of the second *lpf* - like operon. Cm^r	Dr A.G. Torres CVD, University of Maryland, U.S.A.
85-170lpf1-2	Strain 85-170 with deletions in the *lpfA* of *lpf* - like regions 1 and 2	Dr A.G. Torres CVD, University of Maryland, U.S.A.
AGT300lpf1-	The EHEC O157:H7 strain AGT300 with a deletion in the *lpfA* of the first *lpf* - like operon. Cm^r	Dr A.G. Torres CVD, University of Maryland, U.S.A.(Torres *et al.*, 2002)
AGT300lpf1/c	Strain AGT300lpf1- complemented with pBR322*lpfABCC'DE*. Ap^r	Dr A.G. Torres CVD, University of Maryland, U.S.A.(Torres *et al.*, 2002)

Based on homology with the *S. typhimurium lpfABCDE* operon {Baumler *et al* 1995), the first *lpf* - like operon, *lpfABCC'DE* is 5.9 kb in length and corresponds to the 141 O island in the EHEC O157:H7 EDL 933 genome and the second *lpf* – like region, *lpfABCDD'* corresponds to the 154 O island, which is 6.9 kb in length (Perna *et al.*, 2001).

Table 5.11 IVOC of EHEC strains with deletions in their *lpf* - like operons.

	Intestinal region			
Strain	Duodenum	Terminal ileum	PP/T. ileum	Transverse colon
85-170lpf1-	4/5	4/5	3/3	0/3
85-170lpf1/c	0/5	0/5	0/4	0/3
85-170lpf2-	3/8	ND	2/3	ND
85-170lpf1-2	3/7	ND	2/3	ND
AGT300lpf1-	0/3	ND	0/3	ND
AGT300lpf1/c	0/3	ND	0/3	ND
Patient age (months) (median(range))	141 (47-200)	152 (76-206)	121 (39-187)	74, 120, 146

Note: Values correspond to A/E lesion formation as a proportion of explants inoculated. When more than four explants were used the age was given as a median with a range indicated.
Duodenum = fourth part of the duodenum.
PP/T. ileum = Peyer's patches isolated from the terminal ileum.
ND = not done.

The deletion of the *lpf1* region in 85-170 results in this strain adhering to the villous epithelium of the small intestine, in this case the duodenum and terminal ileum, during IVOC. The A/E lesions are similar to those produced by other strains which produce A/E lesions on the villi of the small intestine, such as E2348/69 and ICC170pCVD438. The

deletion of *lpf1* in 85-170 does not prevent 85-170lpf1- from adhering to PP explants and it adheres in a manner similar to 85-170. Strain 85-170lpf1- does not adhere to transverse colon explants during IVOC. The transformation of 85-170lpf1- with pBR322*lpfABCC'DE* did not result in a return to the IVOC adhesion phenotype of 85-170 exemplified by 85-170lpf1/c not adhering to PP explants.

Figure 5.16 A/E lesion formation of 85-170lpf1- on small intestine and PP.

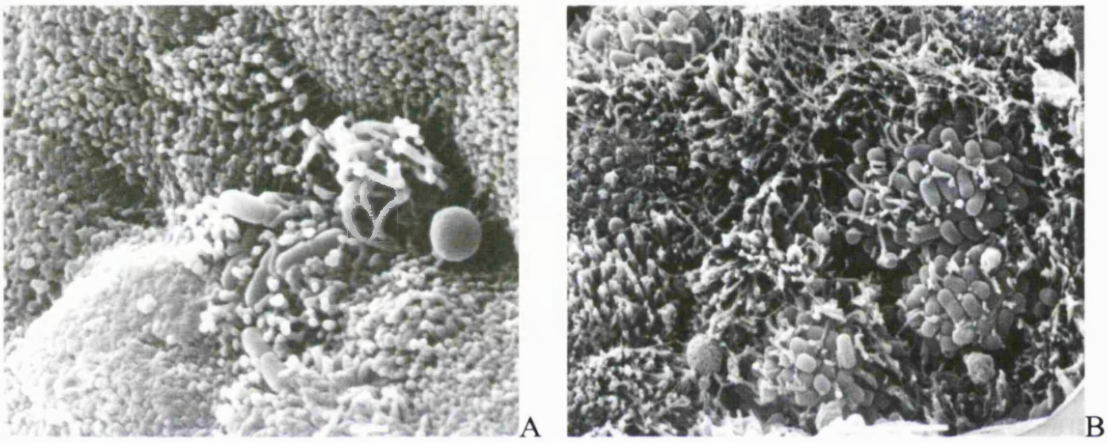


Figure 5.16A is a micrograph of 85-170lpf1- forming A/E lesions on villous epithelium of small intestinal explants during IVOC, bar = 1μm. Figure5.16B is a micrograph of 85-170lpf1- forming A/E lesions on PP explants bar = 5 μm.

The deletion of the second *lpf* - like region in 85-170 to produce 85-170lpf2- did not result in loss of adhesion to PP explants. This strain also produces A/E lesions on duodenal explants similar to those produced by 85-170lpf1- but it does not produce A/E lesions in the same percentage of explants. 85-170lpf1- adheres to 80% of duodenal explants and 85-170lpf2- adheres to 27% of duodenal explants during IVOC. The 85-170lpf1-2- strains contains a double mutation in the *lpfA* of *lpf* - like regions 1 and 2. This strain adhered to both PP explants and duodenal explants and shows a similar percentage of explants adhesion as the 85-170lpf2- strain.

Figure 5.17 The A/E lesions formed by 85-170lpf2- and 85-170lpf1-2- on duodenal and PP explants.

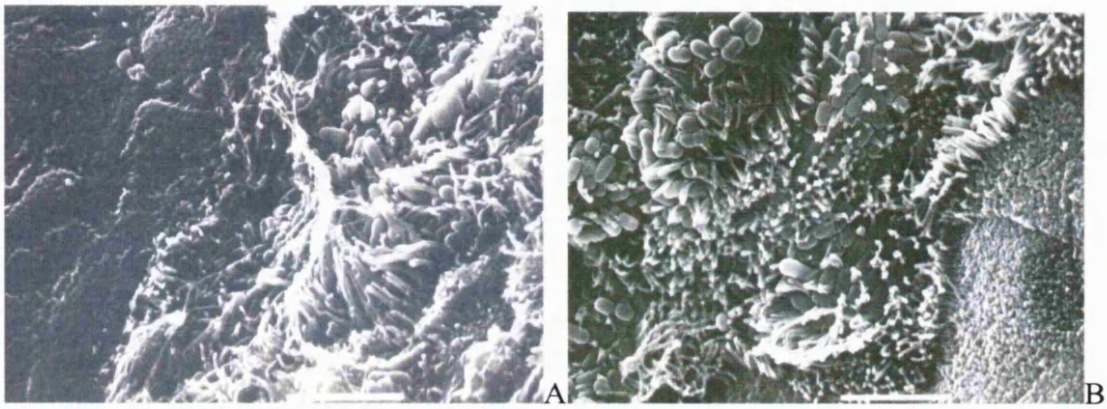


Figure 5.17A is a micrograph of 85-170lpf2- producing A/E lesions on explants during IVOC, bar = 5µm. Figure 5.17B is a micrograph of 8-170lpf1-2- producing A/E lesions on explants during IVOC, bar 5µm.

The AGT300 derivative strains, AGT300lpf1- and AGT300lpf1/c, do not adhere to PP or duodenal explants during IVOC, unlike AGT300 which adheres to PP explants.

Therefore the *lpf* - like regions of certain EHEC strains may play a role in tissue tropism and A/E lesion formation.

5.2.11 The use of supernatants from duodenum adhesion positive strains to promote adhesion of the EHEC strain 85-170 to duodenum explants.

Both E2348/69 and 85-170lpf1- adhere to duodenal explants and may be expressing factor(s) in their supernatants that may promote adhesion to duodenal explants. Therefore the supernatants from these strains were used to incubate the EHEC strain 85-170 with duodenal explants in order to see if this could result in 85-170 binding to duodenum. This was carried out as outlined in section 2.2.9.

Table 5.12 The effect of supernatant from E2348/69 and 85-170lpf1- on the adhesion of EHEC strain 85-170 to duodenal explants.

	Duodenum		
	Incubation conditions		
Strain	Standard assay	E2348/69 S/N	85-170lpf1- S/N
E2348/69	4/4	ND	ND
85-170lpf1-	4/4	ND	ND
85-170	0/4	0/4	0/4
Patient age (months)	43, 44, 127, 110		

Note: Values correspond to A/E lesion formation as a proportion of explants inoculated.
Duodenum = fourth part of the duodenum.
ND = not done.
S/N = supernatant from strain indicated.

The supernatants from E2348/69 and 85-170lpf1- duodenal IVOC incubations did not promote the adhesion or A/E lesion formation of 85-170 to duodenal explants.

5.2.12 The analysis of surface structure expression of the EHEC strains containing deletions in their *lpf* - like regions.

The strains were examined as described in section 2.10.2, using negative staining TEM, in order to determine if the deletion of *lpfA* within the *lpf* – like regions of EHEC strains would influence the expression of fimbrial – like structures and flagella in EHEC strains with deletions in their *lpf* - like regions.

Table 5.13 The expression of fimbrial – like structures and flagella after overnight growth in BHI and during IVOC, by strains with deletions in their *lpf* – like regions.

	Growth conditions	
Strains	Overnight BHI broth bijoux/uni	Duodenal IVOC 2Hr, 4Hr, 6Hr, 8Hr
85-170lpf1-	-ve	-ve, -ve, -ve, -ve
85-170lpf1/c	-ve	ND
85-170lpf2-	FL	ND
85-170lpf1-2-	FL	ND
AGT300lpf1-	-ve	ND
AGT300lpf1/c	FL	ND

Note: -ve = no surface structures observed by TEM

FL = flagella

ND = not done

Uni = universal

As can be seen in table 5.1, the EHEC strains 85-170 and AGT300 express fimbrial - like structures and flagella after overnight growth in BHI broth in bijoux and universals.

Table 5.9 shows that 85-170 may produce fimbrial like structures during IVOC with duodenal explants. However, 85-170 does not produce any surface structures during IVOC with transverse colon explants. After overnight growth in BHI in bijoux and universals fimbrial like structures and flagella are expressed by 85-170 whereas the 85-170lpf1- and 85-170lpf1/c strains do not express such structures under similar conditions. However the 85-170lpf2- and 85-170lpf1-2- strains express flagella, although no fimbrial - like structures, after overnight growth in BHI in bijoux and universals.

Figure 5.18 Expression of flagella by 85-170 *lpfA* deletion derivative strains and AGT300lpf1/c after overnight growth in BHI.

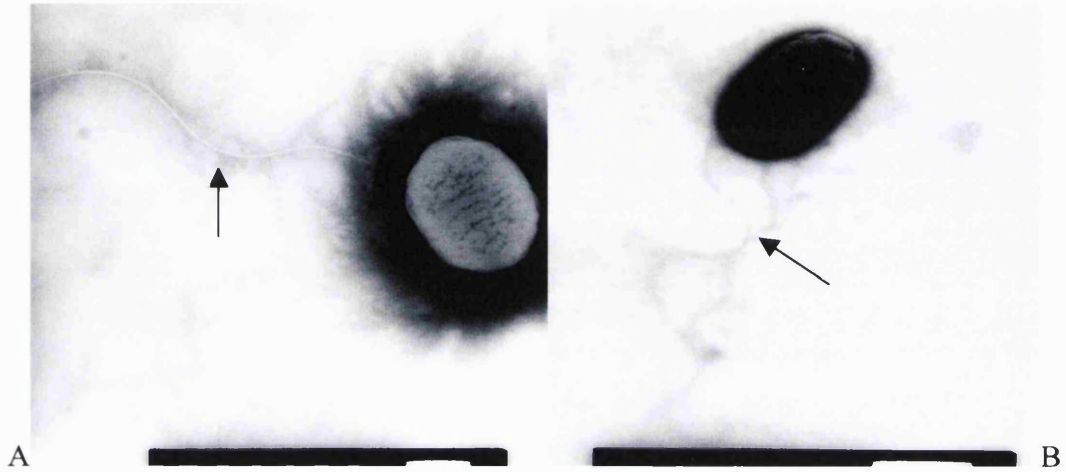


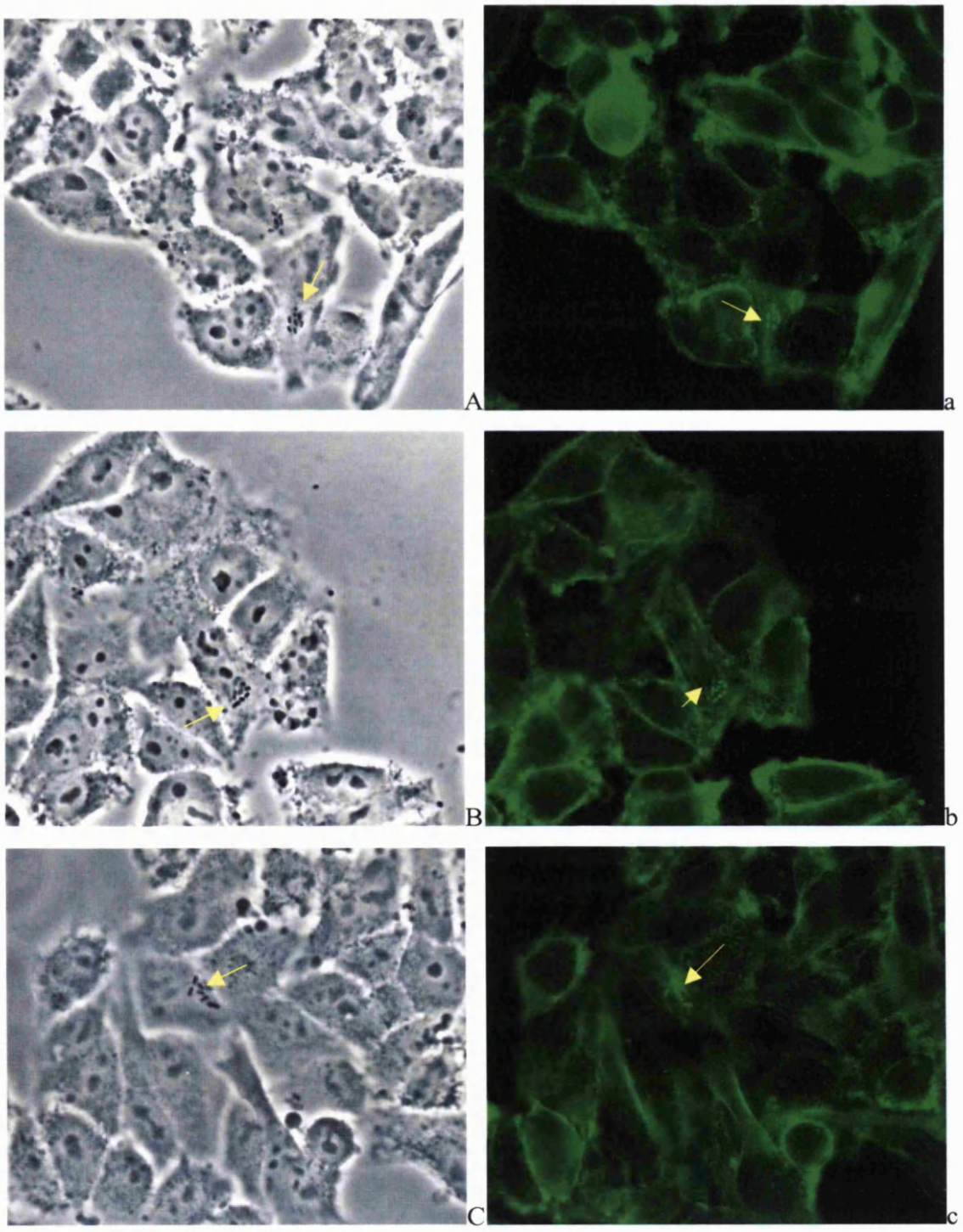
Figure 5.18A is a micrograph of the flagella (black arrow) produced by 85-170 strains with an *lpfA* deletion in the second *lpf* – like operon after overnight growth in BHI, bar = 500nm. Figure 5.18B is a micrograph of the flagella (black arrow) produced by AGT300lpf1/c after overnight growth in BHI, bar = 1μm.

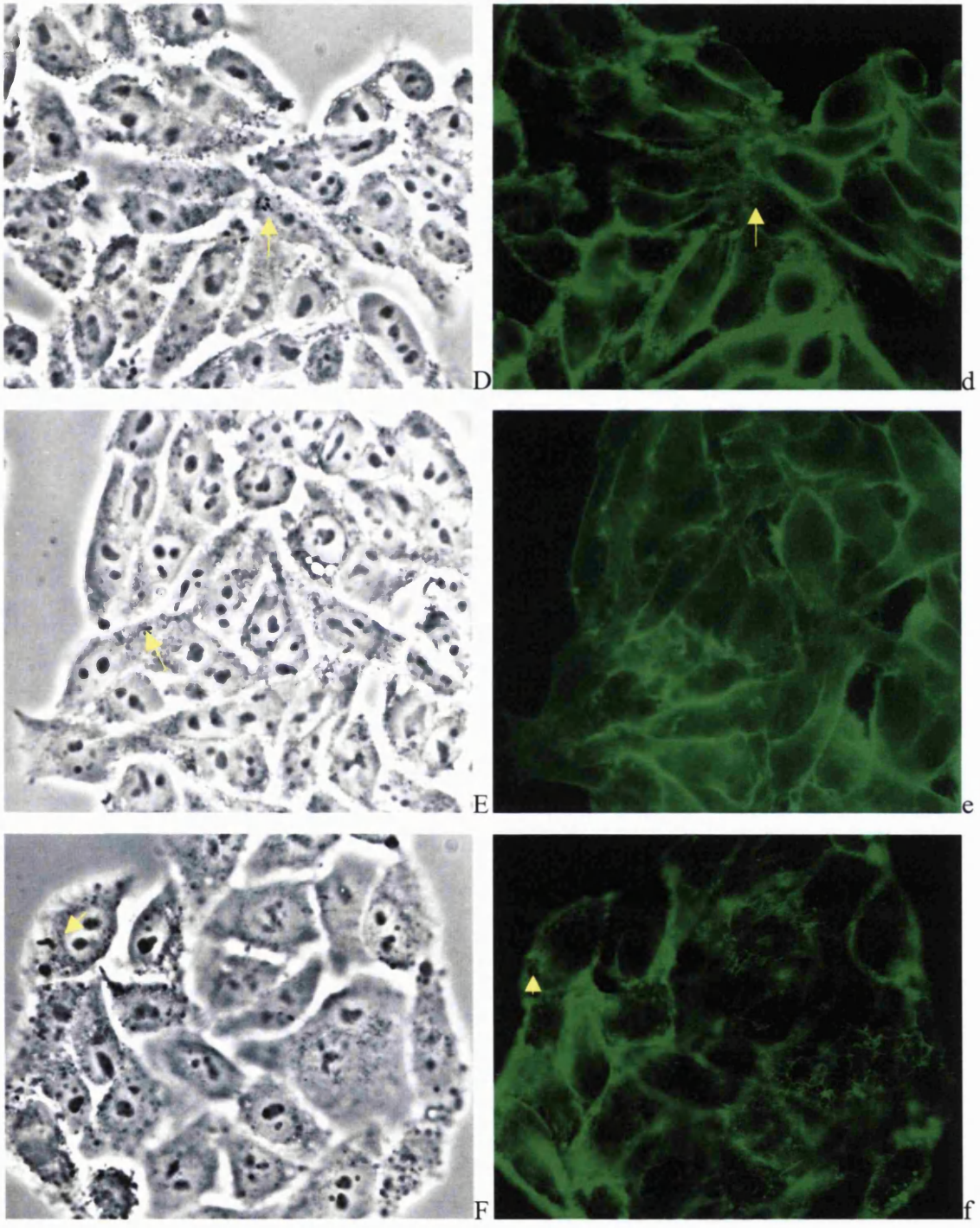
The EHEC strain AGT300 produces fimbrial - like structures and flagella after overnight growth in BHI broth in bijoux and universals. However deletion of the *lpfA* in its first *lpf* - like region results in no expression of surface structures under the same growth conditions. However, unlike 85-170lpf1/c the AGT300lpf1/c strain expresses flagella.

5.2.13 The HEp-2 cell adhesion assay of the EHEC strains containing deletions in their *lpf* like regions.

The HEp-2 cell adhesion assay was carried out as described in section 2.4.2 in order to determine the HEp-2 cell adhesion patterns of the EHEC *lpf* mutant strains.

Figure 5.19 The HEp-2 cell adhesion of the EHEC strains containing deletions in their *lpf* - like regions.





These micrographs are duplicates with the phase contrast image represented by the upper case letters and the fluorescent images represented by the lower case letters. The micrographs are representative images taken after six hours time point in the HEp-2 assay. The yellow arrow indicates examples of bacterial adherence. Magnification is x 80.

The bacterial strain correspond to the following letters, A is 85-170lpf1-, B is 85-170lpf1/c, C is 85-170lpf2-, D is 85-170lpf1-2-, E is AGT300lpf1- and F is AGT300lpf1/c

All the strains showed a decrease in their adhesion to HEp-2 cell when compared with their wildtype strains. All the strains were FAS positive.

The strains 85-170lpf1- and 85-170lpf/c produce the same adherence phenotype, a PA/LAL pattern. They do not produce as many microcolonies as 85-170 after six hours of incubation with HEp-2 cells and little or no adhesion was observed after three hours of incubation. The strains containing a deletion in the *lpfA* of the second *lpf*- like region, strains 85-170lpf2- and 85-170lpf1-2-, show in a decrease in their adhesion when compared to 85-170lpf1-, 85-170lpf1/c and 85-170. They both adhere with a PA pattern of adhesion. 85-170lpf1-2- was FAS positive although not all the bacteria adhering after six hours of incubation with Hep-2 cells were FAS positive (see figures 5.19 A, B, C and D).

The AGT300 derivative strains AGT300lpf1- and AGT300lpf1/c have a PA pattern of adhesion to HEp-2 cells which is same adhesion pattern produced by AGT300 on HEp-2 cells. However, AGT300lpf1- and AGT300lpf1/c adhere less to HEp-2 cells than AGT300. In addition, although both strains were FAS positive not all bacteria adhering were FAS positive after six hours of incubation, as indicated in figure 5.18 E.

5.3 Discussion.

5.3.1 Introduction.

This section has focused on trying to characterise factors, other than intimin, which may be involved in the adhesion of EPEC and EHEC to the host cell. Factors which regulate the adhesion of EPEC and EHEC have been reported elsewhere and have shown that the regulation of adhesion and A/E lesion formation occurs via multiple effectors. In EPEC the expression of intimin and BFP is regulated by the *per* and in addition EPEC and EHEC contain the genes for the *ler*, a LEE encoded regulator, which regulates the expression of the type III secretion pathway, the Esp proteins, Tir and intimin (Elliott *et al.*, 2000;Mellies *et al.*, 1999). The *efa1* gene which is found in O157:H7 and non O157:H7 STEC strains is involved in the colonisation of bovine intestine and its deletion reduces the adhesion of O157:H7 strains to Caco-2 cells (Stevens *et al.*, 2002), (Tatsuno *et al.*, 2000) This gene is 99.9% identical to the *lifA* gene of EPEC which inhibits the production of cytokines and is also homologous to *toxB* which exists on the O157:H7 virulence plasmid pO157. This gene, *toxB*, modulates the production of type III secreted proteins and O157:H7 strains which lack the *toxB* gene on their pO157 show a reduced adherence to Caco-2 cells. The characterisation of adhesins rather than effectors of adhesion during A/E lesion formation has focused on BFP, intimin and the EspA containing needle complex. Of the three, intimin is the only adhesin with a well defined receptor (Kenny *et al.*, 1997). The production of fimbriae in both EPEC and EHEC has yet to be fully characterised. The EPEC E2348/69 strain has been associated with the expression of BFP and type 1 fimbriae (Giron, Ho, and Schoolnik, 1993). The production of type 1 fimbriae in this strain was determined by mannose sensitive agglutination of red blood cells and the fact that this occurred only after growth in standing broth cultures and not after growth in shaken cultures. Haemagglutination increased with the passage of the bacteria (Elliott and Kaper, 1997).

Type 1 fimbriae are involved in the adhesion of UTEC via the FimH lectin, which binds a variety of mannosylated glycoproteins (Schembri *et al.*, 2001). However, type1 fimbriae are not involved in the adhesion process of EPEC and in addition most O157:H7 strains, but not all, do not express type 1 fimbriae (Durno, Soni, and Sherman, 1989). The deletion of the *ler* virulence regulator in both EPEC and EHEC led to the expression of fimbriae in EPEC strain E2348/69 and EHEC strain 85-170 and alteration of their HEp-2 cell adhesion phenotype, with E2348/69 exhibiting diffuse adherence and 85-170 showing a reduction in adherence(Elliott *et al.*, 2000). The expression of fimbriae by the wildtype 85-170 strain was not shown in this study but it was suggested that 85-170 expresses few fimbriae. Other EHEC strains have been shown to possess genes involved in the expression of fimbriae. The pO157 of sorbitol fermenting O157: H- contains an *sfp* gene cluster which when *sfpA* was expressed in non-pathogenic *E. coli* HB101 short fimbriae were produced. The role of this gene cluster in adhesion is not known. However, another set of genes present in EHEC with homology to the *lpf* operon of *S. typhimurium*, *lpfABCC'DE*, may play a role in adhesion to HeLa cells. The expression of this EHEC operon in non - fimbriated *E. coli* strain ORN172 results in the expression of a fimbriae which is morphologically similar to type 1 fimbriae and the fimbriae expressed by a non - fimbriated *E. coli* transformed with the *lpf* operon from *S. typhimurium* (Torres *et al.*, 2002;Baumler and Heffron, 1995). The deletion of this operon in EHEC results in a loss of adhesion to HeLa cells. Other EPEC fimbriae, apart from BFP and type 1 fimbriae, have been assessed using adhesion assays. An EPEC O55 strain has been shown to express an adhesin that is found in UTEC, the mannose resistant Afa adhesin. In O55 this adhesin is expressed as a fimbrial structure that may be involved in diffuse adherence to HEp-2 cells (Keller *et al.*, 2002).

In addition, based on the similarity of the TTSS of EPEC and the flagella type III system involved in flagella assembly and motility, flagella were assessed for their role during EPEC adhesion. Insertional mutation in the flagellin structural *fliC* gene of EPEC strain E2348/69 resulted in impaired microcolony formation and a 60% decrease in adhesion. However, adhesion was not restored by complementation with a *fliC* baring plasmid and

the *fliC* mutant also expressed less BFP. Interestingly wildtype E2348/69 expressed more flagella during adhesion to HeLa cells when compared to liquid culture and this suggested that host cells may stimulate flagella production (Giron *et al.*, 2002).

Therefore both EPEC and EHEC express fimbriae and flagella which may act as adhesins. In section 5, EPEC and EHEC strains were analysed by TEM in order to determine their expression of fimbriae and flagella. The strains were viewed after growth in a series of different media.

5.3.2 Expression of fimbrial – like structures by EPEC and EHEC.

As can be seen in table 5.1 all of the growth media allowed for fimbriae and flagella to be produced when all the strains are grouped together. Both of the agar based media, the plates and slopes, produced the least amount of fimbriae and growth in BHI broth overnight in universals or bijoux was the medium which best supported the expression of flagella and fimbriae. This is underlined by studies which have shown that clinical *E. coli* isolates do not produce type 1 fimbriae when grown on media containing agar (Schwan, Seifert, and Duncan, 1992). The expression of fimbriae, such as type 1 fimbriae, has been shown to be achieved by the growth of strains in nutrient rich anaerobic broth (Biebricher and Duker, 1984). None of the strains in this study were subjected to anaerobic growth although growth in BHI broth in bijoux and universals occurred with a different amount of air present. The bijoux contained less air on inoculation when compared with the universals but this did not appear to affect fimbriae production. The growth of strains in LB and DMEM, both of which contain less nutrients than BHI, did reduce the number of fimbriae positive strains when compared with growth in BHI although even a minimal medium like DMEM still supported the expression of fimbriae and flagella. This suggests that fimbriae can be produced by EPEC and EHEC under varying nutrient and air environments during overnight growth at 37°C. Strain H11 did not produce surface structures and this strain does not adhere to IVOC during the standard IVOC assay. However, H11 adheres to PP explants after passage in BHI broth in bijoux and shows increased HEp-2 cell adherence (see sections 3.2.5.5.1). Therefore the passage of the

bacterial strains may alter the expression of surface structures. This study shows that EPEC and EHEC strains produce fimbriae and that they are present, depending on growth media, before addition to adhesion assays. Fimbrial expression before or during IVOC by EPEC and EHEC strains was not achieved in conditions which replicate the environmental conditions present in the lumen of the gut. This could be achieved by using a gut simulator and when used such an apparatus has been shown to effect gene expression in enteric pathogens (Behrens, Sheikh, and Nataro, 2002).

The fimbrial – like structures expressed by EPEC and EHEC were similar in width with variations occurring in the length of the fimbrial – like structures. They were approximately 7nm wide and between 0.3 and 1.7 μm long and were expressed peritrichously. The range in length is similar to the range of length observed in type 1 fimbriae (Baumler and Heffron, 1995). They were channelled rod like structures that were distinct from flagella. They were shorter than LPF and did not appear to be similar to the fimbriae expressed by EPEC and EHEC *ler* mutants (Elliott *et al.*, 2000;Torres *et al.*, 2002). It is therefore possible that EPEC and EHEC express common fimbriae. The fimbrial – like structures observed were characterised by agglutination phenotype, expression during long term culture and using antibodies to type 1 fimbriae and LPF.

It should be noted that the study of fimbriae is made difficult by the fact that fimbriae or flagella may not respond well to the processing of bacterial strains for TEM analysis. The drying out of the bacterial strains which is necessary for viewing in the vacuum of the TEM, may cause fine structures to collapse and make observation of novel fimbriae difficult to report. In cases when suspected known fimbriae are present in a sample antibodies may be used to try and stabilise the fimbriae structure to allow for viewing and better assessment of the fimbrial morphology. However, this is limited by the availability of antibodies to known fimbriae and does not facilitate the discovery of novel fimbriae. Type 1 fimbriae have been observed without the use of stabilising antibodies and hence may be less prone to damage during processing of bacterial samples for TEM (Mulvey *et al.*, 1998).

5.3.2.1 Yeast and GPRBC agglutination assays.

In an attempt to further characterise the fimbrial - like structures expressed by the EPEC and EHEC strains the yeast and guinea pig red blood cell (GPRBC) agglutination assays were used. Yeast and GPRBC have been used as a ready source of surface expressed sugars in order to determine the presence of bacterial expressed surface structures that act as lectins by binding to sugar residues. For example, *Trypanosoma cruzi* binds to human erythrocytes via a galactose binding protein and GPRBC have been used to determine the presence of mannose binding adhesins in avian pathogenic *E. coli* (da Rocha *et al.*, 2002). Type 1 fimbriae have been shown to bind to yeast in a mannose sensitive manner and this makes the basis of a test for the presence of type 1 fimbriae (Roe *et al.*, 2001). This test was used to categorise the strains expressing fimbriae with type 1 morphology according to their ability to agglutinate yeast and GPRBC in the presence of mannose. Strains that do not agglutinate yeast or GPRBC may be type 2 fimbriae or type 1 fimbriae with a change in receptor affinity (Sokurenko *et al.*, 2001).

The yeast and GPRBC assays give clearly distinguishable agglutination reactions but the yeast assay is the easier of the two to visualise. The EPEC and EHEC strains E2348/69, E77804 and PMK5 showed mannose sensitive agglutination of yeast and GPRBC after overnight growth in BHI in bijoux and universals. 85-170 also agglutinates yeast and GPRBC in a mannose sensitive manner but only after growth in universals. In accordance with previous studies (Elliott and Kaper, 1997), this suggests that E2348/69, E77804, PMK5 and 85-170 produce type 1 fimbriae after overnight growth in BHI broth. All four strains expressed different adhesion patterns during HEp-2 cell assay suggesting that the mannose sensitive adhesins produced during the growth of the strains prior to HEp-2 cell adhesion assay did not define a specific adhesion pattern. The fimbriae produced by the EHEC strains AGT300, TT12B and 3801 after overnight growth in BHI broth in bijoux and universals are not type 1 fimbriae due to their lack of agglutination of yeast and GPRBC but may be type 2 fimbriae or a subset of type 1 fimbriae that no longer bind to yeast or erythrocytes. However, they appear too short and peritrichous to be LPF or type IV fimbriae.

The possible presence of mannose sensitive type 1 fimbriae in the EHEC strains 85-170 and PMK5 suggests that they do not contain a 16 bp deletion in their *fim* switch. To verify this their *fim* genes would have to be sequenced or probed using defined primers (Li, Koch, and Cebula, 1997). This suggests that a probe to identify the 16bp deletion in EHEC O157:H7 stains as a means to detect the presence of pathogens may miss some strains such as 85-170 which is a clinical isolate from a food handler in Canada (Tzipori *et al.*, 1987).

The expression of type 1 fimbriae in certain strains appears to be related to growth conditions. The universals contained more air than the bijoux and this may influence the expression of type 1 fimbriae in 85-170. However, the removal of mannose from the media used during both HEp-2 cell and IVOC assays does not alter the adhesion phenotype of the EPEC and EHEC strains including 85-170. Therefore type 1 fimbriae in EPEC and EHEC do not appear to be involved in adhesion to intestinal explants or HEp-2 cells. However, there is the question of redundancy whereby bacteria have the ability to express several virulence factors, so that if one is knocked out others will compensate. Efa1 has no effect on the adhesion of E2348/69, but in the EAF plasmid cured strain JPN15, the deletion of Efa1 causes a reduction in adhesion of JPN15, suggesting that factors, such as BFP, may have masked the role played by Efa1 in adhesion (Badea *et al.*, 2003). In addition, the expression of type 1 fimbriae may allow the strains to adhere to other surfaces, such as abiotic surfaces or the intestine of animals (Cookson, Cooley, and Woodward, 2002;Durno, Soni, and Sherman, 1989), and their role in pathogenesis is poorly documented for *E. coli* strains that cause pathology in regions other than the urinary tract. In addition to binding to GPRBC the EHEC O157:H7 strain CL-49 adheres to rabbit ileal brush borders via α-linked mannosyl residues present on surface glycoproteins (Durno, Soni, and Sherman, 1989). Thus it is possible that adhesion to the intestinal tract of animals may involve type 1 fimbriae and although they may not be essential for A/E lesion formation in cell culture or during IVOC type 1 fimbriae may allow bacteria to come into closer contact with host cells in animals, and may be an important maintenance mechanism in animal reservoirs(Roe *et al.*, 2001). Type 1 fimbriae may also allow pathogens to adhere to abiotic surfaces and facilitate transmission(Cookson, Cooley, and

Woodward, 2002). The role of type 1 fimbriae in EPEC pathogenesis has yet to be fully resolved and interestingly volunteers challenged with E2348/69 developed a serological response specific to the type 1 fimbriae expressed by E2348/69. The sera from inoculated patients failed to recognise the type 1 fimbriae expressed by other *E. coli* indicating that the type 1 fimbriae expressed by *E. coli* are antigenically distinct (Karch *et al.*, 1987). The basis of this is probably the ability of the *fimH* to valency convert and bind to different receptors (Sokurenko *et al.*, 2001). In addition, the *fim* switch allows type 1 fimbriae to be transiently expressed in response to the environment. Since type 1 fimbriae elicit immune responses this may be a means to avoid immune detection by the host. Such phase variation has been found in commensal *E. coli* strains and UTEC isolates. Therefore it is possible that the ability of 85-170 to vary its expression of type 1 fimbriae according to growth condition may allow it to escape immune detection in an animal or human host. In addition, a UTEC strain with either its type 1 fimbriae expressed at all times or lacking type 1 fimbriae showed that expression of type 1 fimbriae increased the virulence of the strain suggesting that expression of type 1 fimbriae may influence other pathogenic mechanisms (Gunther *et al.*, 2001). This may be partly explained by the ability of FimH to mediate autoaggregation of *E. coli* (Schembri, Christiansen, and Klemm, 2001). Therefore EHEC strains such as 85-170 may use type 1 fimbriae to form interbacterial connections in a manner similar to BFP in EPEC.

5.3.3. The passage of EPEC and EHEC strains and their expression of fimbrial – like structures and flagella.

The growth of bacteria overnight in standing both cultures has been used to prepare bacteria for various virulence assays (Francis *et al.*, 1991;Phillips *et al.*, 2000). In addition, EPEC and EHEC strains were grown overnight in standing broth cultures as described in section 2.2.3 and 2.4.2 before addition to the IVOC assay or HEp-2 cell assay. However it has been suggested that the growth of fimbriae such as type 1 fimbriae may benefit from passage in rich anaerobic standing broth cultures (Biebricher and Duker, 1984;Elliott, Nandapalan, and Chang, 1991;Francis *et al.*, 1991). The passage or daily subculturing of

bacteria has been linked with an increased level of haeamgglutination and the production of type 1 fimbriae by nonpathogenic *E. coli* and the EHEC O157:H7 strain CL-49 (Durno, Soni, and Sherman, 1989;Elliott, Nandapalan, and Chang, 1991). Therefore the bacterial strains used to assess the expression of surface structures by EPEC and EHEC after overnight growth were also passaged in BHI broth for 10, 12 and 14 days in both bijoux and universals. In addition, the bacterial strains were grown in long term standing cultures for 10 days in BHI to determine the effect of not passaging strains during long term cultures. Before observing the strains using TEM the strains were assessed for their ability to agglutinate yeast and GPRBC and an estimate of number of viable bacteria in culture after the incubation periods was determined. In addition to these tests the bacteria were assessed for their ability to express intimin after culture before and after activation in DMEM.

5.3.3.1 Yeast and GPRBC agglutination after passage of EPEC and EHEC.

The passage and NP of the bacterial strains for 10, 12 and 14 days in BHI broth did not alter their agglutination profiles. As after overnight growth, the presence of mannose sensitive adhesins was only detected in EHEC strain 85-170 after growth in universals and not after growth in bijoux. E2348/69, E77804 and PMK5 showed mannose sensitive agglutinated yeast and GPRBC after growth in bijoux and universals. The passage/NP of the bacterial strains did not alter their agglutination phenotype and did not stimulate the agglutination negative strains to produce yeast and GPRBC binding adhesins. It is possible to hypothesise that the bacteria that were not passaged were subject to an environment with diminishing nutrients and a build up of metabolic products. However, this environment did not trigger the expression of mannose binding adhesins in the strains which were agglutination negative and did not alter the expression of the putative type 1 fimbriae of E2348/69, E77804, PMK and 85-170. In addition, growth in bijoux without passage over 10 days may have reduced the level of oxygen in the bijoux. This did not affect the agglutination phenotype of the strains. The assessment of the CFU/ml of the bacterial strains showed that the passage of the strains did not significantly affect the CFU results

when compared to the results after overnight growth. The NP results indicated that NP passage in bijoux showed a decrease in the CFUs of the bacterial strains but one which was not marked and NP in universals showed no significant change in the viability of the bacterial strains. It has been reported that the long term culture of *E. coli* in BHI results in a significant drop in viability after 10 days culture without passage (Fukuda, Nakahigashi, and Inokuchi, 2001). This study used a nonpathogenic *E. coli* strain and it may be that nonpathogenic strains are less adapted to long term growth conditions involving nutrient depletion, changes in pH and changes in O_2 availability. Indeed on closer examination of the results the study shows that between days 6 and 8 of cultivation there is an actual increase in the CFU/ml showing that the bacterial population varies throughout the long term cultivation. A GASP mutation was defined (growth advantage in stationary phase) and the viable bacteria present after long term cultivation harboured this mutation. In addition, strains which survive the long term cultivation do not show a loss of viability in subsequent long term cultivation experiments. Therefore pathogens may have adapted to grow in nutrient depleting environments thus allowing them to survive in multiple environments and may explain the lack of loss of viability of the EPEC and EHEC strains during NP.

5.3.3.2 Intimin expression after passage of EPEC and EHEC strains.

The passage of *E. coli* has been shown to promote fimbrial expression in certain strains. In order to assess the potential changes in bacterial adherence due to passaging and NP EPEC and EHEC strains, the bacterial strain used in this study were probed for intimin expression after growth. As was shown in section 3.2.4, the EHEC strains and the EPEC E77804 strain used in this study do not produce intimin as detectable by western blot after overnight growth in BHI. However, EPEC strain E2348/69 does. In section 3.2.4 and 4.2.1 strains expressing wildtype intimin α (E2348/69, CVD206(pCVD438), ICC170(pCVD438) and strains harbouring the EAF plasmid (G21, G35) expressed intimin, as detected by western blot, after overnight growth in BHI and without activation in DMEM. However these results indicated that the expression of a specific intimin type (G35 expresses intimin γ) or the presence of an EAF plasmid (ICC170(pCVD438) is EAF

negative) is not critical to the expression of intimin after overnight growth in BHI. Therefore this phenotype may be controlled by many elements and may be induced by passage or NP in BHI broth in bijoux or universals.

The passage of the bacterial strains in BHI broth in bijoux did result in intimin being expressed, without activation in DMEM, in E2348/69, E77804, PMK5 and TT12B (see section 3.2.5.5). Therefore the passage of the strains in bijoux changed the dynamics of intimin expression in both EPEC and EHEC strains. Further experiments were carried out in section 5. NP in bijoux and universals and passage of the bacterial strains in universals resulted in a change of phenotype for EPEC strain E77804 when compared to intimin expression after overnight growth. After NP in bijoux for 10 days the EHEC H11 strain lost the ability to express intimin after growth in DMEM, which may have been due to the low O_2 levels in the bijoux after 10 days and the presence of limiting metabolites. However, when this strain was inoculated onto HEp-2 cells it still produced A/E lesions suggesting that the lack of intimin expression could be recovered after passage. The EPEC strains, E77804 and E2348/69, expressed intimin, under the same passage conditions, after NP passage in bijoux and universals and passage and NP in universals, but do not have the same HEp-2 adhesion phenotype or IVOC tissue tropism phenotype. Intimin expression is influenced by growth conditions but EPEC and EHEC do not respond in the same way to different growth conditions.

5.3.3.3 Expression of fimbrial – like structures and flagella after passage.

The bacterial strains were analysed for the expression of possible fimbriae and flagella after passage. Passage promotes the expression of type 1 fimbriae in nonpathogenic *E. coli* strains and reports have suggested that type 1 fimbriae expression varies in broth culture (Schwan, Seifert, and Duncan, 1992). Therefore it may be possible to detect changes in the expression of fimbriae after the passage of bacteria. After passage for 10 days in BHI broth in bijoux and universals changes in the expression of surface structures was observed in EHEC strains AGT300, TT12B and H11 when growth in universals was compared to

overnight growth in BHI, but not for EPEC strains. No fimbriae were expressed by AGT300 and TT12B after passage and H11 produced flagella. Thus in certain strains the expression of fimbriae and flagella may be modulated by passage.

5.3.3.4 HEp-2 cell adhesin of EPEC and EHEC strains after passage.

The passage of the EPEC and EHEC strains altered the expression of intimin, the expression of fimbriae and flagella, as well as adhesion to HEp-2 cells. However, a correlation between these characteristics is difficult to establish as, for example, AGT300 shows an increase in adhesion to HEp-2 cells after passage in BHI with a varying fimbrial and intimin expression profile.

The passage of the strains for 10-14 days in universals appears to have altered the adhesion of EHEC strains to Hep-2 cells when compared to passage in bijoux. After passage in bijoux EHEC strains AGT300, TT12B, H11 and 3801 showed an increase in adhesion to HEp-2 cells whereas only EHEC strains H11 and 3801 showed an increase in adhesion after passage in universals. In addition, passage in universals showed a decrease in the adhesion of PMK5 to HEp-2 cells when compared to passage in bijoux and the standard assay. This reduction in adherence was also observed after the NP of PMK5 in bijoux and universals. The only strain to show an increase in adherence after NP was AGT300. However, AGT300 did not show a change in intimin expression after passage and NP and no fimbriae were observed after passage in universals. Catabolite stress has been implicated in increasing the adhesion of *E. coli* to mouse caecum and EHEC O157:H7 respond to low O_2 by adhering in greater numbers to HEp-2 cells. These studies showed that type 1 fimbriae played a role *in vivo* but that they did not affect the adhesion of O157:H7 to HEp-2 cells in an O_2 limited environment. Of the strains which showed a change in adhesion phenotype due to passage only the EHEC strain PMK5 produces mannose sensitive type 1 fimbriae. PMK5 was yeast/GPRBC agglutination positive after growth in conditions which led to reduced HEp-2 cell adhesion. This suggests that changes in adhesion phenotype do not relate to expression of mannose sensitive adhesins

such as type 1 fimbriae. However, expression of type 1 fimbriae may influence the expression of other virulence factors. In UTEC there is cross talk between the *fim* gene cluster responsible for type 1 fimbriae expression and the *pap* gene cluster responsible for the expression of pyelonephritis-associated pili (Pap). This results in the expression of Pap turning off expression of type 1 fimbriae (Xia *et al.*, 2000). In addition, the histone like nucleoid structuring protein (H-NS) has been found to control a number of environmentally controlled genes in *E. coli* and is also implicated in the control of Pap, type 1 fimbriae and haemolysin in *E. coli* (Madrid *et al.*, 2002;White-Ziegler *et al.*, 2000). This suggests that global regulatory genes may control fimbriae expression and that the expression of one virulence characteristic may affect the expression of other virulence factors. The relationship between fimbriae expression and genes involved in A/E lesion formation is being investigated and some results have shown that deletion of the *ler* contributes to the expression of fimbriae (Elliott *et al.*, 2000).

5.3.4 The use of immuno – EM to type the fimbrial structures expressed by EPEC and EHEC.

The fimbrial - like structures were initially characterised according to their morphology, their potential to agglutinate yeast and GPRBC and their presence under varying growth conditions. In order to further characterise the fimbriae produced, antibodies to type 1 fimbriae and long polar fimbriae were obtained and used to stain the fimbriae and observe them under TEM. The results show that the EPEC and EHEC strains positive for agglutination did not express long polar fimbriae and that binding of anti- type 1 fimbrial antibody to type 1 fimbriae depends on the antibody used. This suggests that there may be different immunogenic differences between type 1 fimbriae and this should be considered when raising antibodies to type 1 fimbriae. The results indicate that E2348/69, E77804, PMK5 and 85-170 express type 1 fimbriae. The type 1 fimbriae expressed by these strains are not necessary for adhesion to HEp2 cells (section 3.2.3) and do not define a common HEp-2 adhesion phenotype or IVOC tissue tropism phenotype. However, their role in carriage, aggregate formation, immune recognition and adhesion *in vivo* remains to be elucidated.

5.3.5 Expression of fimbrial – like structures by EPEC and EHEC during IVOC.

In an effort to highlight such questions E2348/69 and 85-170 were analysed for expression of surface structures during IVOC. E2348/69 adheres to duodenum and fimbriae were only observed after four hours of incubation when A/E lesions have been shown to be present on the mucosal surface (Hicks *et al.*, 1998). The presence of fimbrial – like structures at this time point, on bacteria present in the IVOC medium, may allow the bacteria to form aggregates in order to colonise other regions of the intestine or allow the bacteria to interact with the bacteria adhering at the mucosal surface. 85-170 does not adhere to duodenal explants during the eight hour IVOC and did not express fimbrial – like structures during the IVOC assay with duodenum. However, for both strains fimbriae and flagella expression was more readily observed during incubation with transverse colon explants. Neither strain showed adhesion to transverse colon suggesting that fimbriae and flagella may be expressed during non intimate interactions with certain regions of the host and this is highlighted by the observation that *fliC* E2348/69 mutants produce A/E lesions during IVOC. However, this premise contrast with the results from adhesion to cell lines which show that flagella are expressed during adhesion and involved in adhesion to HeLa cells (Giron *et al.*, 2002). Therefore a role for flagella during IVOC and *in vivo* infections remains to be elucidated. Sampling or staining of bacterial surface structures during intimate contact with the explants may provide useful information as to the role of surface structures during IVOC. Sampling of the bacteria in the IVOC medium may reflect pre or port adhesion events depending of the dynamics of A/E lesion formation.

5.3.6 The characterisation of the *lpf*- like regions of EHEC.

5.3.6.1 The involvement of the *lpf* – operons of EHEC during adhesion to explants in the IVOC assay.

It is clear that EPEC and EHEC strains express fimbriae and flagella and that their role in adhesion and pathogenesis requires further study. The publication of the genome of EHEC strain EDL933 has facilitated the analysis of putative adhesins and factors which may be involved in the regulation of adhesion (Perna *et al.*, 2001). The information generated from the sequencing of this genome was used by Torres A.G et al to characterise an *lpf* - like operon in EHEC. When compared to *E. coli* K-12 EDL 933 contains 1.34 Mb of DNA extra and this additional DNA or O islands are inserted into the common genetic backbone shared by K-12 and O157:H7. These O islands have been classified according to their homology with known virulence factors. EDL 933 has two regions that resemble the *lpf* operon present in *S. typhimurium*. The LPF encoded by these genes are similar to type 1 fimbriae in the organisation of their genes (Baumler and Heffron, 1995) and have been implicated in targeting of *S. typhimurium* to PPs in mice (Baumler, Tsolis, and Heffron, 1996). In addition, the ability of *S. typhimurium* LPF to phase shift may be a possible mechanism in evading cross immunity between *Salmonella* serotypes (Norris and Baumler, 1999). In EHEC O157:H7 strain two putative *lpf* – like operons exist as O islands 141 and 154 (Perna *et al.*, 2001). The 141 O island defined as *lpfABCC'DE* was characterised in EHEC O157:H7 and deletion of the putative major fimbrial subunit *lpfA* resulted in loss of adhesion to HeLa cells. Cloning of this operon into a nonfimbriate *E. coli* strain resulted in the expression of fimbriae which resembled type 1 fimbriae. However, unlike the LPF fimbriae seen after expression of the *S. typhimurium lpfABCDE* operon in a nonpathogenic *E. coli* strain, the fimbriae expressed due to transformation with the EHEC *lpf* - like operon were not polar.

In order to determine the role of LPF in EHEC during IVOC these strains with mutations in the *lpfA* of the *lpf* - like operons of EHEC strains 85-170 and AGT300 were tested for

adhesion to PP explants. The 85-170 strain with a deletion in the *lpf* – like operon (O island 141) characterised by Torres et al (Torres *et al.*, 2002) adhered to PP explants and as a result of this was tested for adhesion to other areas. This strain also adhered to the villous epithelium of duodenal and terminal ileum explants but did not adhere to transverse colon explants. Therefore, this was the first example of a factor, other than intimin, involved in tissue tropism of EHEC during IVOC and is further evidence that intimin γ strains may adhere to the villous epithelium of the small intestine. Such adhesion of intimin γ expressing EHEC strains has been reported in low numbers for strain TT12B (2/11 duodenal explants contained A/E lesions). It is possible that deletion of this *lpf* - like region may have suppressed the expression of LPF fimbriae in 85-170 which may have led to an expansion of its tissue tropism or that the deletion of this operon upregulated other factors involved in adhesion to duodenal and terminal ileum explants. Thus the first *lpf* - like region of 85-170 does not appear to be essential for adhesion to PP explants and unlike adhesion to HeLa cells *in vitro* its deletion promotes A/E lesion formation by 85-170 to duodenal and terminal ileum explants thus expanding the tissue tropism of this strain in comparison to 85-170. Two other 85-170 mutants were then tested for adhesion in IVOC. These strains contained a mutation in the *lpfA* of the second *lpf* - like region *lpfABCDD'* (O island 154). Strain 85-170lpf2- adheres to PP explants suggesting that the second *lpf* - like region is also not essential for adhesion to PP explants during IVOC. This strain also adhered to duodenal explants but with a lower frequency than the 85-170lpf1- strain suggesting that deletion of the second *lpf* - like region does not mediate expansion of tissue tropism in a manner similar to the first region. To test this a third strain was used which contained a mutation in both of the *lpf*- like regions, 85-170lp1-2-. This strain showed the same tissue tropism as 85-170lp2- suggesting that a mutation in the second region affects the mutation in the first region thus limiting the expansion of the tissue tropism of 85-170 but that together the adhesion to PP explants and duodenal explants is not abolished. This suggest that both regions are not essential for adhesion to PP during IVOC and that they play different roles in tissue tropism expansion and that mutation in the first *lpf* - like region appears to have no effect on the second region during IVOC. It was not possible to tell whether the *lpf* - like regions interact with intimin and influence its role in tissue tropism in EHEC during IVOC (all the *lpf* mutants express intimin as detected by western

blot, data not shown). However, these results show that intimin γ expressing EHEC can adhere to the villous epithelium of the small bowl with a high frequency similar to E2348/69 and the intimin α expressing EHEC derivative strain ICC170(pCVD438). This suggests that although intimin is involved in tissue tropism during IVOC other factors play a role.

In order to test the hypothesis that the *lpf* - like regions are involved in tissue tropism in EHEC, another EHEC O157:H7 derivative strain AGT300lpf1- was analysed for adhesion during IVOC. This strain shows reduced adhesion to HeLa cells (Torres *et al.*, 2002). AGT300lpf1- does not adhere to PP explants or duodenal explants during IVOC. This suggests that unlike in 85-170 the first *lpf* - like region of AGT300 is necessary for adhesion to PP, as the wildtype strain AGT300 adheres to PP explants during IVOC. It is possible that the *lpf* - like regions function in different ways in different EHEC isolates and that they may interact with different virulence factors thus producing different IVOC adhesion phenotypes. All the determinants involved in adhesion and A/E lesion formation have not been identified and there may be a degree of heterogenicity amongst EHEC strains and therefore the influence of the *lpf* - like operons may vary. It is clear from the TEM analysis of the strains and from HEp-2 and IVOC data that strains differ in their expression of fimbriae and their adhesion to cells. Thus the loss of a putative EHEC LPF may affect strains in different ways and as can be seen with other fimbriae there may be some cross talk amongst fimbrial operons (Xia *et al.*, 2000).

It should be noted that the fact that the strains could not be returned to their wildtype phenotype by complementation does cause problems when drawing conclusions. It could be that the *lpfABCC'DE* on the pBR322 plasmid does not interact in a wildtype manner with unknown regulators of this operon and that adhesion during IVOC follows a very sensitive pathway.

5.3.6.2 The use of supernatants from the IVOC assays of E2348/69 and 85-170lpf1- to influence the adhesion of 85-170 to duodenal explants during IVOC.

In an effort to further characterise the role of the first *lpf* like operon present in EHEC, the supernatants from a duodenal IVOC assay of E2348/69 and 85-170lpf1- where used to incubate 85-170 with a duodenal explant. The IVOC of 85-170 in the supernatant of E2348/69 and 85-170lpf1- did not result in adhesion of 85-170 to duodenum. This suggests that E2348/69 and 85-170lpf1- do not secrete a biological active substance in sufficient concentrations into the IVOC medium to alter 85-170 adhesion to duodenum. In addition, it may be that the adhering strains produce stimulating molecules that act at a local level or that the activity f the molecule is short lived.

5.3.6.3 The expression of fimbrial – like structures and flagella by strains with deletions in their *lpf* – like regions.

To date the expression of LPF has been viewed by TEM after transformation of nonpathogenic *E. coli* strains (Baumler and Heffron, 1995;Torres *et al.*, 2002). TEM was used in this section to determine if the *lpfA* deleted strains expressed fimbriae or flagella after overnight growth in BHI in bijoux and universals. No fimbriae were observed in the strains containing mutations in the first an second *lpf* - like operon and flagella were observed in the 85-170 strains harbouring mutations in the *lpfA* of the second operon, both operons and the AGT300lpf1/c strain. Thus in the case of the 85-170 flagella were observed in the strains which showed a lower frequency of adhesion to duodenal explants (85-170lpf2- and 85-170lpf1-2-). However the complementation of AGT300lpf1- and 85-170lpf1- was not enough to restore wildtype surface structure expression after overnight growth which in both cases consists of expression of fimbriae and flagella. No fimbriae or flagella were observed on 85-170lpf1- during IVOC with duodenum which is similar 85-170 incubated with duodenum during IVOC. Thus the expression of fimbrial – like structures is influenced by the deletion of both *lpf* – like regions suggesting that the *lpf* - like operons may regulate the expression of other virulence factors.

5.3.6.4. The HEp-2 cell adhesion of EHEC strains with deletions in their *lpf* – like operons.

The HEp-2 cell adhesion of the *lpfA* mutant strains showed that for all strains a decrease in adhesion to HEp-2 cells was noted by qualitative assessment. In the case of the 85-170 derivative strains the decrease in adhesion was manifested by a decrease in the number of microcolonies formed. As in previous studies (Torres *et al.*, 2002) this decrease in adhesion was only a modest decrease as microcolonies were still formed by the 85-170lpf1- and 85-170lpf1/c mutant strains. However in this section we also analysed a strain with mutations in the *lpfA* of the second *lpf* - like operon. The mutation of the second *lpf* - like operon caused a greater reduction in adherence than the mutation in the first operon and the strain with both mutations, 85-170lp1-2-, showed a further decrease in adherence. The complemented strain 85-170lp1f/c did not show full restoration of adhesion to wildtype levels and this has been observed using other EHEC strains (Torres *et al.*, 2002). The AGT300lpf1- and AGT300lpf1/c strains showed a reduction in adhesion to HEp-2 cells when compared to AGT300, however they both shared the same adhesion pattern. However, in addition to a reduction in the number of bacteria adhering bacteria adhered without giving a FAS positive result. Therefore the *lpfA* mutations may have delayed the adhesion of EHEC strains to HEp-2 cells thus limiting the numbers of microcolonies formed after six hours of incubation and in strain AGT300 delayed the onset of a FAS positive result. The adhesion of nonpathogenic *E. coli* expressing LPF to HeLa cells suggests that the reduced adhesion of the strains to HEp-2 cells may be due to the loss of expression of LPF fimbriae and that the adhesion of an intimin negative *E. coli* to HeLa cells suggest a role for LPF during the initial adhesion to HeLa cells or during the adhesion of LEE negative EHEC strains to the intestinal mucosae (Doughty *et al.*, 2002). Evidence for the latter is provided by the involvement of the LPF of EHEC non O157:H7 LEE negative strains during adhesion to Chinese hamster ovary cells (CHO). Therefore, these results suggest that LPF may be involved during adhesion but that LPF is not necessary for A/E lesion formation on cell lines or during A/E lesion formation on explants.

5.3.7 Conclusions.

This section showed that EPEC and EHEC express fimbriae and flagella under different growth conditions and that passage or long term cultivation does not abolish the expression of fimbriae. This indicates that before and in some cases during, adhesion assays, fimbriae and flagella are expressed. Certain EPEC and EHEC strains may express type 1 fimbriae and although it appears they have no role in adhesion during the HEp-2 and IVOC assays their role in pathogenesis remains unresolved due to their expression, immunogenicity and interaction of the *fim* genes with other fimbrial genes. The typing of certain strains as being type 1 fimbriae expressing can be achieved using agglutination assays and immuno-EM. However, type 1 fimbriae can be antigenically distinct and in addition the FimH adhesion can show valency conversion thus resulting in binding to different glycoproteins. Thus type 1 fimbriae may form a very diverse group of fimbriae containing as yet undefined properties and may be expressed by both EPEC and EHEC.

The *lpf* - like operons of EHEC are not required, in the strains tested in this section, for adhesion to PP explants and deletion of these operons can result in expansion of the tissue tropism phenotype of certain EHEC strains. The *lpf* - like operon appears to be involved in the adhesion of EHEC strains to HEp-2, HeLa and CHO cells and *lpfA* homologues are found in a wide variety of EHEC strains (Doughty *et al.*, 2002). Thus factors other than intimin are involved in tissue tropism and fimbrial operons may pay a role during adhesion. The nature of the interaction of LPF with the host cell and the *lpf*- like operon with other virulence operons remains to be elucidated.

Section 6.0 Discussion and Conclusions.

6.1 IVOC of EPEC and EHEC strains.

The study of bacterial pathogenesis relies on the development of appropriate models of infection. *In vitro* organ culture enables intestinal explants to be maintained *in vitro* for up to twenty four hours with continued cell turnover and protein synthesis (Browning and Trier, 1969;Mitchell, Mitchell, and Peters, 1974). Thus IVOC has provided a tool in the study of the interaction of intestinal pathogens with the intestinal mucosae. Its advantage over cell lines is that it provides a more complete model of infection as bacteria can be analysed for their adhesion to fully differentiated enterocytes from selected regions of the intestinal tract. The explants used during IVOC assay will also have glycocalyx and mucus layers which are not present in cell lines such as HEp-2 cell lines. Thus in defining virulence factors that are essential for adhesion to intestinal mucosae *in vivo*, IVOC is closer to the *in vivo* model than cultured intestinal carcinoma cell lines.

IVOC has been used to study the interaction of EPEC and EHEC with selected regions of the intestinal tract (Hicks *et al.*, 1998;Knutton, Lloyd, and McNeish, 1987;Phillips and Frankel, 2000;Phillips *et al.*, 2000). This has confirmed the ability of EPEC to form A/E lesions *in vivo* (Rothbaum *et al.*, 1983;Ulshen and Rollo, 1980) and provided information on the possible roles of BFP and intimin during EPEC adhesion *in vivo* (Hicks *et al.*, 1998). EHEC are thought to have evolved from EPEC through the acquisition of Stx and therefore the ability to form A/E lesions may be central to their pathogenic mechanism (Boerlin *et al.*, 1998;Boerlin, 1999;Boerlin *et al.*, 1999;McGraw *et al.*, 1999;Rumer *et al.*, 2003). However, no A/E lesions have been detected in biopsies of *in vivo* EHEC infections, possibly due to the late collection of biopsies after the manifestation of the cytotoxic effects of Stx (Nataro and Kaper, 1998). EHEC produces A/E lesions in neonatal calves but A/E lesions are rarely observed in cattle, a principle reservoir of EHEC, accounting for the lack of clinical symptoms observed in cattle (Dean-Nystrom *et al.*, 1997;Naylor *et al.*, 2003). Although A/E lesions have been identified in the rectal region where it is considered that EHEC reside in cattle without inducing clinical symptoms (Naylor *et al.*, 2003). It has been shown that enteropathogenic effects in the ligated ileal loops of calves are not related to the

expression of intimin, Tir or Shiga toxin (Stevens *et al.*, 2002), although the responsible virulence factor(s) have not been identified. Thus, in order to study the ability of EHEC to produce A/E lesions Phillips et al (Phillips *et al.*, 1999) used a model relevant to the human infection process, i.e. paediatric intestinal explants in an IVOC assay. This provided evidence that EHEC O157:H7 produce A/E lesions on the intestinal mucosae and adhere preferentially to PP explants. Thus, adherence to PP may be a colonisation mechanism utilised by EHEC as well as *Salmonella* and RDEC-1 (Cantey and Inman, 1981;Clark *et al.*, 1994). In comparison, a prototype EPEC strain bound to small intestinal, PP, and, with a lower frequency, to large intestinal explants during IVOC (Phillips *et al.*, 2000).

Therefore in section 3, EPEC and EHEC strains were tested for their ability to produce A/E lesions during IVOC. The use of EPEC and EHEC strains expressing different intimin types provided information on the tissue tropism associated with different intimin types and used multiple strains to confirm and expand the observations previously made (Phillips *et al.*, 2000). The results showed that other EPEC and EHEC strains can produce A/E lesions during IVOC. In general EHEC strains show preferential adhesion to PP explants but have the ability, in a small number of the explants tested to adhere to duodenal and large intestinal explants. Adhesion to the isolated lymphoid follicles of the duodenum was also noted but further work using this region of the intestine was limited due to the random nature of biopsying areas of the duodenum containing isolated lymphoid follicles. However, this result added to the evidence that the follicular associated epithelium of the intestinal tract may be colonised by EPEC and EHEC and that these regions may contain characteristics that allow them to be preferentially colonised (Autenrieth *et al.*, 1996;Baumler, Tsolis, and Heffron, 1997;Baumler, Tsolis, and Heffron, 1996;Cantey and Inman, 1981;Clark, Hirst, and Jepson, 1998;Clark *et al.*, 1994;Frey *et al.*, 1996;Heel *et al.*, 1997;Jepson *et al.*, 1995;Keren *et al.*, 1978). The tissue tropism of intimin γ and intimin ϵ expressing strains appeared to be similar, in that they both showed preferential adhesion to PP explants during IVOC, and this was matched by the similarities in the carboxy terminus of their intimin proteins (Oswald *et al.*, 2000). The analysis of the tissue tropism of four EHEC O157:H7 strains showed that these strains adhered preferentially to PP explants but that some variation in adhesion during eight hour IVOC does occur. For example TT12B can adhere to duodenal and large intestinal, with a low frequency,

suggesting that expression of intimin γ does not prevent adhesion to these regions. There may be other factors involved in tissue tropism. These factors may be genetic elements shared across a category, such as EHEC, or factors unique to a given serotype or strain. Highlighting the variabilities in tissue tropism of EPEC and EHEC strains will allow such factors to be identified. In addition, environmental factors may influence adhesion during IVOC and tissue tropism. The EHEC O157:H7 strain H11 did not adhere during IVOC but passage of this strain in BHI enabled it to adhere to PP explants. This implies passage related upregulation of factors involved in adhesion to PP explants and this isolate could be screened for genetic elements that are activated following passage and compared with the non adhering H11 isolate. The O26:H11 isolate, 3801, adhered to PP explants but in addition adhered to the villous epithelium of the terminal ileum. Thus this strain had a unique tissue tropism phenotype and was the only EHEC strain to adhere to the villous epithelium of the terminal ileum without the presence of PP in the explant. This may be a characteristic of intimin β strains and this observation would benefit from the analysis of the tissue tropism of other intimin β strains. It is also possible that the presence of PP in the terminal ileal region influences the characteristics of the villous epithelium. It would be of interest to study more proximal ileal tissue where PP are infrequent (Van Kruiningen *et al.*, 2002), however, accessibility for biopsy is extremely limited, e.g. in patients with ileostomies when tissue adaptation may complicate matters further.

Thus this study showed that EHEC and strains adhere to PP explants during IVOC, with very little adhesion to large intestinal explants. EHEC are considered to be colonic pathogens due to the haemorrhagic colitis associated with EHEC infection (Nataro and Kaper, 1998;Griffin, Olmstead, and Petras, 1990;Griffin and Tauxe, 1991). Therefore it is considered logical to presume that EHEC produce A/E lesions on the colon. This study showed for the first time, using IVOC, that EHEC can produce A/E lesions on the colon but that it is with a lower frequency than PP adhesion. This suggests that factors involved in A/E lesion formation on the colon may not be upregulated during the eight hour IVOC assay or that this reflects the *in vivo* situation. The latter would be explained by the adhesion to PP explants and other follicular regions during infection with dissemination of Stx to areas remote from the colonisation environment, thereby producing HC in the colon. It also suggests that PP adhesion may be the initial site of adhesion during EHEC infection and that adhesion to

PPs may be followed by adhesion to other areas such as the colon. This may be due to upregulation of EHEC colonisation-related genes following host interaction, and/or may relate to the time required to downregulate host defences such as LL37 as described in *Shigella* infection in man (Islam *et al.*, 2001). However, this begs the question as to why FAE colonisation is possible, and raises questions of what advantage the host benefits from a pathogen colonisation receptive region that natural selection had maintained.

6.1.1 Adhesion of EHEC to the colon during IVOC.

Experiments were devised to promote the adhesion of EHEC to the colon. Changing the IVOC medium by removing D- mannose, activating the bacteria in DMEM and M9CM before IVOC, passaging of the bacterial strains in BHI and varying the incubation time of the IVOC assay and the medium change time points did not alter the adhesion of EPEC and EHEC to the colon. Overnight IVOC assay of 85-170 and duodenal explants did produce a positive result suggesting that longer IVOC assay incubation times may be a useful model of EPEC and EHEC adherence to intestinal explants. However, the explants showed changes in their structural integrity during prolonged IVOC and further work in this area would require developing a different assay system whereby the build up of molecules or the presence of conditions damaging to the explant would be limited. Using centrifugation to focus the bacteria onto the intestinal explant produced an increase in adhesion of EHEC and EPEC to the colon but did not alter the frequency of A/E lesion formation. Co–incubation of the explants with HEp-2 cells did not alter the tissue tropism of the EPEC and EHEC strains but it did produce A/E lesion on one colonic explant incubated with 85-170.

Therefore the attempts to influence the adhesion of EPEC and EHEC strains to colonic explants during IVOC did not give a reproducible colonic adhesion phenotype. These experiments highlighted some variables that may improve the IVOC assay and for example the co - incubation of colonic explants during or after EHEC and EPEC IVOC with PP explants may provide useful information as to the mechanisms of colonic adhesion. A comparison of gene regulation during PP adhesion and lack of adhesion or low frequency adhesion to colonic regions may provide additional evidence in order to determine the mechanisms involved in IVOC adhesion and the ability of EPEC and

EHEC strains to adhere to the colon during IVOC. One obvious difference between IOVC and the *in vivo* situation is that gassing with 95%O_2/5%CO_2 is required to maintain tissue viability in IVOC, whereas the colonic lumen is anaerobic with a variable pH along its length (more acidic in the caecal region becoming neutral towards the distal regions). It may be worthwhile to reduce the O_2 level to examine if this alters the adhesion phenotype within the confines of maintaining tissue preservation. Alternatively, an investigation of the application of intestinal simulation models on gene activation (Behrens, Sheikh, and Nataro, 2002) may give insights into EHEC pathogenesis.

6.1.2 Intimin expression by EPEC and EHEC.

All of the EPEC and EHEC strains expressed intimin after growth in DMEM. When intimin expression was examined after overnight growth in BHI none of the EHEC strains expressed intimin, whereas some of the EPEC strains were positive for intimin expression. This suggests that EPEC and EHEC bacteria have different intimin regulation mechanisms when inoculated on HEp-2 cells or explants. Passage of the bacterial strains showed a change in intimin expression after overnight growth in EPEC and EHEC strains suggesting that passage can alter virulence factor expression. However, changes in intimin expression due to passage could not be correlated with changes in HEp-2 cell adhesion or fimbrial expression. Not all of the EHEC responded to passage conditions in the same way suggesting that EHEC respond differently to different environments. Passage resulted in the atypical EPEC strain E77804 expressing intimin without activation in a manner similar to E238/69 and this did not vary with changes in passage conditions. The basis for the differences in intimin expression has yet to be defined and it is possible that EPEC and EHEC strains display different sensitivity to environmental signals.

6.1.3 Intimin and tissue tropism during IVOC.

In section 4, the role of intimin in tissue tropism during IVOC was assessed. The expression of different intimin types by EPEC and EHEC may be involved in determining the tissue tropism of EPEC and EHEC strains during IVOC. In earlier experiments using gnotobiotic piglets (Tzipori *et al.*, 1995) intimin α from EPEC was

shown to change the tissue tropism of EHEC during intimin exchange studies. Using IVOC the intimin of EHEC O157:H7 strain 85-170 was able to confer a restricted tissue tropism on EPEC strain E2348/69, whereby E2348/69 expressing the intimin γ of EHEC strain 85-170 adhered only to PP during the eight hour IVOC (Phillips and Frankel, 2000). In section 4 intimin was shown to be required for A/E lesion formation by EHEC O157:H7 during IVOC and the expression of intimin α by EHEC strain 85-170 allowed it to adhere to the villous epithelium of the small intestine in a manner similar to the EPEC strain E2348/69. Thus intimin is involved in the tissue tropism of EHEC O157:H7 during IVOC. This variation in EHEC tissue tropism is Tir type independent. It might be postulated that an ability for EHEC to colonise both the small intestine and PP regions would be advantageous, but there appears to be some selection pressure for restricting adhesion phenotype, and this awaits explanation. The low infective does of EHEC attracts comment, but perhaps this is the norm for host-pathogen contact in the environment, and restricted tropism allows EHEC to get a "foothold" without stimulating host defences, indeed provides an opportunity for the production of bacterial effectors which downregulate host defences. The lack of intimin detection in the EHEC PP IVOC, would also reflect the "stealth" approach of EHEC to initial colonisation.

EPEC strains from the O55 serogroup, expressing intimin α and intimin γ, were used to define the tissue tropism of strains from a given serotype, and to provide further information on the tissue tropism of intimin α strains and new information on the tissue tropism of intimin γ expressing EPEC strains. The results showed that the strains from the O55 serogroup adhered preferentially to PP explants during IVOC, regardless of intimin type expressed. This provided evidence that intimin α expressing typical EPEC strains have a variable tissue tropism and may have different mechanisms of adherence *in vivo*. Thus preferential adhesion to PP explants is not limited to EHEC strains. Mutational analysis of the intimin α of E2348/69 suggested that amino acids in the carboxy terminus of intimin α may be involved in tissue tropism during IVOC (Reece *et al.*, 2001). Thus the intimin α of the O55:H6 strain G21 was sequenced and compared to the intimin α of E2348/69. Two amino acid changes were present, neither of which had been previously characterised. A single mutation was chosen for analysis (V907A) due to its presence in the carboxy terminus Tir and cell binding region of intimin and its proximity to a residue implicated in the tissue tropism of E2348/69

(A911). Mutation of an alanine to valine at position 907 in intimin α of E2348/69 to produce an intimin α protein similar to that expressed by G21 was carried out. The resultant plasmid was placed in both EPEC and EHEC backgrounds and analysed for adhesion to duodenal explants during IVOC. The EPEC and EHEC derivative strains adhered to duodenal explants failing to mimic the tissue tropism of O55:H6 strain G21, indicating that the O55:H6 intimin sequence was not responsible for the restricted phenotype. Thus, it was postulated that factor(s) acting independently of intimin may be involved in tissue tropism.

6.2 HEp-2 cell adhesion of EPEC and EHEC strains.

The *in vitro* cell culture adhesion of EPEC and EHEC strains is used as means of identifying putative enteroadherent *E. coli* strains (Nataro *et al.*, 1987;Knutton *et al.*, 1991;Knutton *et al.*, 2001). HEp-2 cell adhesion patterns are associated with certain virulence factors, i.e. localised adherence and BFP (Nataro *et al.*, 1985). Thus in combination with the FAS test (Knutton *et al.*, 1989), which allows A/E lesion activity to be assessed, typical EPEC strains can be identified by a FAS positive, LA patter of adhesion on HEp-2 cells.

The HEp-2 assay was used to analyse whether the EPEC or EHEC strains had a common adhesion phenotype and whether this correlated with IVOC tissue tropism. The strains expressing BFP adhered to HEp-2 cells with an LA pattern but they did not share the same IVOC tissue tropism (E2348/69, G21 and G35). The intimin α from E2348/69 did not confer the LA adherence phenotype on EHEC strains (ICC170(pCVD438)), but mutations in intimin α may have the ability to confer the LA phenotype on EPEC and EHEC strains (CVD206(pCPG2) and ICC170(pCPG2)), suggesting that not all mutations in intimin α decrease HEp-2 cell adherence. This was the first case of intimin conferring the LA adherence phenotype on an EHEC strain (ICC170(pCPG2)). Intimin γ expressing wildtype strains (85-170, AGT300, H11 and TT12B) were associated with a decreased adhesion to HEp-2 cells when compared to the intimin α expressing wildtype strains (E2348/69 and G21), except in strain G35, but this appeared to be a characteristic particular to this strain and may not have been due to its expression of BFP, as CVD206(pICC55) did not adhere with an LA phenotype. In addition, the intimin γ or the intimin ϵ expressing strains did not share similar

adhesion phenotypes and were only characterised by the lack of LA adherence. Therefore the HEp-2 assay can be used to highlight the adhesion characteristics of EPEC and EHEC strains but the factors involved in adhesion phenotypes remain to be elucidated. However, the use of the HEp-2 assay may miss certain adhesion characteristics as is exemplified by the similar HEp-2 adhesion phenotype of strains E2348/69 and G21 and their different tissue tropism phenotypes during IVOC.

Environmental factors may play a role during adhesion to HEp-2 as passage of the strains in BHI broth showed a change in adhesion phenotype amongst the EHEC strains but did not appear to affect the EPEC strains.

6.3 Intimin and surface structure (fimbrial) expression during IVOC.

In order to further characterise the EPEC and EHEC experiments were designed to test whether intimin could be monitored during IVOC. Intimin was observed by western blot during IVOC. Intimin was expressed by bacteria in the IVOC medium of the E2348/69 incubation during incubation with small intestinal explants and large intestinal explants. The intimin γ expressing strains such as 85-170 and G35 did not appear to express intimin as expressed by the bacteria in the IVOC medium. The conserved antibody used has been shown in this thesis and in previous studies to recognise intimin γ and this result suggest that intimin γ strains may be regulated differently to E2348/69 during IVOC. In addition, it should be noted that G35 and 85-170 shared similar tissue tropism during IVOC and the presence of an EAF plasmid and presumably *per* in G35 did not promote the expression of intimin by this strain in the IVOC medium during IVOC. This suggests that intimin expression may be different in certain strains during IVOC and that some strains such 85-170 may express intimin close to the mucosal surface where it was not possible to sample the bacteria during IVOC.

In addition to monitoring intimin expression during IVOC, a similar method was used in order to determine if surface structures are expressed by bacteria in the IVOC medium during the eight hour IVOC assay. Surface structures could be detected on bacteria during IVOC assay. Fimbrial – like structures and flagella appeared to be expressed across more incubation time points during incubation of E2348/69 and 85-

170 with colon when compared to duodenal explants. This suggests a different regulation of EPEC and EHEC surface structures according to explant used and highlights the possible role of differential eukaryote cell signalling during pathogenesis.

6.4 The expression of surface structures by EPEC and EHEC and the involvement of fimbrial – like operons in adhesion during IVOC.

The EPEC and EHEC strains expressed fimbrial – like structures during growth in various media. Growth in BHI broth at 37°C promoted most expression of fimbrial – like structures and flagella by EPEC and EHEC strains. The fimbrial – like structures expressed by EPEC and EHEC were similar in width (7nm) with some variation in their length (0.3 – 1.7 μm). Thus they had the appearance of type 1 fimbriae (Baumler and Heffron, 1995;Clegg and Gerlach, 1987;Elliott and Kaper, 1997). The yeast and GPRBC assay were used to test for mannose sensitive agglutination, which is a characteristic of type 1 fimbriae (Elliott and Kaper, 1997). The EPEC strain E2348/69 and the intimin ϵ expressing strains E77804 and PMK5 were agglutination positive in similar growth conditions. The EHEC strain 85-170 was the only O157:H7 strain that was agglutination positive, and only after growth in BHI in universals. The presence of mannose sensitive type 1 fimbriae was confirmed using antibodies to type 1 fimbriae and immuno – EM. Therefore EHEC strains such as PMK5 and the EHEC O157:H7 strain 85-170, express type 1 fimbriae. However, the expression of such fimbriae does not appear to be critical during adhesion to IVOC and HEp-2 cells. Their role in pathogenesis has yet to be elucidated but serological responses to type 1 fimbriae from EPEC have been documented as well as type 1 fimbriae mediated inflammation (Elliott and Kaper, 1997;Hedlund *et al.*, 2001;Karch *et al.*, 1987). The expression of type 1 fimbriae was not influenced by passage in BHI broth. The expression of fimbrial – like structures that do not agglutinate yeast or GPRBC by EHEC strains suggests that EHEC may express more than one type of fimbriae. These fimbriae appeared similar and were termed type 2 fimbriae (Baumler and Heffron, 1995;Clegg and Gerlach, 1987). The role of fimbriae in EPEC and EHEC pathogenesis is poorly understood. In an effort to understand the role of fimbriae in EHEC adhesion during IVOC, the role of *lpf* – like operons during adhesion to explants was studied.

LPF have been implicated in the adhesion of *S. typhimurium* to PPs *in vivo* (Baumler, Tsolis, and Heffron, 1997). Due to the sequencing of the genome of EHEC O157:H7 strain EDL933 (Perna *et al.*, 2001), two operons not present in the *E. coli* K12 genome have been designated as being *lpf* – like operons . When the first *lpf* –like operon (O island 141 in EDL933) is expressed in a non – fimbriated *E. coli* strain, peritrichous fimbriae are expressed with a similar morphology to type 1 fimbriae (Torres *et al.*, 2002). Using the same non – fimbriated strain, the expression of the *S. typhimurium lpf* operon, resulted in the expression of fimbriae that were polar and resembled type 4 fimbriae (Baumler and Heffron, 1995). Therefore there appears to be some difference in the *lpf* – like operon of EHEC and the *lpf* operon of *S. typhimurium*. Whether they have similar biological activity *in vivo* remains to be seen but deletion of the *lpf* – like operon in EHEC strains does reduce adherence to HEp-2 cells (Doughty *et al.*, 2002;Torres *et al.*, 2002).

Two EHEC strains with deletions in their *lpf* –like regions were tested for their adhesion to PP explants during IVOC. In 85-170 the deletion of the first *lpf* – like operon did not prevent A/E lesion formation on PP explants. This strain was then tested for adhesion to duodenal explants during IVOC and it produced A/E lesions on duodenal explants, thus showing an expansion of the tissue tropism phenotype when compared to the parent 851-70 strain. This was the first example of a change in tissue tropism during IVOC mediated by a factor other than intimin. Deleting the second *lpf* – like operon in 851-70 (O island 154 in EDL 933) also produced adhesion to duodenal explants but at a lower frequency and an 85-170 derivative strain with mutations in both *lpf* – like operons showed a similar phenotype to the mutation in the second *lpf* – like operon suggesting that the second *lpf* – like operon exerts some influence over the biological activity of the first *lpf* – like region. However as stated in section 4, other factors than intimin may be involved in tissue tropism and it appears that in EHEC strain 85-170 the *lpf* – like regions are involved in tissue tropism but, unlike in *S. typhimurium*, deletion of these regions does not prevent adhesion to PP. The nature of the interaction of the *lpf* – like operons of 85-170 with explants, has yet to be characterised and these operons may be responsible for the expression of adhesins or they may influence the regulation of other virulence factors (Xia *et al.*, 2000).

A second EHEC O157:H7 strain, AGT300, with deletions in the first *lpf* – like region showed that deleting this region in this strain resulted in a loss of adhesion to PP explants. Therefore the *lpf* – like regions appear to have a different activity in different EHEC O157:H7 strains. Understanding the role of these regions during adhesion in IVOC may help define the mechanisms involved in colonisation of the gastrointestinal tract by EHEC O157:H7 but it appears that like intimin proteins certain *lpf* – like operons may promote tissue tropism expansion whilst other *lpf* – like operons may produce a reduction during IVOC.

The *lpf* derivative strains showed decreased adhesion to HEp-2 cells suggesting that the role of the *lpf* – like operon in adhesion to EHp-2 cells may be different to the role of these operons in adhesion during IVOC.

The EHEC strains with deletions in their *lpf* – like operons did not express fimbrial – like structures after overnight growth in BHI, unlike their wildtype strains. This suggests that the deletion of *lpfA* in the *lpf* – like operons of EHEC strains may affect fimbrial expression and possibly the expression of other virulence factors.

6.5 Conclusions.

Human intestinal IVOC has been used to show that EPEC and EHEC strains expressing different intimin types can produce A/E lesions. EHEC O157:H7 strains expressing intimin γ appear to share a PP restricted tissue tropism phenotype. EPEC strains show a variable tissue tropism phenotype. Intimin is involved in the tissue tropism of EHEC O157:H7 during IVOC. However, not all strains expressing the same intimin type show the same tissue tropism suggesting that factors other than intimin are involved in tissue tropism during IVOC, and that these factors may be specific to a serogroup or serotype. Both EPEC and EHEC strains express fimbrial – like structures and the *lpf* – like operons of EHEC O157:H7 strains are involved in adhesion and tissue tropism during IVOC. Thus IVOC is an important model in the study of bacterial pathogenesis. It can be used to highlight the differences in colonising ability of EPEC and EHEC strains and this information can be used to define the biological activity of novel virulence factors.

Appendices.

Appendix 1.

Media and buffers.

IVOC medium

200 ml of DMEM (Sigma)
200ml of double processed tissue culture H_2O (Sigma)
0.44g of sodium bicarbonate (Sigma)
1.34g of NCTC – 135 medium (Sigma)
10% newborn calf serum (Sigma)

The solution was filtered and as required 0.5% D-mannose (Sigma) was added. It was stored at 4°C until use and prewarmed to 37°C before use.

Dulbecco's modified Eagle's medium (DMEM)

D5671 supplied by Sigma. 4500mg glucose/L

Amino acid rich liquid medium without L-glutamine.

NCTC-135 medium

N3262 Supplied by Sigma

Nutrient rich medium used in the maintenance of cultured cell lines containing L – glutamine.

Brain heart infusion broth (BHI)

CM – 225 supplied by Oxoid

BHI broth is used routinely for the growth of fastidious organisms.

Composition in g/L:

Brain heart infusion	250	Sodium chloride	5
Calf brain infusion	200	Disodium phosphate	2.5
Proteose peptone	10	Bactodextrose	2

L-broth (LB)

Lennox L – broth L- 3022 supplied by Sigma.

Tryptone (pancreatic digest of casein)	10g/L
Yeast extract	5g/L
NaCl	5g/L
Glucose	1g/L

LB is used routinely for growth of fastidious organisms.

For making plates or slopes, 1.5 % agar (Agar No 1 Lab M) was used.

Polyacrylamide gel electrophoresis.

Protein marker

Pre stained SDS – PAGE protein marker for molecular weight 30 000 to 120 000 (Sigma P1677)

Stored at -70°C and 10µl added to gel lane.

10 x Running buffer

Glycine (Sigma)	300g
Trizma base (Sigma)	100g
SDS (Sigma)	20g

Make up to 2 litres with dH_2O

Stacking buffer

Trizma base	6.05g
SDS	0.4g

Make up to 80ml with dH_2O and pH6.8 with HCl

Add dH_2O to make final volume of 100ml

Resolving buffer

Trizma base	18.2g
SDS	0.4g

Make up to 80ml with dH_2O and pH8.8 with HCl

Add dH_2O to make final volume of 100ml

30% Acrylamide stock

29.3g acrylamide/0.8g bisacrylamide per 100ml dH_2O (Biorad)

In order to dissolve slightly warm the mixture in the 37°C incubator. Beware volatile > 50°C.

Filter using a 0.45 μm filter

Resolving gel

dH_2O 10ml	
Resolving buffer	7.5ml
Acrylamide (30%)	12.5ml
Ammonium persulphate (10%)	100µl
TEMED	30µl

Add TEMED and ammonium persulphate last

All solutions, except dH_2O and acrylamide, supplied by Sigma.

Stacking gel

dH_20	6.1ml
Acrylamide (30%)	1.3ml
Ammonium persulphate	50µl
TEMED	10µl

All solutions, except dH_2O and acrylamide, supplied by Sigma.

Western blot transfer buffer

Glycine	14.4g
Trizma base	3g
Methanol (Merck BDH)	200ml

Make up to 1 litre using dH_2O

Appendix 2.

Methods and materials.

TEM

Formvar/carbon coating of grids for TEM

0.4% formvar (Agar scientific Ltd) in 1,2-dichloroethane (BDH Chemical Co. Ltd) was placed in a stopped 1.5" diameter boiling tube and clamped at approximately 30°. A clean glass slide was clamped in forceps and lowered into formvar solution for 10 seconds. The slide was removed from the solution and drained on the inside of the tube for 15 seconds and returned to the formvar for a further 10 seconds, followed by removal, drainage and air drying for ten minutes. A sharp scalpel blade was used to score a rectangular area in the formvar on the upper side of the slide. The slide was slowly lowered, at an angle of 30°, into a large diameter glass basin filled with distilled water. Dust particles, which would corrupt the fine layer of formvar, had been previously removed by drawing a piece of velin paper across the top of the water. The silver/grey formvar film separated from the glass slide and floated on the surface of the water.

200 or 400 mesh copper/rhodium grids were placed gently, copper side up, on the floating formvar film using fine pointed jewellers forceps. A rectangular shaped piece of filter paper was cut to a size slightly larger than that of the film and one end bent to angle of 90° to act as a handle. The filter paper was placed over the formvar until soaked and quickly removed, thus taking the formvar coated grids with it. This was placed grid side upwards in a large Petri dish and allowed to completely dry at 60°C.

After drying, the filter paper was trimmed around the grids and coated with a thin layer of carbon by means of carbon evaporation under high vacuum ($< 10^{-4}$ torr). Grids were stored in a dust free environment at room temperature until required.

Preparation of TAAB resin.

The resin used to embed specimens for TEM was made up of the following components: TAAB embedding resin, dodecenylsuccinic anhydride (DDSA),methyl nadic anhydride (MNA) and benzyl dimethylamine (BDMA). All compounds were supplied by TAAB Laboratories Ltd, Reading, UK. The viscosity of TAAB resin is low and this allows it to penetrate well into the tissue specimens. The hardness of the polymerised resin can be determined by varying the relative concentrations of the hardening components; DDSA and MNA. The hardeners are used as an aid to complete infiltration of the resin into the tissue. BDMA acts to accelerate the polymerisation of the resin.

The resin used was made up using the following component proportions (%):

TAAB resin	50
DDSA	26
MNA	22
BDMA	2

The resin, DDSA and MNA were mixed using a magnetic stirrer for 30 minutes and the BDMA added gradually with continued stirring to ensure a thorough mix. Stirring was continued for a further 30 minutes. Mixed resin was stored in 50ml screw top polyethylene containers at -20°C until required. Resin required approximately 45 minutes to reach room temperature before it was ready for use.

Appendix 3.

Research presented to learned societies.

Fitzhenry R.J., Hartland E., Pickard D.J., Dougan G., Frankel G. and Phillips A.D. Intimin and enteropathogenic and enterohaemorrhagic *Escherichia coli* intestinal tissue tropism. World Congress of Paediatric Gastroenterology, Hepatology and Nutrition, Boston, USA. August 2000.

Abstract selected for oral presentation. Awarded Young Investigator Travel Award.

Fitzhenry R.J., Navabpour S., Trabulsi L.R., Frankel G. and Phillips A.D. O55 serogroup and O157:H7 show the same tissue tropism in man. Verotoxigenic *E. coli* 2000 (VTEC 2000), Kyoto, Japan. November 2000.

Publications arising from this thesis.

Fitzhenry R.J., Pickard D.J., Hartland E.L., Reece S., Dougan G., Phillips A.D., Frankel G. Intimin type influences the site of human intestinal mucosal colonisation by enterohaemorrhagic *Escherichia coli* O157:H7. Gut. 2002 Feb; 50(2):180 - 185.

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INTESTINAL MICROFLORA AND INFECTION

Intimin type influences the site of human intestinal mucosal colonisation by enterohaemorrhagic *Escherichia coli* O157:H7

R J Fitzhenry, D J Pickard, E L Hartland, S Reece, G Dougan, A D Phillips, G Frankel

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See end of article for authors' affiliations

Correspondence to: Dr A D Phillips, Centre for Paediatric Gastroenterology, Lower Third Floor, Department of Paediatrics and Child Health, Royal Free Hospital, Pond St, London NW3 2QG, UK; adphill@rfc.ucl.ac.uk

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Background: Enterohaemorrhagic (EHEC) and enteropathogenic (EPEC) *Escherichia coli* epithelial cell adhesion is characterised by intimate attachment, and attaching and effacing (A/E) lesion formation. This event is mediated in part by intimin binding to another bacterial protein, Tir (translocated intimin receptor), which is exported by the bacteria and integrated into the host cell plasma membrane. Importantly, EPEC (O127:H6) and EHEC (O157:H7) express antigenically distinct intimin types known as intimin α and γ, respectively. EHEC (O157:H7) colonises human intestinal explants although adhesion is restricted to the follicle associated epithelium of Peyer's patches. This phenotype is also observed with EPEC O127:H6 engineered to express EHEC intimin γ.

Aims: To investigate the influence of intimin on colonisation of human intestine by *E coli* O157:H7, and intimin types on tissue tropism in humans.

Methods: Human intestinal in vitro organ culture with wild type and mutant strains of O157:H7 were employed.

Results: Introducing a deletion mutation in the *eae* gene encoding intimin γ in EHEC (O157:H7) caused the strain (ICC170) to fail to colonise human intestinal explants. However, colonisation of Peyer's patches and A/E lesion formation were restored with intimin γ expression from a plasmid (ICC170 (pICC55)). In contrast, complementing the mutation with intimin α resulted in a strain (ICC170 (pCVD438)) capable of colonising and producing A/E lesions on both Peyer's patch and other small intestinal explants.

Conclusion: Intimin is necessary for human intestinal mucosal colonisation by *E coli* O157:H7. Intimin type influences the site of colonisation in a Tir type independent mechanism; intimin γ appears to restrict colonisation to human follicle associated epithelium.

Verocytotoxin (or Shiga toxin) producing *Escherichia coli* (VTEC or STEC) are an important example of an emerging group of microbial pathogens associated with food poisoning.[1] Certain VTEC, for example *E coli* O157:H7, can cause haemorrhagic colitis and haemolytic uraemic syndrome in humans and are termed enterohaemorrhagic *E coli* (EHEC).

EHEC, in common with enteropathogenic *E coli* (EPEC), can form characteristic attaching and effacing (A/E) lesions on mammalian cells growing in culture[2] and in animals.[3] Although EHEC O157:H7 was believed to colonise the human large intestinal mucosa,[1] association of EHEC and A/E lesion formation on human tissue had not been demonstrated until recently[4] when we showed, using in vitro organ culture (IVOC) of paediatric intestinal mucosa, that EHEC has a distinct tropism for the follicle associated epithelium (FAE) of ileal Peyer's patches where it causes A/E lesions. In contrast, no adhesion was evident on the mucosal surface of proximal or distal small intestine or colon.[4] This observation contrasted with the adhesion pattern exhibited by EPEC, which included the entire small intestinal mucosa with some colonic colonisation.[4]

The first gene to be associated with A/E activity was the EPEC *eae* gene encoding intimin, an outer membrane adhesion molecule essential for intimate bacterial attachment to eukaryotic host cells.[5] The *eae* gene has also been detected in EHEC O157[6] and other EHEC serogroups,[7] and has been shown to be required for colonisation of O157:H7 in animal models.[8 9]

The intimin encoding *eae* gene is part of a pathogenicity island found in EPEC and EHEC termed the locus of enterocyte effacement (LEE).[10 11] In addition to intimin, the LEE also encodes a type III secretion system (reviewed by Frankel and colleagues[12]) an intimin receptor (Tir/EspE),[13 14] and three secreted proteins EspA, EspB, and EspD, which are required for signal transduction in host cells and A/E lesion formation.[12 15] EspA is a structural protein and a major component of a large filamentous organelle that is transiently expressed on the bacterial surface and interacts with the host cell during the early stage of A/E lesion formation.[16 17] EspA filaments may contribute to bacterial adhesion but of greater significance is that they appear to be a component of a translocation apparatus and as such are essential for the translocation of EspB[16] and Tir[13] into host cells. Of note is the fact that there is a large amount of divergence between EHEC and EPEC LEE encoded gene products which interact directly with the host (that is, intimin, Tir, and the Esps),[11] and that the EPEC LEE is necessary and sufficient for A/E lesion formation[18] whereas EHEC LEE is necessary but not sufficient.[19]

At least five distinct intimin types, designated α, β, γ, δ, and ε, have been identified thus far.[20 21] Importantly, intimin α is specifically associated with one evolutionary branch of EPEC known as EPEC 1, intimin β is associated with both EPEC and

Abbreviations: EHEC, enterohaemorrhagic *Escherichia coli*; EPEC, enteropathogenic *E coli*; A/E, attaching and effacing; Tir, translocated intimin receptor; IVOC, in vitro organ culture; LEE, locus of enterocyte effacement; VTEC, verocytotoxigenic *E coli*; STEC, Shiga toxigenic *E coli*; FAE, follicle associated epithelium; Esp, EPEC secreted protein; FAS, fluorescent actin staining; SEM, scanning electron microscopy; PCR, polymerase chain reaction.

Table 1 Plasmids used in this study

Plasmid	Properties	Reference
pCVD444	pUC18 encoding intimin γ from EHEC O157:H7	Yu and Kaper[6]
pCVD438	pACYC184 encoding intimin α from EPEC O127:H6	Donnenberg and Kaper[29]
pICC55	A pCVD438 derivative encoding recombinant intimin γ	Hartland and colleagues[33]
pCVD438/01	pACYC184 encoding intimin α containing a C937A substitution	Frankel and colleagues[32]

EHEC belonging to their respective clone 2, whereas intimin γ is specifically associated with EHEC O157 and its related strain EPEC O55:H7.[22] Studies on the different intimins from EPEC and EHEC have shown that receptor binding activity is localised to the C terminal 280 amino acids (Int280).[23] A number of groups have reported that intimin can bind directly to uninfected host cells[23–25] and to Tir.[11 14 24] Binding to the host cell but not to Tir is dependent on a disulphide bridge at the carboxy terminus of Int280.[24] However, when expressed on the surface of EPEC, both of these binding activities of intimin are required for intimate bacterial adhesion and A/E lesion formation.

Recent results suggested that different intimin types might play a role in determining the pattern of colonisation and tissue tropism in the host. Among these are intimin exchange studies performed in piglets[26] and our recent investigation, using human intestinal explants, showing that while EPEC expressing intimin α colonised Peyer's patch as well as proximal and distal small intestinal tissue,[4] a restricted pattern of tissue tropism towards Peyer's patches was observed following expression of intimin γ from EHEC in the EPEC background.[27] The aim of this study was to test the importance of intimin in the colonisation of human intestinal mucosa by O157:H7 and to investigate the influence of intimin on tissue tropism when expressed in an EHEC background.

METHODS

Bacterial strains and plasmids

EHEC O157:H7, strain 85-170 expressing intimin γ, is a spontaneous Stx negative derivative of EHEC 84-289 which was originally isolated from a food handler in a Canadian nursing home.[28] CVD206 is an EPEC O127:H6 harbouring a deletion mutation in its *eae* gene encoding intimin α.[29] Prior to adhesion studies, bacteria were subcultured into brain heart infusion broth and incubated aerobically overnight at 37°C without agitation. When appropriate, chloramphenicol was added to a final concentration of 30 μg/ml. The plasmids used in this study are listed in table 1.

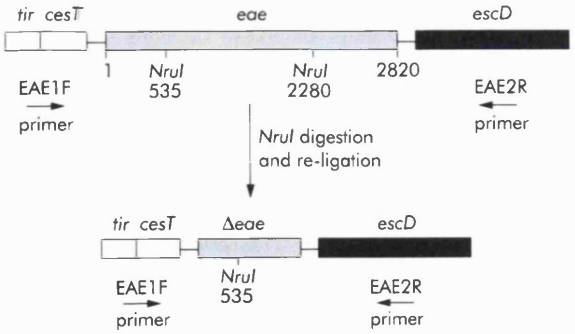

Figure 1 Schematic representation showing construction of the *eae* deletion of enterohaemorrhagic *Escherichia coli* (EHEC) strain 85-170. *Nrul* endonuclease digestion of plasmid pCVD444 was used to introduce deletion in the *eae* gene encoding intimin γ. Following re-ligation and polymerase chain reaction amplification, the DNA fragment containing the deleted form of the *eae* gene was cloned in the suicide vector pCVD442 and the deletion was introduced into EHEC by allelic exchange, as described in experimental procedures. The numbers represent nucleotides within the structural *eae* gene. The genes upstream (3′ end of *tir* and *cesT*) and downstream (*escD*) of *eae* are also indicated. (Not to scale.)

Construction of an *eae* deletion mutant of EHEC strain 85-170

In order to introduce a deletion mutation into the *eae* gene, we took advantage of two unique *NruI* restriction sites in pCVD444[6] which are located in the coding region of the gene (fig 1). Following digestion and gel purification, the plasmid was self ligated, resulting in a plasmid which contains an 1873 bp deletion in the *eae* gene (fig 1). Using polymerase chain reaction (PCR) primers EAE1F (5′ TCTATTCCCGGGAATGAAAACAGATTGTGTTCTTTTGC 3′; positioned at 16 001 to 16 037 bp of the LEE region from EHEC O157:H7) and EAE2R (5′ AGAACATTCCCGGGTACATTTCAGCAGATATTTTTCCC 3′, positioned at 19 759 to 19 722 bp of the LEE region (accession number AF071034)), containing a 5′ *SmaI* site (underlined), the deleted *eae* gene and flanking DNA were recovered on a 1885 bp fragment. This fragment was gel purified and ligated into a *SmaI* digested suicide vector, pCVD442. The recombinant pCVD442 was then used to electroporate EHEC O157:H7 *eae* positive strain 85-170. Ampicillin resistant colonies were isolated and plated onto 10% sucrose L-agar plates, grown overnight at 30°C, to select for double crossover isolates. Ampicillin sensitive clones containing the *eae* deletion were identified by PCR amplification using primers EAE1F and EAE2R. A PCR product of 1885 bp, expected from the *eae* deleted clones, was obtained from over 50% of the sucrose resistant and ampicillin sensitive colonies. The rest of the colonies produced a PCR product of 3758 bp fragment similar to the wild type strain.

Detection of intimin expression by western blotting and fluorescent actin staining (FAS)

Expression of the intimin derivatives was determined by western blots. For immunodetection of intimin in whole cell extracts, stationary L-broth cultures were diluted 1:100 in Dulbecco's modified Eagle's medium and incubated for three hours at 37°C. An equivalent of an optical density 600 (OD_{600}) of 0.5 was loaded onto 7.5% sodium dodecyl sulphate-polyacrylamide gel electrophoresis, as described previously. The electrophoresed polypeptides were transferred onto a nitrocellulose membrane and immunodetection of intimin was performed using a universal rabbit intimin antiserum, raised against a conserved region of intimin ($Int_{387-666}$), diluted 1:500 as described previously.[20 30] FAS test, to detect A/E lesion formation on infected HEp-2 cells, was performed as described previously.[2]

Tissue samples

Human tissue was obtained, with fully informed parental consent and local ethics committee approval, during routine investigation of patients for potential intestinal disorders. Mucosal biopsies of proximal small intestinal mucosa (fourth part duodenum), terminal ileum, Peyer's patches, and transverse colon were taken using a grasp biopsy forceps during routine endoscopy (Olympus PCF paediatric endoscope).

Table 2 Regional adherence of bacterial strains. Number of samples showing attaching/effacing lesions out of the samples tested

Strain used	Region of intestine tested			
	Duodenum	Terminal ileum	Peyer's patch	Colon
85/170	0/3	0/4	3/3	0/4
Age 85/170	43, 164, 212	24, 29, 127, 148	42, 46, 96	38, 82, 149, 198
ICC170	ND	ND	0/3	ND
Age ICC170			82, 82, 170	
ICC170 (pICC55)	0/4	ND	2/3	ND
Age ICC170 (pICC55)	61, 68, 113, 149		80, 133, 149	
ICC170 (pCVD438)	9/9 (4 adhesion only)	4/4 (2 adhesion only)	3/3	0/3
Age ICC170 (pCVD438)	68 (34–175)	24, 29, 38, 131	46, 46, 71	82, 116, 170
CVD206 (pCVD438)	9/9 (3 adhesion only)	ND	ND	ND
Age CVD206 (pCVD438)	68 (34–175)			
85/170 (pCVD438–01)	0/4	ND	0/3	ND
Age 85/170 (pCVD438–01)	66, 106, 131, 175		65, 142, 170	

Age in months, when more than four samples were tested the age is shown as median (range).
ND, not done.

Peyer's patches can be recognised and selectively biopsied during endoscopy, a technique made easier by the application of video endoscopy. Median ages and age ranges of the patients are shown in table 2. All endoscopic biopsies were taken from areas showing no obvious pathology or other abnormality, and all intestinal histology was reported to be normal in the material used in this study.

In vitro organ culture

In vitro organ culture (IVOC) was performed as described previously for eight hours.[31] Each bacterial strain was examined in human IVOC on at least three occasions using tissues from different children. An uninoculated specimen was included with each experimental culture to exclude the possibility of in vivo bacterial colonisation. After incubation with bacteria or appropriate control solutions, IVOC specimens were washed thoroughly three times to remove any non-adherent bacteria and then prepared for scanning electron microscopy (SEM), as described previously.[31] Samples were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer, post-fixed in 1% aqueous osmium tetroxide, and dehydrated in 2,2 dimethoxy-propane. Specimens were transferred to absolute ethanol, critically point dried using liquid carbon dioxide in an Emitech K850 apparatus, mounted on aluminium stubs, sputter coated with gold-palladium using a Polaron E5100 sputter coater, and viewed in a JEOL 5300 SEM at an accelerating voltage of 30 kV.

RESULTS

Construction of an *eae* deletion mutant of EHEC strain 85-170

In a previous study we have shown that EPEC expressing intimin α colonised any region of the IVOC small intestinal mucosa while EHEC expressing intimin γ showed restricted tropism towards the human Peyer's patch mucosa. The aim of this study was to determine the outcome of infection of different regions of the human gut with an EHEC *eae* null mutant (strain ICC170) and with ICC170 complemented with *eae* encoding either intimin γ or intimin α.

For this purpose, we introduced an *eae* deletion mutation in EHEC strain 85-170 (see methods and fig 1), generating strain ICC170. Confirmation of the deletion was achieved by PCR (see methods; data not shown). The *eae* mutation in ICC170 was complemented in trans with pCVD438, encoding intimin α from EPEC.[29] In addition, ICC170 was complemented, as controls, with pCVD438/01 encoding a biologically inactive form of intimin α in which Cys at position 937 was replaced with Ser,[32] or pICC55, a pCVD438 derivative containing the receptor binding domain of intimin γ on a cloned intimin α backbone.[27 33] Expression of the different intimins was determined by western blot analysis of whole cell lysates prepared from the ICC170 derivatives using a universal broad spectrum polyclonal intimin antiserum, reactive with all the different intimin types[30] (fig 2). Lysates from all the recombinant strains, but not from ICC170, reacted similarly with the antiserum (fig 2 and data not shown).

Interaction of the recombinant ICC170 strains with HEp-2 cells and human intestinal IVOC

Before the recombinant ICC170 strains were tested in the human intestinal IVOC model, the ability of the strains to mediate A/E lesion formation on HEp-2 cells was investigated. ICC170 (pCVD438) and ICC170 (pICC55) adhered to the cell monolayers and produced an FAS positive reaction, as did the parent 85-170 strain, while ICC170 and ICC170 (pCVD438/01) were unable to induce reorganisation of the host cell cytoskeleton (data not shown). The results show that ICC170 expressing both intimin α and intimin γ can mediate A/E lesion formation on cultured human epithelial cells.

In previous studies we have shown that the ability of EPEC to induce A/E lesions on human intestinal IVOC is dependent on surface expression of biologically active intimin[31] and that the type of intimin expressed determine tropism to different regions of the gut.[27] To determine the importance of intimin in colonisation and tissue specificity in an EHEC background, we investigated the ability of the recombinant ICC170 strains to mediate A/E lesion formation on mucosal surfaces, using endoscopically and histologically normal paediatric tissue.

The domed mucosal surface overlying individual lymphoid follicles within Peyer's patches can be easily recognised by SEM (fig 3A). Thus bacterial adhesion to FAE can be discriminated from other sites. We repeated experiments reported

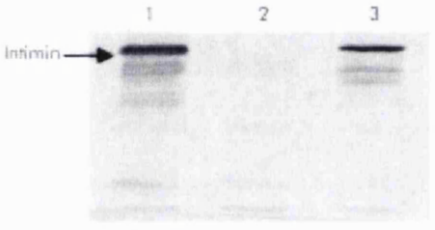

Figure 2 Western blot analysis of whole cell lysates prepared from enterohaemorrhagic *Escherichia coli* (EHEC) 85-170 and the ICC170 derivatives using a universal broad spectrum polyclonal intimin antiserum.[30] Lysates from the wild type (lane 1) and from ICC170 (pCVD438) (lane 3) strains reacted with the intimin antiserum, but not from ICC170 (lane 2).

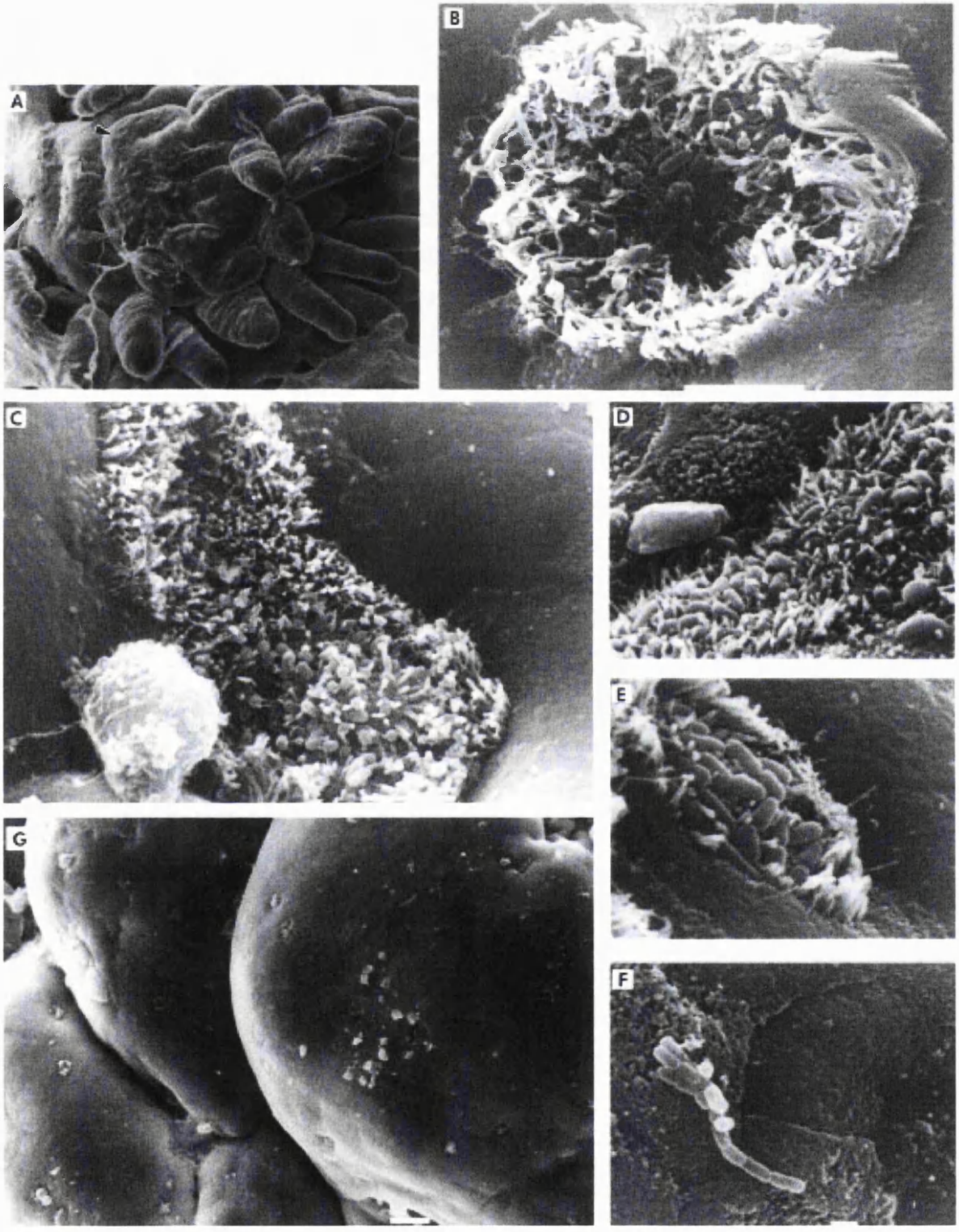

Figure 3 (A) Lymphoid follicles within Peyer's patch of distal ileum (arrow); bar=100 μm. (B) ICC170 (pICC55) adhering to follicle associated epithelium (FAE) and showing attaching and effacing (A/E) lesion formation; bar=5 μm. (C–E) ICC170 (pCVD438) A/E lesion formation on the FAE of the Peyer's patch, duodenum, and ileum, respectively; bar=5 μm, 1 μm, and 1 μm, respectively. (F) ICC170 (pCVD438) adhesion to the duodenum without A/E lesion formation; bar=1 μm. (G) Lack of adhesion of ICC170 (pCVD438/01) to the ileum; bar=10 μm.

previously[4] and again demonstrated a restricted pattern of tissue tropism for O157:H7 strain 85-170 to FAE of Peyer's patches. The *eae* deletion mutation strain ICC170 did not adhere to FAE whereas adhesion of the intimin γ complemented strain ICC170 (pICC55) was, in common with the parent 85-170 strain, limited to FAE (fig 3B; table 2). SEM results were typical of A/E lesion formation. In contrast, the intimin α complemented strain ICC170 (pCVD438) adhered to FAE of Peyer's patches as well as to proximal and distal small intestinal mucosa (fig 3 C–E, respectively). However, unlike E2348/69 expressing intimin α,[4 31] adhesion of ICC170 (pCVD438) (fig 3F) and CVD206 (pCVD438) (data not shown) to duodenum and terminal ileum was not always associated with A/E lesions (table 2). In contrast, and in agreement with previous reports,[31 32] ICC170 (pCVD438/01) (fig 3G), similar to ICC170 itself, did not adhere to intestinal IVOCs (table 2). These results show that intimin, as in EPEC, is essential for colonisation of human mucosa by EHEC and that expressing intimin α in EHEC allows the strain to extend colonisation to include the small intestine in humans.

DISCUSSION

The genetic basis of A/E lesion formation is well documented.[1 10 12 15] However, relatively little is known about the initial intestinal stage of EHEC infection involving colonisation of the gut. The only EHEC adherence factor that has been demonstrated to play a role in intestinal colonisation in vivo in animal models is the 94 to 97 kDa adhesion molecule intimin.[8 9] The importance of intimin in human disease has been shown for EPEC infection by volunteer studies in which an *eae* mutant was significantly attenuated compared with the wild type parent strain[34] and infection of human intestinal IVOC, in which the *eae* mutant was unable to colonise the tissue.[31] The importance of intimin in human disease is also supported by the presence of a high titre of serum intimin antibodies in individuals infected with EHEC[35] and in the colostrum of mothers in Brazil where EPEC infection is endemic.[36] In this study we introduced an *eae* deletion mutation into EHEC strain 85-170 (producing strain ICC170). The *eae* mutation rendered the strain incapable of colonising any region of the human intestinal IVOC mucosa. This result adds yet another layer of evidence for the contribution of intimin to EHEC infection in humans.

Recently, we used IVOC to demonstrate that EHEC O157:H7 shows a distinct tropism for the follicle associated epithelium (FAE) of ileal Peyer's patches where it caused A/E lesions, but no adhesion was evident on the mucosal surface of proximal or distal small intestine or colon.[4] This observation contrasts with the ability of EPEC to efficiently colonise any region of the human small intestinal mucosa and inefficiently the colonic mucosa.[4] The different tissue specificity exhibited by EPEC and EHEC on human intestinal IVOC prompted us to investigate the contribution of intimin types to tissue tropism. We addressed this question using isogenic EPEC derivatives expressing either intimin α or intimin γ and IVOC from different regions of the human gut. We showed that when an *eae* EPEC mutant CVD206[29] was complemented with *eae*α (strain CVD206 (pCVD348)), it efficiently colonised small, but not large, intestine in a similar manner to the intimin α expressing wild type EPEC (E2348/69). In contrast, complementing CVD206 with pBE310 or pICC55 encoding intimin γ resulted in strains which targeted the FAE of Peyer's patches, similarly to intimin γ expressing EHEC.[27] In this paper, we described further work in which ICC170 was complemented with pCVD438 encoding the EPEC intimin α. Using ICC170 (pCVD438) in combination with IVOC, we reported that the change in intimin type resulted in colonisation spreading from the FAE—that is, typical of the parent and ICC170 (pICC55) strains—to villous regions of the proximal and distal small intestine. Importantly, although in most cases adhesion to duodenum and terminal ileum was associated with A/E lesions, in some cases adhering bacteria were seen on the surface of the biopsy with no evidence of A/E lesions. This observation is the first example in which adhesion to the human mucosa can be separated from A/E lesion formation. The reason for this phenomenon is intriguing and is currently under investigation, although it might simply be due to the fact that the dynamics of A/E lesion formation observed in the current study are delayed and the eight hour duration of IVOC was not sufficient to allow the lesion to develop fully. This is supported by the observation that the intimin α expressing EPEC strain (CVD206 (pCVD438)) also showed a similar phenomenon. In addition, attachment of intimin positive bacteria without A/E lesion formation suggests that intimin contributes to initial adhesive events. Although definitive evidence to support this contention is lacking at the present time, the fact that intimin contributes to tissue tropism and host specificity, despite A/E lesions being end points in both instances, suggests that binding of intimin to a host cell receptor during ex vivo and in vivo infections occurs before intimin-Tir binding.[37]

EHEC adhesion to FAE of Peyer's patches appears to be a specific event, and several bacteria express adhesins promoting binding to these regions (for example, long polar fimbriae in *Salmonella*[38] and AF/R1 in RDEC-1[39]; strains not expressing these adhesins show reduced Peyer's patch adhesion and less virulence).[38 40] Indeed, the recently reported complete genome sequence of O157:H7 has identified several fimbrial operons, including with homology to long polar fimbriae,[41] which might therefore, in addition to intimin, contribute to Peyer's patch adhesion. Specialised cells within the FAE, termed M (microfold) cells (reviewed by Neutra and colleagues[42]) have been described as targets of bacterial cell adhesion, invasion, and damage in animals.[43] These cells are thought to function normally as antigen sampling cells, and bacterial pathogens appear to have evolved mechanisms to use this route as a means of colonisation and/or entry into the host. Differences in expression of apical membrane proteins (β-1 integrins[44]) and carbohydrates[45] on M cells in animal species in comparison with absorptive epithelium have provided explanations for this selective adhesion. However, these differences have not been fully established in human tissue[46] and their role in human infectious disease in general, and in *E coli* O157:H7 infection in particular, merits further study. More generally, the FAE has different characteristics to villous epithelium and these may be factors that influence bacterial adhesion. These include differences in the thickness of the glycocalyx,[47] reduction in goblet cell numbers, increased presence of intraepithelial lymphocytes, and the close proximity of lymphoid follicular cells. An important challenge for future work is to determine if any of these factors influence intimin mediated tissue tropism.

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Authors' affiliations

R J Fitzhenry, A D Phillips, Centre for Paediatric Gastroenterology, Department of Paediatrics and Child Health, Royal Free Hospital, London, UK

D J Pickard, E L Hartland†, S Reece, G Dougan, G Frankel, Centre for Molecular Microbiology and Infection, Department of Biochemistry, Imperial College of Science, Technology and Medicine, London, UK

†Current Address: Department of Microbiology, Monash University, Melbourne, Australia

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Tissue Tropism of Enteropathogenic *Escherichia coli* Strains Belonging to the O55 Serogroup

R. J. Fitzhenry,[1] S. Reece,[2] L. R. Trabulsi,[3] R. Heuschkel,[1] S. Murch,[1] M. Thomson,[1] G. Frankel,[2] and A. D. Phillips[1]*

Centre for Paediatric Gastroenterology, Royal Free Hospital and University College Medical School,[1] and Centre for Molecular Microbiology and Infection, Department of Biological Sciences, Imperial College of Science, Technology, and Medicine,[2] London, United Kingdom, and Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, Brazil[3]

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Four enteropathogenic *Escherichia coli* (EPEC) strains belonging to the O55 serogroup (G21 and G30 [both O55:H6], G35 [O55:H−], and G58 [O55:H7]) were tested for their tissue tropism by using human intestinal in vitro organ culture. Strains showed restricted adhesion with attaching-and-effacing activity to follicle-associated epithelium of Peyer's patches, with no apparent adhesion to duodenum or colon. G35 and G58 express intimin γ and show a similar tropism to intimin γ-expressing enterohemorrhagic *E. coli* (EHEC) O157:H7. However, strains G21 and G30 were unusual because they expressed intimin α and had a restricted tissue tropism of intimin γ phenotype. The amino acid sequence of the carboxy-terminal 280 amino acids of intimin from G21 was determined. Comparison with the prototype intimin α from strain E2348/69 (O127:H6) showed a single amino acid difference (corresponding to Val907 and Ala907 in the whole intimins). This mutation was reproduced by site-directed mutagenesis in an intimin α plasmid template, pCVD438, with the hypothesis that it may induce a change in tropism. However, when the mutated plasmid was placed in both EPEC and EHEC backgrounds, duodenal adhesion in a manner similar to strain E2348/69 was evident upon in vitro organ culture. Thus, additional factor(s) unrelated to intimin exist in the O55:H6 genome that influence human intestinal tissue tropism.

Enteropathogenic *Escherichia coli* (EPEC) are an important cause of acute and prolonged diarrhea in the developing world with between 30 to 40% of diarrhea in the first year of life being attributed to EPEC (31).

E. coli is a clonal species, with clones identified on a combination of O and H antigens. The O antigen is part of the lipopolysaccharide present in the outer membrane of gram-negative bacteria (42, 45). *E. coli* serogroup O55 is the third most frequent EPEC O serogroup involved in infantile diarrhea, and O55:H6, O55:H7, and O55:H− account for 90% of the strains isolated (37). EPEC and enterohemorrhagic *E. coli* (EHEC) can be divided into two major groups of related clones, designated EPEC clones 1 and 2 and EHEC clones 1 and 2. EPEC clone 1 typically expresses flagellar antigen H6, whereas EPEC clone 2 expresses flagellar antigens H2 or H−. EHEC clone 1 includes the O157:H7 serogroup, whereas EHEC clone 2 contains other Shiga toxin-positive *E. coli* serogroups. In addition, EHEC strains belonging to clone 2 are closely related to the typical EPEC strains, whereas EHEC O157:H7 is related to the atypical EPEC O55:H7 (12).

Typical EPEC strains are classified as being positive for gene probes to *eae* (encoding intimin), EAF (EPEC adherence factor plasmid), and *bfp*A (encoding bundlin, the subunit pillin of the bundle-forming pilus) (20). The O55:H− strains are considered to have lost their flagella by mutation, since the nonmotile derivatives and the motile progenitor usually have the same biochemical properties. Therefore, it has been suggested that H− strains of the O55 serogroup are derived from H6 or H7 strains (37).

EPEC and EHEC have the ability to produce attaching-and-effacing (A/E) lesions on enterocytes (30, 35, 44). The A/E lesion is characterized by localized destruction of the brush border microvilli with intimate attachment of the bacteria to the enterocyte. The area underlying the bacteria consists of polymerized actin, ezrin, talin, and myosin (14) and the actin-regulating proteins WASP (Wiskott-Aldrich syndrome family of proteins) and Arp 2/3 (actin-related proteins 2 and 3 complex) (19). This host cell-bacterium interface may be raised above the host cell surface producing a pedestal. The genes necessary and sufficient for the production of an A/E lesion by EPEC are located on a pathogenicity island termed the locus of enterocyte effacement (LEE) (28). EHEC and other A/E bacteria, such as *Citrobacter rodentium*, also contain the LEE (4, 27) but in EHEC the LEE is necessary but not sufficient for A/E lesion production (8). Intimin, a 94- to 97-kDa protein encoded by the *eae* gene within the LEE, is an adherence factor, which has been shown to play a key role in intestinal colonization (12). Intimin mediates intimate attachment to the epithelial cell by binding to another LEE-encoded protein, the translocated intimin receptor (Tir), which is delivered into the host cell by a type III secretion system (22). The importance of intimin in human disease is highlighted by an *eae* mutant being significantly attenuated in human volunteers compared to the wild-type parent strain (7) and by the presence of a high titer of serum intimin antibodies in both individuals infected with EHEC (18) and in the colostrum of mothers in Brazil where EPEC infection is endemic (25).

* Corresponding author. Mailing address: Centre for Paediatric Gastroenterology, Lower 3rd Floor, Royal Free Campus, Royal Free Hospital and University College Medical School, Rowland Hill St., London NW3 2PF, United Kingdom. Phone: 44-20-7830-2783. Fax: 44-20-7830-2146. E-mail: adphill@rfc.ucl.ac.uk.

TABLE 1. List of strains and plasmids used during this study

Strain or plasmid	Description	Source or reference
XL1-Blue	*EndA* -ve competent *E. coli* K-12 strain	Stratagene
CVD206	*eae* deletion mutant of O126:H7 strain E2348/69	5
ICC170	*eae* deletion mutant of O157:H7 strain 85/170	9
G21	Intimin α, EAF+, Bfp+, O55:H6	1, 37
G30	Intimin α, EAF+, Bfp+, O55:H6	1, 37
G35	Intimin γ, EAF+, Bfp+, O55:H−	1, 37
G58	Intimin γ, EAF−, Bfp+, O55:H7	1, 37
pCVD438	pACYC184 encoding intimin α	5
pCPG1	pMAL-c2 encoding intimin $\alpha_{O55:H6}$	This study
pCPG2	pACYC184 encoding intimin α_{A907V}	This study

To date, five intimin types have been identified and designated α, β, γ, δ, and ε (1, 32). Intimin α and intimin γ are associated with different evolutionary branches of EPEC and EHEC. Intimin α is associated with EPEC branch 1 and intimin γ is specifically associated with EHEC O157:H7 and the related EPEC O55:H7 (46). Intimin exchange studies in piglets have shown that varying the intimin type can alter the site of intestinal colonization by EHEC (43). This activity of intimin was further characterized by using in vitro organ culture (IVOC). The prototype intimin α-expressing strain O127:H6 E2348/69 produces A/E lesions on duodenum, terminal ileum, Peyer's patches and, in a small percentage of incubations, on the colon (33). In contrast, intimin γ-expressing EHEC exhibited a restricted tropism toward the follicle-associated epithelium (FAE) of Peyer's patches (35). When intimin γ from O157:H7 was placed in an *eae* mutant strain of E2348/69, adherence to the FAE predominated, thus mimicking the restricted tissue tropism of O157:H7 (35). In contrast, placing intimin α in an *eae* mutant strain of O157:H7 resulted in colonization of the small intestine, as well as Peyer's patches (9). Thus, intimin is able to modulate the tissue tropism of EPEC and EHEC on human intestinal explants, producing characteristic intimin α- and γ-related phenotypes.

The receptor binding activity of intimin has been localized to the C-terminal 280 amino acids (Int280) (10). The global fold of Int280α was determined by nuclear magnetic resonance (21) and crystallography (26), which showed that it is built from three globular domains with the first two comprising β-sheet sandwiches. The third C-terminal domain (residues 183 to 280) has a topology that resembles C-type lectin domains (CTLD), a family of proteins responsible for cell surface carbohydrate recognition. Analysis of intimin-Tir complexes revealed amino acids likely to be involved in binding (3, 26) and showed these residues to be concentrated in a solvent-exposed region of the CTLD. Site-directed mutagenesis of residues within the CTLD was able to modulate the biological activity of intimin; in particular, substituting a valine at 911 with alanine in intimin α produced a derivative, which bound to intestinal mucosa in an intimin γ-like phenotype (36).

We studied the EPEC O55 serogroup, which expresses different intimin types, by using in vitro organ culture to further elucidate the mechanisms involved in tissue tropism.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids used in this study are listed in Table 1. Strains and plasmids were grown overnight at 37°C with shaking (250 rpm) in L broth (LB; Sigma) for DNA sequencing and site-directed mutagenesis; for infection assays, bacterial strains were grown overnight without shaking in brain heart infusion broth.

Human intestinal in vitro organ culture adhesion assay. Tissue was obtained with fully informed parental consent and local ethical committee approval by using grasp forceps during routine endoscopic (Olympus PCF pediatric endoscope) investigation of intestinal disorders. Duodenal, terminal ileal, Peyer's patch, and transverse colonic biopsies were taken from areas showing no macroscopic abnormalities. Light microscopy subsequently showed no histological abnormality. IVOC infections were performed as described previously (15). Briefly, 25 μl of an overnight bacterial culture was inoculated onto the biopsy samples from the various regions of the gut. The IVOC medium was changed every 2 h, and the assay was terminated at 8 h. Each bacterial strain was examined on several occasions by using tissue from different patients. Samples were fixed with 2.5% glutaraldehyde and postfixed in 1% aqueous osmium tetroxide and viewed by scanning electron microscopy (SEM) and transmission electron microscopy (TEM).

DNA sequencing of intimin from O55:H6 strain G21. Genomic DNA from G21 was isolated by using the DNAeasy tissue kit (Qiagen), according to manufacturer's instructions. The 3′ end of the *eae* gene encoding Int280α from O55:H6 was amplified by PCR by using high-fidelity *Pfu* DNA polymerase (1 cycle of 94°C for 1 min, followed by 25 cycles of 94°C for 30 s, 50°C for 1 min, and 74°C for 1 min, followed by 1 cycle of 74°C for 5 min; forward primer, 5′-GGA ATTCATTACTGAGATTAAGGCT-3′; reverse primer, 5′-CGGGATCCTTAT TTTACACAAGTGGC-3′). The amplified DNA and the plasmid pMAL-c2 were cut by using the restriction enzymes *Eco*RI and *Bam*HI. The amplified DNA was inserted into cut pMAL-c2 by ligation with T4 DNA ligase. pMAL-c2 was transformed into *E. coli* XL1-Blue cells. Ampicillin resistant transformants were randomly selected and screened for the appropriate insert. XL1-Blue colonies containing the recombinant plasmid were grown overnight in LB, the plasmids isolated by using the plasmid miniprep kit (Qiagen) and ethanol precipitation. DNA sequencing was performed with 1 to 2 μg of plasmid DNA, and the Int280 forward and reverse primers (36).

Site-directed mutagenesis. Site-directed mutagenesis of intimin α was performed by using the QuickChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions. Plasmid pCVD438 encoding intimin α (5) was used as a template. A complementary mutagenesis oligonucleotide pair incorporating a single amino acid substitution was designed. Primers were as follows: forward primer (5′-CAGCTCAAGATGTGAAGAGTGGTGT TGC-3′) and reverse primer (5′-GCAACACCACTCTTCACATCTTGAGCT G-3′).

Mutated plasmid containing staggered nicks was generated by extension of primers annealed to opposite strands of the denatured plasmid by temperature cycling in the presence of the high-fidelity *Pfu* DNA polymerase. Synthesized DNA containing the desired mutation was selected from the original DNA template by incubation with the enzyme *Dpn*I at 37°C for 1 h, which cleaves the parental DNA at *dam* methylated GATC sequences, leaving the unmethylated newly synthesized mutated plasmid intact. Nicks in the plasmid were repaired after transformation of 1 μl of the synthesized DNA into competent *E. coli* XL1-Blue cells. Chloramphenicol-resistant transformants were randomly selected and grown overnight in LB. Plasmid DNA was isolated by using the plasmid miniprep kit (Qiagen) and ethanol precipitation. Correct incorporation of the mutation was monitored by DNA sequencing by using an automated DNA sequencer. The mutated plasmids were then transformed into *eae* deletion mutants of EPEC strain CVD206 (5) and EHEC strain ICC170 (9).

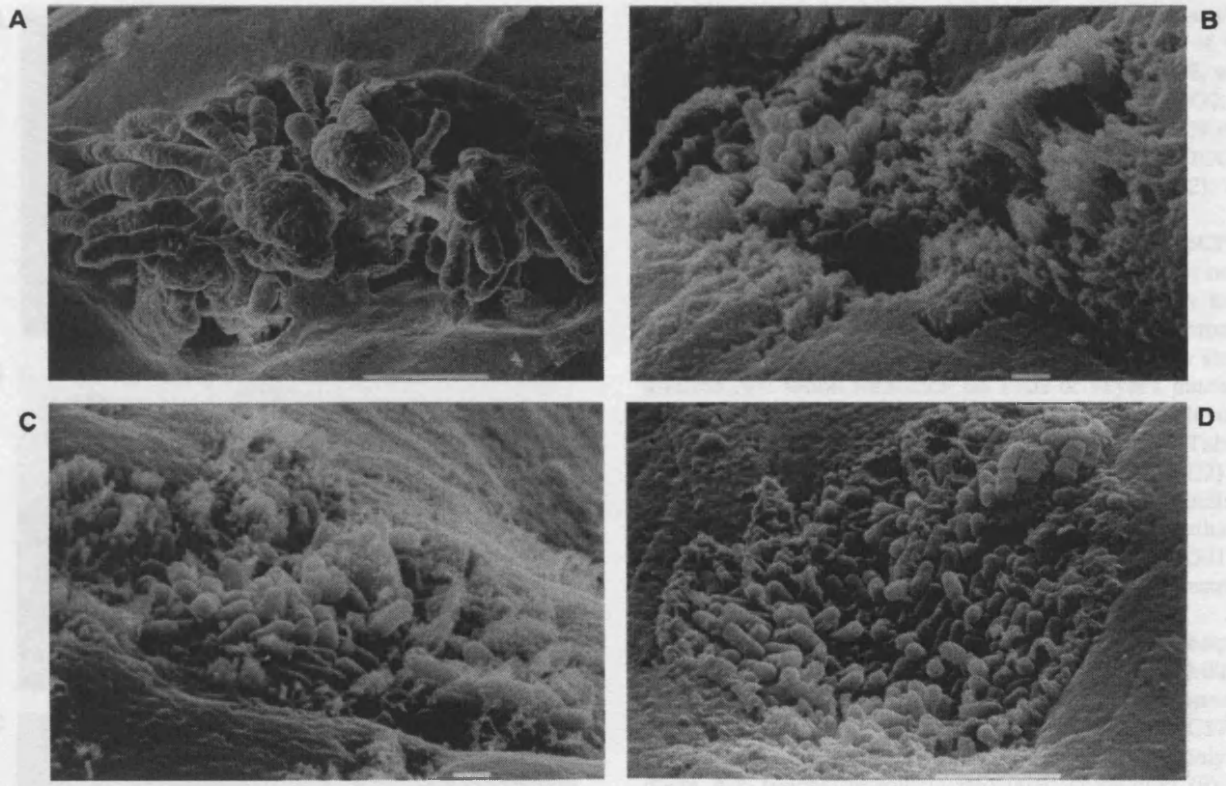

FIG. 1. SEM of IVOC of O55 Strains. (A) Low-power view of lymphoid follicles within IVOC sample from ileal Peyer's patch region. (B to D) O55:H6 (B), O55:H− (C), and O55:H7 (D), showing adhesion of strains to the FAE of an ileal Peyer's patch. Bars: 100 μm (A), 0.5 μm (B and C), 5 μm (D).

RESULTS

IVOC tissue tropism of O55 serogroup strains G21, G30, G35, and G58. Intimin, from EPEC and EHEC, is essential for A/E lesion formation in IVOC (9, 15). All of the EPEC strains expressed intimin, as shown by Western blotting with a universal intimin antiserum (2) (data not shown). SEM of the uninoculated controls showed good preservation of mucosal architecture, the epithelium was intact and there was no evidence of excess extrusion of enterocytes or mucus. All O55 strains showed a restricted pattern of A/E lesion formation, which was limited to FAE overlying ileal Peyer's patches (Fig. 1 and Table 2). Thus, the intimin γ-expressing strains G35 (O55:H−) and G58 (O55:H7) showed the same tissue tropism as EHEC O157:H7 (35), which expresses intimin γ. Strains expressing intimin α can produce A/E lesions on duodenum and ileum, as well as Peyer's patches (9, 33). However, O55:H6 strain G21, which expresses intimin α, did not reproduce this phenotype, forming A/E lesions on Peyer's patches alone. In order to investigate if this phenomenon is unique to strain G21 or common to other O55:H6 strains, a second isolate, strain G30 was tested in IVOC. Like G21, strain G30 did not adhere to duodenum, terminal ileum, or colon. These results indicate that intimin α strains may show a variable pattern of tissue tropism.

TEM was used to confirm the presence of A/E lesions on intestinal Peyer's patch explants (Fig. 2). In all three O55 strains, intimate adherence of bacteria to the enterocyte with effacement of microvilli could be seen. The bacterium-enterocyte interface was primarily seen as a depression, with only a few pedestal-like structures observed. No bacteria were observed within enterocytes.

Sequencing of intimin α from O55:H6 strain G21. Site-directed mutagenesis within the CTLD region of intimin α has shown that the mutation of a specific residue (Val911) can lead to the production of an intimin α-expressing EPEC derivative with a restricted colonization phenotype (36). To determine whether the *eae* gene of G21 contained any variation in this

TABLE 2. Patient age and number of biopsies with adherent bacteria expressed as a proportion of biopsies inoculated

Strain	No. of biopsies with adherent bacteria/no. of biopsies inoculated[a]		
	Small intestine	Peyer's patch	Colon
G21	0/4	2/3	0/3
G30	0/3	ND	0/3
G35	0/4	4/4	0/3
G58	0/7	3/3	0/3
CVD206	0/5	ND	ND
CVD206(pCPG2)	5/5	ND	ND
ICC170(pCPG2)	3/3	ND	ND

[a] The median patient ages (in months) for each group were as follows: small intestine, 115 (range, 9 to 188); Peyer's patch, 73 (range, 30 to 171); and colon, 119 (range, 103 to 167). ND, not done.

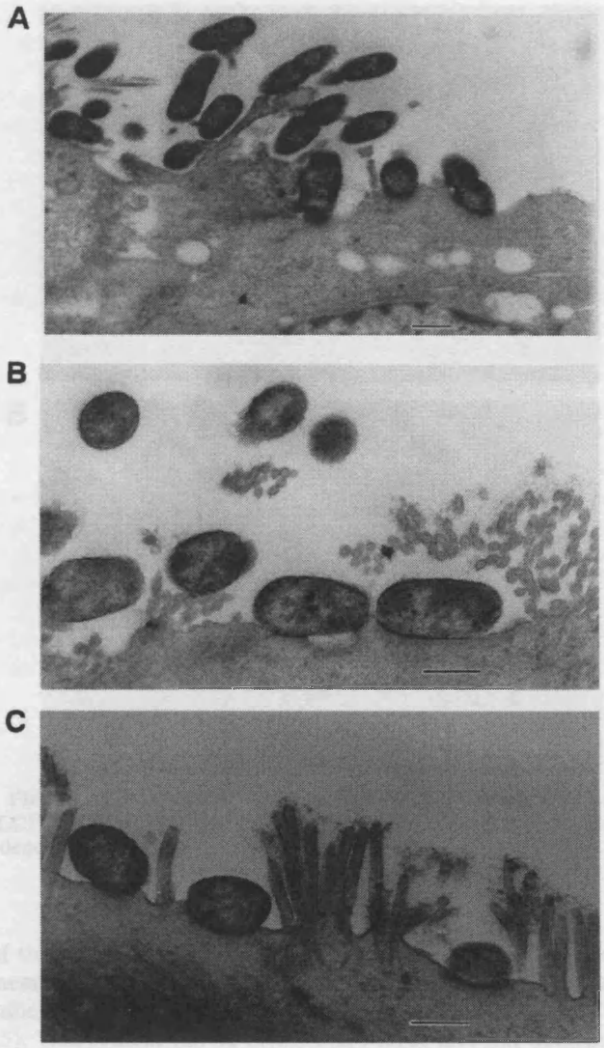

FIG. 2. TEM of IVOC of O55 Strains. (A to C) O55:H6 (A), O55:H− (B), and O55:H7 (C), showing A/E lesions on the FAE of an ileal Peyer's patch. Note the lack of pedestal formation. Bar, 0.5 μm.

region, the carboxy-terminal region encoding Int280 was compared to that of the prototype intimin α from O127:H6 strain E2348/69. This revealed a single amino acid change at position 907 (Fig. 3), i.e., a valine residue in G21 instead of an alanine residue as in E2384/69. In order to assess the influence of the presence of valine at position 907 in the intimin α of G21, site-directed mutagenesis was performed on pCVD438, which contains *eae*α from E2348/69, generating plasmid pCPG2. The plasmid was transformed into the *eae* mutant E2348/69 strain CVD206 and the *eae* mutant O157:H7 85/170 strain ICC170. These strains, CVD206(pCPG2) and ICC170(pCPG2), were tested by using the IVOC assay.

IVOC assay of CVD206(pCPG2) and ICC170(pCPG2). CVD206(pCPG2) was tested for A/E lesion formation on duodenum by using the IVOC assay. It was important to use duodenal explants as A/E lesion formation on the proximal small intestine is a reported characteristic of intimin α strains, whereas A/E lesion formation on FAE of Peyer's patches is synonymous with strains expressing intimin α and with strains expressing intimin γ. CVD206 showed no adhesion (Table 2) as reported earlier in qualitative form. CVD206(pCPG2) produced A/E lesions on the duodenum in a manner similar to that of E2348/69 (Table 2 and Fig. 4A). This indicates that the difference in intimin amino acid sequence between G21 and E2348/69 cannot be the determining factor in the restricted tissue tropism of G21.

The use of the EPEC derived strain CVD206 as a recipient, for pCPG2, may have been a factor in the inability of CVD206(pCPG2) to reproduce the restricted phenotype associated with G21. To test this, pCPG2 was placed in ICC170, an *eae* mutant strain derived from EHEC 85/170 which only produces A/E lesions on follicle-associated epithelium (9). The resultant strain, ICC170(pCPG2), produced A/E lesions on duodenum in a manner similar to that of CVD206(pCPG2) (Table 2 and Fig. 4B). Therefore, the restricted tissue tropism of O55:H6 G21 is associated with a factor distinct from intimin, which is not in the EPEC E2348/69 or the EHEC 85-170 backgrounds.

DISCUSSION

The *E. coli* O55 serogroup is a prominent cause of infantile diarrhea. Each EPEC serogroup is made up of clones with distinct combinations of virulence factors. In the case of the O55 serogroup this results in some strains belonging to the classic EPEC genotype and others having an atypical EPEC genotype with close links to EHEC O157:H7. The similarity between O55:H7 and O157:H7 is demonstrated by their nearly identical sequences for the H7 flagellin gene and the similarity

E69 (741)	VDVKAPEVEFFTTLTIDDGNIEIVGTGVKGKLPTVWLQYGQVNLKASGGN
G21	VDVKAPEVEFFTTLTIDDGNIEIVGTGVKGKLPTVWLQYGQVNLKASGGN
E69 (791)	GKYTWRSANPAIASVDASSGQVTLKEKGTTTISVISSDNQTATYTIATPN
G21	GKYTWRSANPAIASVDASSGQVTLKEKGTTTISVISSDNQTATYTIATPN
E69 (841)	SLIVPNMSKRVTYNDAVNTCKNFGGKLPSSQNELENVFKAWGAANKYEYY
G21	SLIVPNMSKRVTYNDAVNTCKNFGGKLPSSQNELENVFKAWGAANKYEYY
E69 (891)	KSSQTIISWVQQTAQDAKSGVASTYDLVKQNPLNNIKASESNAYATCVK **(939)**
G21	KSSQTIISWVQQTAQDVKSGVASTYDLVKQNPLNNIKASESNAYATCVK

FIG. 3. Comparison of carboxy-terminal amino acid sequences of intimin α from E2348/69 (AAC 38392) and G21 (O55:H6). Amino acids are numbered according to the position in intimin α of E2348/69.

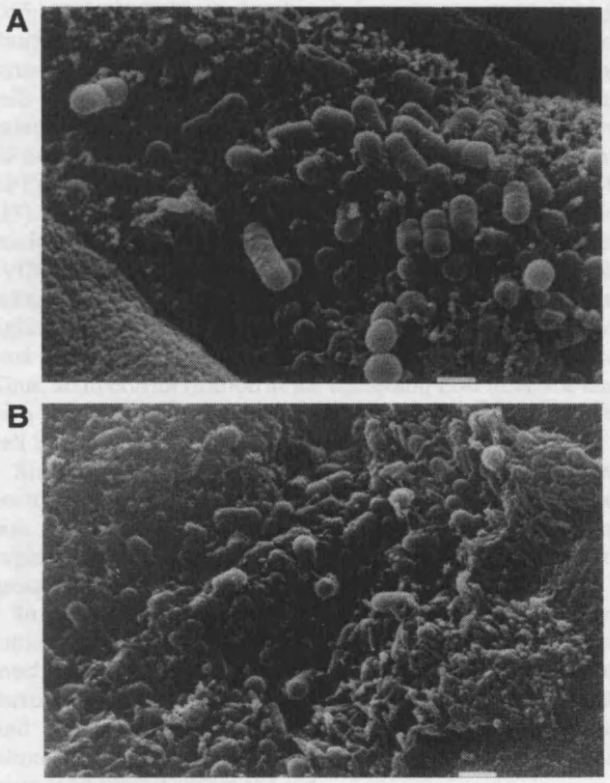

FIG. 4. SEM of IVOC. (A and B) CVD206(pCPG2) (A) and ICC170(pCPG2) (B), both showing A/E lesion formation on the duodenum. Bar, 0.5 μm.

of their *eae* alleles (29, 37). The *eae* gene encodes the outer membrane protein intimin, which allows EPEC and EHEC to adhere to human intestinal cells and produce A/E lesions (9, 15). This protein can be divided into three globular domains. The CTLD, at the carboxy terminus of intimin, is responsible for binding of intimin to its translocated receptor, Tir. The 280-amino-acid carboxy-terminal region also allows intimin to induce microvillus-like processes on Hep-2 cells (34), acts as an immune modulator (16), and may bind a host cell receptor (12). Intimin α has been show to bind β1 integrin in vitro (11), although this process is not essential for A/E lesion formation (23). In addition, intimin γ binds nucleolin, which may act as a host-derived cell surface receptor during the initial stages of EHEC adhesion (40).

Intimin can be divided according to antigenicity, and so far five distinct subtypes have been published (1, 32). The significance of the different subtypes in pathogenesis remains to be fully elucidated. Recent studies have shown a correlation between intimin type and tissue tropism (9, 33). The data showed that intimin α and intimin γ produce different tropism phenotypes in a manner that is Tir type independent. Using IVOC, intimin α has been shown to allow both EPEC and EHEC to produce A/E lesions on duodenum and Peyer's patches, whereas intimin γ limits A/E lesion formation to Peyer's patches.

In this study we have shown that EPEC strains from the O55 serogroup, expressing intimin α or intimin γ, adhered preferentially to the FAE of Peyer's patches. In the case of O55:H6 strains G21 and G30, this is the first example of intimin α-expressing strains showing the lack of adherence to the small intestinal villous surface.

A recent study of the biological activity of intimin α highlighted certain important residues involved in intimin-Tir interaction (36). The valine at position 911 is hydrophobic and lies at the top end of the Tir-binding site. It is solvent exposed and as such is ideally placed to interact with Tir (21). Its mutation to an alanine residue resulted in restricted tissue tropism, with A/E lesion formation being limited to the FAE of Peyer's patches. An equivalent alanine substitution at position 906 in intimin γ led to disruption of Tir-binding activity (24). Thus, the removal of a hydrophobic valine residue, within the Tir-binding site of intimin α and intimin γ, and substitution with alanine led to altered binding activity and in the case of intimin α, restricted A/E lesion formation in IVOC. The intimin α of G21 was sequenced and showed a single amino acid mutation at position 907 compared to the intimin α of O127:H6 strain E2348/69. Using the intimin α template plasmid, pCVD438, we constructed an intimin α with a valine at position 907, thus matching G21. When this plasmid (pCPG2) was placed in an EPEC or an EHEC background, it showed a typical intimin α phenotype and adhered to duodenum. Thus, the mutation at position 907 was not a factor involved in the restricted adherence of G21 to FAE of Peyer's patches. It is theoretically possible that residues outside the C-terminal domain of intimin are involved in the determination of tissue tropism, but these residues show >90% homology between the various intimin types, so it remains a remote possibility. It is more likely that the restricted tissue tropism of G21 is determined by other genes which are specific to the O55:H6 genome. Several different infection models have shown that intimin is involved in tissue tropism (9, 33, 36, 43) and thus it appears that an O55:H6 factor is operating to prevent colonization of small intestinal villous surfaces despite the possession of intimin α. If this factor can be identified it will be interesting to see whether it is also present in the intimin γ-positive O55:H−, O55:H7, and O157:H7 strains, which would suggest a multifactorial control of restricted adhesion to the FAE of Peyer's patches.

The restricted adhesion of strain G35 (O55:H−) to Peyer's patches is in line with the fact that it expresses intimin γ, although it also possesses the EAF plasmid (37) and hence the genes for BFP production (13), i.e., it is a typical EPEC (20). Thus, the presence of BFP, which has been considered to be an initial adhesion for EPEC colonization (6), is not synonymous with villous adhesion. Indeed, in a previous study with IVOC, BFP was suggested to be involved in three-dimensional colony formation and not initial adherence (15). Such a role would remain compatible with adhesion restricted to the FAE, i.e., to establish colony units within the gut environment, allowing nonintimate bacterium-to-bacterium adhesion and, once a quorum of bacteria have been established, to permit disaggregation and colonization of other regions of the gut.

TEM showed limited evidence of pedestals (19, 38) being formed by the three O55 strains. Rather, the intimate adhesion between bacterium and host cell membrane resulted, generally, in slight depressions in the surface of the enterocyte. A similar appearance was shown in newborn pigs infected with

O55:H7 (41), indicating it is not just due to the relatively short-term length of incubation in IVOC. Some pedestal-like structures were illustrated, but these were located on extruding cells where cytoplasmic extrusion gives the appearance of elongated pedestals (41). In addition, pedestal formation in in vivo EPEC infections (17, 39, 44) and in animal models of human EPEC infection (30) are similar to those produced by IVOC (15). The lack of elongated pedestal or pseudopod (38) formation on gut explants highlights the difference between IVOC and cell line adhesion studies. Nonpolarized epithelial cells, derived from the larynx, lung, and cervix do not possess tight junctions, lack a terminal web, and do not have the complex actin-based cytoskeleton of the intestinal brush border. Thus, actin polymerization at the bacterium-host interface may be a more tightly regulated process in gut epithelium than in cell lines.

Strain O55:H7 was shown to enter the enterocytes of newborn pigs, implying an invasive potential (41). This phenomenon was not apparent after 8 h IVOC with Peyer's patch explants and has not been reported in other, non-O55 serogroup, in vivo EPEC infections in humans.

In summary, EPEC strains from the O55 serogroup show initial adherence to the FAE overlying Peyer's patches. The mechanism of this tissue tropism includes intimin and other factor(s) specific to the O55 genome. Further analysis of EPEC and EHEC genomes will allow us to determine the mechanisms by which these pathogens adhere, maintain their presence, and spread within the gut.

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Human intestinal tissue tropism of intimin epsilon O103 *Escherichia coli*

Robert J. Fitzhenry [a], Mark P. Stevens [b], Claire Jenkins [c,d], Timothy S. Wallis [b], Robert Heuschkel [a], Simon Murch [a], Michael Thomson [a], Gad Frankel [d], Alan D. Phillips [a,*]

[a] *Centre for Paediatric Gastroenterology, Lower 3rd Floor, Royal Free Hospital, Pond St, London NW3 2QG, UK*
[b] *Institute for Animal Health, Compton, UK*
[c] *Division of Enteric Pathogens, Central Public Health Laboratory, London, UK*
[d] *Centre for Molecular Microbiology and Infection, Department of Biological Sciences, Imperial College of Science, Technology and Medicine, London, UK*

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Abstract

Human intestinal in vitro organ culture was used to assess the tissue tropism of human isolates of *Escherichia coli* O103:H2 and O103:H- that express intimin ε. Both strains showed tropism for follicle associated epithelium and limited adhesion to other regions of the small and large intestine. This is similar to the tissue tropism shown by intimin γ enterohaemorrhagic (EHEC) O157:H7, but distinct from that of intimin α enteropathogenic (EPEC) O127:H6.

Keywords: Intimin; Enteropathogenic; Enterohaemorrhagic; Human; O103 *Escherichia coli*

1. Introduction

The characteristic intestinal epithelial cell lesion produced by enteropathogenic (EPEC) and enterohaemorrhagic (EHEC) *Escherichia coli* is termed the 'attaching/effacing' (A/E) lesion (reviewed in Frankel et al. [1]). The first gene to be associated with A/E activity, the *eae* gene, encodes the intimate bacterial adhesin intimin [2]. This gene is part of the locus of enterocyte effacement (LEE) pathogenicity island, which also encodes a type III secretion system, an intimin receptor (Tir) that is translocated into host cells, and other EPEC secreted proteins (EspA, EspB, and EspD, EspF, Map) [3]. Intimin can also bind to β-1 integrins [4], and to cell surface-located nucleolin [5], although the in vivo consequences of this are unknown. The Tir receptor binding activity of intimin is localised at the carboxy-terminal 280 amino acids of the polypeptide, and at least five different intimin types, designated α, β, γ, δ, and ε have been described [6,7], although other, as yet unpublished, intimin types have been identified and their sequences deposited in GenBank (ζ, accession number: AJ298279; η, accession number: AJ271407; ι (*sic*), accession number: AJ308551; and κ, accession number: AJ308552).

Intimin α is specifically expressed by human EPEC strains belonging to one evolutionary branch known as EPEC clone 1, while intimin β is mainly associated with human and animal EPEC and EHEC strains belonging to their respective clone 2. Intimin γ is associated with EHEC O157:H7, EPEC O55:H7 and O55:H-, while intimin δ is expressed by EPEC O86:H34 [6]. Intimin ε is expressed by human and bovine EHEC strains of serogroups O8, O11, O45, O103, O121 and O165 [7]. Intimin types ζ and η have been identified in O84:NM and O84:H2 respectively, although their sequences appear identical, intimin ι in O145:H4, and intimin κ in O118:H5.

Intimin is required for colonisation and pathogenesis following experimental infection of humans by EPEC [8], and by EHEC O157:H7 in calves [9] and pigs [10]. Recent results show that different intimin types play a role in determining the pattern of colonisation and tissue tropism

* Corresponding author. Tel.: +44 (20) 7830-2783; Fax: +44 (20) 7830-2146.
E-mail address: adphill@rfc.ucl.ac.uk (A.D. Phillips).

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in the host. Among these are the observations of intimin γ O157:H7 adhesion being restricted to follicle associated epithelium (FAE) [11], whereas intimin α EPEC colonise both small intestine and FAE [11]. Furthermore intimin exchange studies using human intestinal explants show that EPEC O127:H6 engineered to express intimin γ instead of intimin α, change tropism to a FAE restricted phenotype [12] and that intimin γ EHEC O157:H7 engineered to express intimin α changes to an EPEC-like tropism phenotype [13]. Intimin ε shows overall sequence similarity to intimin β, but the last 280 C-terminal amino acids which contain the Tir and cell binding region show a greater similarity with intimins α and γ [7].

In this paper we have investigated the human intestinal tissue tropism shown by two O103 intimin ε strains.

2. Methods

2.1. Bacterial strains

Intimin ε positive O103:H2 strain PMK5, was isolated from a patient with haemolytic uremic syndrome (HUS) in France [14]. In order to perform IVOC in our category two laboratory a derivative of PMK5 that does not express Shiga toxin 1 was generated from the parent wild-type strain in category three facilities at the Institute for Animal Health (see below). The other intimin ε positive strain E77804 (O103:H-) was isolated from an adult male with diarrhoea in the UK. This strain is *stx* negative and EPEC adherence factor probe (EAF) negative.

2.2. Bionumeric analysis

Amino acid sequences of the different intimin types were retrieved from GenBank. These included human EPEC O127:H6 strain E2348/69 (M58154), calf EHEC O26:H- strain 413/89 (AJ223063), human EHEC O157:H7 strain EDL933 (Z11541), dog EPEC strain 4221 (U66102), human EHEC O103:H2 strain PMK5 (AF116899) and bovine EHEC O84:H4 strain 4795/97 (AJ308551). Multiple alignment and cluster analysis were carried out on these sequences using Bionumerics software (Applied Maths, Belgium). A dendogram was constructed by the unweighted pair group method using arithmetic averages (UPGMA). Bootstrap analysis was performed with 1000 resampled data sets.

2.3. Construction of an EHEC O103:H2 stx1 mutant [15]

Sequences flanking the *stx1A* gene were amplified and the products were gel-purified and combined in an overlapping polymerase chain reaction (PCR) [16]. The secondary PCR product was cloned into the suicide vector pCVD442 [17] and the resulting plasmid was introduced into a nalidixic acid resistant derivative of PMK5 by conjugation from *E. coli* S17-1λ*pir* [18] and a merodiploid isolated on LB agar containing ampicillin and nalidixic acid. Double recombinants were selected on medium containing 5% sucrose at 30°C, screened for the deletion by colony PCR and verified by Southern hybridisation. The resulting PMK5 Δ*stx1* mutant contained a large internal deletion of the *stx1A* gene that also removed the start codon for the B subunit. Culture supernatants of the PMK5 Δ*stx1* mutant showed no cytotoxicity for VERO cells but PMK5 Δ*stx1* still demonstrated attaching and effacing activity on cultured epithelial cells (data not shown).

2.4. Tissue samples

Samples were obtained, with fully informed parental consent and ethical approval, during routine investigation of patients for intestinal disorders. Mucosal biopsies of small intestine, ileal Peyer's patches and colon were taken during endoscopy (Fujinon EG/EC-41 paediatric endoscope). Only macroscopically normal appearing areas were sampled, and results were only used when subsequent intestinal histology was reported to be normal. Patients' ages are listed in Table 1.

2.5. Organ culture adhesion assay

8 h IVOC was performed [19]. Each bacterial strain was examined on at least three occasions using tissue from different children. An uninoculated specimen was included each time to exclude in vivo bacterial colonisation. After incubation, specimens were washed thoroughly to remove non-adherent bacteria and prepared for scanning electron microscopy (SEM). Samples were fixed with 2.5% glutaraldehyde, post-fixed in osmium tetroxide, critically point dried using liquid carbon dioxide, and viewed in a JEOL 5300 scanning EM. Some SEM samples of proximal intestinal incubations were reprocessed for light microscopy to visualise internal detail. This was to resolve the question whether bacterial adhesion was to a follicular area or to a mounded villus. Samples were placed in 2,2-dimethoxypropane, infiltrated with resin, polymerised and then ultramicrotome sectioned. 0.5 μm sections were stained with 1% aqueous toluidine blue in 1% sodium tetraborate.

3. Results

Fig. 1 shows the similarity matrix and dendogram constructed from the multiple alignment and cluster analysis using the UPGMA method. Analysis of the similarity matrix values of the complete amino acid sequence of the various intimins showed that intimins ε and γ were more similar to each other (86%) than to the other intimins (81.1–84.8%). In addition, the similarity between intimins α and η is higher (92.9%) than with the other intimins

Table 1
Attaching/effacing lesion formation in intestinal regions expressed as a proportion of biopsies inoculated

Strain	Intestinal region				
	Small intestine		Large intestine	FAE	
	D4	Ileum		D4 Isolated	Ileal PP
O103:H- (E77804)	3/9	0/8	0/17	2/2	2/3
Age (months)	140	151	142	103, 111	34, 110, 181
Median (range)	(19–185)	(83–181)	(46–191)		
O103:H2 (PMK5 Δ*stx1*)	2/11	0/7	1/16	NA	5/6
Age (months)	122	166	141	–	141
Median (range)	(19–185)	(83–181)	(46–191)		(46–191)

Age is expressed individually, unless more than three samples were used when median and range are given.
Abbreviations: FAE, follicle associated epithelium; D4, fourth part of the duodenum; PP, Peyer's patch; NA, not available.

(81.1–87.9%). The dendogram illustrates these relationships. The similarity values between the different intimin sequences are all generally high because the sequences are highly conserved in the N-terminal region.

The IVOC results are shown in Table 1. Both O103 strains showed some degree of attaching/effacing lesion formation on duodenal mucosa. However, in four of the five instances when this occurred, the bacteria adhered to mounded areas of the sample which may have represented follicular regions, rather than the villous surface, although this was not clear on SEM (Fig. 2a). Sections cut through the region of adhesion showed no evidence of follicular morphology (data not shown), so it was deduced that the regions were villous. No adhesion was evident on distal small intestine. By chance, two duodenal samples that were incubated with strain E77804 (O103:H-), were clearly observed to contain isolated lymphoid follicles (Fig. 2b). Both samples showed bacterial adhesion with attaching/effacing lesion formation on FAE (Fig. 2c), but not on neighbouring villous epithelium. Both intimin ε strains demonstrated A/E lesions on FAE of ileal Peyer's patches (Fig. 2d). Biopsy samples were taken from multiple levels of the colon (caecum, ascending, transverse, descending, and sigmoid) and only one sample (descending colon) incubated with PMK5 showed A/E lesion formation. Thus both strains showed a distinct tropism towards FAE overlying lymphoid follicles with limited adhesion to other intestinal regions.

4. Discussion

The application of IVOC to the study of EPEC and EHEC human intestinal tract adhesion has demonstrated two distinct phenotypes of tissue tropism. Firstly, a more widespread pattern of adhesion to the small intestine and FAE of Peyer's patches, with limited adhesion to the large bowel epithelium; this is typically shown by intimin α expressing EPEC [11]. Secondly, a restricted range of adhesion to only FAE of Peyer's patches, typified by intimin γ positive O157:H7 [11]. This suggested that intimin type influenced tissue tropism. This had also been indicated by animal based data where the expression of intimin α instead of intimin γ by O157:H7 was associated with the spread of infection from the colonic region into the small intestine in neonatal piglets [20]. Similar experiments, performed using the human IVOC model, demonstrated that O157:H7 expressing intimin α colonised the small intestine as well as FAE of Peyer's patches [13], and that O127:H6 EPEC showed a FAE restricted phenotype when expressing intimin γ [12]. The observation of some O103 strain adhesion to duodenal mucosa, in addition to the more pronounced follicular adhesion, is similar to that shown by O127:H6 when mutated to express intimin γ [12]. The current study has shown that intimin ε strains O103:H- and O103:H2 have a similar human intestinal tropism to intimin γ expressing strains, and also show adherence to FAE of isolated lymphoid follicles of the duodenum. Lymphoid follicles are spread along the length of human intestine [21,22], indicating the potential colonisation of intimin ε O103 *E. coli* along the length of the gut, not just in the terminal ileal region.

It has not been established whether this tropism changes during the infection, as IVOC has not been extended beyond an 8 h period. There may be parallels with animal pathogens which show an initial restricted adhesion to

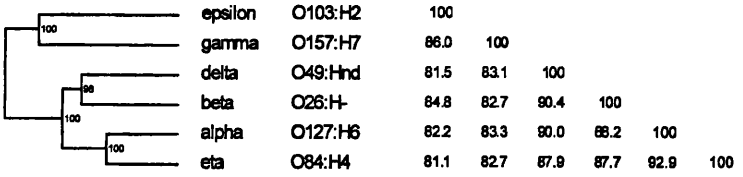

Fig. 1. A dendogram and similarity matrix of the amino acid sequences of α, β, γ, δ, ε and η intimins, constructed using the UPMGA method. Numbers at branch points indicate bootstrap values (as a percentage) performed with 1000 resampled data sets.

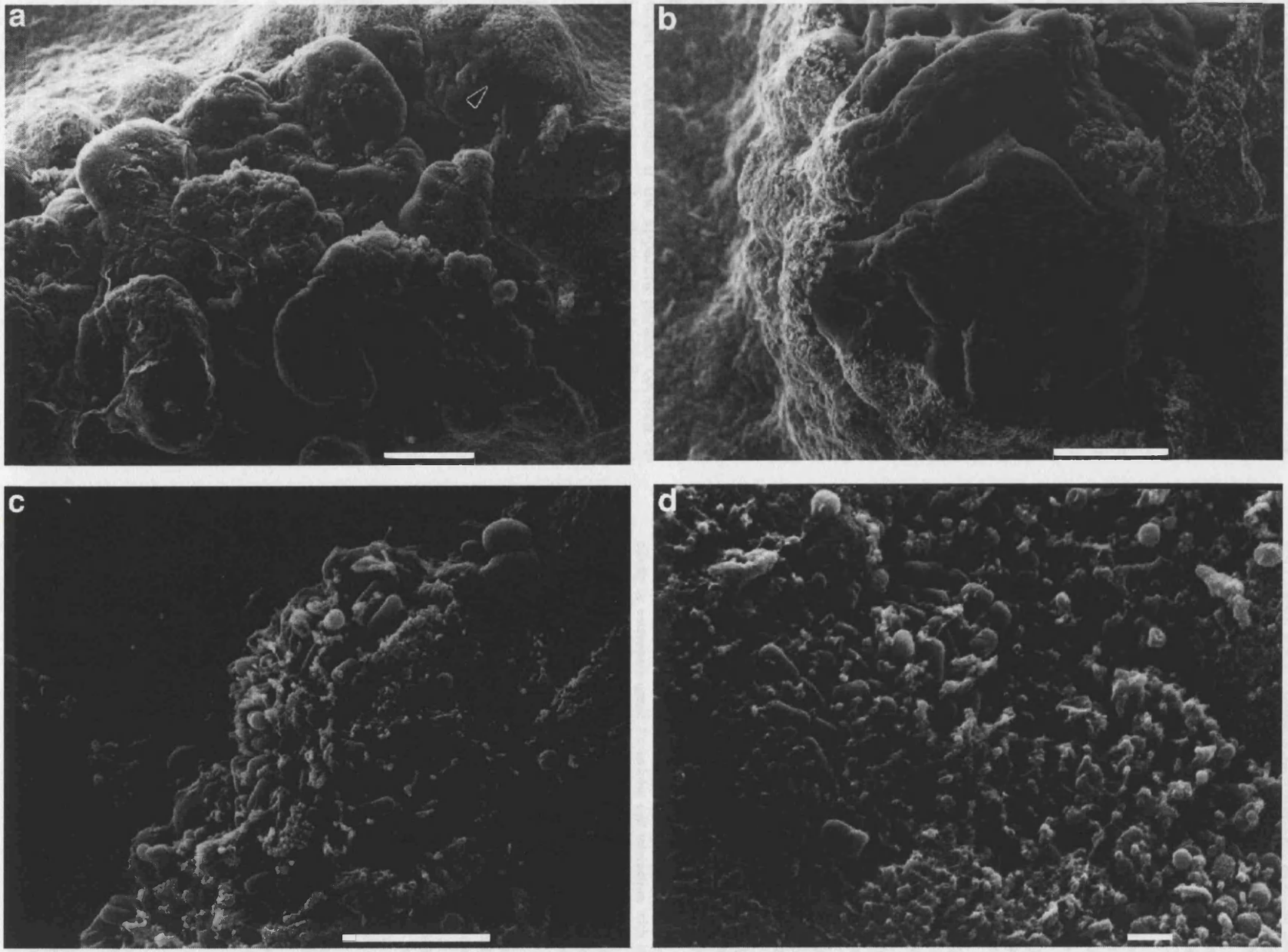

Fig. 2. SEM of IVOC. a: Follicular-like region (arrow) within duodenal sample. b: Isolated lymphoid follicle within duodenum. c: Strain E77804 showing A/E lesion on follicular-like epithelium in duodenum. d: Strain PMK5 showing A/E lesion on FAE of Peyer's patch. Bar = 100 μm (a,b), 5 μm (c), and 1 μm (d).

FAE overlying Peyer's patches, but which, with time, spread to other intestinal surfaces, e.g. the intimin β expressing rabbit strains RDEC-1 *E. coli* O15:H- [23] and *E. coli* O103:K-:H2 [24]. RDEC-1 adheres to Peyer's patch lymphoid follicles within 24 h, but does not attach to ileal or colonic mucosa until 3 days after inoculation. The authors deduced that the intestinal mucosa was probably colonised by bacteria shed from the Peyer's patches. Similar observations in rabbit EPEC O103:H2 led the authors to suggest that this might be a phenomenon common to all EPEC infections [24]. The difference in tropism we have observed between O127:H6 and O157:H7 [11] indicates that some strains may be able to colonise the small intestinal mucosa directly without an intermediate stage of FAE colonisation. Although we have evidence that intimin type influences initial tissue tropism, the factors that are responsible for the change in tropism that occurs during an infection have not been determined. Furthermore, it is unclear how intimin influences tissue tropism and it will be of interest to study the distribution of host cell receptors for intimin in the intestine.

The similarity matrix values of the complete amino acid sequences of the various intimins showed that intimins ε and γ are more 'related' to each other than to the other intimins and we have found that strains expressing these intimins show a similar human intestinal tissue tropism. The reasons for this are unclear, however, it will be of interest to determine if the apparent parallel between relatedness and tissue tropism holds true for intimin α and η strains.

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