

CXC Chemokine Responses of Intestinal Epithelial Cells to Shiga-toxigenic *Escherichia coli*



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Abstract

Since Shiga-toxigenic *Escherichia coli* (STEC) strains are not considered to be entero-invasive, the mechanism(s) by which Shiga toxin (Stx) gains access to the circulation and to target tissues expressing its target receptor Gb₃ is crucial to the disease process. There is increasing evidence that by facilitating translocation of Stx across the intestinal epithelium and by transporting bound toxin to remote sites such as the renal endothelium, polymorphonuclear leucocytes (PMNs) play a key role in the pathogenesis of serious STEC disease. Plasma levels of PMN-attracting CXC chemokines such as IL-8 also appear to correlate in humans with the severity of disease. Thus, the capacity of STEC strains to elicit CXC chemokine responses in intestinal epithelial cells may be a crucial step in pathogenesis.

In order to determine which STEC factor(s) are responsible for the induction of CXC chemokine responses by intestinal epithelial (HCT-8) cells, a real-time reverse transcription PCR assay was developed to quantitatively measure relative expression of chemokine mRNA for IL-8, ENA-78, GCP-2, MGSA, MIP-2 α and MIP-2 β . Similarly, a commercially available sandwich ELISA was used to measure levels of IL-8 protein secreted by HCT-8 cells in response to infection with STEC. When HCT-8 cells were infected with the well-characterised locus of enterocyte effacement (LEE)-negative O113:H21 strain 98NK2 or the LEE-positive STEC strain EDL933, there were significant differences in the levels of chemokine mRNA and IL-8 protein expression. In particular, the LEE-negative strain 98NK2 induced significantly higher and earlier levels of chemokine mRNAs, including IL-8, MIP-2 α and MIP-2 β at 1 and 4 h, and ENA-78 at 4 h. However, EDL933 elicited no significant upregulation of any of the chemokine mRNAs at 1 h, and only modest increases in IL-8, MIP-2 α and MIP-2 β by 4 h, post-infection. These results were confirmed by IL-8 ELISA which showed that 98NK2 elicited significant levels of IL-8 protein by 2 h post-infection, and remained high until 4 h post-infection. In comparison, EDL933 did not elicit significant IL-8 induction over that of control cells, even at 4 h post-infection.

When a range of STEC isolates from clinical samples were tested for their capacity to induce chemokine production in HCT-8 cells, highly significant differences were observed between the strains. Infection of HCT-8 cells with a range of LEE-negative STEC strains isolated from patients with severe STEC disease resulted in significantly higher and earlier upregulation of IL-8 and MIP-2 α mRNA than that elicited by several LEE-positive STEC strains. Similarly, levels of IL-8 protein in LEE-negative STEC-infected HCT-8 culture supernatants were significantly higher than in LEE-positive STEC-infected culture supernatants. Only one LEE-positive strain, an O26 strain 95ZG1, was capable of inducing chemokine responses comparable to that induced by infection with the LEE-negative STEC strains. These results were also shown not to be attributable to differences in the adherence, initial doses or growth of the strains during the assay, or to a loss of viability of the HCT-8 cells. These results, therefore, suggest that there may be interesting differences in the ability of STEC strains to induce chemokine production in intestinal epithelial cells.

The factor(s) that contribute to chemokine induction by epithelial cells in response to STEC were then examined. The difference in responses could not be attributed to the expression or non-expression of LEE genes, the presence or absence of an STEC megaplasmid or to differences in O serogroup. Although purified Stx1 and Stx2 were able to induce IL-8 and MIP-2 α mRNA, and IL-8 protein, the levels of chemokine induction in response to wild-type STEC did not correlate with the type or amount of Stx produced by these strains *in vitro*. Similarly, deletion of the single *stx*₂ gene from 98NK2 had no significant effect on chemokine induction compared to wild-type 98NK2-infected HCT-8 cells. Interestingly, several of the LEE-negative STEC strains eliciting the strongest chemokine responses belonged to flagellar serotype H21. Incubation of HCT-8 cells with purified H21 flagella elicited IL-8 and MIP-2 α mRNA responses similar to those seen in the presence of the most potent LEE-negative STEC strains. Deletion of the *fliC* gene largely abolished the capacity of 98NK2 to elicit IL-8 and MIP-2 α mRNA and IL-8 protein responses in HCT-8 cells. Similarly, deletion of both *stx*₂ and *fliC* from 98NK2 elicited a response similar to that observed with deletion of *fliC* alone.

Flagella were then purified from the high chemokine-inducing STEC strains 95HE4 (O91:H7) and 95ZG1 (O26:H11). Purified H7 and H11 flagella were similarly able to induce

both IL-8 and MIP-2 α mRNA, and IL-8 protein, in HCT-8 cells at levels similar to their respective wild-type strains. Deletion of *fliC* from two other STEC strains, 97MW1 (O113:H21) and 86-24 (O157:H7), confirmed that flagellin was responsible for the majority of chemokine responses in these wild-type strains. However, an inability of EDL933 to induce these responses was unexpected and later found to be due to a lack of expression of H7 flagella by this strain. Purified H21 FliC (His₆-FliC) alone was able to induce chemokine production (including IL-8, MIP-2 α and MIP-2 β at 1 and 4 h, and ENA-78 at 4 h) by HCT-8 cells at similar levels to that observed for 98NK2. Taken together, these data suggest that although Stx is capable of inducing CXC chemokine responses, the elevated responses observed in cells infected with certain STEC strains are largely attributable to the production of flagellin.

Purified His₆-H21 flagellin was also able to induce p38 MAPK activation *in vitro* and IL-8 and MIP-2 α mRNA were superinduced in the presence of both Stx2 and H21 flagellin. Blockade of the p38 pathway with SB203580 resulted in a down-regulation of IL-8 protein levels (by up to 61%) in response to H21 flagellin, but not IL-8 mRNA, suggesting that this inhibition may occur post-transcriptionally. Blocking the ERK and JNK pathways similarly decreased IL-8 secretion in response to H21 flagellin, suggesting that all three MAPK pathways are involved in this response. Indeed, concurrent inhibition of all three pathways resulted in virtually complete inhibition of IL-8 protein production (98%). Transfected HeLa and MDCK cells stably expressing TLR5 activated p38 in the presence of purified H21 flagellin, whereas dominant-negative (DN) TLR5-expressing cells did not, supporting previous studies that show that flagellin acts via TLR5. These data suggest that TLR5 and the p38, ERK and JNK MAPK pathways all play an important role in the response of intestinal epithelial cells to H21 flagellin from STEC, and that the combined effects of Stx and flagellin on host intestinal epithelial cells may result in an augmented inflammatory response.

A role for flagellin in virulence was then investigated. BALB/c mice were orally inoculated with wild-type 98NK2 or 98NK2 Δ *fliC*. Of the 16 mice challenged with the wild-type strain 98NK2, 9 (56%) died during the experiment (median survival time 7.6 days). However, only 3 of 16 mice (19%) challenged with 98NK2 Δ *fliC* died (median survival time > 14 days). The difference in survival rate was statistically significant. No significant

differences in the level of intestinal colonisation of 98NK2 or 98NK2 Δ *fliC* were observed. Thus, flagellin directly contributes to the virulence of STEC in streptomycin-treated mice. Since the streptomycin-treated mouse is a model for systemic Stx-mediated pathology, these results suggest that the pro-inflammatory effects of flagellin play an important role in the pathogenesis of Stx-mediated STEC disease *in vivo*.

Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

Trisha Jayne Rogers, 14 September 2004.

Dedication

*I would like to dedicate this thesis to my grandfather,
William Dudley Cottle,
who passed away during its creation.*

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List of Abbreviations

Abbreviations that are acceptable to the Journal of Bacteriology are used in this thesis without definition in the text. Additional abbreviations are defined when first used in the text and are listed below.

A _{260, 405, 570, 600}	Absorbance at 260, 405, 570 or 600 nm, respectively
α-DIG	Anti-Digoxigenin-Fab fragment
A/E	Attaching and effacing
α-Hly	<i>E. coli</i> α-haemolysin
AIEC	Adherent-invasive <i>E. coli</i>
Amp	Ampicillin
AP	Alkaline phosphatase
AP-1	Activator protein-1
astA	EAggEC heat-stable enterotoxin (EAST1) gene
BCIP	5-Bromo-4-chloro-3-indoyl-phosphate (or X-phosphate)
Bfp	Bundle forming pili
BL-3	Bovine lymphoma cells
BSA	Bovine serum albumin
Caco-2	Human colonic epithelial cells
cat	Chloramphenicol acetyl transferase gene cassette
CDEC	Cell-detaching <i>E. coli</i>
Ces	Chaperone for <i>E. coli</i> secreted proteins
CFU	Colony-forming unit
CHO	Chinese hamster ovary
Cml	Chloramphenicol
C-terminus	Carboxy terminus
Cu	Plasmid-cured
CXC	Cysteine (C)-X-C motif
CXCR1/2	CXC receptor 1/2 (or IL-8RA/B)
DAEC	Diffusely-adherent <i>E. coli</i>
Daudi	Human lymphoma cells
DIG	Digoxigenin
DMEM	Dulbecco's modified Eagle's medium
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
DN	Dominant-negative
DTT	Dithiothreitol
eae	<i>E. coli</i> attaching and effacing (intimin) gene
Eaf	EPEC adherence factor
EAggEC	Enteraggregative <i>E. coli</i>
EAST1	EAggEC heat-stable enterotoxin 1
Efa1	EHEC factor for adherence
EHEC	Enterohaemorrhagic <i>E. coli</i>

EHEC-Hly	EHEC-enterohaemolysin (or Ehx)
Ehx	EHEC-enterohaemolysin (or EHEC-Hly)
EIEC	Enteroinvasive <i>E. coli</i>
ELISA	Enzyme-linked immunosorbent assay
ELR	Glutamine (E)-leucine (L)-arginine (R) motif
ENA-78	Epithelial-derived neutrophil activating peptide-78
EpeA	EHEC plasmid-encoded autotransporter
EPEC	Enteropathogenic <i>E. coli</i>
ERK	Extracellular regulated kinases
Esc	<i>E. coli</i> secretion system protein
Esp	<i>E. coli</i> secreted protein
EspI	<i>E. coli</i> secreted protease, island encoded
EspP	Extracellular serine protease, plasmid encoded
ETEC	Enterotoxigenic <i>E. coli</i> (ETEC)
Etp	EHEC type II secretion pathway protein
ExPEC	Extraintestinal pathogenic <i>E. coli</i>
F-actin	Filamentous actin
FCS	Foetal calf (bovine) serum
FRT	FLP recombinase recognition target site
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
Gb ₃	Globotriaosylceramide (Gal α [1 \rightarrow 4]Gal β [1 \rightarrow 4]Glc-ceramide)
Gb ₄	Globotetraosylceramide (GalNAc β [1 \rightarrow 3]Gal α [1 \rightarrow 4]Gal β [1 \rightarrow 4]-Glc-ceramide)
GCP-2	Granulocyte chemotactic protein-2
G-CSF	Granulocyte colony stimulating factor
GMVEC	Glomerular microvascular endothelial cells
Gro	Growth related oncogene
h	Hour(s)
HBSS	Hanks' balanced salt solution
HC	Haemorrhagic colitis
HCA-7	Human colon carcinoma cells
HCT-8	Human colonic epithelial cells
HeLa	Human cervical epithelial cells
Henle 407	Human intestinal epithelial cells
HEp-2	Human laryngeal epithelial cells
HepA3	Hepoxilin A3
HI	Heat-inactivated
HIMEC	Human intestinal microvascular endothelial cells
HRMEC	Human renal glomerular microvascular endothelial cells
HRP	Horseradish peroxidase
HRTEC	Human renal tubular epithelial cells
HT-29	Human colonic epithelial cells
HUS	Haemolytic uraemic syndrome
HUVEC	Human umbilical vein endothelial cells
ICAM-1	Intracellular cell adhesion molecule type 1
Ig	Immunoglobulin
Iha	IrgA homologue adhesin
I κ B- α	Inhibitory subunit of κ B
IL	Interleukin
IL-8RA/B	Interleukin-8 receptor A/B (or CXCR1/2)
IP	Immunoprecipitation
IPTG	Isopropyl- β -D-thiogalactopyranoside

IRAK	IL-1 receptor-associated kinase
IrgA	Iron regulated gene A
JNK	c-Jun N-terminal kinases
Kan	Kanamycin
KatP	Catalase-peroxidase, plasmid encoded
LB	Luria-Bertani
LCT	Large clostridial toxins
LD ₅₀	50% lethal dose
LEE	Locus for enterocyte effacement
Ler	LEE-encoded regulator
<i>lifA</i>	Lymphostatin gene
LIM	Locus for improving microcolony formation
LIX	LPS-induced CXC chemokine
LPA	Locus of proteolysis activity
LPF	Long polar fimbriae
LPS	Lipopolysaccharide
LRR	Leucine rich repeat
MAPK	Mitogen activated protein kinase
mCXCR2	Murine CXCR2 homologue
MDBK	Madin-Darby bovine kidney cells
MDCK	Madin-Darby canine kidney cells
MEK	Mitogen-activated protein kinase kinase
MEKK-1	Mitogen-activated protein kinase kinase kinase 1
MGSA	Melanocyte growth stimulating activity (or Gro- α)
MHA-T	Microangiopathic haemolytic anaemia and thrombocytopenia
mIL-8Rh	Murine IL-8 receptor homologue
MIP-2 α	Macrophage inflammatory protein-2 α (or Gro- β)
MIP-2 β	Macrophage inflammatory protein-2 β (or Gro- γ)
MNEC	Meningitis (neonatal)/sepsis-associated <i>E. coli</i>
MOPS	3-(N-morpholino)-propanesulphonic acid
MPO	Myeloperoxidase
MyD88	Myeloid differentiation primary response gene 88
NBT	4-Nitroblue tetrazolium chloride
NF- κ B	Nuclear factor- κ B
Ni-NTA	Nickel-nitrilotriacetic acid
nt	Nucleotide(s)
NTEC	Necrotic <i>E. coli</i>
N-terminus	Amino terminus
ORF	Open reading frame
PAMP	Pathogen-associated molecular pattern
Pap	P fimbrial proteins
PBS	Phosphate buffered saline
PD	PD98059
PEEC	Pathogen-elicited epithelial chemoattractant
Per	Plasmid-encoded regulator
Pil	Type IV pilus biosynthesis locus
PMN	Polymorphonuclear leucocyte
PMSF	Phenylmethylsulfonyl fluoride
pO113	Megaplasmid of STEC O113:H21 strain 98NK2
pO157	Megaplasmid of O157:H7 STEC
PRR	Pattern recognition receptor
pSFO157	Megaplasmid of sorbitol fermenting STEC O157:H ⁻

PssA _R	Protease secreted by STEC Resistant
Raji	Human lymphoma cells
RDEC	Rabbit diarrhoeagenic <i>E. coli</i>
REPEC	Rabbit enteropathogenic <i>E. coli</i>
RT-PCR	Reverse-transcription polymerase chain reaction
RTX _S	Repeats in toxin Sensitive
Saa	STEC autoagglutinating adhesin
SAPK	Stress activated protein kinases
SB	SB203580
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	Standard error of the means
Sep	Secretion of <i>E. coli</i> proteins
Sfp	Sorbitol-fermenting EHEC O157 fimbriae, plasmid-encoded
SLT	Shiga-like toxin
SLTEC	Shiga-like-toxin producing <i>E. coli</i>
SP	SP600125
STa	Heat-stable enterotoxin a
STEC	Shiga-toxigenic <i>E. coli</i> or Shiga toxin-producing <i>E. coli</i>
Str	Streptomycin
Stx	Shiga toxin
T84	Human colonic epithelial cells
TAI	Tellurite resistance- and adherence-conferring island
TAK-1	Transforming growth factor- β -activated kinase-1
TBE	Tris-borate-EDTA buffer
TCP	Toxin coregulated pilus
TE	Tris-EDTA buffer
TEER	Trans-epithelial electrical resistance
TEMED	N,N,N',N'-tetramethyl-ethylene-diamine
THP-1	Human monocytic cells
Tir	Translocated intimin receptor
TIR	Toll/interleukin-1 receptor
TLR	Toll-like receptor
TNF- α	Tumour necrosis factor α
ToxB	Toxin B
Tra	Conjugal transfer region
TRAF6	TNF-receptor-associated factor 6
TTBS	Tween-Tris buffered saline
TTP	Thrombotic thrombocytopenic purpura
UPEC	Uropathogenic <i>E. coli</i>
VCAM-1	Vascular cell adhesion molecule type 1
Vero	African green monkey kidney epithelial cells
VT	Verocytotoxin
VTEC	Verocytotoxin-producing <i>E. coli</i>
X-gal	5-bromo-4-chloro-3-indoyl- β -D-galacto-pyranoside
X-pho	5-Bromo-4-chloro-3-indoyl-phosphate (or BCIP)

CHAPTER 1: General Introduction

1.1 General background

Escherichia coli comprise a diverse group of Gram-negative bacteria that can be distinguished serologically on the basis of their O (lipopolysaccharide) and H (flagellar) antigens, and sometimes, by their K (capsular) antigens. *E. coli* is a commensal organism that is abundant in the gastrointestinal flora of both humans and animals. The majority of *E. coli* that are present in the gastrointestinal tract rarely cause disease in humans, except when the host is otherwise immunocompromised, or if the intestinal barrier is breached. However, some strains have acquired pathogenic and/or toxigenic ‘virulence factors’ that have enabled them to cause disease in humans. These pathogenic strains are responsible for enteric diseases as well as extraintestinal infections in humans.

There are six well-characterised classes of diarrhoeagenic *E. coli*: diffusely-adherent *E. coli* (DAEC), enteroaggregative *E. coli* (EAggEC); enteroinvasive *E. coli* (EIEC), enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC) and Shiga-toxigenic *E. coli* (STEC; a subgroup of which is also referred to as enterohaemorrhagic *E. coli* [EHEC]) all of which are reviewed in Nataro and Kaper (1998). Newly described groups include cell-detaching *E. coli* (CDEC) which causes diarrhoea in children, and adherent-invasive *E. coli* (AIEC) which is associated with Crohn’s disease (Gunzberg *et al.*, 1993; Nataro and Kaper, 1998; Darfeuille-Michaud, 2002). Pathogenic *E. coli* that are responsible for extraintestinal infections include: uropathogenic *E. coli* (UPEC) and necrotoxic *E. coli* (NTEC), which are

both responsible for urinary tract infections and other extraintestinal infections (De Rycke *et al.*, 1999; Johnson and Russo, 2002); and meningitis (neonatal)/sepsis-associated *E. coli* (MNEC) and extraintestinal pathogenic *E. coli* (ExPEC) (Unhanand *et al.*, 1993; De Rycke *et al.*, 1999; Russo and Johnson, 2000), which are responsible for sepsis/meningitis and other extraintestinal infections. The focus of this thesis will be on the STEC group.

STEC strains are responsible for considerable morbidity and mortality worldwide. Konowalchuk *et al.* (1977) first described the feature that distinguishes STEC from other pathogenic *E. coli*; that is, the production of a toxin that is irreversibly cytotoxic for African green monkey kidney (Vero) cells, and which is antigenically distinct from the heat-labile enterotoxin of ETEC. Due to the cytotoxic activity on Vero cells, this toxin was named Verocytotoxin (VT), and the strains of *E. coli* that produced this toxin were called Verocytotoxin-producing *E. coli* (VTEC). VTEC strains were found to be associated with both haemorrhagic colitis (HC) (Riley *et al.*, 1983) and the haemolytic uraemic syndrome (HUS) (Karmali *et al.*, 1983b). A previously rare serotype of *E. coli* (O157:H7) was also associated with these conditions (CDC, 1982; Johnson *et al.*, 1983; Karmali *et al.*, 1983b; Riley *et al.*, 1983; Wells *et al.*, 1983).

The cytotoxin from one of Konowalchuk's isolates, along with other VTEC isolates, was purified and found to be similar in structure and biological activity to the Shiga toxin (Stx) produced by *Shigella dysenteriae* type 1 (O'Brien *et al.*, 1982; O'Brien and LaVeck, 1983). This VT was found to be cytotoxic for human cervical epithelial (HeLa) cells and could be neutralised by anti-Stx from *S. dysenteriae*, resulting in the nomenclature of Shiga-like toxin (SLT) (O'Brien *et al.*, 1982; O'Brien and LaVeck, 1983; O'Brien *et al.*, 1983). The strains producing SLT were thereafter described as Shiga-like toxin producing *E. coli* (SLTEC), or as either Shiga-toxigenic or Shiga toxin-producing *E. coli* (STEC). Production of VT/SLT by *E. coli* was then identified as the factor common to strains capable of causing both HC and HUS, including *S. dysenteriae*, and the toxin was found to be responsible for damage to intestinal and renal tissue (Karmali *et al.*, 1983a). Thus, VTEC and STEC are essentially synonymous and refer to *E. coli* strains that can produce one or more toxins from the Stx family (Calderwood *et al.*, 1996). For the purpose of this thesis, all VTEC or SLTEC strains will be referred to as STEC, and the toxin produced by STEC will be referred to as

Stx, rather than VT or SLT. The STEC class represents a large group of bacteria including over 200 O:H serotypes. However, the production of Stx alone is not sufficient to cause disease in humans, since only a subset of these serotypes has been associated with human disease. The term EHEC is often used to describe a further subset of STEC strains that are capable of causing severe disease, including HC and HUS.

1.2 Clinical spectrum and management of STEC disease

1.2.1 Clinical spectrum of STEC disease

STEC strains are responsible for a broad spectrum of clinical disease resulting in significant morbidity and mortality worldwide. STEC-mediated disease may involve either sporadic cases or small to large outbreaks. Manifestations can range from asymptomatic carriage, diarrhoea and/or bloody diarrhoea and HC, to the life-threatening conditions HUS and thrombotic thrombocytopenic purpura (TTP). Following ingestion and gut colonisation, patients usually suffer watery diarrhoea; in some cases this progresses to bloody diarrhoea (up to 70% of patients) within 1 to 2 days and HC with severe abdominal pain (Fig. 1.1) (Slutsker *et al.*, 1997; Mead and Griffin, 1998). Patients may suffer from vomiting (up to 60%) and may have low-grade fever, although this is less common (up to 30%) (Griffin *et al.*, 1988; Mead and Griffin, 1998). The first described cases of bloody diarrhoea and HC from O157:H7 infection were in 1982 (Riley *et al.*, 1983). Riley *et al.* (1983) described two outbreaks of HC that were associated with the ingestion of undercooked hamburger meat from a fast food restaurant in Oregon (USA). This outbreak was traced to *E. coli* O157:H7 (Riley *et al.*, 1983); since then, STEC have been shown to be important aetiologic agents of HC, as well as HUS and TTP (CDC, 1982; Karmali *et al.*, 1983b; Remis *et al.*, 1984; CDC, 1985; Karmali *et al.*, 1985; Neill *et al.*, 1985; Morrison *et al.*, 1986; Riley, 1987; Griffin *et al.*, 1990; Banatvala *et al.*, 2001; Elliott *et al.*, 2001; Andreoli *et al.*, 2002).

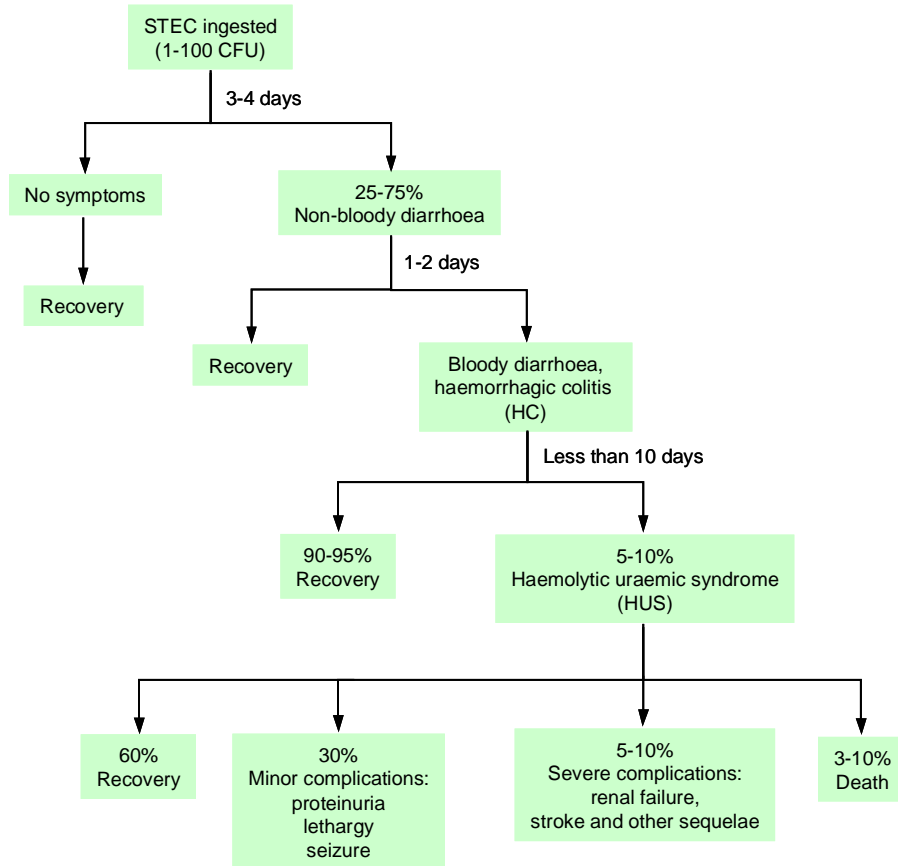


Figure 1.1 Clinical manifestations of STEC infection. Modified from Mead and Griffin (1998).

In a proportion of patients (usually 5-10%, but up to 20% or more during some outbreaks), STEC infection may progress to HUS (Fig. 1.1) (Mead and Griffin, 1998). Gasser *et al.* (1955) first described HUS in five children presenting primarily with renal failure. However, the link with STEC infection was first reported in 1983 when Karmali *et al.* (1983b) described sporadic cases of HUS in patients positive for STEC strains belonging to serogroups O26, O111, O113 and O157. HUS as a result of STEC infections and which have a diarrhoeal prodrome are referred to as diarrhoea associated HUS (D+HUS), as opposed to the atypical non-diarrhoeal associated HUS (D-HUS), which is not STEC-related (Siegler, 1995; Proesmans, 1996). Over 90% of HUS cases are D+HUS and the majority of these cases are caused by STEC strains (Siegler, 1995).

HUS is defined as a triad of acute renal failure, thrombocytopenia and microangiopathic haemolytic anaemia (Gasser *et al.*, 1955; Gianantonio *et al.*, 1964; Mead and Griffin, 1998; Paton and Paton, 1998b). Of patients who progress to HUS, approximately 50% require dialysis and 25% develop acute neurological symptoms including stroke, seizure, encephalopathy and coma (Mead and Griffin, 1998; Paton and Paton, 1998b). Other complications may include colonic perforation or stricture, intussusception, rectal prolapse, appendicitis, pancreatitis, cholecystitis, hepatitis, haemorrhagic cystitis, pulmonary oedema, myocardial dysfunction, diabetes mellitus, and pleural and/or pericardial effusions (Tarr, 1995; Mead and Griffin, 1998; Nataro and Kaper, 1998; Banatvala *et al.*, 2001). HUS is the leading cause of acute renal failure in children (Karmali, 1989; Siegler, 1995; Paton and Paton, 1998b). Death occurs in approximately 3-10% of HUS patients (Mead and Griffin, 1998; Nataro and Kaper, 1998; Paton and Paton, 1998b). Of those that survive, up to 30% may suffer permanent disabilities or late complications including renal insufficiency, hypertension and neurological deficits (Karmali, 1989; Tarr, 1995; Mead and Griffin, 1998; Paton and Paton, 1998b).

Specific prognostic markers for the development of HUS include young age, fever, bloody diarrhoea, elevated white blood cell count, thrombocytopenia, vomiting, anuria and low (interleukin [IL]-10) or high (IL-1 β , IL-6, IL-8, tumour necrosis factor- α [TNF- α] and granulocyte colony stimulating factor [G-CSF]) cytokine levels (Robson *et al.*, 1989; Robson *et al.*, 1991; Karpman *et al.*, 1995; Bell *et al.*, 1997; Inward *et al.*, 1997b; Murata *et al.*, 1998; Honda, 1999; Litalien *et al.*, 1999; Buteau *et al.*, 2000; Westerholt *et al.*, 2000; Wong *et al.*, 2000; Elliott *et al.*, 2001; Gerber *et al.*, 2002). The use of antimotility or antimicrobial agents is also correlated with a poorer prognosis (Bell *et al.*, 1997; Honda, 1999; Wong *et al.*, 2000).

Another sequela of STEC infection related to HUS is TTP. TTP was first described by Moschcowitz (1925), who presented the case of a 16 year old female with acute febrile pleiochromic anaemia, petechiae, paralysis and coma, who later died of this illness. This condition, originally termed Moschcowitz syndrome, has similar pathological features to HUS, including microangiopathic haemolytic anaemia and thrombocytopenia (Ruggenti *et al.*, 2001; Hosler *et al.*, 2003). However, unlike HUS, it is more common in adults than in children (Hosler *et al.*, 2003). Patients presenting with TTP are more often febrile and have

more severe neurological involvement than patients with HUS, but are less likely to have renal dysfunction (Karmali, 1989; Ruggerenti *et al.*, 2001; Hosler *et al.*, 2003). The formation and localisation of platelet-rich thrombi, and the difference in organ distribution, account for the predominantly neurological symptoms in TTP rather than renal dysfunction seen in HUS (Brain and Neame, 1982; Hosler *et al.*, 2003). Patients with TTP, but not HUS, may also have deficiencies in von Willebrand factor-cleaving protease (Furlan *et al.*, 1998; Tsai and Lian, 1998). Cases of TTP associated with *E. coli* O157:H7 were first reported in 1986 (CDC, 1986; Morrison *et al.*, 1986).

1.2.2 Management of patients with STEC disease

The treatment of patients with HUS or TTP largely involves supportive therapy. This includes dialysis, maintenance of fluid balance, treatment of hypertension, transfusion of packed erythrocytes, platelet infusions and plasma exchange or infusion (Rock *et al.*, 1991; Nataro and Kaper, 1998; Paton and Paton, 1998b). Some patients may require a renal transplant or hemi-colectomy (Richardson *et al.*, 1988; Nataro and Kaper, 1998). The use of antibiotic therapy is generally contraindicated since some studies have suggested that treatment of STEC with antibiotics either has no beneficial effect or may actually increase the risk of progressing from diarrhoea to HUS (Riley, 1987; Nataro and Kaper, 1998; Paton and Paton, 1998b; Wong *et al.*, 2000; Andreoli *et al.*, 2002). Stx is encoded on bacteriophages infecting the STEC strain, and it is thought that the use of antibiotics may be harmful due to the induction of late phage promoters, which increases Stx production (Neely and Friedman, 1998; Plunkett *et al.*, 1999; Zhang *et al.*, 2000b; Wagner *et al.*, 2002). Patients with STEC disease or HUS therefore need to be monitored closely for potential complications.

1.3 Epidemiology

1.3.1 Incidence of STEC disease

Human infections associated with STEC have been reported in over 30 countries from six continents (Mead and Griffin, 1998). STEC O157:H7 was among the first STEC serotypes associated with HC, HUS and TTP (CDC, 1982; Johnson *et al.*, 1983; Riley *et al.*, 1983; Wells *et al.*, 1983; Remis *et al.*, 1984; CDC, 1985; Neill *et al.*, 1985; CDC, 1986; Morrison *et al.*, 1986). However, although O157:H7 is the most prevalent serotype (Siegler, 1995; Mead and Griffin, 1998; Gerber *et al.*, 2002; Werber *et al.*, 2003), in many parts of the world other serogroups/serotypes have also repeatedly been associated with human disease, including O26, O91, O103, O111, O113, O145 and O157:H⁻ (Karmali *et al.*, 1985; Verweyen *et al.*, 2000; Elliott *et al.*, 2001).

In the United States, *E. coli* O157:H7 was isolated from stool specimens of patients with diarrhoea more frequently than *Shigella* spp. and was the third most commonly isolated pathogen after *Campylobacter* and/or *Salmonella* spp. (Park *et al.*, 1994; Griffin, 1995; Nataro and Kaper, 1998). O157:H7 was also the most commonly isolated pathogen from stool specimens containing visible blood (Slutsker *et al.*, 1997). In the United States alone, O157:H7 strains are responsible for approximately 74,000 cases of diarrhoea and over 2,000 hospitalisations per year (Mead *et al.*, 1999). Non-O157:H7 are not routinely reported in the United States, but are estimated to occur in an extra 36,000 cases and over 1,000 hospitalisations per year (Mead *et al.*, 1999). In Australia, the serotypes O111:H⁻, O16:H21, O26:H11, O91:H10, ONT:H41 and O157:H⁻ have been reported in cases of paediatric diarrhoea and haemorrhagic colitis (Robins-Browne *et al.*, 1998).

In Australia, O157:H7 was rarely isolated from patients presenting with D+HUS; instead O111:H⁻ was the most common serotype isolated (Elliott *et al.*, 2001). Furthermore, both outbreaks and sporadic cases of HUS in Australia have been associated with several non-O157:H7 serotypes including O48:H21, O111:H⁻, O113:H21 and O157:H⁻ (Paton *et al.*, 1995b; Paton *et al.*, 1996; Henning *et al.*, 1998; Paton *et al.*, 1999; Elliott *et al.*, 2001). The incidence of STEC-mediated HUS in children varies from country to country but is in the

range of 0.2 to 22 per 100,000 children, with the highest rate being found in Argentina (Lopez *et al.*, 1995; Verweyen *et al.*, 2000). In most countries, including Australia, the United States, and throughout most of Europe, the incidence of HUS ranges from 0.4 to 3.4 per 100,000 children (Caprioli *et al.*, 1994; Walters *et al.*, 1994; Caprioli and Tozzi, 1998; Verweyen *et al.*, 2000; Elliott *et al.*, 2001).

1.3.2 Sources of STEC

Cattle are regarded as the principal reservoir of STEC strains, including the serotypes that are responsible for human disease (Nataro and Kaper, 1998; Paton and Paton, 1998b). STEC are thought to occur ubiquitously in cattle (between 10 to 60%) and faecal shedding is higher during the warmer months, which coincides with peaks of STEC infection in the human population (Hancock *et al.*, 1998; Nataro and Kaper, 1998; Paton and Paton, 1998b). However, STEC are also prevalent in the gastrointestinal tracts of many other animals including sheep, pigs, goats, deer, horses, rabbits, dogs, cats and chickens (Mead and Griffin, 1998; Nataro and Kaper, 1998; Paton and Paton, 1998b; Mainil, 1999). While most STEC-carrying animals are asymptomatic, some serotypes may cause watery diarrhoea in calves, cats and dogs (Nataro and Kaper, 1998; Paton and Paton, 1998b). However, infection of piglets with STEC may result in a serious and often fatal infection referred to as piglet oedema disease (DeGrandis *et al.*, 1989; Samuel *et al.*, 1990; Mainil, 1999). Piglet oedema disease is characterised by neurological symptoms including ataxia, convulsions, paralysis, and oedema of the brain, stomach, colon and intestine, and is caused by STEC strains that produce a variant of Stx called Stx2e (Section 1.4.1) (Gyles, 1998; Paton and Paton, 1998b).

1.3.3 Transmission of STEC

Most cases of STEC disease are caused by the ingestion of contaminated food. STEC generally enters the food chain via the contamination of food and/or water with faecal matter, particularly that of cattle. STEC contamination has occurred in meat (e.g. ground beef,

mettwurst, salami), unpasteurised dairy products, fresh vegetables (radish sprouts, lettuce, alfalfa sprouts), unpasteurised apple juice or cider and water (Riley *et al.*, 1983; Paton *et al.*, 1996; Akashi *et al.*, 1994; Mead and Griffin, 1998; Nataro and Kaper, 1998; Paton and Paton, 1998b). Contamination is usually the result of improper handling procedures during manufacturing, marketing, storage or cooking (Nataro and Kaper, 1998; Verweyen *et al.*, 2000). However person-to-person transmission has occurred in families with infected individual(s), day care centres, nursing homes and kindergartens (Riley, 1987; Griffin *et al.*, 1988). STEC can also be acquired from animals in petting zoos and from swimming in contaminated water (Friedman *et al.*, 1999; Paunio *et al.*, 1999; Heuvelink *et al.*, 2002).

Faecal shedding in HUS patients has been reported for several weeks, and in some cases up to two months, after the resolution of infection (Karch *et al.*, 1995; Mead and Griffin, 1998; Nataro and Kaper, 1998; Paton and Paton, 1998b). The infectious dose for STEC is believed to be extremely low and has been reported to be less than 100 colony-forming units (CFU) for some strains (Paton and Paton, 1998b). This has obvious implications for the degree of hygiene required to limit person-to-person spread, particularly during outbreaks.

1.4 Shiga toxin

1.4.1 Structure and mode of action of Stx

The clinical manifestations of STEC disease are principally mediated by the action of Stx on susceptible host cells. The Stxs are AB₅ holotoxins consisting of a pentameric B subunit (7.7-kDa monomers) and a catalytic A subunit (32-kDa) (Fig. 1.2) (Fraser *et al.*, 1994; Sandvig and van Deurs, 2000). There are two main types of Stx: Stx1 and Stx2. Stx1 is virtually identical to the Stx produced by *S. dysenteriae*, whereas Stx2 shows only 55% and 57% amino acid identity to Stx1 in the A and B subunits, respectively (Jackson *et al.*, 1987; Nataro and Kaper, 1998). STEC strains may carry either Stx1 or Stx2, or both. Whereas Stx1

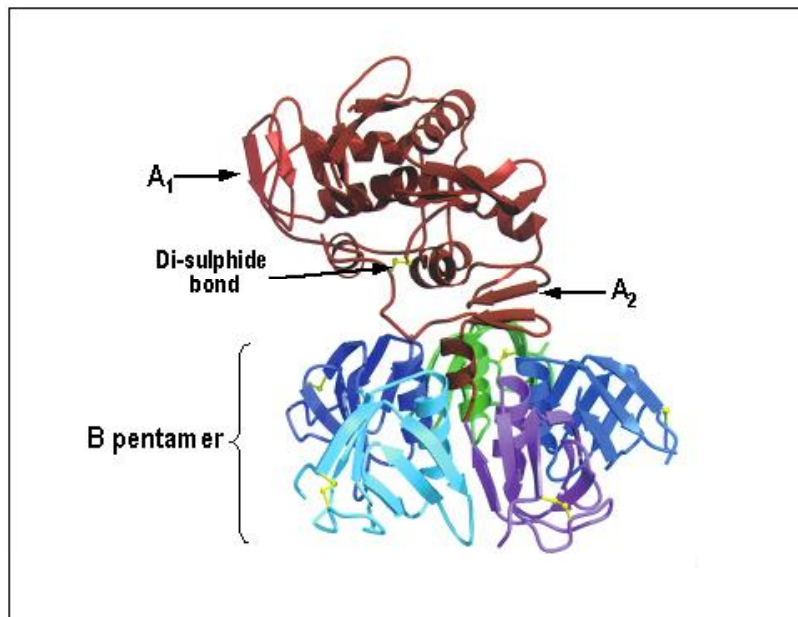


Figure 1.2 Shiga toxin structure. The A subunit is shown in red. The five B-subunit monomers, which comprise the binding pentamer, are shown in blue, cyan, green and purple. Reproduced from Fraser *et al.* (1994).

is highly conserved in terms of its amino acid sequence, Stx2 is highly variable. Indeed, the Stx2 group has been divided into subtypes on the basis of differences in sequence that correlate with distinct biological properties, e.g. Stx2c, Stx2d and Stx2e (O'Brien and Holmes, 1987; Nataro and Kaper, 1998; Paton and Paton, 1998b). Nevertheless, they share a common structure and mode of action.

The Stx B component consists of five subunit monomers that form a pentameric ring and are involved in binding to the glycolipid receptor, globotriaosylceramide (Gb₃; Gal α [1→4]Gal β [1→4]Glc-ceramide) (Waddell *et al.*, 1988), which is present on eukaryotic cell membranes (Lindberg *et al.*, 1987; Lingwood *et al.*, 1987). The exception to this is the variant toxin Stx2e that binds preferentially to the receptor globotetraosylceramide (Gb₄; GalNAc β [1→3]Gal α [1→4]Gal β [1→4]Glc-ceramide) and is produced by a subset of STEC strains that are responsible for piglet oedema disease (DeGrandis *et al.*, 1989; Samuel *et al.*, 1990). The genes encoding Stx1 and Stx2 are generally carried on bacteriophages integrated into the bacterial genome (Scotland *et al.*, 1983; O'Brien *et al.*, 1992; Nataro and Kaper, 1998;

Plunkett *et al.*, 1999; Kaper *et al.*, 2004). A single STEC isolate can carry one or more Stx-converting bacteriophages and consequently may produce more than one type of Stx (O'Brien *et al.*, 1992).

Once Stx binds to Gb₃, it undergoes a process of internalisation via receptor-mediated endocytosis in clathrin-coated pits (Sandvig *et al.*, 1989; Sandvig and van Deurs, 1996; Sandvig and van Deurs, 2000). In some cell types, the endosome undergoes fusion with lysosomes resulting in toxin degradation (Fig. 1.3) (Sandvig and van Deurs, 1996). However, in susceptible cells, endocytosis is followed by retrograde transport via the Golgi apparatus to the endoplasmic reticulum, followed by translocation to the cytosol (Sandvig *et al.*, 1989; Sandvig *et al.*, 1992; Sandvig *et al.*, 1994; Sandvig and van Deurs, 1996). During this

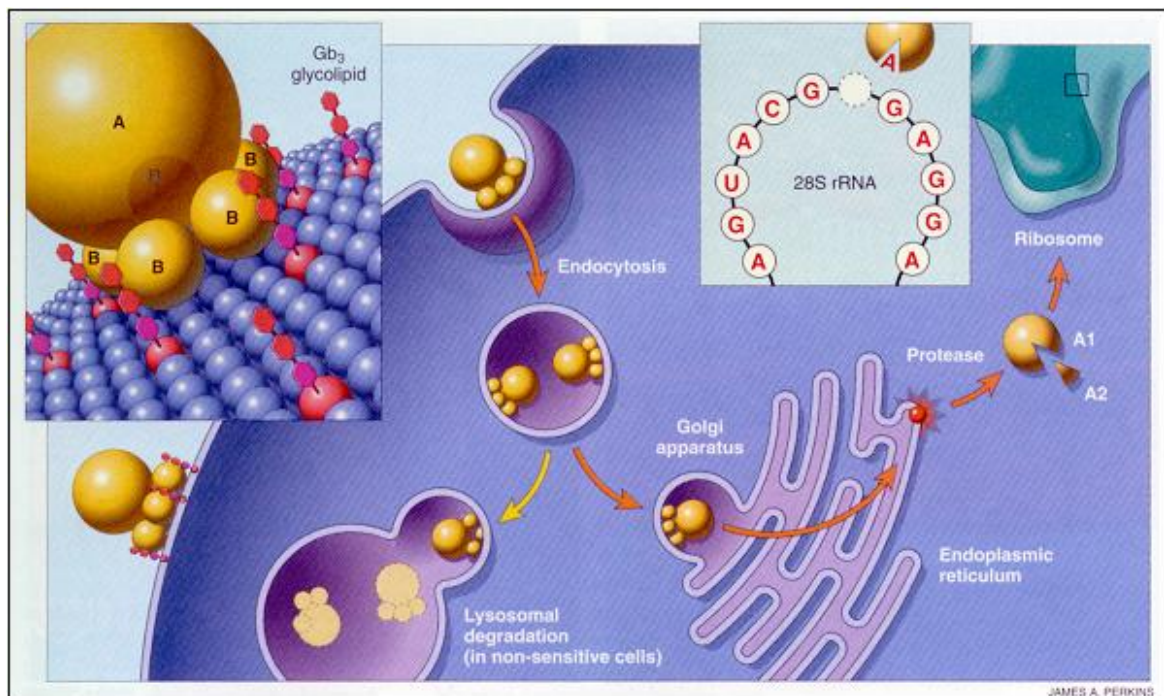


Figure 1.3 Intracellular trafficking of Stx. The holotoxin binds to the glycolipid Gb₃ on the cell surface via its B pentamer and is internalised by endocytosis. In non-sensitive cells, sealed vesicles containing toxin fuse with lysosomes and toxin is degraded (yellow arrow). In sensitive cells, vesicles undergo retrograde transport via the Golgi apparatus to the endoplasmic reticulum. The A subunit of the toxin is nicked by a protease and translocated to the cytosol. The catalytic A1 fragment cleaves a particular adenine base from 28S ribosomal RNA, inhibiting protein synthesis and leading to cell death. Reproduced from Paton and Paton (2000).

process, the A subunit is nicked by a protease (furin) generating a catalytically active 27-kDa amino (N)-terminal A1 fragment and a 4-kDa carboxy (C)-terminal A2 fragment (Garred *et al.*, 1995; Sandvig and van Deurs, 1996). The active A1 subunit has RNA-*N*-glycosidase activity and subsequently cleaves a specific *N*-glycosidic bond in the 28S rRNA (Endo *et al.*, 1988). The resultant loss of an adenine base prevents elongation factor 1-dependent aminoacyl t-RNA binding to the 60S ribosomal subunit (Igarashi *et al.*, 1987; Ogasawara *et al.*, 1988; Saxena *et al.*, 1989), thereby inhibiting the elongation step of protein synthesis, which results in cell death.

1.4.2 Role of Stx in the pathogenesis of HC and HUS

Much of the pathology seen in STEC disease is a result of the systemic effect of Stx. Although production of Stx2 is correlated highly with the development of more serious disease, including HUS (Ostroff *et al.*, 1989; Boerlin *et al.*, 1999; Gerber *et al.*, 2002; Werber *et al.*, 2003), it is important to note that STEC strains producing only Stx1 are also capable of causing both bloody diarrhoea and HUS in humans, albeit less commonly than strains producing Stx2 (Banatvala *et al.*, 2001; Gerber *et al.*, 2002). This is further highlighted by the fact that *Shigella* strains that produce Stx1 only are also capable of causing HUS (O'Brien and Holmes, 1987; Kovitangkoon *et al.*, 1990; Srivastava *et al.*, 1991). The pathological features and clinical manifestations of Stx-induced disease are dependent upon the distribution of the target receptor. Gb₃ is particularly concentrated in renal tissue and in microvascular endothelial cells of the kidney, intestine, pancreas and brain, consistent with the pathological features of both bloody diarrhoea and HUS (Fontaine *et al.*, 1988; Obrig *et al.*, 1993).

The role of Stx in pathogenesis has been extensively examined in several animal models. When macaque monkeys were infected intragastrically with either a wild-type *S. dysenteriae* 1 strain producing Stx, or an isogenic mutant lacking Stx, only the strain producing Stx induced a combination of bloody diarrhoea, destruction of colonic mucosa, severe inflammatory vasculitis of the peritoneal mesothelium, and an efflux of inflammatory

cells into the intestinal lumen (Fontaine *et al.*, 1988). In streptomycin-treated mice, oral challenge with *E. coli* strains expressing Stx2 (but not Stx1) resulted in acute renal cortical necrosis and death (Wadolkowski *et al.*, 1990a; Wadolkowski *et al.*, 1990b). However, unlike that observed in HUS patients (Richardson *et al.*, 1988), thrombotic microangiopathy of the glomeruli was not observed in this mouse model of Stx-mediated disease (Wadolkowski *et al.*, 1990b). Tesh *et al.* (1993) also reported renal cortical epithelial cell damage in the kidneys of mice injected intraperitoneally or intravenously with either purified Stx1 or Stx2. Stx2 had a 50% lethal dose (LD₅₀) that was approximately 400-fold lower than that of Stx1, even though Stx1 was found to have a 10-fold higher affinity for the cellular receptor Gb₃ compared to Stx2 (Tesh *et al.*, 1993). The role of Stx in renal damage is further supported by data showing that the administration of antibodies directed against Stx protects mice from Stx-mediated kidney damage and death (Wadolkowski *et al.*, 1990b; Tesh *et al.*, 1993). These data highlight differences in the toxicity of Stx and pathology of Stx-mediated disease in different animal models.

Further support for the role of Stx in the pathogenesis of HC and HUS has been observed in a rabbit model of infection. Rabbits infected with a rabbit diarrhoeagenic *E. coli* (RDEC) strain transfected with a Stx1-converting bacteriophage were shown to have more severe disease than those infected with the wild-type RDEC strain that did not express Stx1 (Sjogren *et al.*, 1994). RDEC expressing Stx1 also caused more serious histological lesions in infected rabbits, including vascular changes, oedema and inflammation that resembled HC in humans (Sjogren *et al.*, 1994). Similarly, in a baboon model, it was shown that animals injected with Stx2 intravenously developed thrombocytopenia, haemolytic anaemia and glomerular thrombotic microangiopathy (Siegler *et al.*, 2003) similar to patients presenting with HUS (Richardson *et al.*, 1988). In contrast, Stx1 produced no histological signs of HUS in this model (Siegler *et al.*, 2003). However, Taylor *et al.* (1999) showed that purified Stx1 was able to induce renal damage and microvascular thrombosis, thrombocytopenia and anaemia in baboons. Other animals, including dogs (Fenwick and Cowan, 1998) and gnotobiotic piglets (Moxley and Francis, 1998) have also been shown to develop some of the pathological features of HUS in response to challenge with either STEC or Stx alone.

Another role for Stx in the pathogenesis of HUS is the development of thrombosis and platelet aggregation. Stx1 is capable of increasing the adherence of leucocytes to human umbilical vein endothelial cells (HUVEC) under physiologic flow conditions, in a dose-dependent manner, most likely by increasing the expression of the leucocyte adhesion molecules E selectin, vascular cell adhesion molecule type 1 (VCAM-1) and intracellular cell adhesion molecule type 1 (ICAM-1) on endothelial membranes (Morigi *et al.*, 1995). Leucocyte adhesion to HUVEC was subsequently inhibited by pre-incubation with antibodies against VCAM-1, ICAM-1 or E selectin (Morigi *et al.*, 1995). Other studies have also shown increased leucocyte adhesion 18 hours (h) after intraperitoneal injection of Stx1 into rats, which resulted in decreased erythrocyte velocity (O'Loughlin and Robins-Browne, 2001). Stx may therefore enhance leucocyte-mediated damage by increasing leucocyte adherence to endothelial cells, reducing blood-flow through the capillaries, and contributing to thrombosis and platelet aggregation, which is a defining characteristic of HUS.

There are several differences between the *in vitro* effects of Stx1 and Stx2 that may correlate with disease progression and severity. Stx2 was found to be 1,000 times more cytotoxic than Stx1 toward human renal glomerular microvascular endothelial cells (HRMEC) despite a 10-fold greater binding capacity of Stx1 (Louise and Obrig, 1995). It has also been suggested that there are differences in the number of available binding sites for Stx1 and Stx2 on the surface of HRMEC, suggesting that the two toxin types engage distinct receptor populations (Louise and Obrig, 1995). This may be due to differences in the fatty acid composition of the ceramide moiety of Gb₃ on HRMEC, with that preferred by Stx1 being more abundant (Louise and Obrig, 1995). This effect appears to be specific for renal endothelial cells, since HUVEC are equally affected by both Stx1 and Stx2 (Louise and Obrig, 1995), although it should be noted that renal endothelial cells are the primary cell target *in vivo*. These studies are further supported by work using human intestinal microvascular endothelial cells (HIMEC); although Stx1 can bind 50 times more effectively than Stx2, these cells are more susceptible to protein synthesis inhibition by Stx2 (Jacewicz *et al.*, 1999). Therefore, these studies suggest that differences in the binding and action of Stx1 and Stx2 on endothelial cells may explain why Stx2-producing STEC strains are more often associated with disease progression. The association between the severity of STEC disease in

humans and the production of Stx2, rather than Stx1, may be related to an enhanced capacity for Stx2 to damage renal glomerular microvasculature during the development of HUS. Endothelial damage can result in platelet adhesion and consumption leading to the thrombocytopenia and thrombosis (Morigi *et al.*, 1995), which is characteristic of HUS.

Cytokines may also play an important role in enhancing the cytotoxicity of Stxs *in vivo*. Co-administration of Stx with TNF- α increases the sensitivity of HUVEC to the effects of the toxin, thereby resulting in increased endothelial damage (Louise and Obrig, 1991). Stx1 is also capable of inducing the synthesis of several pro-inflammatory cytokines, including IL-1 β , TNF- α , IL-6 and IL-8, in a lipopolysaccharide (LPS)-independent manner (van Setten *et al.*, 1996). In the presence of the cytokines TNF- α , IL-1 and IL-6, endothelial cell sensitivity to Stx toxicity is increased one million-fold in murine peritoneal macrophages *in vitro* (Tesh *et al.*, 1994). Similarly, in glomerular epithelial cells, IL-1 and TNF- α , as well as LPS, are able to increase Stx1 toxicity (Hughes *et al.*, 2000). It has also been shown that several cytokines, including IL-1 and TNF- α , as well as LPS, have the ability to upregulate expression of Gb₃ on endothelial cells resulting in a 10- to 100-fold increase in binding sites for Stx1 (van de Kar *et al.*, 1992; Hughes *et al.*, 2000). Taken together, these results suggest that pro-inflammatory cytokine production, including IL-6 and TNF- α , may contribute to endothelial damage by upregulating the concentration of Gb₃ receptors, thereby increasing the sensitivity of cells to the effects of Stx, and may therefore be important in causing endothelial damage.

Apoptosis may also play a role in mediating the cellular damage characteristic of HUS. Apoptotic changes have been observed in renal cortices obtained from children with HUS, from mice infected with *E. coli* O157:H7 and in paediatric renal tubular epithelial cells treated with Stx2 (Isogai *et al.*, 1998; Karpman *et al.*, 1998; Sakiri *et al.*, 1998). Another study also showed apoptotic damage in a renal tubule biopsy specimen obtained from a child with Stx2-mediated HUS (Kaneko *et al.*, 2001). Apoptosis was more severe in human renal tubular epithelial cells (HRTEC) treated with Stx2-producing STEC than in those treated with Stx1-producing STEC, and could also be enhanced by the pre-stimulation of cells with TNF- α (Karpman *et al.*, 1998). In a monocytic (THP-1) cell line Stx1 upregulated TNF- α via transcriptional regulation (Sakiri *et al.*, 1998). Increased levels of TNF- α mRNA were

preceded by the nuclear translocation of the transcriptional activators nuclear factor- κ B (NF- κ B) and activator protein-1 (AP-1) and loss of the cytoplasmic transcriptional inhibitory subunit of κ B (I κ B- α) (Sakiri *et al.*, 1998). The importance of TNF- α is supported in studies using a gnotobiotic mouse model of infection with *E. coli* O157:H7. In this model, mice treated with TNF- α developed severe neurotoxic symptoms and had more severe systemic symptoms and glomerular damage than mice that did not receive TNF- α (Isogai *et al.*, 1998). Administration of a TNF- α inhibitor was also able to prevent the development of these symptoms (Isogai *et al.*, 1998).

1.4.3 Interaction of Stx with intestinal epithelial cells

Since STEC are generally considered to be non-invasive, the ability of Stx to cause systemic disease must be the result of translocation of Stx from the intestinal lumen to the circulation and then to target organs expressing Gb₃. The mechanism by which Stx gains access to the circulation and target tissues is one of the most crucial steps in the pathogenesis of disease. Stx1 and Stx2 have both been shown to translocate across intact, polarised epithelial cells, whilst still remaining biologically active. Acheson *et al.* (1996) described the ability of Stx1 to translocate across polarised human colonic epithelial Caco-2 and T84 cells. This process was found to be energy dependent and did not disrupt tight junction integrity, as measured by transepithelial resistance (Acheson *et al.*, 1996). Translocation of Stx was also saturable, suggesting that the process may be receptor-mediated, but is not related to the binding of Stx1 to Gb₃ since T84 cells do not express this glycolipid (Acheson *et al.*, 1996). Thus, Stx1 is capable of crossing polarised epithelial cells by a transcellular pathway (Acheson *et al.*, 1996). This is supported by the observation of Stx1 within endosomes, and in association with the Golgi network and endoplasmic reticulum of T84 cells (Philpott *et al.*, 1997).

Hurley *et al.* (1999) were the first to study the transepithelial movement of both Stx1 and Stx2. It was found that Stx1 movement was directional, favouring translocation in the physiologically relevant apical-to-basolateral direction, whereas Stx2 movement was not

directional (Hurley *et al.*, 1999). In addition, 40-fold less Stx2 translocated across Caco-2 monolayers in a 24 h period compared to Stx1. However, both toxins remained biologically active after translocation (Hurley *et al.*, 1999). The two toxins therefore appeared to be using different mechanisms for translocation across epithelial cells. This is consistent with the observation that the addition of a 1,000-fold excess of Stx2 to Caco-2 cells did not inhibit the translocation of Stx1 (Hurley *et al.*, 1999). It has been proposed that Stx1 utilises a microtubule-dependent (receptor-mediated endocytosis) vesicle pathway, whereas Stx2 is using a microtubule-independent pathway for translocation (Hurley *et al.*, 1999). Although less Stx2 was shown to translocate across epithelial cells than Stx1 (Hurley *et al.*, 1999), intestinal microvascular endothelial cells are more sensitive to Stx2 than Stx1, which may compensate for the reduced translocation of Stx2 (Jacewicz *et al.*, 1999). These may be important mechanisms whereby Stx traverses the epithelial barrier of the intestine gaining access to the bloodstream and to the kidney and other Gb₃ rich tissues of the body. Another possibility is that Stx is absorbed directly into the blood stream through a break down of the intestinal barrier due to damage caused by the adherence of STEC to epithelial cells. However, no free Stx has been detected in the blood or serum of patients with HUS or STEC infections (Acheson *et al.*, 1998; Paton and Paton, 1998b; te Loo *et al.*, 2000b).

In addition to renal epithelial and endothelial cells (Section 1.4.2), Stx may also induce apoptosis in intestinal epithelial cells. Stx1-induced apoptosis was found to be dependent upon the presence of Gb₃ on Caco-2 and human laryngeal epithelial (HEp-2) cells, but apoptosis was not observed in Gb₃-negative T84 cells (Jones *et al.*, 2000). The B subunit of Stx1 alone is also sufficient to mediate Gb₃-dependent apoptosis, and this process may be regulated via the Bcl-2 family (Jones *et al.*, 2000). Therefore, apoptotic damage of the intestinal epithelial barrier may provide another mechanism whereby Stx can gain access to underlying tissues and the systemic circulation.

To date, conclusive evidence that Gb₃ is expressed on the human intestinal epithelium has not been forthcoming (Bjork *et al.*, 1987; Paton and Paton, 1998b). However, several human-derived colonic epithelial cell lines do express Gb₃, including Caco-2 (Jacewicz *et al.*, 1995), HCT-8 (Thorpe *et al.*, 1999) and HT-29 cells (Jacewicz *et al.*, 1995), but not T84 cells (Philpott *et al.*, 1997). Until Gb₃ receptors are conclusively found in normal human intestinal

epithelium, the relevance of the above *in vitro* findings to human disease should be interpreted with caution.

1.5 Adherence mechanisms

Although production of Stx is a *sine qua non* of STEC virulence, production of Stx alone is not sufficient to cause serious disease. There are over 200 O:H serotypes of STEC which by definition all produce Stx, but not all serotypes have been associated with disease in humans. Therefore, factors other than the production of Stx must be important. Factors that enhance adherence to the intestinal epithelium are likely to be crucial to the disease process. This is supported by data that show that HUS-related STEC strains adhere 2.6-fold more efficiently to human intestinal epithelial (Henle 407) cells than non-human isolates (Paton *et al.*, 1997).

1.5.1 Attaching and effacing (A/E) adherence

Many STEC serogroups capable of causing serious disease in humans (O157, O111 and O26) are capable of forming attaching and effacing (A/E) lesions on enterocytes similar to those produced by EPEC strains (Francis *et al.*, 1986; Sherman *et al.*, 1988; Dytoc *et al.*, 1994a; Vallance and Finlay, 2000), rabbit EPEC (REPEC) (Cantey and Blake, 1977; Marches *et al.*, 2000), and the mouse pathogen *Citrobacter rodentium* (Schauer and Falkow, 1993a; Schauer and Falkow, 1993b; Luperchio and Schauer, 2001). These A/E lesions are characterised by several ultrastructural changes including loss of enterocyte microvilli and the intimate attachment of the bacteria to the host cell surface (Knutton *et al.*, 1989; Donnenberg *et al.*, 1997). Beneath the adherent bacteria, there is an accumulation of cytoskeletal components, including actin and myosin light chain, resulting in the loss of microvilli (effacement) and the formation of pedestals on the epithelial cell surface (Fig. 1.4) (Knutton *et al.*, 1989; Donnenberg *et al.*, 1997; Goosney *et al.*, 2001).

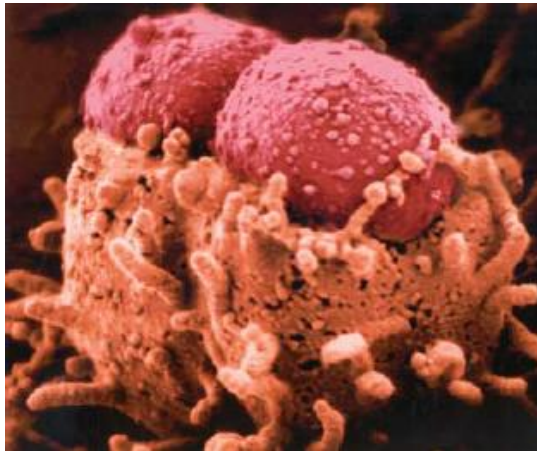


Figure 1.4 Attaching and effacing (A/E) histopathology caused by EPEC and STEC. The attaching and effacing histopathology results in pedestal-like structures, which rise up from the epithelial cell on which the bacteria perch. Reproduced from Kaper *et al.* (2004).

All of the genes responsible for the generation of A/E lesions are located on a 35.5-kb chromosomal pathogenicity island termed the locus for enterocyte effacement (LEE), which encodes for 41 proteins (Fig. 1.5) (McDaniel *et al.*, 1995). Like the LEE locus in EPEC, in O157:H7 STEC the LEE pathogenicity island is inserted at the *selC* locus and all 41 genes are present, in the same order, in both the EPEC O127:H6 strain E2348/69 and the STEC O157:H7 strain EDL933 LEE (McDaniel *et al.*, 1995; Frankel *et al.*, 1998; Perna *et al.*, 1998). The overall nucleotide identity between the EPEC LEE and the STEC O157:H7 LEE is 93.9% (Frankel *et al.*, 1998).

The majority of genes within the LEE island are organised into five polycistronic operons: *LEE1*, *LEE2*, *LEE3*, *tir* and *LEE4* (Fig. 1.5) (Elliott *et al.*, 1988; Mellies *et al.*, 1999). The LEE locus encodes a type III secretion system (secretion of *E. coli* proteins [*sep*] A to *sepI*) that is involved in the secretion of a variety of LEE-encoded proteins (Jarvis *et al.*, 1995; Jarvis and Kaper, 1996). The LEE also encodes several secreted proteins, including *E. coli* secreted protein A (EspA), EspB, EspD and EspF, which are required for host cell signal transduction and the rearrangement of actin and other cytoskeletal proteins essential for pedestal formation (Jarvis and Kaper, 1996; Lai *et al.*, 1997), as well as various chaperone for

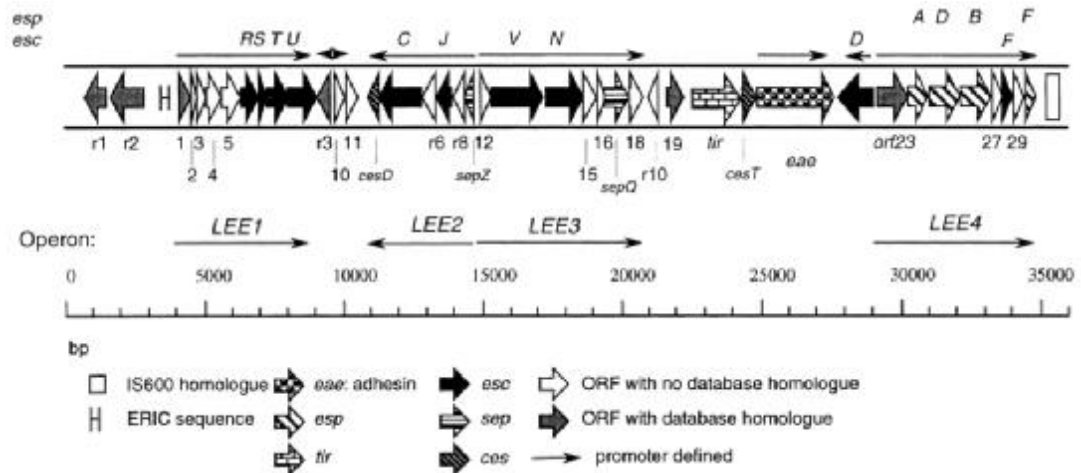


Figure 1.5 Genetic organisation of the LEE pathogenicity island of EPEC strain E2345/69. Block arrows show the open reading frames and direction of transcription. The *esp*, *E. coli* secretion (*esc*) and *ces* genes are labelled. The thin arrows indicate the putative operons, LEE1, LEE2, LEE3 and LEE4. This figure is a modified version of the genetic map of EPEC E2348/69 LEE and is essentially analogous to that of STEC LEE. Reproduced from Mellies *et al.* (1999).

E. coli secreted (Ces) proteins (Abe *et al.*, 1998; Collington *et al.*, 1998; Ebel *et al.*, 1998; Kresse *et al.*, 1999; Mellies *et al.*, 1999). The LEE locus also includes the *E. coli* attaching and effacing (*eae*) gene, which encodes intimin, a 97-kDa outer membrane protein required for the intimate attachment of bacteria to epithelial cells (Jerse *et al.*, 1990; Jerse and Kaper, 1991; Beebakhee *et al.*, 1992; McKee *et al.*, 1995), and *tir* which encodes the translocated intimin receptor (Tir) which is a 72- to 78-kDa protein produced by the bacteria which is then translocated into the host cell membrane where it acts as a receptor for intimin (Kenny *et al.*, 1997; Diebel *et al.*, 1998). The EPEC and STEC LEE proteins that encode the type III secretion system are highly conserved in terms of their amino acid sequence, but intimin and the secreted proteins (Esp and Tir), are more divergent (Paton *et al.*, 1998; Perna *et al.*, 1998).

In EPEC, the initial attachment to epithelial cells is thought to be mediated by the EPEC adherence factor (Eaf) plasmid-encoded bundle forming pili (Bfp) (Tobe *et al.*, 1996; Bustamante *et al.*, 1998). However, no Bfp homologue has been found in STEC, and the

initial attachment of bacteria to epithelial cells is thought to be mediated by EspA (Fig. 1.6) (Ebel *et al.*, 1998; Delahay *et al.*, 1999; Hartland *et al.*, 2000a). EspA is believed to form the terminal part of the translocation apparatus, and EspB and EspD are thought to form a pore structure in the host cell membrane that allows the passage of secreted proteins (Frankel *et al.*, 1998; Wolff *et al.*, 1998; Kresse *et al.*, 1999; Wachter *et al.*, 1999; Hartland *et al.*, 2000a; Vallance and Finlay, 2000; Daniell *et al.*, 2001). EspA and EspD are essential for the translocation of Tir into host cells (Kenny *et al.*, 1997). EspB is translocated into the cytoplasm of epithelial cells in an EspA- and EspD-dependent manner, where it is thought to be involved in the localisation of Tir into the host cell membrane and in signalling pathways leading to cytoskeletal rearrangement and pedestal formation (Kenny *et al.*, 1997; Abe *et al.*, 1998; Knutton *et al.*, 1998; Taylor *et al.*, 1998; Hartland *et al.*, 2000a). Other secreted proteins include EspF, which may affect intestinal epithelial barriers by redistributing the

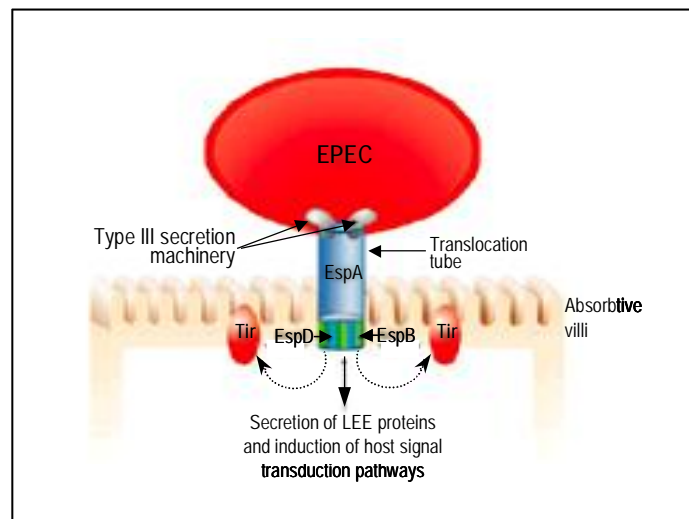


Figure 1.6 Initial adherence and signal transduction stage of EPEC infection. EPEC bind to intestinal epithelial cells and EspA is then secreted by the type III secretion system and forms a putative filamentous translocation tube. EspB and EspD, which are part of the translocation tube, are also secreted by the type III secretion system and inserted into the host cell membrane. These proteins are predicted to form a pore structure, facilitating the translocation of Tir and other LEE secreted proteins (EspS) into the host cell, resulting in induction of host cell signal transduction pathways. Modified from Vallance and Finlay (2000).

tight junction protein occludin, thereby resulting in an increase in intestinal epithelial permeability (McNamara *et al.*, 2001), and may also induce apoptosis in intestinal epithelial cells (Crane *et al.*, 2001).

After the initial attachment involving EspA, subsequent binding to epithelial cells is mediated by the intimate adherence of intimin with its receptor Tir on the epithelial cell surface, and the rearrangement of cytoskeletal proteins including filamentous (F)-actin, α -actinin, talin, ezrin, gelsolin, myosin II, tropomyosin and villin, resulting in the formation of pedestals (Fig. 1.7) (Knutton *et al.*, 1989; Finlay *et al.*, 1992; Kenny *et al.*, 1997; Vallance and Finlay, 2000; Goosney *et al.*, 2001). In EPEC, Tir is tyrosine-phosphorylated once it is delivered into the host cell (Kenny and Finlay, 1997; Diebel *et al.*, 1998), but Tir is not tyrosine-phosphorylated in O157:H7 strains (DeVinney *et al.*, 1999; DeVinney *et al.*, 2001). However, Tir in other STEC serogroups, including O26, are tyrosine-phosphorylated similar to that in EPEC strains (Diebel *et al.*, 1998). In EPEC, A/E lesion formation is absolutely dependent upon tyrosine-phosphorylation of Tir, and EPEC *tir* mutants cannot be

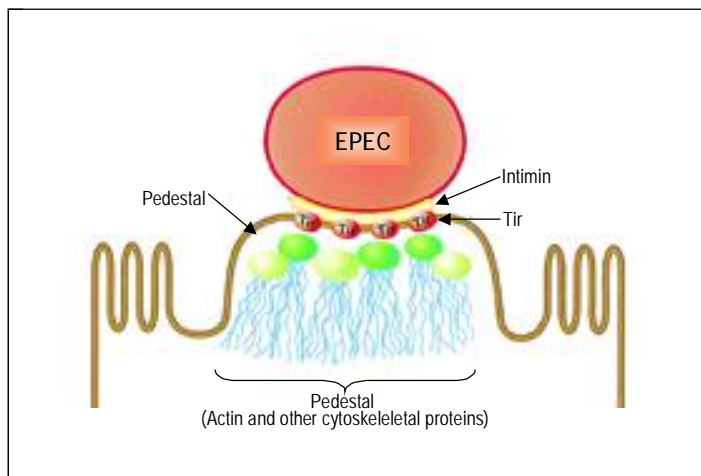


Figure 1.7 The intimate attachment of EPEC to epithelial cells. In the final step of adherence, EPEC intimately attaches to the host cell via intimin-Tir binding and the collapse of the type III secretion machinery. Actin and other cytoskeletal components are then recruited and rearranged beneath the intimately attached bacteria, resulting in pedestal formation. Modified from Vallance and Finlay (2000).

complemented by EHEC *tir* (DeVinney *et al.*, 2001). This suggests that there may be differences in the signalling pathways involved in A/E lesion formation between these pathogens, and within STEC.

There are several differences in the A/E lesions of EPEC and STEC. Firstly, the lesions occur in different sections of the human intestine, which may be related to differences in the type of intimin expressed by the bacteria. The C-terminal end of intimin may be critical, since it is involved in receptor binding (Beebakhee *et al.*, 1992; Yu and Kaper, 1992). There are at least five antigenically distinct subtypes of intimin that have been identified (α , β , γ , δ and ϵ) and each subtype is associated with particular serotypes of EPEC and/or STEC (Adu-Bobie *et al.*, 1998; Oswald *et al.*, 2000). This may account for the differences seen in binding of EPEC and STEC to the small bowel and colon, respectively (McGraw *et al.*, 1999; Oswald *et al.*, 2000). STEC O157:H7 A/E lesion formation was shown to be restricted to the follicle-associated epithelium of Peyer's patches in paediatric intestinal cells, whereas EPEC strains were shown to bind to all regions of the small intestinal mucosa (Phillips *et al.*, 2000b). When an EPEC intimin mutant was complemented with intimin from STEC, the resulting EPEC strain showed increased binding to the follicle-associated epithelium of Peyer's patches in a manner similar to that associated with wild-type STEC (Phillips *et al.*, 2000a). Interestingly, this occurred without the replacement of the original EPEC *tir* suggesting that another receptor may be involved in intimin binding (Phillips *et al.*, 2000a). Other studies have also demonstrated that the intimin subtype is associated with differences in binding in a Tir-independent manner (Hartland *et al.*, 1999; Fitzhenry *et al.*, 2002). The eukaryotic receptor for intimin α , β and γ on HEp-2 cells has now been identified as nucleolin (Sinclair and O'Brien, 2002, Sinclair and O'Brien, 2004).

These results have also been supported by studies investigating the impact of expression of different intimin types in the *C. rodentium* mouse model (Reece *et al.*, 2001). A mutation in the V252/911 residues in intimin α (which is associated with EPEC) did not change the ability to cause A/E lesions on HEp-2 cells (Reece *et al.*, 2001). However, in the mouse model of infection, adherence was limited to the follicle-associated epithelium of the Peyer's patch, similar to that observed with intimin γ -producing STEC strains (Reece *et al.*, 2001). Likewise, when *C. rodentium* was engineered to express intimin γ from STEC (instead

of intimin β), the strain could not colonise orally infected mice and did not induce colonic hyperplasia (Hartland *et al.*, 2000b). Similarly, when an STEC O157:H7 strain (86-24) lacking *eae* was engineered to express intimin from an EPEC O127 strain and then used to challenge gnotobiotic piglets, the STEC strain behaved more like EPEC and was found to colonise the distal half of the small intestine and the surface of the large intestine, but not the colon (Tzipori *et al.*, 1995). These results suggest that a host cell receptor, in addition to Tir, may be responsible for the binding of intimin to host cells *in vivo*, and helps explain the tissue tropism associated with these pathogens in humans (Tzipori *et al.*, 1995; Phillips *et al.*, 2000a).

Other differences exist between the LEE loci of STEC and EPEC. When the entire LEE locus from EPEC was cloned into *E. coli* K-12, A/E lesions were observed suggesting that the LEE locus of EPEC contains all the genes required for the formation of A/E lesions on epithelial cells (McDaniel and Kaper, 1997). In contrast, when the entire LEE locus from STEC O157:H7 was cloned into *E. coli* K-12, the A/E phenotype was not observed (Elliott *et al.*, 1999). This suggests that in STEC a factor(s) outside of the LEE locus is required for A/E lesion formation that is not present in *E. coli* K-12, and therefore that functional and/or regulatory differences exist between the EPEC and STEC LEE loci (Elliott *et al.*, 1999). In EPEC, *eae*, as well as the *bfp* operon, is regulated by the plasmid-encoded regulator (*per*) locus (Gomez-Duarte and Kaper, 1995; Tobe *et al.*, 1996; Bustamante *et al.*, 1998). In EPEC, Per was shown to transcriptionally activate both *eae* and *bfp* (Gomez-Duarte and Kaper, 1995; Tobe *et al.*, 1996; Bustamante *et al.*, 1998). Per is now thought to be a global regulator of EPEC LEE and induces the production of a transcriptional activator, the LEE-encoded regulator (Ler) (Mellies *et al.*, 1999). Together Per and Ler activate the expression of the LEE genes in EPEC (Perna *et al.*, 1998; Mellies *et al.*, 1999). LEE-positive STEC also have a Ler homologue that regulates the expression of the Esps, intimin and Tir, and is required for A/E lesion formation in HEp-2 cells (Elliott *et al.*, 2000). A naturally occurring O157:H7 STEC strain (95SF2) with a defective Ler was unable to adhere to and form A/E lesions on HEp-2 cells (Ogierman *et al.*, 2000). However, when a functional Ler from O157:H7 strain EDL933 was introduced into this strain, it was able to adhere to and form A/E lesions on HEp-2 cells similar to wild-type EDL933 (Ogierman *et al.*, 2000). To date, no Per

homologue has been found in STEC, although an as yet unidentified analogous regulator may exist in these strains (Gomez-Duarte and Kaper, 1995; Mellies *et al.*, 1999).

To date, other than Stx, LEE-mediated adherence is the only STEC trait that has been associated with an increased capacity to cause disease in both humans and animals. In STEC O157:H7, *eae* has been shown to be crucial for colonisation, A/E lesion formation and diarrhoea in both gnotobiotic piglets (McKee *et al.*, 1995; Tzipori *et al.*, 1995) and in calves (Dean-Nystrom *et al.*, 1998). An *eae* deletion mutant of STEC O157:H7 strain 86-24 lacked intimate attachment to colonic epithelial cells of newborn piglets (Donnenberg *et al.*, 1993). Similarly, in EPEC, mutants lacking EspA and EspB are unable to cause A/E lesion formation in the rabbit intestine (Abe *et al.*, 1998). In the mouse, a $\Delta espB$ mutant of the A/E pathogen *C. rodentium* failed to colonise the intestine (Newman *et al.*, 1999), but an *eae*-deficient *C. rodentium* strain that had been complemented with EPEC intimin exhibited restored virulence (Frankel *et al.*, 1996). In humans, antibody responses in STEC patients to several LEE-encoded factors have been reported, including Tir, EspA and intimin (Jerse and Kaper, 1991; Paton *et al.*, 1998; Voss *et al.*, 1998; Jenkins *et al.*, 2000; Sanches *et al.*, 2000), indicating that these LEE-encoded proteins are expressed during human STEC infection.

1.5.2 Non-A/E lesion-mediated adherence mechanisms

Factors other than A/E lesion-mediated adherence may be important in the ability of STEC to adhere to the intestine. This is supported by the observation that many LEE-negative STEC strains are also capable of causing severe disease, including HUS (Paton and Paton, 1998b), and must therefore utilise different mechanisms to adhere to the intestine in order for colonisation to occur. The LEE-negative O113:H21 STEC strain CL-15 has been shown to adhere to both HEp-2 cells and rabbit intestine causing microvillus effacement (Fig. 1.8), this occurs in the absence of the *eae* gene, and without the recruitment of α -actinin or the polymerisation of actin (Dytoc *et al.*, 1994b).



Figure 1.8 Adherence of STEC O113:H21 strain CL-15. Transmission electron micrograph of organisms (arrows) adhering to surface epithelial cells in the caecum of a rabbit challenged 10 days earlier with CL-15. Normal microvilli appear in the top right of the diagram, whereas adherent STEC (arrows) cause effacement of microvilli without the formation of pedestals. Approximate magnification, $\times 20,000$. Reproduced from Dytoc *et al.* (1994b).

Similarly, LEE-positive strains have other putative adhesins. One study screened the ability of over 4,000 transposon mutants in an O157:H7 strain to adhere to Caco-2 cells (Tatsuno *et al.*, 2000). The less adherent mutants were then divided into three distinct groups: (i) non-adherent (10 mutants), (ii) less adherent than wild-type (16 mutants) and (iii) unable to form microcolonies but able to adhere in a diffuse manner (1 mutant) (Tatsuno *et al.*, 2000). Although some of these strains had mutations in the LEE locus (group [i] mutants were unable to secrete type III proteins into supernatants and the group [iii] mutant did not produce Tir or intimin), the group (ii) mutants were able to secrete type III proteins and to produce A/E lesions, suggesting that the defects in adherence occurred outside of the LEE locus (Tatsuno *et al.*, 2000). These group (ii) mutants included Efa1 (Section 1.5.2.3.3), open reading frame (ORF) 3 of a region homologous to an EPEC pathogenicity island (the locus for improving microcolony formation [LIM]) (Tobe *et al.*, 1999), and several putative or hypothetical membrane proteins, among others (Tatsuno *et al.*, 2000). These data suggest that other factors are important in mediating adherence in addition to, or *in lieu of*, the LEE locus.

1.5.2.1 Role of STEC megaplasms in adherence

Most STEC strains associated with human disease possess a large virulence plasmid (or megaplasmid). All O157:H7 strains possess a well-characterised 92- to 104-kb plasmid, designated pO157 (Schmidt *et al.*, 1994; Schmidt *et al.*, 1996a; Burland *et al.*, 1998; Makino *et al.*, 1998; Nataro and Kaper, 1998). Similar large plasmids are also found in many non-O157:H7 STEC strains (Schmidt *et al.*, 1999). Megaplasms from the same STEC serotype are usually similar in size. However, megaplasms from LEE-negative STEC strains are usually larger than those carried by the LEE-positive strains (Boerlin *et al.*, 1998). These megaplasms carry a variety of genes that may be involved in adherence, or encode putative accessory virulence factors. Thus, gene(s) encoded on these plasmids may aid in the pathogenesis of disease caused by these strains.

Studies investigating the role of the 60-MDa O157:H7 plasmid (pO157) in the adherence of O157:H7 initially linked this property to the presence of fimbriae (Karch *et al.*, 1987). STEC O157:H7 strains that possessed pO157 were shown to express fimbriae and were able to adhere to Henle 407 cells, but not HEp-2 cells (Karch *et al.*, 1987). When O157:H7 was cured of pO157, the expression of fimbriae, as well as adherence to Henle 407 cells, was reduced compared to the wild-type strain (Karch *et al.*, 1987). Similarly, the introduction of pO157 into *E. coli* K-12 conferred the production of fimbriae and induced an adherent phenotype when tested on Henle 407 cells (Karch *et al.*, 1987). However, sequence analysis of pO157 has shown that there are no fimbrial loci on pO157 (Burland *et al.*, 1998; Makino *et al.*, 1998). Other studies have also suggested that although the presence of fimbriae increases the number of bacteria adhering to cells, non-fimbriated bacteria are still capable of adhering *in vitro* to HEp-2 and Henle 407 cells (Sherman *et al.*, 1987; Toth *et al.*, 1990), and *in vivo* to the rabbit intestinal tract (Ashkenazi *et al.*, 1992).

Subsequent studies have provided conflicting results on the role of pO157 in the adherence of O157:H7 strains. In one study, curing of pO157 resulted in a three-fold increase adherence to Henle 407 cells compared to the wild-type strain (Junkins and Doyle, 1989). However, other studies have shown either no difference (Fratamico *et al.*, 1993) or reduced (Toth *et al.*, 1990) adherence of plasmid-cured O157:H7 to Henle 407 or HEp-2 cells.

Studies in animal models have also yielded conflicting results on the role of pO157. In a gnotobiotic piglet model of infection, pO157 was found to be unimportant for adherence and virulence in piglets (Tzipori *et al.*, 1987; Tesh and O'Brien, 1992) and curing of pO157 had no effect on diarrhoea or intestinal pathology in rabbits (Li *et al.*, 1993). Similarly, when streptomycin-treated mice were challenged with either O157:H7 EDL933 or its plasmid-cured (Cu) derivative (EDL933-Cu) both strains were capable of colonising the mouse at a similar level (Wadolowski *et al.*, 1990a). However, when both EDL933 and EDL933-Cu were administered to mice at the same time, the wild-type strain out competed EDL933-Cu, suggesting that a factor(s) encoded on the plasmid may be important in colonisation of the mouse intestine and in establishing an infection (Wadolowski *et al.*, 1990a). In rabbits, an O157:H7 strain and its plasmid-cured derivative both adhered to intestinal ileal tissue to a similar degree (Dytoc *et al.*, 1993). Thus there is no clear and unequivocal role for pO157 in the adherence of O157:H7 strains either *in vitro* or *in vivo* and further studies are needed to determine the role, if any, of pO157 in adherence.

1.5.2.2 Fimbrial-mediated adherence

1.5.2.2.1 Sorbitol-fermenting pili

In addition to non-sorbitol fermenting STEC strains such as O157:H7, sorbitol-fermenting O157:H⁻ strains are also important human pathogens that are responsible for diarrhoea as well as outbreaks of HUS (Karch and Bielaszewska, 2001). Sorbitol-fermenting O157 STEC have a distinct megaplasmid, pSFO157, which differs markedly from that carried by non-sorbitol-fermenting O157:H7 strains, pO157 (Brunner *et al.*, 1999; Brunner *et al.*, 2001). A novel 32-kb locus in pSFO157, designated sorbitol-fermenting EHEC O157 fimbriae, plasmid-encoded (*sfp*) was identified that encodes proteins with similarity to the P-fimbrial proteins (Pap) of UPEC, but lacks homologues of genes encoding the subunits of a tip fibrillum and the regulatory genes (Fig. 1.9) (Marklund *et al.*, 1992; Brunner *et al.*, 2001).

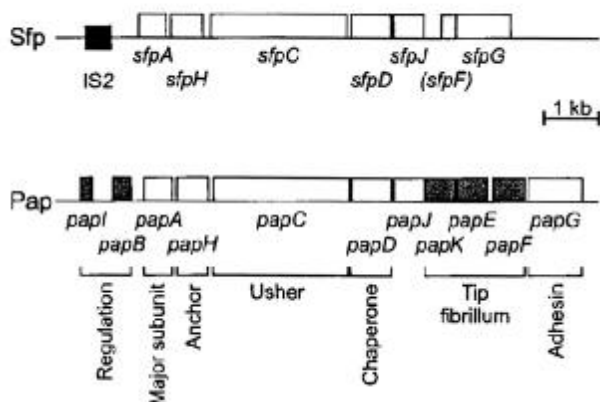


Fig. 1.9 Genetic organisation of the sorbitol-fermenting pili (*sfp*) operon of pSFO157 from sorbitol-fermenting STEC O157:H⁻ strain 3072/96 and comparison with the P-fimbrial (*pap*) operon of UPEC. The white boxes represent ORFs with homology to the *pap* operon; shaded boxes represent ORFs only present in the *pap* operon. The black box depicts a DNA region with high homology to IS2. Modified from Brunder *et al.* (2001).

The *sfp* locus contains six ORFs (*sfpA*, *sfpH*, *sfpC*, *sfpD*, *sfpJ* and *sfpG*), and was determined by PCR analysis to be unique to sorbitol-fermenting O157:H⁻ STEC (Brunder *et al.*, 2001). Although the putative major pilin subunit SfpA has 62% amino acid identity to the major pilin PapA of UPEC, it is more closely related to the PmpA pilin of *Proteus mirabilis* (Bijlsma *et al.*, 1995; Brunder *et al.*, 2001). The putative adhesin is SfpG, but it lacks homology to other known fimbrial proteins (Brunder *et al.*, 2001). An *sfpG* deletion mutant was unable to agglutinate human erythrocytes when compared to the wild-type strain (Brunder *et al.*, 2001). However, as demonstrated by Western blotting with anti-SfpA serum, this mutant was still able to express SfpA on the surface (Brunder *et al.*, 2001). Therefore, SfpG is likely to be the adhesin necessary for binding to erythrocytes, and may also be important in attachment to intestinal epithelia (Brunder *et al.*, 2001).

1.5.2.2.2 Long polar fimbriae

Long polar fimbriae (LPF) have also been implicated in the adherence of both LEE-positive and LEE-negative STEC. Homologues of the LPF of *Salmonella enterica* serovar Typhimurium (*S. typhimurium*) have been identified in the chromosome of both LEE-negative O113:H21 and LEE-positive O157:H7 STEC strains (Doughty *et al.*, 2002; Torres *et al.*, 2002). In O113:H21 the locus consists of four ORFs, *lpfA*_{O113} to *lpfD*_{O113}, and homologues of *lpfA*_{O113} were found in most LEE-negative (26 of 28) and LEE-positive (8 of 11) STEC strains (Doughty *et al.*, 2002). Based on similarity to other fimbrial proteins, LpfA_{O113} is predicted to be the major fimbrial subunit protein, LpfB_{O113} a putative chaperone, LpfC_{O113} an outer membrane usher and LpfD_{O113} a putative minor fimbrial subunit (Doughty *et al.*, 2002). In O157:H7, the locus contains six putative ORFs, *lpfA*, *B*, *C*, *C'*, *D* and *E* (Fig. 1.10) (Torres *et al.*, 2002).

Deletion of *lpfA*_{O113} resulted in reduced adherence to Chinese hamster ovary (CHO) cells compared with the wild-type O113:H21 strain (Doughty *et al.*, 2002). Similarly, when the *lpf* genes from O157:H7 strain EDL933 were introduced into a non-fimbriated *E. coli* K-12 strain, the transformed strain produced fimbriae and adhered to HeLa and Madin-Darby bovine kidney (MDBK) cells (Torres *et al.*, 2002). A Δ *lpfA*_{O157} mutant of O157:H7 also exhibited reduced adherence to HeLa and MDBK cells and formed fewer microcolonies than the respective wild-type strain (Torres *et al.*, 2002). When *lpfA*_{O157} was reintroduced into the

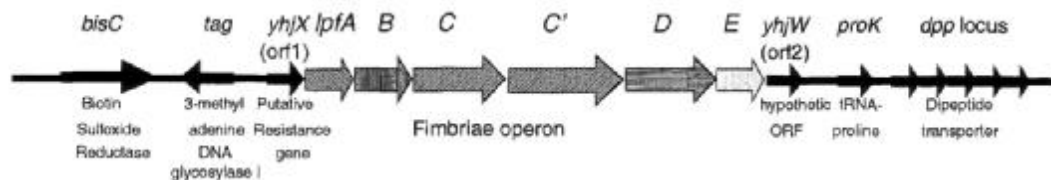


Figure 1.10 Genetic organisation of the long polar fimbriae (*lpf*) operon of STEC O157:H7 strain EDL933. The *lpfA*, *B*, *C*, *C'*, *D* and *E* genes are shown as block shaded arrows and the genes with homology to *E. coli* K-12 genes are represented by solid arrows. Reproduced from Torres *et al.* (2002).

Δlpf mutant on a plasmid, the adherence and microcolony formation phenotypes were restored to that of the wild-type strain (Torres *et al.*, 2002). These data suggest that LPF of STEC may aid in microcolony formation and in adherence to host epithelial cells. Interestingly, homologues of both of these loci were found in the REPEC O15:H⁻ strain 83/39, and mutagenesis of the *lpf_{O157}* homologue resulted in reduced early colonisation by 83/39 and severity of diarrhoea in infected rabbits (Newton *et al.*, 2004).

1.5.2.2.3 Type IV pili

LEE-negative STEC O113 strains carry a large virulence megaplasmid, pO113, that has previously been shown to encode the STEC autoagglutinating adhesin (Saa) (Section 1.5.2.3.1). Sequence analysis of pO113 from 98NK2 also revealed a novel type IV pilus biosynthesis locus (*pil*) approximately 11-kb downstream from *saa* (Srimanote *et al.*, 2002). Type IV pili are important in mediating adherence in a variety of pathogenic bacteria, including the Bfp of EPEC (Giron *et al.*, 1991) and the toxin coregulated pilus (TCP) of *Vibrio cholerae* (Sengupta *et al.*, 1993). The pO113 *pil* locus was found to have greatest deduced amino acid similarity to those of the IncI plasmids Collb-P9 from *Shigella sonnei* and R64 from *S. typhimurium*, and to *E. coli* plasmid R721 (Srimanote *et al.*, 2002).

The *pil* locus is approximately 11.5-kb and contains 11 closely linked genes, designated *pilL* through *pilV*, and an additional separately transcribed gene *pilI* which is upstream of *pilL* (Fig. 1.11) (Srimanote *et al.*, 2002). Among other LEE-negative STEC strains, *pilS* homologues were found in all O113 strains and in 11 of 14 strains belonging to other serogroups (Srimanote *et al.*, 2002). Curing of pO113 from 98NK2 resulted in a loss of long thin pili on the surface of the bacteria, and a loss of haemagglutination of guinea pig erythrocytes (Srimanote *et al.*, 2002). Like Collb9, R64 and R721, pO113 could be transferred by conjugation into the plasmid-cured 98NK2 derivative, 98NK2-Cu (Srimanote *et al.*, 2002). Similarly, pO113 from STEC strain EH41 was recently shown to have a conjugal transfer region (Tra), but was unable to mobilise the IncQ plasmid RSF1010 (Leyton

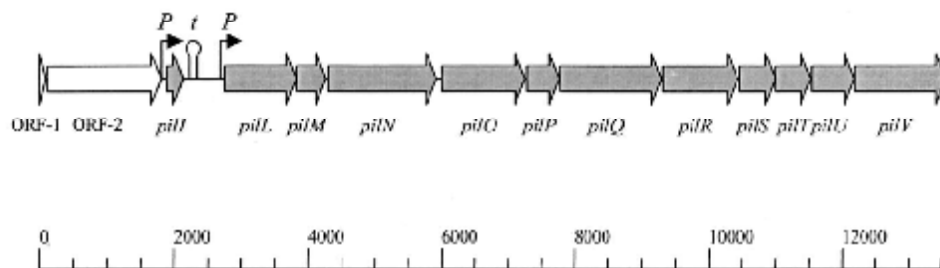


Fig. 1.11 The type IV pili locus from STEC O113:H21 strain 98NK2. Block arrows show the ORFs, and the putative promoter (*P*) and transcription termination (*t*) sites are labelled. The scale below the figure is in base pairs. Reproduced from Srimanote *et al.* (2002).

et al., 2003). This is in contrast to pO157, which lacks *pil* loci or *tra* genes and is therefore non-conjugative (Burland *et al.*, 1998; Makino *et al.*, 1998; Srimanote *et al.*, 2002). Although these type IV pili agglutinate guinea pig erythrocytes, they were not involved in mediating adherence of 98NK2 to HEP-2 cells or HCT-8 cells (Srimanote *et al.*, 2002). Therefore, the role of type IV pili in STEC adherence remains to be elucidated.

1.5.2.3 Putative non-fimbrial adherence mechanisms

1.5.2.3.1 STEC autoagglutinating adhesin

STEC autoagglutinating adhesin (Saa) is a unique adhesin produced only in LEE-negative STEC. The prototype Saa is a 516-amino acid protein, which is encoded on the large megaplasmid of an O113:H21 strain 98NK2 (pO113) that was responsible for an outbreak of HUS in Adelaide, Australia (Paton *et al.*, 1999; Paton *et al.*, 2001a). The Saa protein was found to have a moderate degree of similarity (25% amino acid identity) to the YadA protein of *Yersinia enterocolitica* (Paton *et al.*, 2001a). In *Y. enterocolitica*, YadA contributes to autoagglutination, serum resistance, complement activation, epithelial cell adherence, and resistance to phagocytosis and binding to extracellular matrix proteins (China *et al.*, 1993;

Tamm *et al.*, 1993; Tahir *et al.*, 2000), and is also encoded on a large virulence plasmid (Cornelis *et al.*, 1998).

Mutations in *saa*, or curing of the virulence plasmid pO113, resulted in significantly reduced adherence of 98NK2 to HEp-2 cells (Paton *et al.*, 2001a). Similarly, when cloned *saa* was expressed in *E. coli* K-12, there was a 9.7-fold increase in adherence to HEp-2 cells and the strain showed a semi-localised adherence pattern (Paton *et al.*, 2001a). *Saa* homologues were also detected in many other LEE-negative STEC serotypes, including O48:H21 and O91:H21, but not in LEE-positive STEC isolates (Paton *et al.*, 2001a). The presence of *saa* also correlated with the presence of the enterohaemolysin gene *ehxA* (Section 1.6.1) in these strains (Paton *et al.*, 2001a). *Saa* produced by other LEE-negative isolates, ranged in size from 460- to 534-amino acids, and this was due principally to variation in the number (2–4) of 37-amino acid repeats towards the C-termini of the proteins (Paton *et al.*, 2001a). *Saa* was the first adhesin shown to be specific for LEE-negative strains, and may be important *in vivo* for non-A/E lesion-mediated adherence to intestinal epithelial cells.

1.5.2.3.2 *IrgA* homologue adhesin

Another putative adhesin is *IrgA* homologue adhesin (*Iha*), which was first described in *E. coli* O157:H7 strains (Tarr *et al.*, 2000). As its name implies, *Iha* is 53% identical at the amino acid level to the iron regulated gene A (*IrgA*) of *V. cholerae* (Goldberg *et al.*, 1992), which has been shown to be important for the adherence and colonisation of mice by *V. cholerae* (Goldberg *et al.*, 1990). *Iha* is chromosomally encoded in O157:H7 and homologues have been found in both LEE-positive (O157:H7) and LEE-negative (including O104:H21 and O113:H21) STEC strains, as well as in a RDEC-1 strain (Tarr *et al.*, 2000). An *iha* homologue has been detected on both the chromosome and the pO113 megaplasmid from STEC strain 98NK2 (Srimanote, 2003; GenBank accession number AF399919).

DNA adjacent to *iha* in the O157:H7 genome contains the tellurite resistance locus that is conserved in pathogenic *E. coli*, but which is absent from non-toxigenic *E. coli* O55:H7, sorbitol-fermenting STEC O157:H⁻ and *E. coli* K-12 (Tarr *et al.*, 2000). This region

was therefore termed the tellurite resistance- and adherence-conferring island (TAI island) (Tarr *et al.*, 2000). When *iha*_{O157} was cloned into *E. coli* K-12 it conferred adherence to both HeLa and MDBK cells (Tarr *et al.*, 2000). *E. coli* K-12 containing cloned *iha*_{O157} was found to express a 78-kDa outer membrane protein, but in the wild-type O157:H7, Iha was detected as a 67-kDa outer membrane protein (Tarr *et al.*, 2000). This suggests that the full-length Iha may be cleaved in O157:H7 (Tarr *et al.*, 2000).

However, in LEE-negative O113:H21 and O104:H21 strains, Iha was detected as a 56-kDa outer membrane protein (Tarr *et al.*, 2000). Thus, significant size heterogeneity exists between Iha of different serotypes of STEC, and may represent differences in proteolytic cleavage or degradation (Tarr *et al.*, 2000). When *iha*_{O113} was deleted from pO113 in 98NK2, no reduction in adherence to HEp-2 cells was observed (Srimanote, 2003). Similarly, when both the chromosomal- and pO113-encoded copies of *iha*_{O113} were deleted from 98NK2, no decrease in adherence to HEp-2 cells was observed compared to the wild-type strain (unpublished results from our laboratory). Therefore, neither chromosomal- nor plasmid-encoded Iha_{O113} appear to play a role in adherence of 98NK2 to HEp-2 cells. However, Iha may contribute to adherence of other STEC serotypes, including O157:H7, to epithelial cells of either human or bovine origin.

1.5.2.3.3 Toxin B and EHEC factor for adherence

Sequence analysis of pO157 revealed an ORF, the N-terminal 700-amino acids of which exhibited significant similarity to the toxin family known as large clostridial toxins (LCT), which includes Toxin A and Toxin B from *Clostridium difficile* (Lyerly *et al.*, 1988; Burland *et al.*, 1998). Interestingly, the intestinal lesions produced in O157:H7 colitis, and the toxin-mediated damage to intestinal cells in *C. difficile* patients, are very similar, suggesting that this toxin may play an important role in the intestinal pathology during STEC infection (Griffin *et al.*, 1990). This large ORF (3,169-amino acids) in pO157 was named toxin B (*tox*B) (Burland *et al.*, 1998). A homologue of *tox*B has also been found on the

chromosome of non-O157:H7 LEE-positive STEC strains and named EHEC factor for adherence (Efa1).

Efa1 is a novel protein that is associated with increased adherence in STEC. Efa1 is encoded by a large (9.7-kb) gene on the chromosome of O111:H⁻, O55:H7, O157:H⁻ and other non-O157:H7 LEE-positive STEC strains (Nicholls *et al.*, 2000; Janka *et al.*, 2002; Stevens *et al.*, 2002). Efa1 is not required for A/E lesion formation and is not physically linked to the LEE pathogenicity island (Nicholls *et al.*, 2000). Efa1 has 97.4% amino acid identity to the EPEC *lifA* gene product lymphostatin (Stevens *et al.*, 2002) and also has low amino acid identity (28%) to ToxB of pO157 (Burland *et al.*, 1998; Makino *et al.*, 1998). An *efa1* mutant of STEC O111:H⁻ was found to have a 7-fold reduction in adherence to CHO cells, and exhibited a loss of both autoaggregation and haemagglutination of human erythrocytes (Nicholls *et al.*, 2000). Likewise, a 19-fold reduction in HeLa cell adherence of an STEC O5 $\Delta eaf1$ strain was observed compared to the wild-type strain (Stevens *et al.*, 2002). Although the *efa1* mutant retained the ability to form A/E lesions on HeLa cells it had a reduced ability to express and secrete the LEE-encoded proteins EspA and Tir, which are required for A/E lesion formation (Stevens *et al.*, 2002). Recently, the contribution of Efa1 to virulence was also determined *in vivo* in a bovine model of infection. Efa1 was found to be essential for efficient colonisation of the bovine intestinal epithelium (Stevens *et al.*, 2002). Calves that received the mutant strain did not develop bloody diarrhoea or other clinical signs of infection, whereas calves receiving the wild-type STEC developed bloody diarrhoea three days post inoculation (Stevens *et al.*, 2002). Thus, Efa1 may also be important in adherence to the human intestinal tract.

Similarly, with ToxB, an O157 strain cured of plasmid pO157 showed a reduced adherence to Caco-2 cells and lower secretion of the LEE-encoded proteins EspA, EspB and Tir compared to the wild-type strain (Tatsuno *et al.*, 2001). When *toxB* was reintroduced on a plasmid into the plasmid-cured strain, the adherence phenotype and secretion of LEE-encoded proteins was restored (Tatsuno *et al.*, 2001). Similarly, a $\Delta toxB$ mutant also showed reduced adherence and lower levels of secretion of the LEE-encoded proteins (Tatsuno *et al.*, 2001). These results are similar to those reported with the chromosomally encoded homologue Efa1

(Stevens *et al.*, 2002). Thus, ToxB and Efa1 may be important in both adherence and the secretion of LEE-encoded proteins.

1.5.2.3.4 Locus of proteolysis activity

Another novel locus found only in LEE-negative Stx2d-positive STEC, is the locus of proteolysis activity (LPA). This 33-kb locus, which contains 24 ORFs, was discovered in an O91:H⁻ strain and is integrated 15-bp downstream of *selC* (Schmidt *et al.*, 2001), the integration site of the LEE pathogenicity island (Section 1.5.1) (McDaniel *et al.*, 1995; Perna *et al.*, 1998). This locus encodes a novel serine protease (EspI) (Section 1.6.2.2), an adherence locus similar to *iha* of O157:H7 strains (Section 1.5.2.3.2), an *E. coli* vitamin B12 receptor (BtuB), an AraC-type regulatory molecule, and four homologues of *E. coli* phosphotransferase proteins (Schmidt *et al.*, 2001). It also contains complete and incomplete insertion sequences, prophage sequences, and a phage integrase gene downstream of *selC* (Schmidt *et al.*, 2001). The LPA was named according to the proposed function of the serine protease, EspI (*E. coli* secreted protease, island encoded) (Section 1.6.6.2).

The *Iha* homologue from LPA, designated *Iha*₄₇₉₇, has 91% amino acid identity to the *Iha* of *E. coli* O157:H7 (Section 1.5.2.3.2). Although no role for adherence was determined (Schmidt *et al.*, 2001), *Iha*₄₇₉₇ may play a role in adherence of STEC due to its homology to the putative adhesin, *Iha*, of O157:H7. Nine of 35 (26%) LEE-negative *stx*_{2d}-positive STEC strains possessed *iha*, including serotypes O128:H2/H⁻, O62:H⁻, O96:H⁻ and ONT:H⁻ (Schmidt *et al.*, 2001). All STEC O26:H⁻/H11, O111:H11/H, O145:H⁻ and O157:H7 strains also tested positive for *iha*, as well as 42% of ETEC, EAEC, EIEC, and UPEC strains tested (Schmidt *et al.*, 2001).

1.6 Putative accessory virulence factors

In addition to the production of Stx and the ability of strains to adhere to and colonise the intestinal tract, many STEC strains also carry several putative accessory virulence factors that may be important in the pathogenesis of serious disease.

1.6.1 Enterohaemolysin

A significant proportion (89%) of STEC O157:H7, O113:H21, O116:H21 and rough:H⁻ strains isolated from cattle and O139:K12:H1 strains isolated from pigs were observed to have a haemolytic phenotype that was distinct from the *E. coli* α -haemolysin (α -Hly), and was designated EHEC-enterohaemolysin (EHEC-Hly) or Ehx (Beutin *et al.*, 1989; Bauer and Welch, 1996b). Similarly, all O157:H7 strains and the majority (77 to 81%) of non-O157 STEC strains isolated from patients were positive for Ehx, including O157:H⁻, O145:H⁻, O111:H8 and O26:H11 serotypes (Beutin *et al.*, 1994; Schmidt *et al.*, 1995). The Ehx haemolytic phenotype was found to be genetically and serologically unrelated to α -Hly and could be distinguished by the appearance of a lysis zone on plates containing washed sheep erythrocytes after 18–24 h incubation, but not on conventional blood agar (Beutin *et al.*, 1989).

Unlike α -Hly, which is chromosomally encoded, EhxA is encoded on pO157 in O157:H7 strains (Schmidt *et al.*, 1994; Schmidt *et al.*, 1995; Burland *et al.*, 1998; Makino *et al.*, 1998). Sequence analysis of pO157 showed an operon with four ORFs, designated *ehxC*, *A*, *B* and *D*, based on their similarity (60% deduced amino acid identity overall) to the *hlyC*, *A*, *B* and *D* genes of the α -Hly operon (Schmidt *et al.*, 1994; Schmidt *et al.*, 1995; Schmidt *et al.*, 1996a; Burland *et al.*, 1998). This operon was found to have similarity to members of the repeats in toxin (RTX) family of toxins (Schmidt *et al.*, 1996a; Burland *et al.*, 1998). In the case of α -Hly, the HlyA protein is synthesised as an inactive precursor and converted to its active form by the product of the *hlyC* gene (Welch, 1991). The products of *ehxC* and *ehxA* from EDL933 are 19.9-kDa and 107-kDa proteins, respectively, and these share approximately 61% and 66% amino acid identity with α -HlyC and α -HlyA, respectively

(Schmidt *et al.*, 1995). The major difference between EhxA and HlyA is a 70-amino acid region at the N-terminus that is believed to be important for target cell specificity (Bauer and Welch, 1996a).

EhxA has been found to lyse a bovine lymphoma (BL-3) cell line as well as both sheep and human erythrocytes in a calcium-dependent manner (Bauer and Welch, 1996a; Bauer and Welch, 1996b). However, another study found that EhxA was able to lyse guinea pig, mouse and ferret erythrocytes, but not human, rabbit, rat, turkey or chicken erythrocytes (Chart *et al.*, 1998). Unlike α -HlyA, EhxA was shown not to lyse two human lymphoma (Raji and Daudi) cell lines, which may be a result of differences in the N-terminus of these two proteins (Bauer and Welch, 1996b). EhxA formed transient ion-permeable channels, with a diameter of approximately 2.6 nm, by integration into lipid bilayer membranes, in a similar manner to α -HlyA (Schmidt *et al.*, 1996b). EhxA is thought to contribute to pathogenicity by releasing haem and haemoglobin which may act as a source of iron in the gut, thereby aiding the growth and colonisation of STEC (Law and Kelly, 1995).

Eighty eight percent of O111 isolates from HUS cases, but only 22% of O111 isolates from patients with diarrhoea, had a haemolytic phenotype, suggesting that EhxA may play an important role in the progression to severe disease (Schmidt and Karch, 1996). EhxA is highly immunogenic, since 19 of 20 convalescent-phase sera from patients recovering from O157-related HUS had antibodies to EhxA, indicating that this protein is expressed during serious STEC infections (Schmidt *et al.*, 1995). In Ehx-positive STEC, the haemolysin operon is highly conserved (Boerlin *et al.*, 1998) and may be associated, along with *eae* and *stx₂*, with the capacity to cause severe disease in humans (Boerlin *et al.*, 1999).

1.6.2 Serine protease autotransporter of Enterobacteriaceae (SPATE) family

1.6.2.1 Extracellular serine protease P

Another putative virulence factor encoded on pO157 is the extracellular serine protease, plasmid encoded (EspP) (Brunner *et al.*, 1997; Burland *et al.*, 1998; Makino *et al.*, 1998). The *espP* gene encodes a large 141.8-kDa protein, which undergoes N- and C-terminal processing during secretion, resulting in a 104.5-kDa mature protein (Brunner *et al.*, 1997). EspP is 64% identical to the N-terminal end of EspC of EPEC (Stein *et al.*, 1996; Brunner *et al.*, 1997) and 45% identical to the immunoglobulin A1 (IgA1) proteases from *Haemophilus influenzae* and *Neisseria* spp. (Poulsen *et al.*, 1992; Lomholt *et al.*, 1995; Brunner *et al.*, 1997). The amino acid sequence of EspP showed that it was the same protein as the 104-kDa protein found in O26:H⁻ STEC strains (Ebel *et al.*, 1996), which had previously been designated PssA (protease secreted by STEC) (Djafari *et al.*, 1997).

Southern hybridisation analysis initially showed that *espP* homologues were widespread in O157 and O26 STEC serogroups (Brunner *et al.*, 1997). Homologues of *espP* are also present in up to 57% of STEC strains belonging to over 16 different serotypes, although it is unique to STEC (Brunner *et al.*, 1999). EspP is a serine protease and was shown to cleave both pepsin A and human coagulation factor V, and may be an important contributing factor to the intestinal haemorrhage observed in patients (Brunner *et al.*, 1997). EspP was also shown to be cytotoxic to Vero cells (Djafari *et al.*, 1997). EspP may be important in the pathogenesis of STEC disease since patients infected with STEC develop antibodies against EspP (Brunner *et al.*, 1997), indicating that this protease is expressed *in vivo*.

1.6.2.2 Extracellular serine protease I

As previously discussed in Section 1.5.2.3.4, some LEE-negative Stx2d-positive STEC contains a novel locus called the locus of proteolysis activity (LPA). The LPA was

named according to the proposed function of the serine protease, EspI. The first 842 nucleotides of *espI* is 94% identical to the 3' end of the serine protease gene of O157:H7, *espP* (Section 1.6.2.1) (Brunder *et al.*, 1997). EspI also has 51% amino acid identity to SepA of *Shigella flexneri*, which is involved in the invasion and destruction of host epithelial cells (Benjelloun-Touimi *et al.*, 1998; Schmidt *et al.*, 2001).

When *espI* was expressed in *E. coli* K-12, production of a protein of 110.4-kDa that is not normally present in *E. coli* K-12 strains was observed (Schmidt *et al.*, 2001). Using pepsin A and human apolipoprotein A-1 as substrates, *E. coli* K-12 expressing EspI demonstrated serine protease activity (Schmidt *et al.*, 2001). Nine of 35 LEE-negative Stx_{2d}-positive STEC strains possessed *espI*, *iha* (Section 1.5.2.3.2) and *btuB*, including serotypes O128:H₂/H⁻, O62:H⁻, O96:H⁻ and ONT:H⁻ (Schmidt *et al.*, 2001). This is a newly discovered pathogenicity island in LEE-negative Stx_{2d}-positive STEC strains that may function in the pathogenicity of these strains.

1.6.2.3 EHEC plasmid-encoded autotransporter

Leyton *et al.* (2003) recently identified a serine protease, EHEC plasmid-encoded autotransporter (EpeA) in an O113:H₂₁ strain (EH41). The *epeA* gene was located near the transfer region of pO113 and was found to encode a novel high molecular mass protein of 1,359-amino acids, which has 58% amino acid identity to the serine protease EspI (Section 1.6.2.2) (Leyton *et al.*, 2003). Wild-type EH41 was found to secrete two high molecular weight proteins of 104- and 112-kDa, respectively, which were common to several O113:H₂₁ strains and were also found in some other LEE-negative STEC strains (Leyton *et al.*, 2003).

Wild-type EH41, and *E. coli* K-12 containing cloned *epeA*, both secreted a 112-kDa protein into culture supernatants, and demonstrated protease and mucinase activity, whereas wild-type *E. coli* K-12 and EH41 Δ *epeA* did not (Leyton *et al.*, 2003). Homologues of *epeA* were found in all (11 of 11) O113:H₂₁ strains, and 63% (15 of 24 strains) of other LEE-negative STEC strains, but not in LEE-positive STEC, EPEC, EAaggEC, EIEC or ETEC (Leyton *et al.*, 2003). However, 85% of LEE-negative STEC strains isolated from patients

with HUS, TTP or HC were positive for *epeA* (Leyton *et al.*, 2003), suggesting that this protease may be important in the pathogenesis of severe LEE-negative STEC disease.

1.6.3 Catalase-peroxidase

Sequence analysis of pO157 revealed an ORF of 2,208-bp, encoding a predicted 736-amino acid protein with substantial similarity to members of the bacterial bifunctional catalase-peroxidase family, including PerA of *Bacillus stearothermophilus* (Loprasert *et al.*, 1989), MI85 from *Mycobacterium intracellulare* (Morris *et al.*, 1992), and KatG of *E. coli* (Triggs-Raine *et al.*, 1988), *Mycobacterium tuberculosis* (Heym *et al.*, 1993) and *S. typhimurium* (Loewen and Stauffer, 1990). This was the first plasmid-encoded catalase-peroxidase and was therefore designated KatP (catalase-peroxidase, plasmid encoded), to distinguish it from KatG and KatE of *E. coli* (Brunner *et al.*, 1996).

Amino acid analysis found peroxidase 1 and 2 motifs, in addition to a N-terminal signal sequence, suggesting that KatP is transported through the cytoplasmic membrane (Brunner *et al.*, 1996). Catalase-peroxidase activity was analysed in both crude cytoplasmic and periplasmic extracts of EDL933 and its plasmid-cured derivative, EDL933-Cu. Activity was found primarily in the periplasmic extract of EDL933 but not in that of EDL933-Cu (Brunner *et al.*, 1996). Similarly, cloning of *katP* into an *E. coli* K-12 strain lacking KatG (UM202) resulted in peroxidase expression, and disruption of *katP* with a *kan* cassette abrogated this activity (Brunner *et al.*, 1996).

PCR analysis identified homologues of *katP* in a range of STEC, including O157, O26 and O111 serogroups and the gene was found to be closely associated with the plasmid-encoded haemolysin operon, *ehx* (Section 1.6.1) (Brunner *et al.*, 1996; Burland *et al.*, 1998). However, *katP* homologues were not detected in a range of other pathogenic *E. coli* including ETEC, EPEC, EAaggEC and EIEC (Brunner *et al.*, 1996). Although a role for KatP has yet to be determined, it may be important *in vivo* for the neutralisation of superoxides produced by neutrophils and macrophages during infection.

1.6.4 Enteroaggregative heat-stable enterotoxin

A heat stable, plasmid-encoded enterotoxin was first identified in EA_gEC and named EA_gEC heat-stable enterotoxin 1 (EAST1) (Savarino *et al.*, 1993). EAST1 is encoded by the EA_gEC heat-stable enterotoxin (*astA*) gene (Savarino *et al.*, 1993). EAST1 is a small peptide of only 4.1-kDa (38-amino acids) that has 50% identity with the ETEC heat-stable enterotoxin a (STa) (Schulz *et al.*, 1990; Savarino *et al.*, 1993).

Chemically synthesised EAST1 was shown to have enterotoxic activity on rabbit ileal tissue *in vitro* (Savarino *et al.*, 1993). Similarly, when *astA* was cloned into *E. coli* K-12, enterotoxic activity was acquired, whereas inactivation of the *astA* gene in the *E. coli* K-12 clone resulted in a loss of this activity (Savarino *et al.*, 1993). Homologues of *astA* have been found in up to 57% of EA_gEC (Savarino *et al.*, 1993), as well as in 100% of O157:H7, 88% of O26 and 52% of other STEC serotypes, and in 41% of ETEC, 73% of EPEC and 7% of DAEC, but were not observed in EIEC (Savarino *et al.*, 1996; Yamamoto *et al.*, 1997). The presence of *astA* also correlates with enterotoxic activity in these strains (Savarino *et al.*, 1996). These studies suggest that EAST1 may have enterotoxic activity and is commonly distributed amongst most pathogenic *E. coli*.

1.6.5 Type II secretion system

Another putative virulence determinant encoded on the large virulence plasmid pO157 is a type II secretion system, which is located 5' to the haemolysin operon, *ehx* (Section 1.6.1) (Schmidt *et al.*, 1997; Burland *et al.*, 1998; Makino *et al.*, 1998). This type II locus comprises 13 ORFs (Fig. 1.12) with homology to the pullulanase operons of *Klebsiella pneumoniae* and *Klebsiella oxytoca* (Pugsley *et al.*, 1990; Pugsley and Reyss, 1990; Reyss and Pugsley, 1990), the *out* operon of *Erwinia chrysanthemi* (Condemine *et al.*, 1992), and the *exe* operons of *Aeromonas hydrophilia* and *Aeromonas salmonicida* (Howard *et al.*, 1993; Karlyshev and MacIntyre, 1995), as well as other type II secretion systems in Gram-negative bacteria (Pugsley, 1993; Schmidt *et al.*, 1997). The ORFs were subsequently named EHEC type II

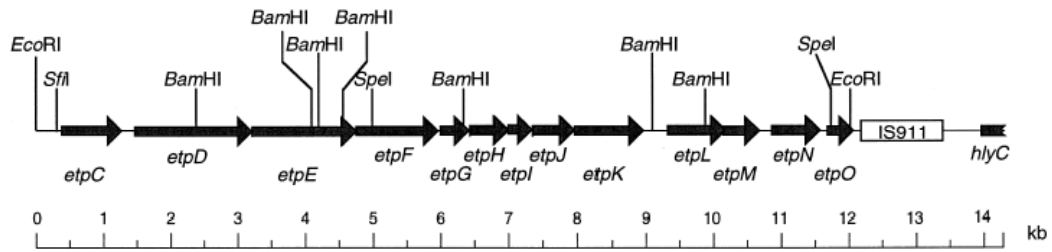


Figure 1.12 Genetic organisation of the type II secretion pathway from STEC O157:H7 strain EDL933. The arrows indicate the length and direction of the ORFs belonging to the EHEC type II secretion pathway (*etp*) operon. Restriction enzyme sites are indicated above. The last ORF at the 3' end, which appears truncated, represents the 5' end of the haemolysin operon (*ehxC/hlyC*) gene. Reproduced from Schmidt *et al.* (1997).

secretion pathway (*etp*), *etpC* to *etpO*, and were separated from the *ehx* operon by an insertion element (Fig. 1.12) (Schmidt *et al.*, 1997).

Homologues of the *etp* genes were found in 100% of O157 strains and 60% of non-O157 STEC strains including O26, O103 and O111 serogroups isolated from patients with HUS or diarrhoea (Schmidt *et al.*, 1997). However, *etp* homologues were not detected in EPEC, EAaggEC, ETEC or EIEC strains (Schmidt *et al.*, 1997). This operon appears to contain all of the genes necessary for secretion of proteins. However, no study has demonstrated the actual secretion of a protein(s) by this pathway and therefore its function and role in pathogenesis remains uncertain.

1.6.6 Subtilase cytotoxin

The STEC O113:H21 strain 98NK2 was recently reported to produce a novel cytotoxin encoded on the megaplasmid pO113 (Srimanote, 2003; Paton *et al.*, 2004). This AB₅ toxin comprises a single 35-kDa A subunit and a pentamer of 13-kDa B subunits, and was designated subtilase cytotoxin (Paton *et al.*, 2004). The A subunit has similarity to a subtilase-like serine protease of *Bacillus anthrax* (26% amino acid identity) whereas the B subunit has similarity to putative exported proteins from *Yersinia pestis* (56% amino acid

identity) and *Salmonella typhi* (50% amino acid identity) (Paton *et al.*, 2004). Both the A and B subunits were required for cytotoxicity in CHO, HCT-8 and Vero cells, and in Vero cells this cytotoxicity was dependent on the subtilase-like activity of the protein (Paton *et al.*, 2004). Intraperitoneal injection of purified subtilase cytotoxin was lethal to mice and resulted in extensive microvascular thrombosis and necrosis of the brain, kidneys and liver (Paton *et al.*, 2004). Oral challenge of mice with *E. coli* K-12 strains expressing the cloned toxin operon resulted in dramatic weight loss (Paton *et al.*, 2004). Homologues of the subtilase cytotoxin were found in 32 of 68 STEC strains tested (Paton *et al.*, 2004). Subtilase may therefore contribute to the pathogenesis of disease in humans, but further studies are required in order to determine whether the subtilase cytotoxin has a direct role in human disease.

1.7 Epithelial cell responses to STEC infection

STEC are generally considered to be non-invasive pathogens; after ingestion and establishment of intestinal colonisation, they release Stx into the gut lumen. The toxin is then absorbed across the epithelium into the circulation, where it targets host cells expressing the specific glycolipid receptor Gb₃. In humans, Gb₃ receptors are present at high levels in renal tubular epithelium; significant levels are also expressed in microvascular endothelial cells of the kidney, intestine, pancreas and brain, particularly after exposure to inflammatory cytokines. Stx-mediated damage at these sites accounts for the pathological features of STEC disease and HUS (Obrig *et al.*, 1993; Paton and Paton, 1998b). Clearly, the penetration of Stx into underlying tissues is a crucial step in pathogenesis. The interaction between STEC and intestinal epithelial cells is likely to be critical to the pathogenesis of disease, since they are the primary cell contacts for colonisation. Polymorphonuclear leucocytes (PMNs) are now thought to be important in this process as well.

1.7.1 Role of PMNs in the pathogenesis of serious STEC disease

An association between poor prognosis, including the development of HUS, and elevated levels of PMNs in blood of STEC-infected patients has been well established (Robson *et al.*, 1989; Walters *et al.*, 1989; Robson *et al.*, 1991; Fitzpatrick *et al.*, 1992b; Bell *et al.*, 1997; Inward *et al.*, 1997a; Buteau *et al.*, 2000). Walters *et al.* (1989) originally described this phenomenon; in a review of 79 children with HUS, significantly higher counts of PMNs were found in the blood of children who had a poorer outcome than those children who completely recovered from the illness (Walters *et al.*, 1989). Other studies now support these data, and children with high levels of PMNs ($> 12,000/\mu\text{l}$) have a poorer prognosis, including a 7-fold increased chance of developing HUS, an increased duration of anuria, dialysis and hospitalisation, and an increased risk of death (Walters *et al.*, 1989; Robson *et al.*, 1991; Bell *et al.*, 1997; Inward *et al.*, 1997a; Buteau *et al.*, 2000). Faecal leucocytes were also observed more frequently in patients presenting with STEC infections than in patients with *Salmonella*, *Campylobacter* or *Shigella* infections (Slutsker *et al.*, 1997). Autopsies from patients that died from HUS have also shown high levels of PMNs in their glomeruli (Inward *et al.*, 1997a). Histological studies of gastrointestinal tissues of STEC patients also show PMN infiltration in the lamina propria and intestinal crypts (Kelly *et al.*, 1987; Richardson *et al.*, 1988; Griffin *et al.*, 1990; Kelly *et al.*, 1990). Similarly, several animal models, including mice (Isogai *et al.*, 1998), rabbits (Heczko *et al.*, 2000), piglets (Francis *et al.*, 1986; McKee *et al.*, 1995), calves (Dean-Nystrom *et al.*, 1997; Dean-Nystrom *et al.*, 1998) and monkeys (Kang *et al.*, 2001), have also demonstrated the recruitment of PMNs in response to STEC infection.

PMNs are now thought to have several influences on STEC disease pathogenesis, both locally and systemically. At the time that this project commenced, Hurley *et al.* (2001) demonstrated that the amount of Stx crossing polarised T84 monolayers was proportional to the degree of PMN transmigration. Migration to the gut epithelium, and the subsequent translocation of PMNs from the basolateral surface to the apical (luminal) surface may break down the tight junction barrier resulting in increased absorption of Stx from the lumen into underlying tissues and subsequently the blood stream (Hurley *et al.*, 2001). The presence of

low doses of Stx1 (10 pg/ml) can induce both the respiratory burst and superoxide production in PMNs, resulting in localised tissue damage, increased absorption of Stx and also the induction of Stx2-encoding bacteriophages (King *et al.*, 1999; Wagner *et al.*, 2001).

However, no free Stx has been detected in the blood or serum of patients with HUS or STEC infections (Acheson *et al.*, 1998; Paton and Paton, 1998b; te Loo *et al.*, 2000b). Interestingly, Stx has been observed to bind exclusively to human PMNs when incubated in whole blood *in vitro*, and not to erythrocytes, lymphocytes, monocytes or lipoproteins (te Loo *et al.*, 2000a). Similarly, in HUS patients, Stx2 was exclusively bound to PMNs in up to 90% of patients (te Loo *et al.*, 2000b). These data may explain the previous inability to detect free Stx in the serum of patients. The binding of Stx2 to PMNs in HUS patients is also correlated with the simultaneous presence of bloody diarrhoea, and this breakdown of the intestinal barrier may help Stx to traverse the epithelium into underlying tissues and PMNs (te Loo *et al.*, 2000b).

In vitro, binding of Stx to PMNs was shown to occur via a unique receptor with 100-fold lower affinity than that of Stx to Gb₃ (te Loo *et al.*, 2000a). Bound toxin was subsequently released on contact with glomerular microvascular endothelial cells (GMVECs), which express the high-affinity receptor Gb₃ (te Loo *et al.*, 2000a). Remarkably, only Stx bound to PMNs, and not Stx or PMNs alone, were able to cause the inhibition of protein synthesis and cell death in endothelial cells (te Loo *et al.*, 2000a). These processes would therefore provide a mechanism whereby PMNs may locally increase intestinal permeability and the absorption of Stx into underlying tissues, and then also bind and transport Stx systemically to target tissues expressing Gb₃, such as the microvascular endothelial cells of the kidney, intestine, pancreas and brain.

1.7.2 Recruitment of PMNs by intestinal epithelial cells

Since PMNs are likely to be crucial in the development of HUS, the role of cytokines in the recruitment and migration of PMNs is also likely to be important in the pathogenesis of serious STEC disease. In HUS patients, several cytokines are known to be upregulated during

infection, including IL-1 β , IL-6, IL-8 and TNF- α , and have been detected in the serum, urine and/or stools (Fitzpatrick *et al.*, 1992b; Karpman *et al.*, 1995; Raqib *et al.*, 1995; Murata *et al.*, 1998; Litalien *et al.*, 1999). Upregulation of IL-6 and TNF- α may occur for up to 10 days after infection and are correlated with the severity of renal injury (Karpman *et al.*, 1995; Raqib *et al.*, 1995). Similarly, levels of IL-1 and IL-8 remain increased in the stools of patients longer than those of IL-6 and TNF- α (Raqib *et al.*, 1995). The level of IL-8 in patients also correlates with leucocytosis and PMN counts, as well as with the severity of disease, including the development of HUS (Fitzpatrick *et al.*, 1992b; Murata *et al.*, 1998; Litalien *et al.*, 1999; Westerholt *et al.*, 2000). Litalien *et al.* (1999) have also shown that children with severe renal dysfunction have a 10-fold increase in IL-6 levels and a greater than 10-fold increase in concentrations of IL-1, IL-8 and IL-10.

Pathogenic bacteria including *Salmonella* spp., *S. dysenteriae*, *Y. enterocolitica*, *Listeria monocytogenes* and EIEC, have long been associated with the upregulation of a number of pro-inflammatory cytokines and chemokines (Jung *et al.*, 1995; Schulte *et al.*, 1996; Yang *et al.*, 1997; Coates and McColl, 2001). Purified Stx1 and/or Stx2 are also capable of inducing the synthesis of cytokines such as IL-1 β , TNF- α , IL-6 and IL-8 by Caco-2 and HCT-8 cells (Section 1.4.2) (van Setten *et al.*, 1996; Thorpe *et al.*, 1999) and human monocytes (Yamasaki *et al.*, 1999). Migration of PMNs and the production of IL-8 by polarised T84 cells were induced by the apical application of STEC strains, but the intensity of this effect was higher in LEE-negative than in LEE-positive STEC strains (Hurley *et al.*, 2001) and therefore may involve factors other than Stx production.

The above data imply a role for PMNs in the pathogenesis of serious STEC disease and an important role for cytokines in mediating the recruitment and migration of PMNs in this process. However, the exact mechanisms involved are poorly understood. IL-8 is a member of the CXC chemokine family (that contains an ELR [glutamine (E)-leucine (L)-arginine (R)] motif preceding the CXC [cysteine (C)-X-C] motif) and is a potent PMN chemoattractant, as are other members of this family including epithelial-derived PMN activating peptide-78 (ENA-78), granulocyte chemotactic protein-2 (GCP-2), melanocyte growth stimulating activity (MGSA, also called growth related oncogene [Gro]- α),

macrophage inflammatory protein-2 α (MIP-2 α or Gro- β) and MIP-2 β (or Gro- γ) (reviewed in Baggiolini *et al.*, 1997; Gale and McColl, 1999).

Purified Stx1 is capable of inducing IL-8, MGSA, MIP-2 α , MIP-2 β and ENA-78 mRNA (as well as IL-8 and MGSA protein expression) via a ribotoxic stress response pathway in a dose-dependent manner in HCT-8 cells (Thorpe *et al.*, 2001). This would be expected to lead to the recruitment of inflammatory cells (including PMNs), damage to the intestinal barrier and therefore, increased Stx absorption. IL-8, and the IL-8 receptor CXC receptor 1 (CXCR1 or IL-8 receptor A [IL-8RA]), but not CXCR2 (IL-8RB) have been shown to be important for PMN recruitment in response to UPEC infection (Godaly *et al.*, 1997; Godaly *et al.*, 2000). Furthermore, IL-8 also plays an important role in *in vivo* PMN recruitment and migration across the urinary tract of mice and in the upregulation of CXCR1 in response to UPEC (Godaly *et al.*, 2001). IL-8 receptor knockout mice similarly failed to recruit PMNs to the lumen and PMNs were unable to cross the infected mucosa of the urinary tract (Godaly *et al.*, 2000). Thus CXC chemokines may be important in the recruitment of PMNs in serious STEC infection and therefore may play a pivotal role in the pathogenesis of HUS.

1.8 Aims of this thesis

Recent data referred to above suggest a key role for PMNs in the pathogenesis of STEC disease by increasing the permeability of the intestinal barrier, and in the binding and transporting of Stx throughout the body. However the factor(s) mediating this process remain unknown. The aim of this thesis was to characterise the responses of intestinal epithelial cells to STEC and to determine the factor(s) involved in mediating chemokine induction and therefore PMN recruitment. Although some role for IL-8 has been observed in patients with STEC disease, the role of other PMN-attracting chemokines is uncertain. Characterisation of the STEC factor(s) responsible for mediating these chemokine responses in intestinal epithelial cells will provide a better understanding of the STEC-epithelial cell interaction, which may have important implications for the pathogenesis of HUS.

Accordingly, the specific aims of the work described in this thesis are:

1. To develop a procedure for the detection and quantitation of chemokine mRNA induction in intestinal epithelial (HCT-8) cells in response to infection with STEC.
2. To determine the chemokine responses elicited in HCT-8 cells *in vitro* by a range of STEC isolates.
3. To elucidate the factor(s) of STEC responsible for the induction of chemokines by HCT-8 cells *in vitro*.
4. To determine the role of these factor(s) in the recruitment of PMNs and their contribution to the pathogenesis of STEC-mediated disease.

CHAPTER 2: Materials and Methods

2.1 Bacterial strains and plasmids

The wild-type *E. coli* strains and *E. coli* K-12 strains used in this study are shown in Table 2.1. Most wild-type STEC strains used were clinical isolates from the Women's and Children's Hospital, Adelaide, S.A., Australia. Plasmids used in this study are described in Table 2.2.

2.2 Bacterial culture media and growth conditions

Luria-Bertani (LB) broth consisted of 10 g/l Bacto™ tryptone (Becton, Dickinson and Company, Sparks, MD, USA), 5 g/l Bacto™ yeast extract (Becton, Dickinson and Co.) and 30 mM NaCl, pH 7.5. LB agar is identical to LB broth but includes 15 g/l Bacto™ agar (Becton, Dickinson and Co.), unless otherwise indicated. Terrific broth, used for the over-expression of His₆ constructs, consisted of 24 g/l yeast extract, 12 g/l tryptone, 0.4% (v/v) glycerol, 170 mM KH₂PO₄ and 720 mM K₂HPO₄. GYT medium, for making electro-competent *E. coli*, consisted of 10% (v/v) glycerol, 1.25 g/l yeast extract and 2.5 g/l tryptone. SOC medium, for the recovery of electro-transformed *E. coli*, consisted of 20 g/l tryptone, 5 g/l yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂ and 10 mM MgSO₄. Glycerol medium, for the long-term storage of *E. coli* at -80°C, consisted of 30% (v/v) glycerol and 10 g/l tryptone.

Table 2.1 *E. coli* strains used in this study

Strain	Description	Reference or Source
86-24	STEC, serotype O157:H7	Griffin <i>et al.</i> (1988)
90-103	STEC, serotype O157:H7	R. M. Robins-Browne, University of Melbourne, Vic., Australia
94-6752	STEC, serotype O157:H7	R. M. Robins-Browne
94CR	STEC, serotype O48:H21	Paton <i>et al.</i> (1997)
95HE4	STEC, serotype O91	Paton <i>et al.</i> (1997)
95NR1	STEC, serotype O111:H ⁻	Paton <i>et al.</i> (1997)
95SF2	STEC, serotype O157:H ⁻	Paton <i>et al.</i> (1997)
95ZG1	STEC, serotype O26	Paton <i>et al.</i> (1997)
97MW1	STEC, serotype O113:H21	Paton <i>et al.</i> (1999)
97MW1 ^{SR}	Streptomycin resistant derivative of 97MW1	Paton <i>et al.</i> (2000)
98NK2	STEC, serotype O113:H21	Paton <i>et al.</i> (1999)
98NK2-Cu	Megaplasmid cured derivative of 98NK2	Paton <i>et al.</i> (2001a)
98NK2 ^{SR}	Streptomycin resistant derivative of 98NK2	Paton <i>et al.</i> (2001b)
B2F1	STEC, serotype O91:H21	Melton-Celsa <i>et al.</i> (1996)
DH1	<i>E. coli</i> K-12 derivative: F ⁻ , <i>glnV44 recA1 endA1 gyrA96</i> (Nal ^R) <i>thi1 hsdR17</i> (r _k ⁻ m _k ⁺) <i>relA1 spoT1?</i> <i>rfbD1?</i>	Hanahan (1983)
DH1[pJCP591]	DH1 containing O113 <i>rfb</i> cloned in pHC79	Paton and Paton (1999)
DH5 α	<i>E. coli</i> K-12 derivative: F ⁻ , <i>deoR, supE44 D(lacZYA-argF) U169 [f80 dlacD(lacZ M15)] hsdR17recA1 endA1 gyrA96 thi-1 relA1, I⁻</i>	Bethesda Research Laboratories, Gaithersburg, MD, USA
EDL933	STEC, serotype O157:H7	Tzipori <i>et al.</i> (1987)
EDL933-Cu	Megaplasmid cured derivative of EDL933	Tzipori <i>et al.</i> (1987)
EDL933 Δeae	EDL933 containing a deletion in <i>eae</i>	Ogierman <i>et al.</i> (2000)
EDL933[pJCP710]	EDL933 containing EDL933 <i>ler</i> over-expressed in pBluescript KS	Ogierman <i>et al.</i> (2000)
EDL933[pJCP711]	EDL933 containing 95SF2 <i>ler</i> over-expressed in pBluescript KS	Ogierman <i>et al.</i> (2000)
EPEC 87A	EPEC, serotype O111	Paton <i>et al.</i> (1997)

Table 2.1 *E. coli* strains used in this study (continued)

Strain	Description	Reference or Source
JM109	<i>E. coli</i> K-12 derivative: F ⁺ , <i>traD36 proA</i> ⁺ <i>B</i> ⁺ <i>lacI</i> ^f <i>D(lacZ)M15/ D(lac-proAB) glnV44 e14</i> ⁻ <i>gyrA96 recA1 relA1 endA1 thi-1 hsdR17</i>	Yanisch-Perron <i>et al.</i> (1985)
JM109[pJCP525]	JM109 containing <i>stx</i> ₁ cloned in pBluescript	Paton <i>et al.</i> (1995a)
JM109[pJCP539]	JM109 containing <i>stx</i> ₂ cloned in pBluescript	Paton <i>et al.</i> (1999)
JM109 [P4586]	JM109 containing O111 <i>rfb</i> cloned in pPR691	Bastin <i>et al.</i> (1991)
M15[pREP4]	<i>E. coli</i> K-12 derivative: NaI ^S , Str ^S , Rif ^S , F ⁻ , <i>th</i> ⁻ , <i>lac</i> ⁻ , <i>ara</i> ⁺ , <i>gal</i> ⁺ , <i>mtl</i> ⁻ , <i>recA</i> ⁺ , <i>uvr</i> ⁺ , <i>lon</i> ⁺ , carrying pREP4	QIAGEN GmbH

Table 2.2 Plasmids used in this study

Plasmid	Description	Reference or Source
pBluescript K/S +/-	Cloning vector (Amp ^R)	Promega
pEF6/V5-His	Cloning vector for transfection of mammalian cell lines (Amp ^R , Blasticidin ^R)	Invitrogen-Life Technologies
pGEM-T easy	PCR product cloning vector (T-tailed; Amp ^R)	Promega
pHC79	Cosmid cloning vector (Amp ^R)	Hohn and Collins (1980)
pKD3	Template for amplification of <i>cat</i> cassette for red recombinase-mediated recombination (Cml ^R , Amp ^R)	Datsenko and Wanner (2000)
pKD4	Template for amplification of <i>kan</i> cassette for red recombinase-mediated recombination (Kan ^R , Amp ^R)	Datsenko and Wanner (2000)
pKD46	Temperature sensitive, red recombinase plasmid (Amp ^R)	Datsenko and Wanner (2000)
pPR691	Cosmid cloning vector (Kan ^R , Spec ^R , Str ^R)	Jiang <i>et al.</i> (1987)
pQE-30	His ₆ -tag expression vector (Amp ^R)	QIAexpressionist TM (QIAGEN GmbH)
pREP4	Encoding <i>lacI</i> , Kan ^R	QIAexpressionist TM (QIAGEN GmbH)
DN TLR5	pEF6/V5-His containing cloned DN TLR5 (Δ 695-858) (Amp ^R , Blasticidin ^R)	A. T. Gewirtz; Epithelial Pathobiology Unit, Emory University, Atlanta, GA, USA (Zhou <i>et al.</i> , 2003).
TLR5	pEF6/V5-His containing cloned TLR5 (Amp ^R , Blasticidin ^R)	A. T. Gewirtz (Zhou <i>et al.</i> , 2003).

Antibiotics were added to broth and solid media at the following concentrations: ampicillin (Amp) 50 µg/ml, chloramphenicol (Cml) 25 µg/ml, kanamycin (Kan) 50 µg/ml and streptomycin (Str) 50 µg/ml, unless otherwise specified. Other additives were added to solid media at the following concentrations: isopropyl-β-D-thiogalactopyranoside (IPTG) 48 µg/ml and 5-bromo-4-chloro-3-indoyl-β-D-galacto-pyranoside (X-gal) 40 µg/ml, unless otherwise specified.

Liquid cultures of *E. coli* were routinely grown in LB broth in 30 ml McCartney bottles and were incubated at 37°C (with agitation), unless otherwise specified. All *E. coli* strains used in this study were preserved by resuspension of cells from a fresh overnight LB agar culture in Glycerol medium and stored at -80°C.

2.3 Cell lines, tissue culture media and growth conditions

Cell lines used in this study are listed in Table 2.3. All tissue culture media and reagents were obtained from Gibco BRL-Life Technologies, Grand Island, NY, USA, unless otherwise indicated. All cells were grown at 37°C in 95% air/5% CO₂, unless otherwise indicated.

HCT-8 cells were routinely grown in RPMI 1640 medium supplemented with 10 mM HEPES, 1 mM sodium pyruvate, 10% (v/v) heat-inactivated (HI) foetal calf serum (FCS), 50 IU/ml penicillin and 50 µg/ml streptomycin, unless otherwise specified. HEp-2 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% (v/v) HI FCS, 50 IU/ml penicillin and 50 µg/ml streptomycin at 37°C, unless otherwise specified. T84 cells were grown in a 1:1 mixture of DMEM/Hams F-12 medium supplemented with 10% (v/v) HI FCS, 50 IU/ml penicillin and 50 µg/ml streptomycin, unless otherwise specified. Vero cells were grown in DMEM supplemented with 10% (v/v) HI FCS, 50 IU/ml penicillin and 50 µg/ml streptomycin at 37°C, unless otherwise specified.

Table 2.3 Cell lines used in this study

Cell line	Description	Source
HCT-8	Human colonic epithelial; ileocecal colorectal adenocarcinoma	Division of Veterinary and Biomedical Sciences, Murdoch University, Murdoch, W.A., Australia
HeLa	Human cervical epithelial adenocarcinoma	A.T. Gewirtz
HeLa DN TLR5	HeLa cells stably transfected with DN TLR5 plasmid (Table 2.2)	A.T. Gewirtz
HeLa TLR5	HeLa cells stably transfected with TLR5 plasmid (Table 2.2)	A.T. Gewirtz
HEp-2	Human laryngeal carcinoma	Womens and Childrens Hospital, Adelaide, S.A., Australia
MDCK	Madin-Darby canine (<i>Canis familiaris</i>) kidney epithelial	A.T. Gewirtz
MDCK DN TLR5	MDCK cells stably transfected with DN TLR5 plasmid (Table 2.2)	A.T. Gewirtz
MDCK TLR5	MDCK cells stably transfected with TLR5 plasmid (Table 2.2)	A.T. Gewirtz
T84	Human colonic epithelial; metastatic site: lung colorectal carcinoma	Pediatric Gastroenterology and Nutrition, Massachusetts General Hospital, Boston, MA, USA
Vero	African green monkey (<i>Cercopithecus aethiops</i>) kidney epithelial	Institute of Medical and Veterinary Science (IMVS), Adelaide, S.A., Australia

For TLR5 experiments, HeLa cells were grown in McCoy's 5A medium (Cambrex Bio Science, Walkersville, MD, USA) supplemented with 10% (v/v) HI FCS, 50 IU/ml penicillin and 50 µg/ml streptomycin, unless otherwise specified. MDCK cells were grown in DMEM supplemented with 10% (v/v) HI FCS, 10 mM HEPES, 50 IU/ml penicillin and 50 µg/ml streptomycin, unless otherwise specified. For stably transfected Toll-like receptor 5 (TLR5) and dominant-negative (DN) TLR5 HeLa and Madin-Darby Canine Kidney (MDCK) cells, Blasticidine S hydrochloride (Sigma-Aldrich, St. Louis, MO, USA) was added to media at 10 µg/ml.

2.4 Chemicals and reagents

2.4.1 General chemicals

All chemicals used were analytical grade. Most chemicals were purchased from Ajax Chemicals, Auburn, N.S.W., Australia, or from Sigma-Aldrich, unless otherwise indicated. All amino acids and vitamins used were purchased from Sigma-Aldrich. Blocking reagent, X-Gal, 5-bromo-4-chloro-3-indoyl-phosphate (BCIP or X-Pho), deoxynucleoside triphosphates (dNTPs), anti-Digoxigenin-Fab fragment alkaline phosphatase-conjugate (α -DIG-AP), DNase, glycogen, Klenow fragment of DNA polymerase I, lysozyme, 4-nitroblue tetrazolium chloride (NBT), pronase, RNaseA, T4 DNA ligase and Taq DNA polymerase were purchased from Roche Diagnostics Pty. Ltd., Sydney, N.S.W., Australia. IPTG was obtained from Inalco Pharmaceuticals, San Francisco, CA, USA. Acrylamide solution, alkaline phosphatase-conjugated goat anti-mouse and goat anti-rabbit IgG, and ammonium persulphate were obtained from Bio-Rad Laboratories, Richmond, CA, USA. SuperSignal[®] West Pico Chemiluminescent substrate was obtained from Pierce Biotechnology Inc., Rockford, IL, USA. RNasin[®] ribonuclease inhibitor and RQ1 RNase-free DNase were obtained from Promega, Madison, WI, USA.

Biotinylated anti-human IL-8 antibody, monoclonal anti-human IL-8/CXCL8 antibody, and recombinant human IL-8 were from R&D systems Inc., Minneapolis, MN, USA. SYBR[®] green I nucleic acid stain and recombinant human TNF- α were obtained from Invitrogen Life Technologies, Grand Island, NY, USA. Peroxidase conjugated streptavidin was obtained from Rockland, Gilbertsville, PA, USA. Stx1 and Stx2 were obtained from Toxin Technologies Inc., Sarasota, FL, USA. ATF-2 fusion protein, immobilised phospho-p38 mitogen activated protein kinase (MAPK; Thr180/Tyr182) monoclonal antibody and phospho-ATF-2 (Thr71) antibody were obtained from Cell Signaling Technology Inc., Beverly, MA, USA. The p38 inhibitor SB203580 and the MAP kinase kinase (MEK)-1 and -2 inhibitor PD98059 were obtained from Calbiochem, La Jolla, CA, USA. The c-Jun N-terminal kinase (JNK)-1 and -2 inhibitor SP600125 was obtained from A. G. Scientific Inc., San Diego, CA, USA.

All restriction enzymes were purchased from New England Biolabs Inc., Beverly, MA, USA or Roche Diagnostics Pty. Ltd. and used according to the manufacturer's instructions. All reagent kits were used according to the manufacturer's instructions.

Ampicillin was purchased from CSL, Parkville, Vic., Australia, chloramphenicol and kanamycin sulphate were from Roche Diagnostics Pty. Ltd., and streptomycin sulphate was purchased from Sigma-Aldrich. Blasticidine S hydrochloride was also obtained from Sigma-Aldrich.

2.4.2 Oligonucleotides

The oligonucleotides used in this study are listed in Table 2.4 and were purchased from Sigma Genosys (Sigma-Aldrich).

2.5 DNA extraction procedures

2.5.1 Plasmid DNA extraction procedures

2.5.1.1 Method 1

Plasmid DNA was isolated from *E. coli* grown in LB broth supplemented with the appropriate antibiotic and incubated at 37°C overnight with shaking, as previously described but with some modifications (Birnboim and Doly, 1979). Plasmid DNA was normally isolated from 2 ml overnight culture for high copy number plasmid vectors and yields of plasmid obtained were normally in the range of 100 µg to 500 µg. Low copy number plasmid vectors were normally isolated from 10 ml overnight cultures. The culture was transferred to a 10 ml centrifuge tube and sedimented in a Sigma 2-4 centrifuge at 4,000 × *g* for 10 min. The cell pellet was resuspended in 300 µl P1 buffer (50 mM Tris-HCl, 10 mM EDTA, pH 8.0), and transferred to a microcentrifuge tube. P2 buffer (300 µl; 0.2 M NaOH, 0.1% [w/v]

Table 2.4 Oligonucleotides used in this study

Oligo Name	Sequence (5'-3')	GenBank Accession No./ Description /Reference
IL-8 Fwd	GAAGGAACCATTCTCACTGTGTGTA	M28130 (IL-8 mRNA nucleotide [nt] 75-99)
IL-8 Rev	TTATGAATTCTCAGCCCTCTCAAAAAC	M28130 (IL-8 mRNA complementary nt 402-375)
ENA-78 Fwd	GAACCCGCGACCGCTCGC	XM_003507 (ENA-78 mRNA nt 62-79)
ENA-78 Rev	AGAAAAGGGGCTTCTGGATCAA	XM_003507 (ENA-78 mRNA complementary nt 393-372)
GCP-2 Fwd	CTCCACCCAGCTCAGGAACC	XM_003502 (GCP-2 mRNA nt 14-33)
GCP-2 Rev	GAAAAGGGGCTTCCGGGTCCA	XM_003502 (GCP-2 mRNA complementary nt 351-331)
MGSA Fwd	AGCCACACTCAAGAATGGGCG	XM_003504 (MSGa mRNA nt 304-324)
MGSA Rev	TGGCATGTTGCAGGCTCCTC	XM_003504 (MSGa mRNA complementary nt 758-777)
MIP-2 α Fwd	ATTTGTTAATATTTCTTCGTGATGACATATCA	X53799 (MIP-2 α mRNA nt 709-740)
MIP-2 α Rev	TCGAAACCTCTCTGCTCTAACAC	X53799 (MIP-2 α mRNA complementary nt 1,010-1,032)
MIP-2 β Fwd	AGAACATCCAAAGTGTGAATGTAAGG	X53800 (MIP-2 β mRNA nt 198-223)
MIP-2 β Rev	TCCTTTCCAGCTGTCCCTAGAA	X53800 (MIP-2 β mRNA complementary nt 458-479)
GAPDH Fwd	TCCTTGAGGCCATGTGGGCCAT	XM_033258 (GAPDH mRNA nt 206-228)
GAPDH Rev	TGATGACATCAAGAAGGTGGTGAAG	XM_033258 (GAPDH mRNA complementary nt 445-421)
c1	TTATACGCAAGGCGACAAGG	Datsenko and Wanner (2000)
k1	CAGTCATAGCCGAATAGCCT	Datsenko and Wanner (2000)
EcoHI	AATACCAACAGCCTCTCGCT	Reid <i>et al.</i> (1999)
EcoH2	AGAGACAGAACCTGCTGC	Reid <i>et al.</i> (1999)
F-Flc1	ATGGCACAAGTCATTAATACCCAAC	Botelho <i>et al.</i> (2003)
R-Flc2	CTAACCCCTGCAGCAGAGACA	Botelho <i>et al.</i> (2003)

Table 2.4 Oligonucleotides used in this study (continued)

Oligo Name	Sequence (5'–3')	GenBank Accession No./ Description /Reference
M13 Fwd	CGCCAGGGTTTTCCCAGTCACGAC	M13/pUC sequencing primer (Stratagene, La Jolla, CA, USA)
M13 Rev	AGCGGATAACAATTTACACAGGA	M13/pUC sequencing primer (Stratagene)
pKD46 Fwd	TCGCCCCTGAAGAGAAAGAG	AY048746 (pKD46 nt 1,428-1,447)
pKD46 Rev	TGCACCTGGGCCATGTAAG	AY048746 (pKD46 complementary nt 2,923-2,905)
stx2F	GGCACTGTCTGAAACTGCTCC	Paton and Paton (1998a)
stx2R	TCGCCAGTTATCTGACATTCTG	Paton and Paton (1998a)
TR27	CGACCCAACAAAGTTATGTCTCTTCGTTAAA- TAGTATACGGGTGTAGGCTGGAGCTGCTTC	<i>stx</i> ₂ (nt 89-129) incorporating pKD3/4 priming site 1 (underlined)
TR28	TTTATAACGGGCCTGTGCCAGTTATCTGAC- ATTCTGGTTGCATATGAATATCCTCCTTAG	<i>stx</i> ₂ (complementary nt 875-835) incorporating pKD3/4 priming site 2 (underlined)
TR30	CCATTGAACGTCTCTTCTTGGCCTGCGTAT- TAACAGTGCTGTGTAGGCTGGAGCTGCTTC	H21 <i>fliC</i> (AF517663 nt 23-63) incorporating pKD3/4 priming site 1 (underlined)
TR31	GTTAGACACTTCGGTCGCGTAGTCAGCATCT- TGATACGGCTCATATGAATATCCTCCTTAG	H21 <i>fliC</i> (AF517663 complementary nt 1,323-1,282) incorporating pKD3/4 priming site 2 (underlined)
TR34	CCGGGTCTGGTGCTGATTAC	nt -25 to -44 of <i>stx</i> ₂
TR36	TCTATCGAGCGTCTGTCTTCTGGCTTGC GTA- TTAACAGCGCGGTGTAGGCTGGAGCTGCTTC	H7 <i>fliC</i> (AE005415 nt 82-123) incorporating pKD3/4 priming site 1 (underlined)
TR37	GTTGGACACTTCGGTCGCATAGTCGGCGTCC- TGAATACGGGACATATGAATATCCTCCTTAG	H7 <i>fliC</i> (AE005415 complementary nt 1,662-1,621) incorporating pKD3/4 priming site 2 (underlined)
TR45	ACATGCGCATGCATGGCACAAGTCATTAAT- ACCCAAC	F-FliC1 (incorporating <i>SphI</i> restriction site [underlined])
TR46	CATCGGTCGACCTCTAACCCCTGCAGCAGAG- ACA	R-FliC2 (incorporating <i>SalI</i> restriction site [underlined])

sodium dodecyl sulphate [SDS]) was added, the tube was inverted 2–3 times to mix, and the mixture was incubated at room temperature for no longer than 5 min. P3 buffer (300 μ l; 3M KAc, pH 5.5) was added, the tube was inverted several times to mix, and then incubated on ice for 10 to 15 min. The precipitate was sedimented in a Sigma 1-15 centrifuge at $14,900 \times g$ for 5 min, the supernatant was transferred to a fresh tube, extracted with 400 μ l chloroform, vortexed for 30 seconds, and then centrifuged at $14,900 \times g$ for 1 min. The upper aqueous phase was collected and added to 700 μ l of cold isopropanol. The mixture was chilled at -80°C for 30 min and plasmid DNA was sedimented at $14,900 \times g$ for 15 min. The supernatant was discarded and the pellet was rinsed in 1 ml of 70% (v/v) ethanol. The pellet was re-sedimented at $14,900 \times g$ for 2 min then dried at 65°C for 10 min. The pellet was subsequently dissolved in 40 μ l sterile Milli-Q water. The plasmid DNA was restriction endonuclease digested (Section 2.6.2) and analysed by agarose gel electrophoresis (Section 2.6.4).

2.5.1.2 Method 2

Plasmid DNA, for sequencing or cloning, was purified using the QIAprep[®] Miniprep kit (QIAGEN GmbH, Hilden, Germany), as described in the manufacturer's instructions. Briefly, strains were cultured as previously described in Section 2.5.1.1 above. A 5–10 ml culture was transferred to a 10 ml centrifuge tube and sedimented in a Sigma 2-4 centrifuge at $4,000 \times g$ for 10 min. The cell pellet was resuspended in 250 μ l Buffer P1 and transferred to a microcentrifuge tube. Buffer P2 (250 μ l) was added and the tube was gently inverted 4–6 times to mix. Buffer N3 (350 μ l) was added and the tube was inverted immediately but gently 4–6 times. The precipitate was sedimented in a microcentrifuge at $14,900 \times g$ for 10 min, after which the supernatant was applied to a QIAprep[®] column by pipetting. The column was centrifuged at $14,900 \times g$ for 1 min, washed with 500 μ l Buffer PB and centrifuged at $14,900 \times g$ for 1 min. The column was washed with 750 μ l Buffer PE, centrifuged at $14,900 \times g$ for 1 min, and then the eluate was removed and centrifuged for a further 1 min to remove residual wash buffer. The column was placed into a clean microcentrifuge tube, 30–50 μ l Milli-Q

water was added to the column and left to stand for 1 min, and then centrifuged at $14,900 \times g$ for 1 min to elute plasmid DNA. The plasmid DNA was restriction endonuclease digested (Section 2.6.2) and analysed by agarose gel electrophoresis (Section 2.6.4).

2.5.2 Preparation of genomic DNA

Genomic DNA was isolated using methods modified from Manning *et al.* (1986). Bacteria were cultivated in 20 ml LB broth and incubated at 37°C overnight with shaking. Cells were pelleted in a Sigma 2-4 centrifuge at $4,000 \times g$ for 10 min and the pellet resuspended in 2 ml of 25% (w/v) sucrose, 50 mM Tris-HCl (pH 8.0) and 1 ml of fresh lysozyme (10 mg/ml in 0.25 M EDTA, pH 8.0). After 20 min incubation on ice, 750 μ l Tris-EDTA (TE) buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and 250 μ l lysis solution (62.5 mM EDTA, 5% [w/v] Sarkosyl, 50 mM Tris-HCl, pH 8.0) were added, followed by 2 mg of solid pronase. After 1 h incubation at 60°C, the solution was extracted three times with TE-saturated phenol (or until the aqueous phase was clear). The aqueous phase was subsequently extracted with diethyl ether and precipitated with 8 ml of cold absolute ethanol. The precipitated DNA was harvested with a Pasteur pipette hook, quickly rinsed in cold 70% (v/v) ethanol and dissolved overnight in 1 ml of TE buffer or Milli-Q water.

2.6 Analysis and manipulation of DNA

2.6.1 DNA quantitation

DNA concentration was determined by measuring the Absorbance at 260 nm (A_{260}). For double stranded DNA, a solution containing 50 μ g/ml has an A_{260} of 1.0. For single stranded DNA, a solution containing 33 μ g/ml has an A_{260} of 1.0.

2.6.2 Restriction endonuclease digestion of DNA

Restriction endonuclease digestion of plasmid or chromosomal DNA was performed using restriction enzymes and the restriction buffers specified and provided by the manufacturer. Typically, 0.1 to 1 µg of DNA was incubated with 2 U of restriction endonuclease in a final volume of 10–20 µl. The reaction mixture was incubated at 37°C for 2–16 h, or as otherwise specified by the manufacturer.

2.6.3 Calculation of DNA restriction fragment size

Restriction fragment sizes were calculated by comparing their relative mobility in agarose gels with that of DNA size markers. *HpaII*-restricted pUC19 plasmid DNA (Geneworks, Adelaide, S.A., Australia), *EcoRI*-digested *Bacillus subtilis* bacteriophage SPP-1 DNA (Geneworks) or *HindIII*-restricted bacteriophage lambda DNA (Geneworks) were used as DNA size markers. DNA fragment sizes in each of these digests were as follows: pUC19 plasmid DNA/*HpaII* (501, 404, 331, 242, 190, 147, 111, 110, 67, 34 and 26 bp); bacteriophage SPP-1 DNA *EcoRI* (8.51, 7.35, 6.11, 4.84, 3.59, 2.81, 1.95, 1.86, 1.51, 1.39, 1.16, 0.98, 0.72, 0.48 and 0.36 kb); and bacteriophage lambda DNA/*HindIII* (23.1, 9.4, 6.6, 4.37, 2.3, 2.0, 0.564 and 0.125 kb).

2.6.4 Agarose gel electrophoresis

Electrophoresis was carried out at room temperature on horizontal 0.8% to 2% (w/v) agarose gels (Progen Industries, Darra, Qld., Australia). Gels were run at 170 V for 1–3 h in Tris-borate-EDTA (TBE) buffer (67 mM Tris, 22 mM boric acid, 2 mM EDTA, pH 8.0), containing 0.5 µg/ml ethidium bromide. Prior to electrophoresis, DNA was mixed with 0.1 volumes of loading buffer (25% [w/v] Ficoll, 0.25% [w/v] bromophenol blue, 0.25% [w/v] xylene cyanol). DNA fragments were visualised by trans-illumination with UV light (wavelength 312 nm) and photographed using either a Polaroid MP4 camera with type 667

positive film or a Tractel GDS-2 Gel Documentation Video Imager and Thermal Printer system (K65HM).

2.6.5 Isolation and purification of restriction fragments from agarose gels

DNA restriction fragments of interest were excised from agarose gels and purified using the UltraClean™ GelSpin DNA purification kit (Mo Bio, Solana, CA, USA), according to the manufacturer's instructions. Briefly, the agarose gel was melted at 65°C for 2 min in three volumes of GelBind Buffer, then microcentrifuged at $14,900 \times g$ for 15 s. The column was washed with 300 μ l GelWash Buffer, the eluate was discarded and the column re-centrifuged for 1 min. The DNA was eluted with 30–50 μ l of Milli-Q water by centrifugation at $14,900 \times g$ for 1 min.

2.6.6 Ligation of restriction endonuclease-digested DNA fragments to vector DNA

Plasmid vector and foreign DNA were cleaved by either single or double restriction enzyme digestion (Section 2.6.2) and the restriction enzymes were inactivated by heating at 65–85°C (as specified by the manufacturer) for 5–15 min. If necessary, the fragments were isolated by gel electrophoresis (Section 2.6.4) and purified (Section 2.6.5). Purified, digested DNA fragments were then combined either at a molar ratio of 3:1 (insert:vector) or according to the following formula (Maniatis *et al.*, 1989):

$$\text{Insert (ng)} = \frac{\text{vector (ng)} \times \text{insert (kb)} \times (\text{insert/vector ratio})}{\text{vector (kb)}}$$

The ligation mixture consisted of DNA to be ligated, 1 μ l T4 DNA ligase (1 U/ μ l, Roche Diagnostics Pty. Ltd.), and 2 μ l 10 \times ligation buffer, made up to a total volume of 20 μ l with Milli-Q water. The 1 \times ligation buffer comprises 20 mM Tris-HCl (pH 7.5), 10 mM

MgCl₂, 10 mM dithiothreitol (DTT) and 0.6 mM ATP. The reaction mixture was incubated overnight at 4°C. Ligated DNA was then used to transform an appropriate competent *E. coli* strain (Section 2.7.3).

2.7 Preparation of competent cells and transformation of *E. coli*

2.7.1 Preparation of super-competent cells for chemical transformation

E. coli strains were grown in 10 ml LB broth to mid-exponential phase (A_{600} of 0.6, ca. 4×10^8 cells/ml). The bacteria were chilled on ice for 5–10 min and pelleted in a Sigma 3K18C centrifuge at $4,000 \times g$ for 10 min at 4°C. The pellet was resuspended in 10 ml ice-cold sterile Solution α (30 mM KAc, 100 mM KCl, 10 mM CaCl₂, 50 mM MnCl₂, 15% [v/v] glycerol) and re-centrifuged. The pellet was resuspended in 1 ml ice-cold sterile Solution β (10 mM 3-[N-morpholino]-propanesulphonic acid [MOPS], 75 mM CaCl₂, 10 mM KAc, 15% [v/v] glycerol). After incubation on ice for 60 min these competent cell suspensions were dispensed in 100 μ l volumes and used immediately or stored at -80°C until use.

2.7.2 Preparation of CaCl₂-MgCl₂ competent cells for chemical transformation

Competent cells were also prepared according to the method described by Hanahan (1983). Briefly, *E. coli* strains were grown in 20 ml LB broth to mid-exponential phase (A_{600} of 0.6). The bacteria were left on ice for 15–20 min and pelleted in a Sigma 3K18C centrifuge at $4,000 \times g$ for 10 min at 4°C. The pellet was resuspended in 10 ml sterile ice-cold 100 mM MgCl₂ and re-centrifuged. The pellet was resuspended in 2 ml of sterile ice-cold 100 mM CaCl₂ and incubated on ice for 60 min. Glycerol was added to a final concentration of 15% (v/v). These competent cell suspensions were dispensed in 100 μ l volumes and used immediately or stored at -80°C until use.

2.7.3 Chemical transformation

As required, an aliquot of competent cells (from Section 2.7.1 or Section 2.7.2) was removed from -80°C and allowed to thaw on ice. A 10 μl volume of the DNA to be transformed was added to 100 μl of competent cells (Section 2.5.1.2). After 30 min incubation on ice, the mixture was heat-shocked at 42°C for 2 min and incubated for a further 20 min on ice. LB broth (1 ml) was added and the cells were incubated at 37°C to allow expression of the relevant antibiotic resistance gene (30 to 60 min). The transformation mixture was then directly plated on the appropriate antibiotic selection plates and incubated overnight at 37°C .

2.7.4 Preparation of electro-competent *E. coli*

A 10 ml overnight culture in LB broth was sub-cultured 1/10 into 100 ml fresh LB broth and incubated at 37°C with shaking until cells reached an A_{600} of 0.6. After chilling on ice for 30 min, the culture was pelleted in a Sigma 3K18 centrifuge at $4,000 \times g$ for 15 min at 4°C and washed twice in 50 ml ice-cold sterile 10% (v/v) glycerol. The competent cells were finally resuspended in 2 ml of ice-cold GYT medium, and 100 μl aliquots were stored at -80°C until use. Alternatively, a 10 ml culture was grown to $A_{600} = 0.6$, pelleted, then the cells were washed twice in 10 ml 10% (v/v) glycerol and resuspended in 100 μl of 10% (v/v) glycerol and used immediately (Section 2.7.5).

2.7.5 Electroporation of plasmid DNA into *E. coli*

DNA (0.4 μg plasmid DNA or 10–100 ng linear DNA) to be transformed was desalted by drop-dialysis against Milli-Q water using a VSWP type filter (13 mm, Millipore Corp., Billerica, MA, USA). Desalted DNA was added to 100 μl of electro-competent cells (Section 2.7.4). The DNA-cell mixture was gently mixed and transferred to an ice-cold sterile 0.2 cm gap electroporation cuvette (Invitrogen Life Technologies). Electro-transformation was

carried out using an ECM[®] 600 Electro Cell Manipulator (BTX Genetronics, San Diego, CA, USA) at a setting of R5 (25 μ F and 129 Ω), 2.5 kV and pulse length at 4–6 msec. Immediately after electroporation, cells were resuspended in 1 ml of SOC Medium. The cells were incubated at 37°C for 1 h then plated onto a selective LB agar plate and incubated overnight at 37°C.

2.8 Lambda red recombinase mutagenesis of STEC strains

The lambda red recombinase method (Datsenko and Wanner, 2000; Yu *et al.*, 2000) was used to make deletion mutations in STEC strains by targeted homologous recombination. The system employs the phage lambda red recombinase synthesised under the control of an inducible promoter in the temperature sensitive plasmid pKD46, to catalyse homologous recombination between linear fragments of DNA and the *E. coli* chromosome (Datsenko and Wanner, 2000; Yu *et al.*, 2000). This system is efficient with regions of DNA homology as short as 30–50 bp (Yu *et al.*, 2000). The plasmids pKD4 and pKD3 (Table 2.2) carry antibiotic resistance cassettes (*kan* and *cat*, respectively) that are flanked by FRT (FLP recombinase recognition target) sites. Each primer includes 40-bp of sequence (at the 5' end) homologous to regions flanking the gene of interest, followed by 20-bp of sequence (at the 3' end of the primer) homologous to the template plasmid priming sites (priming site 1 or 2) flanking the antibiotic resistance cassette. The PCR product is then electroporated into the host strain, which has previously been transformed with the lambda red recombinase expression vector, pKD46 (Table 2.2), and recombination occurs between the homologous sequences on the ends of the PCR product, and the antibiotic resistance cassette replaces the target gene.

For lambda red recombinase mutagenesis, overnight cultures of the host STEC strain harbouring pKD46, were diluted 1/10 in LB broth containing 100 μ g/ml Amp, and the red recombinase was induced in the presence of 100 mM L-arabinose at 30°C until the culture reached an A_{600} of 0.6. The culture was incubated on ice for 30 min, pelleted in a Sigma 3K18 centrifuge at 4,000 $\times g$ for 15 min at 4°C, and washed twice in ice-cold sterile 10%

(v/v) glycerol. The pellet was resuspended in a final volume of 100 μ l of 10% (v/v) glycerol. For electroporation, 50 μ l of cells and 40 μ l desalted PCR product were mixed, and electroporated into the host STEC strain harbouring pKD46, as described in Section 2.7.5. Transformants were allowed to recover at 37°C for 2 h, plated onto Kan or Cml plates and incubated at 37°C overnight to induce the loss of pKD46. Amp^S (indicating curing of pKD46) and Kan or Cml resistant transformants were then screened for the insertion of *kan* or *cat* into the appropriate gene by PCR (Section 2.9.1) and sequencing (Section 2.10), using primers specific for the *kan* (k1) or *cat* (c1) (Table 2.4) cassette and the gene of interest.

2.9 Polymerase chain reaction (PCR)

2.9.1 Standard PCR reaction using Taq polymerase

Standard PCR was performed using Taq DNA polymerase according to the manufacturer's instructions (Roche Diagnostics Pty. Ltd.). The PCR reaction was performed in 0.5 ml microcentrifuge tubes containing 2 mM each of dATP, dCTP, dGTP and dTTP, 100 pmol each of forward and reverse primer, 1 \times PCR buffer (10 mM Tris-HCl, 1.5 mM MgCl₂, 50 mM KCl, pH 8.3), 1.5 U Taq DNA polymerase (Roche Diagnostics Pty. Ltd.) and 100 ng template DNA. The reaction mixture was made up to 50 μ l with Milli-Q water.

Template DNA was initially denatured at 95°C for 3 min followed by 25–35 cycles of amplification (30 sec denaturation at 95°C, 30 sec annealing at 50–60°C [depending on the melting temperature of the primers], and extension for 1–3 min at 72°C [depending on the predicted size of the product; approximately 60 s per kb]) using a Hybaid PCR Sprint thermal cycler (Integrated Sciences, Willoughby, N.S.W., Australia). Reaction products (2–10 μ l) were analysed by agarose gel electrophoresis, as described in Section 2.6.4.

2.9.2 High fidelity PCR

High fidelity PCR was performed using the Expand™ long template PCR system 3 (Roche Diagnostics Pty. Ltd.), according to the manufacturer's instructions. Briefly, PCR reactions were performed in 0.5 ml microcentrifuge tubes containing 500 μM each of dATP, dCTP, dGTP and dTTP, 100 pmol each of forward and reverse primer, 1 × PCR buffer 3, 3.75 U Expand™ enzyme mix and 100 ng template DNA. The reaction mixture was made up to 50 μl with Milli-Q water and amplified as described in Section 2.9.1, but with extension at 68°C for 1–10 min (depending on the predicted size of the product).

2.9.3 Purification of PCR products

PCR products were purified using a MiniElute™ PCR Purification Kit, according to the manufacturer's instructions (QIAGEN GmbH). Briefly, PCR reactions were mixed with 5 volumes of Buffer PB, loaded onto a MiniElute™ column and microcentrifuged at 14,900 × *g* for 1 min. The column was washed with 750 μl Buffer PE and centrifuged at 14,900 × *g* for 1 min. The eluate was removed and the column centrifuged for 1 min to remove residual wash buffer. The column was placed into a clean microcentrifuge tube, 30–50 μl Milli-Q water was added to the column and left to stand for 1 min, and then the DNA was eluted by centrifugation at 14,900 × *g* for 1 min. Alternatively, PCR products were purified using an UltraClean™ PCR Clean-up DNA purification kit (Mo Bio), according to the manufacturer's instructions.

2.9.4 Cloning of PCR products

For cloning, PCR products were directly ligated into pGEM®-T Easy as described in the manufacturer's specifications (Promega). Briefly, the ligation mixture consisted of the purified PCR product (Section 2.9.3), 50 ng pGEM®-T Easy vector DNA, 3 U T4 DNA ligase

and 1 × ligation buffer, made up to a total volume of 10–15 µl with Milli-Q water. The reaction mixture was incubated overnight at 4°C. Ligation mixtures were then transformed into *E. coli* DH5α as described in Section 2.7.3.

2.10 DNA sequencing and analysis

For DNA sequencing, plasmid DNA template or PCR products were purified as described in Sections 2.5.1.2 and Section 2.9.3, respectively. The sequencing procedures used were those described in the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit manual (Applied Biosystems, Perkin-Elmer, Vic., Australia). Each reaction mixture contained DNA template (200 to 500 ng of plasmid DNA or 10 to 100 ng of PCR product), 3.2 pmol of the appropriate primer and 4 µl Big Dye reaction mix. The reaction was adjusted to 20 µl by the addition of Milli-Q water. The mixture was subjected to 25 cycles of amplification (96°C for 30 sec, 55–60°C for 15 sec, followed by 60°C for 4 min) using a Hybaid PCR Sprint thermal cycler.

The extension product was purified by the addition of 80 µl of 75% (v/v) isopropanol and 20 µg glycogen. The precipitate was collected by centrifugation at 14,900 × *g* for 30 min, washed in 75% (v/v) isopropanol, and dried at 65°C for 10 min. Sequencing was performed by the Molecular Pathology Unit at the IMVS, Adelaide, S.A., Australia using an Applied Biosystems 3700 DNA sequencing analyser. DNA sequence data were analysed using the DNAMAN program (Lynnon Biosoft®, Vaudreuil, Quebec, Canada), and BLAST searches were used to identify homologies with sequence data from the National Center for Biotechnology Information (Bethesda, MD, USA [www.ncbi.nlm.nih.gov/blast]) (Altschul *et al.*, 1997).

2.11 Preparation of DNA probes and DNA hybridisation

2.11.1 Preparation of digoxigenin labelled probe

DNA probes were labelled with digoxigenin (DIG) using the DNA labelling kit, according to the manufacturer's instructions (Roche Diagnostics Pty. Ltd.). Briefly, 15 μl of the DNA (10 ng to 3 μg) to be labelled was added to a microfuge tube and heat-denatured at 95°C for 10 min. The denatured DNA was immediately cooled on ice for 1 min, after which 2 μl hexanucleotide mixture, 2 μl dNTP mixture (containing DIG-11-dUTP) and 1 μl Klenow fragment were added. The solution was subsequently incubated overnight at 37°C. The labelled DNA was then denatured at 95°C for 10 min and immediately cooled on ice before adding to the hybridisation mixture (Section 2.11.2).

2.11.2 Southern hybridisation analysis

Southern transfer of DNA was carried out by a method previously described by Southern (1975) and modified by Maniatis *et al.* (1989). Digested genomic or plasmid DNA samples (0.1 to 100 μg) were subjected to agarose gel electrophoresis (Section 2.6.4). The DNA was denatured by soaking the gel in 0.5 M NaOH, 1.5 M NaCl for 30 min, followed by 30 min incubation in 0.5 M Tris-HCl, 1.5 M NaCl (pH 7.5) at room temperature. The DNA was transferred onto positively-charged nylon membrane (Hydond-N⁺) (Amersham Biosciences, Sydney, N.S.W., Australia) by capillary transfer for 4–12 h using 10 \times SSC (1 \times SSC is 0.15 M NaCl, 0.15 M sodium citrate) as a transfer buffer. After transfer, DNA was fixed to the membrane by placing on 3 MM Whatmann No. 1 filter paper soaked with 0.4 M NaOH for 20 min, and then washed briefly in 2 \times SSC.

Filters were prehybridised at 42°C for 90 min in pre-hybridisation solution (50% [v/v] formamide, 1% [w/v] SDS, 6 \times SSC, 5 \times Denhardt's solution [1 \times Denhardt's solution is 2% (w/v) Ficoll, 2% (w/v) polyvinyl pyrrolidone, 2% (w/v) bovine serum albumin (BSA)], 100 $\mu\text{g}/\text{ml}$ herring sperm DNA). This was discarded and replaced with the labelled DNA probe in hybridisation solution (50% [v/v] formamide, 1% [w/v] SDS, 6 \times SSC, 1 \times Denhardt's

solution, 100 µg/ml herring sperm DNA, 100 µg/ml heparin). The probe solution was denatured by incubation at 80–90°C for 10 min prior to adding to the filter. Hybridisation was carried out for 16–24 h at 42°C. The membrane was washed twice with shaking at 65°C for 15 min in $2 \times$ SSC followed by two additional 20 min washes at 65°C with $0.2 \times$ SSC, containing 0.1% (w/v) SDS. The probe solution was stored at –20°C for reuse.

Following the washes, the membrane was rinsed briefly in Buffer 1 (100 mM Tris-HCl, 150 mM NaCl, pH 7.5), followed by incubation in Buffer 2 (1% [w/v] blocking reagent in Buffer 1) for 30 min at room temperature. Filters were washed twice in Buffer 1 for 15 min at room temperature followed by 1 h incubation with α -DIG-AP (diluted 1:5,000 in Buffer 1). After 3 washes in Buffer 1 to remove unbound antibody-conjugate, and equilibration of the membranes in Buffer 3 (100 mM Tris-HCl, 100 mM NaCl, 50 mM MgCl₂, pH 9.5), filters were developed by incubation in the dark using the colour detection solution (10 ml Buffer 3 containing 45 µl of 75 mg/ml NBT in 70% [v/v] dimethylformamide [DMF] and 35 µl of 50 mg/ml X-pho in DMF). The colour reaction was stopped with TE buffer.

2.11.3 Preparation of filters for Dot-blot hybridisation analysis of bacterial lysates

Strains were subcultured in 96-well microtitre trays containing 200 µl per well of LB broth supplemented with an appropriate antibiotic, and incubated for 16 h at 37°C. The cells were pelleted in a Sigma 3K18C centrifuge at $2,000 \times g$ for 10 min. The pellets in each well were resuspended in 10 µl TE buffer and 10 µl of 10% (w/v) SDS followed by 50 µl of 0.5 M NaOH, 1.5 M NaCl. The tray was centrifuged at $2,000 \times g$ for 10 min and 3–5 µl of supernatant in each well was applied to Hybond N⁺ membrane (Amersham Biosciences). The membranes were subsequently fixed with 0.4 M NaOH and hybridised as described in Section 2.11.2.

2.12 RNA extraction procedures

Eukaryotic RNA was isolated by one of the following procedures:

2.12.1 RNA extraction using TRIZOL[®] Reagent

RNA was extracted from HCT-8 cells using TRIZOL[®] Reagent according to the manufacturer's instructions (Invitrogen Life Technologies). All procedures were carried out at room temperature, unless specified otherwise. Briefly, 1 ml TRIZOL[®] Reagent was added to the cell monolayer, in a 6-well tissue culture tray, and the cell lysate was passed through a pipette several times. The homogenised samples were incubated for 5 min to permit the complete dissociation of nucleoprotein complexes, and then 200 μ l chloroform was added. The tubes were shaken vigorously by hand for 15 seconds, and after an incubation of 2–3 min, the samples were microcentrifuged at $12,000 \times g$ for 15 min (after which the RNA remains exclusively in the aqueous phase). The RNA was precipitated with the addition of 500 μ l isopropanol for 10 min, and then centrifuged at $12,000 \times g$ for 10 min. The RNA pellet was washed with 1 ml of 75% (v/v) ethanol and centrifuged at $7,500 \times g$ for 5 min. The RNA pellet was air-dried for 10 min, resuspended in 40 μ l nuclease-free water, and heated for 10 min at 55–60°C. RNA was re-precipitated in 1/10 volume sodium acetate (pH 4.8) and 2 volumes absolute ethanol and stored at -80°C overnight. The following day, RNA was pelleted by centrifugation at $12,000 \times g$ for 30 min at 4°C , washed in 75% (v/v) ethanol, and resuspended in nuclease-free water. RNasin[®] Ribonuclease Inhibitor was added to RNA, and contaminating DNA was digested with RQ1 RNase-free DNase, followed by DNase stop solution, according to the manufacturer's instructions (Promega).

2.12.2 RNA extraction using RNeasy[®] Mini kit

RNA was also extracted from HCT-8 cells using a RNeasy[®] Mini kit according to the manufacturer's instructions (QIAGEN GmbH). Briefly, cells were disrupted by the addition

of 700 μl buffer RLT and mixed by pipetting. The lysate was homogenised by microcentrifugation in a QIAshredder spin column for 2 min at $13,000 \times g$. One volume of 70% (v/v) ethanol was added to the lysate, mixed by pipetting, loaded onto an RNeasy mini column and centrifuged for 15 sec at $8,000 \times g$. The column was washed with 700 μl buffer RW1, followed by washing with 500 μl buffer RPE, and then centrifuged for 15 sec at $8,000 \times g$. The column was washed a second time with 500 μl buffer RPE, and centrifuged at $13,000 \times g$ for 2 min. The RNA was eluted in 40 μl nuclease-free water by centrifugation at $8,000 \times g$ for 1 min.

2.13 Reverse-transcription-PCR (RT-PCR) and real-time RT-PCR

2.13.1 RT-PCR

RT-PCR was performed using the One-step Access RT-PCR system (Promega) according to the manufacturer's instructions. Briefly, each reaction was performed in a final volume of 25 μl , containing 1 \times AMV/*Tfl* reaction buffer, 0.2 mM dNTPs, 20 nmol of each oligonucleotide, 1 mM MgSO_4 , 2.5 U AMV reverse transcriptase, 2.5 U *Tfl* DNA polymerase, and 1 ng to 1 μg RNA. Reactions were performed on a Hybaid PCR Sprint thermal cycler and included the following steps: 45 min of reverse transcription at 48°C , followed by 2 min denaturation at 94°C and 30 cycles of amplification (denaturation at 94°C for 30 seconds, annealing at $56\text{--}60^\circ\text{C}$ for 30 seconds and extension at 72°C for 45 seconds), unless indicated otherwise. The absence of DNA contamination in all RNA preparations was confirmed by RT-PCR analysis using primers specific for the gene encoding the house-keeping enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Table 2.4). The gene encoding GAPDH contains an intron such that mRNA template directs amplification of a 239-bp product, whereas chromosomal DNA template directs amplification of a 341-bp product. The absence of DNA contamination was determined by electrophoresis of 10 μl of each PCR product on a 2% (w/v) agarose gel (Section 2.6.4).

2.13.2 Real-time RT-PCR

The comparative levels of chemokine mRNA produced by HCT-8 cells after stimulation with various *E. coli* strains were determined using quantitative real-time RT-PCR. Oligonucleotide primer pairs used are specified in Table 2.4. RT-PCR was performed using the one-step access RT-PCR system (Promega) according to the manufacturer's instructions. Each reaction was performed in a final volume of 20 μ l, essentially as described in Section 2.13.1, but also containing a 1/20,000 dilution of SYBR[®] Green I nucleic acid stain. The quantitative RT-PCR was performed on a Rotorgene RG-2000 cycler (Corbett Research, Mortlake, N.S.W., Australia) and included the following steps: 45 min of reverse transcription at 48°C, followed by 2 min denaturation at 94°C and 40 cycles of amplification (denaturation at 94°C for 30 seconds, annealing at 56–60°C for 30 seconds and extension at 72°C for 45 seconds), unless indicated otherwise. Each RNA sample was assayed in triplicate using primers specific for the various chemokine mRNAs, or mRNA for GAPDH, which was used as an internal control.

Results were calculated using the comparative cycle threshold ($2^{\Delta\Delta C_t}$) method (User Bulletin no.2 [<http://docs.appliedbiosystems.com/pebi/docs/04303859.pdf>]; Applied Biosystems), in which the amount of target mRNA is normalized to a reference (0 h control) relative to an internal control (GAPDH mRNA). Results are expressed as relative changes in chemokine mRNA levels compared to 0 h control levels. Standard deviations (SD) were initially determined as the $\sqrt{((SD \text{ sample})^2 + (SD \text{ GAPDH})^2)}$, and this was then applied to the formulas: $SD_+ = 2^{\Delta\Delta C_t - SD} - 2^{\Delta\Delta C_t}$ and $SD_- = 2^{\Delta\Delta C_t} - 2^{\Delta\Delta C_t + SD}$.

2.14 General protein analysis

2.14.1 Preparation of bacterial whole cell lysates

E. coli strains to be run on SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gels were cultured in 10 ml LB broth overnight at 37°C with shaking. The bacteria were pelleted in a Sigma 2-4 centrifuge at 4,000 \times g for 10 min. The bacterial pellet was resuspended in

200–500 μ l LUG buffer (5% [v/v] β -mercaptoethanol, 62.5 mM Tris-HCl [pH 6.8], 2% [w/v] SDS, 10% [v/v] glycerol, 0.05% [w/v] bromophenol blue), heated at 100°C for 10 min, and 5–10 μ l was loaded on an acrylamide gel. Alternatively, samples were stored at –20°C for future analysis.

2.14.2 SDS-PAGE

SDS-PAGE was carried out essentially as described by Laemmli (1970). Samples heated in LUG buffer were loaded on acrylamide gels and subjected to electrophoresis on a Mighty[®] Small II SE250 gel apparatus (Hofer[®], Amersham Biosciences) at 170 V for 90 min. Separating gels contained 12% (v/v) acrylamide, 142 mM Tris-HCl (pH 8.8), 0.05% (w/v) SDS, 0.04% (v/v) ammonium persulphate and 0.014% (v/v) N,N,N',N'-tetramethylethylene-diamine (TEMED). Stacking gels contained 3% (v/v) acrylamide, 62 mM Tris-HCl (pH 6.8), 0.05% (w/v) SDS, 0.04% (v/v) ammonium persulphate and 0.11% (w/v) TEMED. Proteins were stained by overnight incubation at room temperature, or for 1 h at 65°C, with gentle agitation in staining solution (0.1% [w/v] Coomassie Brilliant Blue R250 in 25% [v/v] methanol, 25% [v/v] acetic acid), unless otherwise specified. De-staining was accomplished by washing with several changes of a solution containing 10% (v/v) isopropanol and 10% (v/v) acetic acid with gentle agitation either at room temperature or at 65°C for 30–60 min.

The SDS-PAGE molecular weight markers used were either broad range markers (Bio-Rad Laboratories), BenchMark[®] Pre-stained protein ladder (Invitrogen Life Technologies), PhosphoPlus[®] biotinylated protein ladder (Cell Signaling Technology Inc.), or SeeBlue[®] Plus2 Pre-stained protein standard (Invitrogen Life Technologies). Sizes of broad range markers are 200.0, 112.5, 97.4, 66.2, 45.0, 31.0, 21.5, 14.4, and 6.5 kDa. Sizes of BenchMark[®] Pre-stained protein ladder are 172.0, 110.2, 79.0, 62.4, 48.0, 36.6, 24.5, 19.0, 13.5 and 5.3 kDa. Sizes of PhosphoPlus[®] biotinylated protein ladder markers are 200, 140, 100, 80, 60, 50, 40, 30, 20 and 10 kDa. Sizes of SeeBlue[®] Plus2 Pre-stained protein standard markers are 250, 148, 98, 64, 50, 36, 22, 16, 6 and 4 kDa.

2.14.3 Silver staining of SDS-PAGE

SDS-PAGE gels for silver staining were electrophoresed on pre-cast 12% NuPage gels (Invitrogen Life Technologies). Silver staining was performed as follows: the gel was removed from the plastic casing and fixed for 60 min in fixing solution (40% [v/v] ethanol, 5% (v/v) glacial acetic acid), and then oxidised for 5 min in oxidising solution (40% [v/v] ethanol, 5% [v/v] glacial acetic acid, 0.7% [w/v] periodic acid). The oxidising solution was removed from the gel by rinsing three times for 10 min in Milli-Q water, and then stained for 10 min in staining solution (1% [w/v] NaOH, 1.4% [v/v] NH₄OH, 3.3% [v/v] of 20% [w/v] AgNO₃). Excess staining solution was removed by washing gels three times for 10 min in Milli-Q water and gels were then developed in developing solution (0.005% [w/v] citric acid, 0.05% [v/v] 37% formaldehyde) until a brown precipitate formed, after which the solution was decanted and development terminated by the addition of 4% (v/v) acetic acid for 15 min.

2.14.4 Western blot analysis

The method used was a modification of that described by Towbin *et al.* (1979). Samples were run on 12% (v/v) polyacrylamide gels (Section 2.14.2) and transferred to nitrocellulose (BioTrace[®]NT, Pall Corporation, Ann Arbor, MI, USA) at 300 mA for 1 h in transfer buffer (25 mM Tris-HCl pH 8.3, 192 mM glycine, 10% [v/v] methanol) using a Transphor electrophoresis unit TE223 (Hoefer[®], Amersham Biosciences).

Following transfer, the nitrocellulose sheet was blocked by incubating for 30 min in 5% (w/v) skim milk powder in Tween-Tris buffered saline (TTBS) (0.05% [v/v] Tween-20, 20 mM Tris-HCl, 0.9% [w/v] NaCl, pH 7.4), followed by incubation in antiserum (appropriately diluted in TTBS containing 0.02% [w/v] skim milk powder), for 2–16 h. The antiserum was removed and the nitrocellulose filter was washed three times for 5 min in TTBS with gentle agitation. Alkaline phosphatase (AP)-conjugated goat anti-mouse or goat anti-rabbit IgG was added at a dilution of 1:3,000 in TTBS and incubated for 1–2 h with gentle agitation at room temperature. The nitrocellulose filter was washed four times for 20

min in TTBS to remove unbound antiserum. Antigen-antibody complexes were detected by the addition of NBT and BCIP in DIG buffer 3 as previously described (Section 2.12.2).

For p38 MAPK assays only (Section 2.22), following transfer, nitrocellulose was blocked in blocking buffer (5% [w/v] skim milk powder in TTBS) for 1 h at room temperature, followed by washing three times in TTBS for 5 min. The nitrocellulose filter was incubated in primary antibody dilution buffer (5% [w/v] BSA in TTBS) with phospho-ATF-2 antibody (1:1,000) overnight at 4°C with gentle agitation. The antiserum was removed and the nitrocellulose sheet was washed three times for 5 min in TTBS with gentle agitation. Horseradish peroxidase (HRP)-conjugated anti-rabbit IgG antibody (1:2,000) and HRP-conjugated anti-biotin antibody (1:1,000; to detect biotinylated protein markers) were incubated in blocking buffer for 1 h at room temperature. The membrane was washed three times for 5 min with TTBS to remove unbound secondary antibodies. Antigen-antibody complexes were detected by the addition of SuperSignal[®] West Pico Chemiluminescent substrate for 1 min according to the manufacturer's instructions (Pierce Biotechnology Inc.), drained, covered with clear plastic, and then exposed to X-ray film (Kodak BioMax MR Film, Amersham Biosciences).

2.15 Infection of HCT-8 cells with *E. coli*

For chemokine assays, HCT-8 cells were seeded in 6-well tissue culture trays and allowed to attach overnight. Cells were used at 90–100% confluence. Cells were washed twice with phosphate buffered saline (PBS) (0.137 M NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄, 1.5 mM KH₂PO₄, pH 7.4), 1 ml RPMI 1640 (without antibiotics or FCS) was added to each well, and cells were left at 37°C in 5% CO₂ for at least 2 h. One ml of an overnight culture of *E. coli* in LB broth was pelleted at 4,000 × *g* for 10 min in a Sigma 2-4 centrifuge, resuspended in 10 ml FCS-, antibiotic- and phenol red-free RPMI 1640 medium, and incubated for approximately 1–2 h at 37°C, or until the culture reached an A₆₀₀ of 0.21–0.22 (ca. 3 × 10⁸ CFU/ml). *E. coli* strains were centrifuged at 4,000 × *g* for 10 min and resuspended in an equal volume of FCS-, antibiotic- and phenol red-free RPMI 1640 medium.

HCT-8 monolayers were subsequently infected with 100 μl of *E. coli* suspension (approximately 3×10^7 CFU/ml as confirmed by viable count), or medium (for controls), and incubated at 37°C in 95% air/5% CO₂ for 1 or 4 h, at which time the supernatant was collected and stored at -20°C for the IL-8 enzyme linked immunosorbent assay (ELISA) (Section 2.20), and the monolayer was lysed in 1 ml TRIzol[®] Reagent for RNA extraction (Section 2.12.1). Samples were also collected at 0 h to determine baseline chemokine expression in HCT-8 cells before stimulation with *E. coli*.

2.16 Establishment of polarised intestinal epithelial cell monolayers

Intestinal epithelial cells (HCT-8 or T84 cells) were grown on the surface of polycarbonate filter inserts (0.33cm², 5 μm pore size Transwell[®] membranes; Costar, Cambridge, MA, USA) in order to establish polarised monolayers, essentially as described previously (Hurley *et al.*, 1999). Transwell[®] filter inserts were pre-coated with 5 $\mu\text{g/ml}$ rat tail collagen. Briefly, 500 $\mu\text{g/ml}$ rat tail collagen was diluted 1:100 in 60% (v/v) ethanol, and 100 μl was plated onto the surface of a Transwell[®] filter, and left overnight to dry. Intestinal epithelial cells were seeded at a density of $2\text{--}5 \times 10^6$ cells/ml, and 100 μl of cells were added to the upper chamber of collagen-coated Transwell[®] filters and 600 μl media was added to the bottom chamber. These volumes were used as per the manufacturer's instructions to avoid the effects of hydrostatic pressure. For inverted monolayers, in which cells were grown on the underside of the collagen-coated Transwell[®] filters, 70 μl of the cell suspension were added to the underside of filters that were placed upside down overnight in the incubator, which is a sufficient amount of time to allow cell attachment. The next day, Transwell[®] filter membranes were flipped back upright in a 24-well plate and 100 μl media was added to the top chamber with 600 μl media added to the bottom chamber.

Cells were fed every 2 days by changing the media in the top and bottom chamber. The cells polarise and become ready for use at 6–10 days post-seeding. The development of tight junctions was measured using trans-epithelial electrical resistance (TEER) (Section

2.16.1). TEER is a measurement of passive ion permeability; the higher the TEER the less permeable the epithelium is to passive flow of ions across the monolayer. Thus, measurement of TEER represents a highly sensitive means of establishing barrier function. Cells were used at a minimum TEER of 1,000 Ohms (Ω) cm^{-2} .

2.16.1 Electrical resistance measurements

The Millicell-ERS apparatus (Millipore Corporation) was used to measure TEER of the intestinal epithelial cells grown on Transwell[®] filters. Before measuring TEER, Transwell[®] trays were removed from the incubator and allowed to reach room temperature. The electrode was placed in the Transwell[®], one electrode per chamber, and the reading in Ω was noted. To ensure that measurements represent a stable reading, Transwells[®] were all tested at least twice. To convert the measured Ω value into a $\Omega \text{ cm}^{-2}$ value, the Ω measurement from a cell-free Transwell[®] (usually around 150 Ω) was subtracted from the measured Ω value of the monolayer, and this value was multiplied by the surface area of the Transwell[®] (0.33 cm^2).

2.17 PMN transmigration assays

2.17.1 Isolation of PMNs

Human PMNs were isolated from normal volunteers as described previously (Parkos *et al.*, 1991). Briefly, whole blood (150 to 500 ml) was collected by venipuncture from normal donors of both sexes into an anticoagulation solution containing sodium citrate (13.2 g) and dextrose (11.2 g) in 500 ml of water (pH 6.5). The buffy coat was separated by centrifugation at $300 \times g$ at room temperature. The plasma, platelets and mononuclear cells were removed by aspiration. The PMN and erythrocytes were resuspended in 2% (w/v) gelatin in Hanks' balanced salt solution (HBSS) without Ca^{2+} and Mg^{2+} (HBSS⁻) (Sigma-Aldrich) and incubated at 37°C for 25 min. The supernatant was collected and centrifuged at

300 × g for 10 min. The residual erythrocytes were lysed in cold lysis buffer (0.829% [w/v] NH₄Cl, 0.1% [w/v] NaHCO₃, 0.0038% [w/v] EDTA) and the PMNs were pelleted at 300 × g for 10 min at 4°C. The PMNs were washed in HBSS⁻ and resuspended at a final density of 5 × 10⁷ PMN per ml. This technique allows for the rapid isolation of PMNs (viability > 98% as determined by trypan blue exclusion) at greater than 90% purity.

2.17.2 PMN transmigration assay

PMN transmigration assays were performed essentially as described previously (Parkos *et al.*, 1991; Hurley *et al.*, 2001), with some modifications. Intestinal epithelial T84 or HCT-8 cells were grown as inverted monolayers on the surface of polycarbonate filter inserts (0.33cm², 5 µm pore size Transwell[®] membranes, Costar) as described in Section 2.16. Cells were used at a minimum TEER of 1,000 Ω cm⁻² (Section 2.16.1). Monolayers were washed twice in HBSS with Ca²⁺ and Mg²⁺ (HBSS⁺) (1 ml in bottom chamber; 200 µl in top chamber) then incubated for 1 h at 37°C in DMEM/Hams F-12 medium. STEC strains were cultured in RPMI 1640 medium (without phenol red) for approximately 2 h, and adjusted to a concentration of 8 × 10⁹ bacteria per ml in RPMI 1640 medium, as determined by CFU counts. Transwells[®] were flipped upside down in a moist chamber, and infected on the apical (bottom) surface with 2 × 10⁸ bacteria/monolayer for 1 h at 37°C, to allow bacteria to attach. Transwells[®] were washed 3 × in HBSS⁺ to remove non-adherent bacteria then placed in a clean 24-well tray containing 1 ml medium in the bottom (apical) chamber and 100 µl in the top (basolateral) chamber. PMNs were added to the basolateral (top) chamber at a concentration of 1 × 10⁶ per monolayer, and allowed to transmigrate for 2 h at 37°C. The number of PMNs that transmigrated was determined by assaying for the PMN-specific azurophilic granule marker myeloperoxidase (MPO) as described in Section 2.17.3.

2.17.3 Myeloperoxidase assay

MPO assays were performed essentially as described previously (Parkos *et al.*, 1992; Parkos *et al.*, 1991). After each transmigration assay, still adherent, but transmigrated, PMNs were removed from the apical surface of the monolayer by gentle tapping of the insert. To the samples (bottom reservoir) or standards (2×10^3 to 2×10^6 PMNs), 50 μ l of 10% (v/v) Triton X-100 was added and gently agitated for 25 min at 4°C. An equal volume of citrate buffer (18.4% [w/v] sodium citrate, 13% [w/v] citric acid, pH 4.2) was added to the wells. Peroxidase activity was assayed by the addition of 50 μ l substrate solution (1 mM 2,2'-azino-di-[3-ethyl] dithiazoline sulphonic acid, 10 mM H₂O₂ in 5% [v/v] citrate buffer, pH 4.2) per well, allowed to develop at room temperature for 5–20 min, then A₄₀₅ was measured in a Beckman Coulter spectrophotometer (Beckman Coulter Inc., Fullerton, CA, USA). PMN cell equivalents were estimated from PMN standards (2×10^3 to 2×10^6).

2.18 Adherence assay

Adherence assays were performed essentially as described previously on HEp-2 or HCT-8 cells (Paton *et al.*, 1997). Briefly, *E. coli* strains were grown overnight at 37°C in LB broth, and diluted to a density of 1×10^4 CFU/ml (as confirmed by viable count) in the appropriate medium (without antibiotics or FCS). HEp-2 or HCT-8 cell monolayers in 24-well tissue culture plates were washed with 2 ml PBS, and then infected with one ml aliquots of the diluted bacterial suspension. After incubation at 37°C for 3 h, the culture medium was removed and the monolayers were washed four times with PBS to remove non-adherent bacteria. The cell monolayers were detached from the plate by treatment with 100 μ l of 0.025% (v/v) trypsin, 0.02% (w/v) EDTA. Cells were lysed by the addition of 400 μ l of 0.025% (v/v) Triton X-100 and, after vigorous resuspension, 50 μ l aliquots of 10^{-1} , 10^{-2} and 10^{-3} dilutions of the lysate were plated on LB agar to determine the total number of adherent bacteria per well. Assays were carried out in quadruplicate and results were expressed as the mean (\pm SD) CFU/well. The significance of differences in adherence was analysed using Student's unpaired *t*-test (2-tailed).

2.19 Vero cell cytotoxicity assay

Toxin titres were measured by Vero cell cytotoxicity assay as described previously (Paton *et al.*, 1995b). Briefly, Vero cells were seeded into 96-well flat bottom trays and incubated overnight at 37°C until confluent. Confluent monolayers were washed twice with PBS, treated with 50 µl filter-sterilised 4 h culture supernatants which had been serially diluted in DMEM (without FCS), and then incubated at 37°C for 30 min. After incubation, 150 µl of DMEM supplemented with 2% (v/v) HI FCS was added per well. Cytotoxicity was assessed after 3 days of incubation at 37°C. The toxin titre expressed as CD₅₀ per ml was defined as the reciprocal of the maximum dilution producing a cytopathic effect on at least 50% of the cells in each well. Purified Stx1 and Stx2 (Toxin Technologies Inc.) were used as standards.

2.20 IL-8 ELISA

The levels of IL-8 in culture supernatants at 0, 1 and 4 h were assayed in duplicate using a commercial sandwich enzyme linked immunosorbent assay (ELISA) (R&D Systems Inc.). ELISAs were performed in 96-well trays (Maxisorp Nunc-immuno plates, Nunc, Roskilde, Denmark) and all steps were carried out at room temperature, unless otherwise indicated. Briefly, plates were coated overnight with 100 µl of 2 µg/ml monoclonal anti-human IL-8/CXCL8 capture antibody diluted in PBS. After overnight incubation, plates were washed 3 times with wash buffer (0.05% Tween 20 in PBS, pH 7.4), and blocked for 1 h with 300 µl of PBS containing 1% (w/v) BSA, 5% (w/v) sucrose, 0.05% (w/v) NaN₃. Plates were washed three times in wash buffer, and 100 µl of standard (recombinant human IL-8 [31.25 pg/ml to 2,000 pg/ml]), or sample (neat, 1/10 and 1/100 dilution) diluted in ELISA diluent (0.1% [w/v] BSA, 0.05% [w/v] Tween 20 in TBS [20 mM Tris, 150 mM NaCl], pH 7.3) was added to duplicate wells and incubated for 2 h. Plates were washed three times in wash buffer, and then 100 µl of 20 ng/ml biotinylated anti-human IL-8 antibody diluted in ELISA diluent was added per well and incubated for 2 h. Plates were washed 3 times with wash buffer, 100 µl of 1/10,000 dilution of peroxidase conjugated streptavidin (in TBS containing

0.1% [w/v] BSA) per well was added, and then incubated for 20 minutes. Plates were washed three times in wash buffer and 100 μ l of Sigma fast *o*-phenylenediamine dihydrochloride substrate solution was added per well, and incubated for 20–30 minutes. Stop solution (50 μ l of 1 M H₂SO₄) was added to each well and A₄₅₀ was read on a Dynatech MR5000 (Dynatech Industries, Inc. McLean, VA, USA), using wavelength correction at A₅₇₀ nm. The sensitivity limit of the ELISA is 31.25 pg/ml.

2.21 Preparation of cell extracts

Cell extracts for immunoprecipitation (IP) kinase assays (Section 2.22) and Western blotting (Section 2.14.4) were prepared essentially as previously described (Smith *et al.*, 2003b). Briefly, following treatment and removal of culture media, cell culture plates were transferred to ice and the cells were washed twice with cold PBS (1 ml/well for 6-well plates or 2 ml/well for 10 cm plates). Cells were scraped into cold PBS (600 μ l) to which 10 μ g/ml of leupeptin, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 0.5 mM DTT were added. Cells were pelleted by centrifugation at 10,000 \times g for 2 min at 4°C, the supernatant was removed, and pelleted cells were resuspended in Triton lysis buffer (25 mM HEPES [pH 7.5], 300 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA [pH 8.0], 0.1% Triton X-100, 0.1 mM sodium orthovanadate [Na₃VO₄], 20 mM β -glycerol phosphate, 10 μ g/ml leupeptin, 1 mM PMSF, 0.5 mM DTT). The resuspended cells were gently rocked at 4°C for 30 min and cellular debris was subsequently removed by centrifugation at 13,000 \times g for 15 min at 4°C. The supernatants were collected and frozen at –80°C. The protein concentrations in the cell extracts were determined according to the method of Bradford (1976) using the using the Bio-Rad Protein assay kit (Bio-Rad Laboratories).

2.22 p38 MAPK assay

p38 MAPK activity was assessed using the p38 MAPK assay kit according to the manufacturer's instructions (Cell Signaling Technology Inc.). All protein kinase assays were

performed on cell extracts (Section 2.21) of equal protein concentration. Briefly, 200 μg of protein extract was mixed with 20 μl monoclonal antibody specific for phosphorylated p38 (Thr180/Tyr182) immobilized on Sepharose beads. These mixtures were incubated overnight at 4°C with gentle rocking. Immunoprecipitates were washed twice with lysis buffer (to which 1 mM PMSF had been added), washed twice with kinase buffer, and centrifuged for 30 sec at 13,000 $\times g$ between washes. Immunoprecipitates were resuspended in 50 μl of kinase buffer supplemented with 200 μM ATP and 2 μg ATF-2 fusion protein. Kinase reaction mixtures were incubated for 30 min at 30°C, and the reaction was terminated by the addition of 3 \times SDS-PAGE sample buffer (187.5 mM Tris-HCl [pH 6.8], 6% [w/v] SDS, 30% [v/v] glycerol, 0.03% [w/v] bromophenol blue, 150 mM DTT), followed by heating samples at 100°C for 10 min and centrifugation at 13,000 $\times g$ for 10 min. Samples were analysed by SDS-PAGE (Section 2.14.2) and Western blotting (Section 2.14.4) using antibody specific for ATF-2 phosphorylated at Thr71 (1:1,000).

2.23 Isolation of LPS

An overnight culture of the desired strain was subcultured 1/10 in 1 litre LB broth and incubated with shaking for approximately 2 h, or until A_{600} is 0.6–0.8. The culture was centrifuged in a Beckman J2-M1 centrifuge at 8,000 $\times g$ for 10 min at 4°C, the supernatant was discarded, and the pellet was stored at –80°C overnight, or until use. The bacterial pellet was thawed and resuspended in 15 ml of 2 mM MgCl_2 , 10 mM Tris-HCl (pH 8.0), to which 50 μl DNase (30 mg/ml stock) and 100 μl RNase (3.75 mg/ml stock) was added, and the sample was subsequently sonicated 3 \times 30 seconds on ice. An additional 50 μl DNase and 100 μl RNase was added to this solution and incubated at 37°C for 2 h, then 5 ml 0.5 M EDTA (in 10 mM Tris-HCl, pH 8.0), 2.5 ml 20% (w/v) SDS (in 10 mM Tris-HCl, pH 8.0) and 2.5 ml 10 mM Tris-HCl (pH 8.0) was added and the solution adjusted to a pH of 9.5. The solution was mixed, incubated at 37°C for 1 h, and centrifuged at 20,000 $\times g$ for 30 min at 15°C to remove peptidoglycan. Pronase (200 μl of 25 mg/ml stock) was added to the supernatant and incubated at 37°C with shaking overnight.

The LPS was precipitated by the addition of 50 ml 0.375 M MgCl₂ in 95% (v/v) ethanol and then cooling to 0°C in a dry ice-ethanol bath. The precipitate was centrifuged at 9,000 × g for 30 min at 0°C, the pellet was resuspended in 25 ml 0.1 M EDTA, 2% (w/v) SDS in 10 mM Tris-HCl, pH 8.0, then sonicated 3 × 30 seconds on ice. After sonication, the solution was adjusted to a pH of 8.0 by the dropwise addition of NaOH, and incubated at 85°C for 30 min. The solution was cooled to room temperature and the pH adjusted to 9.5 with NaOH. Pronase (25 µl) was added and incubated at 37°C overnight.

The LPS was precipitated by the addition of 50 ml 0.375 M MgCl₂ in 95% (v/v) ethanol, cooled to 0°C in a dry ice-ethanol bath, and then centrifuged for 30 min at 15,000 × g at 0°C. The pellet was resuspended in 20 ml 10 mM Tris-HCl (pH 8.0), sonicated 2 × 30 seconds on ice, 675 µl of 1 M MgCl₂ was added, and the LPS was pelleted in a Beckman L8-80 ultracentrifuge at 30,000 × g for 4 h at 15°C. The LPS was finally resuspend in Milli-Q water and dialysed against Milli-Q water for three days. The LPS was lyophilised and stored at 4°C. The purity and concentration of the LPS preparation was confirmed by electrophoresis on pre-cast Bis-Tris PAGE gels (Invitrogen Life Technologies) and silver staining (Section 2.14.3).

2.24 Crude preparation of flagella

Flagella were prepared from STEC strains as described previously (Steiner *et al.*, 2000). Briefly, bacteria were pelleted at 8,000 × g for 10 min, resuspended in 30 ml 500 mM Tris-HCl (pH 8.0) and sheared in a Sorvall Omni mixer at maximum speed for 1 min (Sorvall Products, Norwalk, CT, USA). Bacterial cells and debris were removed by centrifugation at 8,000 × g for 15 min. The supernatant was clarified by filtration (0.8 µm pore size), and flagella were pelleted by centrifugation at 100,000 × g for 60–90 min then resuspended in PBS. Purity was assessed by SDS-PAGE (Section 2.14.2) and protein concentration was assayed according to the method of Bradford (Bradford, 1976). H21 and H7 antiserum was obtained from the *Salmonella* Reference Laboratory, IMVS, Adelaide, S.A., Australia.

2.25 Expression and purification of flagellin

2.25.1 Expression of His₆-FliC

High level protein expression was engineered using the QIAexpressionist™ His₆ fusion protein system (QIAGEN GmbH). The gene of interest (*fliC*) was PCR amplified, incorporating appropriate restriction sites into the oligonucleotides (Section 2.9.2; Table 2.4). The PCR product was digested with restriction enzymes and ligated into pQE-30, pQE-31, or pQE-32 (Table 2.2) depending on the frame of translation. This placed the His₆ tag and up to 10 linker amino acids at the N-terminus of the fusion protein expressed by such constructs. The construct was transformed into *E. coli* strain M15[pREP4] (Table 2.1) and selected by plating on LB agar supplemented with 50 µg/ml Kan and 100 µg/ml Amp. Recombinant pQE constructs were sequenced in order to confirm correct in-frame fusion.

For large-scale purification of His₆-FliC, M15[pQE-30/*fliC*] was grown in 50 ml LB broth overnight at 37°C with agitation in the presence of 50 µg/ml Kan and 100 µg/ml Amp. This starter culture was diluted 1/10 into 500 ml Terrific broth and incubated at 37°C until an A₆₀₀ of 0.75 had been reached. High-level expression of His₆-FliC was induced by the addition of 2 mM IPTG, and incubation at 37°C for a further 3 h with agitation. The culture was then centrifuged at 4°C for 10 min at 11,440 × *g*. The pellet was washed in PBS, resuspended in 10 ml of lysis buffer (50 mM sodium-phosphate, 300 mM NaCl, 10 mM imidazole, pH 8.0), and the cells were lysed using a French pressure cell (Aminco-SLM Instruments, Urbana, IL, USA) operated at 12,000 psi. The lysate was centrifuged at 8,000 × *g* for 15 min at 4°C to remove cellular debris. The supernatant was centrifuged at 100,000 × *g* for 1 h at 4°C and the soluble supernatant used for purification by Nickel-nitrilotriacetic acid purification (Section 2.25.2).

2.25.2 Purification of His₆-FliC by Ni-NTA chromatography

His₆-FliC was purified by nickel-nitrilotriacetic acid (Ni-NTA) affinity chromatography (QIAGEN GmbH). A 2 ml Ni-NTA column was equilibrated with 20 ml

lysis buffer (50 mM NaH₂PO₄, 30 mM NaCl, 10 mM imidazole, pH 8.0). M15[pQE-30/*fliC*] lysate supernatant was treated with 5 µg/ml DNase and 10 µg/ml RNase for 10 min on ice and was loaded onto the column at a rate of 15 ml/h. After the entire supernatant had been loaded, the column was washed with 15 ml wash buffer (50 mM sodium-phosphate, 300 mM NaCl, 10% [v/v] glycerol, pH 6.0). Bound His₆-FliC was eluted from the column with a 0–500 mM imidazole gradient in wash buffer (50 mM sodium-phosphate, 300 mM NaCl, 10% [v/v] glycerol, pH 6.0). Two ml fractions were collected and immediately placed on ice, 10 µl from each fraction was subject to SDS-PAGE analysis (Section 2.14.2) to confirm the presence and purity of FliC. Fractions containing His₆-FliC were dialysed overnight against PBS, and then stored in 50% (v/v) glycerol at –20°C, or at 4°C for immediate use. Protein concentration was determined according to the method of Bradford (1976). Where required, HI His₆-FliC was prepared by heating at 100°C for 4 h.

2.26 Colonisation of streptomycin-treated mice by STEC

Mouse colonisation studies were carried out essentially as described previously (Wadolowski *et al.*, 1990a). Five to six week old BALB/c mice were treated with streptomycin sulphate (Str, 5 g/l) in their drinking water for 24 h prior to the oral administration of STEC, and throughout the remainder of the experiment. Food was withdrawn from the mice 15 to 18 h prior to the administration of STEC. Str resistant derivatives of various strains were grown with aeration overnight at 37°C in 10 ml LB broth, pelleted at 4,000 × *g* for 10 min, and resuspended in 2 ml of 20% (w/v) sucrose, 10% (w/v) NaHCO₃. Each mouse was given a 50 µl volume of STEC administered orally (approximately 10⁸ CFU/mouse), after which the food was re-introduced. The actual dose administered was determined by viable counts on LB plates supplemented with Str. Faecal samples were collected and levels of colonisation by STEC were determined by plating serial dilutions of faecal homogenates on LB agar supplemented with 50 µg/ml Str. Results were expressed as CFU/g faeces.

CHAPTER 3: Initial Characterisation of Epithelial Cell Chemokine Responses to a Range of STEC Isolates

3.1 Introduction

After ingestion and establishment of intestinal colonisation, STEC release Stx into the gut lumen. The toxin is then absorbed across the gut epithelium into the circulation, where it targets host cells expressing the specific glycolipid receptor Gb₃ (Section 1.4.1). Since STEC are non-invasive, the penetration of Stx into underlying tissues is a crucial step in pathogenesis, and PMNs are now thought to be important in this process (Section 1.7). Recently it was observed that the amount of Stx1 and Stx2 crossing polarised T84 cell monolayers in an apical to basolateral direction was proportional to the number of PMNs migrating in the opposite direction (Hurley *et al.*, 2001). This was due to an increase in paracellular permeability and the break down of the tight junction barrier (Hurley *et al.*, 2001). PMNs have also been shown to directly bind Stx2 *in vitro* and *in vivo* via a receptor with a 100-fold lower affinity than Gb₃ (te Loo *et al.*, 2000a; te Loo *et al.*, 2000b). Bound toxin was subsequently released on contact with target cells expressing Gb₃ (te Loo *et al.*, 2000a). Thus, PMNs may function in the pathogenesis of STEC disease by contributing to the break down of the intestinal epithelial barrier, and in the transport of Stx to target tissues (Section 1.7.1).

Intestinal epithelial cells play an important role in mediating inflammatory responses since they are the first cells to contact enteric pathogens. Pathogenic bacteria including *Salmonella* spp., *S. dysenteriae*, *Y. enterocolitica*, *L. monocytogenes* and EIEC have long

been associated with the upregulation of a number of pro-inflammatory cytokines and chemokines in host cells (Jung *et al.*, 1995; Schulte *et al.*, 1996; Yang *et al.*, 1997; Coates and McColl, 2001). In cases of human STEC disease, elevated IL-8 levels have been shown to correlate both with leucocytosis and poor prognosis (Fitzpatrick *et al.*, 1992b; Karpman *et al.*, 1995; Murata *et al.*, 1998; Litalien *et al.*, 1999; Westerholt *et al.*, 2000; King, 2002). IL-8 is a member of the CXC chemokine family and is a potent PMN chemoattractant, as are other members of this family including ENA-78, GCP-2, MGSA, MIP-2 α and MIP-2 β (Section 1.7.2) (Baggiolini, 1997; Gale and McColl, 1999). Thus, CXC chemokines may be important in the recruitment of PMNs in serious STEC infection, and may play a pivotal role in the pathogenesis of HUS.

In this chapter, procedures for the detection and quantitation of chemokine mRNA in HCT-8 cells, and IL-8 protein, in HCT-8 culture supernatants in response to infection with STEC were developed. These were used to quantitate the relative mRNA levels of IL-8, ENA-78, GCP-2, MGSA, MIP-2 α and MIP-2 β mRNA using the house-keeping gene mRNA for GAPDH as an internal standard. A commercial ELISA was also used to detect IL-8 protein levels in STEC-infected HCT-8 monolayers.

3.2 Results

3.2.1 Design of oligonucleotides for real-time RT-PCR

To measure mRNA transcripts for the CXC chemokines induced in response to infection with STEC, a quantitative real-time RT-PCR assay was developed. Initially primer pairs were designed for each of the target mRNAs, based on multiple alignment with human mRNA nucleotide sequences deposited in GenBank (primer sequences and accession numbers are listed in Table 2.4), including IL-8, ENA-78, GCP-2, MGSA, MIP-2 α and MIP-2 β , as well as GAPDH. GAPDH has previously been shown to be suitable for use as an internal standard for mRNA extraction, since it is constitutively expressed and its levels are unaffected by the presence of either EPEC or Stx (de Grado *et al.*, 2001; Thorpe *et al.*, 2001). The CXC

chemokines have high homology at the nucleotide level and since SYBR green nucleic acid stain, which binds double-stranded DNA, was being used for product detection in the real-time RT-PCR assay, it was important to get a single and specific product for each primer pair. For accurate quantitation in real-time RT-PCR it is important that all products that are being compared are approximately the same size, and that they exhibit similar amplification kinetics. For this reason, primers were initially designed to amplify fragments of approximately 300 bp. However, given the high homology between the CXC chemokine genes, it was not always possible to generate both a single product, and a product of the same size. Under these circumstances, slight differences in amplicon length were tolerated, so as to maintain specificity.

To obtain chemokine mRNA to test the chemokine primers, HCT-8 cells were treated with 100 ng/ml TNF- α for 4 or 24 h, in order to induce a range of chemokines. RNA was extracted (Section 2.12.1) from HCT-8 monolayers and the primers were tested using RT-PCR (Section 2.13.1). Initially, a single amplification product was obtained only with the primer pairs: MIP-2 α Fwd/Rev (323 bp) and MIP-2 β Fwd/Rev (281 bp). Primers for IL-8, ENA-78 and GCP-2 were re-designed and re-tested until a single product was obtained. This was achieved using the following primer pairs: IL-8 Fwd/Rev (327 bp), ENA-78 Fwd/Rev (331 bp) and GCP-2 Fwd/Rev (337 bp) (Fig. 3.1). For MGSA Fwd/Rev, no PCR product was detected with TNF- α stimulated cells at 4 (result not shown) or 24 h (Fig. 3.1). However, when HCT-8 cells were stimulated with the STEC strain 98NK2 (Section 3.2.2.1) a PCR product of the correct size (473 bp) was obtained (Fig. 3.1). The absence of DNA contamination in all RNA preparations was confirmed by RT-PCR analysis using GAPDH Fwd/Rev primers (Table 2.4). The gene encoding GAPDH contains an intron such that mRNA template directs amplification of a 239-bp product, whereas chromosomal DNA template directs amplification of a 341-bp product. Only RNA samples that amplified a single band of 239 bp with GAPDH primers were used. The above chemokine primers were checked by real-time RT-PCR, and all yielded single bands of the correct size, and were shown to have a single product as confirmed by both melt-curve analysis on the real-time RT-PCR machine, and by agarose gel electrophoresis (Section 2.6.4). The primer pairs used are listed in Table 2.4 along with their respective nucleotide sequence.

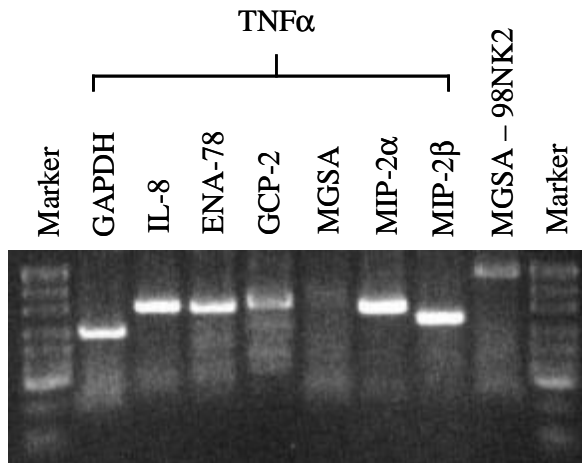


Figure 3.1 RT-PCR analysis using chemokine primers. HCT-8 cells were stimulated with 100 ng/ml TNF- α for 24 h or with STEC strain 98NK2 (O113:H21) for 4 h. RNA was extracted (Section 2.12.1) and RT-PCR performed (Section 2.13.1). The expected sizes of the products are: GAPDH (239 bp), IL-8 (327 bp), ENA-78 (331 bp), GCP-2 (337 bp), MGSA (473 bp), MIP-2 α (323 bp) and MIP-2 β (281 bp). The mobilities of *Hpa*II-digested pUC19 DNA size markers are 501, 404, 331, 242, 190, 147, 111, 110, 67, 34 and 26 bp.

3.2.2 Chemokine induction by 98NK2 and EDL933

3.2.2.1 Chemokine mRNA induction in HCT-8 cells in response to 98NK2 and EDL933

Initial experiments involved identification of a range of principal chemokines expressed by HCT-8 cells in response to the well-characterised LEE-positive O157:H7 STEC strain EDL933 and the LEE-negative O113:H21 STEC strain 98NK2 (Table 2.1). HCT-8 monolayers were incubated with approximately 3×10^7 STEC for either 1 or 4 h, after which total cellular RNA was extracted (Section 2.12.1) and analysed for the presence of mRNA specific for IL-8, ENA-78, GCP-2, MGSA, MIP-2 α and MIP-2 β by real time RT-PCR, as described in Section 2.13.2 (Fig. 3.2).

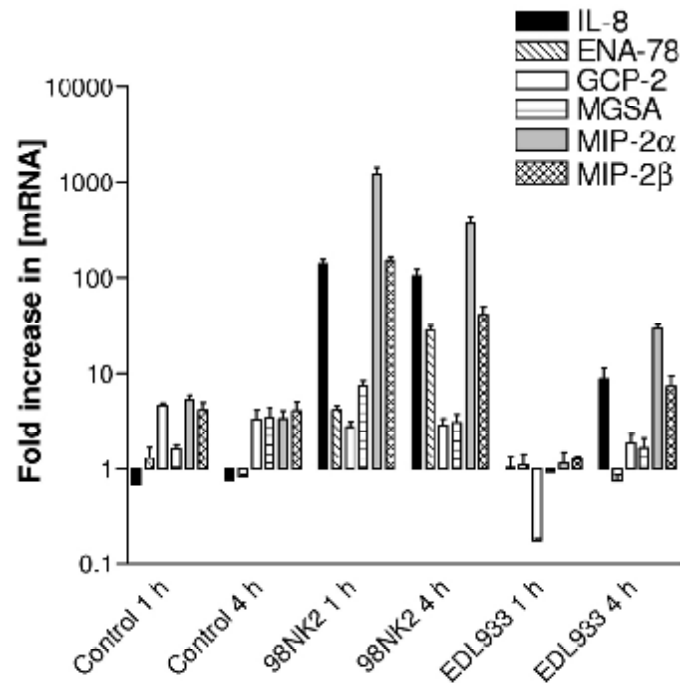


Figure 3.2 Induction of CXC chemokine mRNA in HCT-8 cells infected with strains 98NK2 and EDL933. HCT-8 cells were stimulated with 3×10^7 CFU/ml of STEC strains 98NK2 (O113:H21) or EDL933 (O157:H7). At 1 or 4 h, total RNA was extracted from cells (Section 2.12.1) and chemokine mRNA was quantitated by real-time RT-PCR (Section 2.13.2). Results are expressed as the fold increase in [mRNA] relative to levels at 0 h, and data are shown as the means \pm SD for triplicate assays.

At 1 h, there was no clear difference in the levels of mRNA for any of the six chemokines between unstimulated (control) cells and those infected with EDL933 (Fig. 3.2). However, cells infected with the same dose of 98NK2 exhibited marked upregulation of mRNA for IL-8, MIP-2 α , and MIP-2 β but not for ENA-78, GCP-2 or MGSA (Fig. 3.2). The greatest responses were observed for IL-8, MIP-2 α and MIP-2 β mRNAs, which were upregulated 197-, 227- and 36-fold, respectively, relative to control cells. At 4 h, similar responses were observed in cells infected with 98NK2, except that ENA-78 mRNA was also upregulated at this time. However, at this time point, upregulation of mRNA for IL-8, MIP-2 α and MIP-2 β was also observed in HCT-8 cells infected with EDL933, although the degree of upregulation (11-, 9-, and 2-fold, respectively) remained lower than that seen in cells

infected with 98NK2 (Fig. 3.2). Since upregulation of both IL-8 and MIP-2 α occurred consistently, future experiments focused on these two chemokines as a representative indication of chemokine upregulation.

3.2.2.2 98NK2 and EDL933 stimulate secretion of IL-8 by HCT-8 cells

To confirm that upregulation of IL-8 mRNA also resulted in an increased secretion of the chemokine itself, levels of IL-8 in 98NK2- and EDL933-infected HCT-8 culture supernatants were measured by ELISA, as described in Section 2.20. IL-8 was studied because there is a reliable and commercially available ELISA for this chemokine. Commercial ELISA assays are not available for the other high-induced chemokines MIP-2 α or MIP-2 β (R&D Systems Inc.). The ELISA data indicate that cells exposed to 98NK2 induced significant levels of IL-8 protein at 2, 3 and 4 h ($9,024 \pm 3,224$, $13,800 \pm 3,300$ and $15,060 \pm 7,759$ pg/ml IL-8, respectively), but at 1 h, levels were similar to that in control cells (< 60 pg/ml IL-8) (Fig. 3.3). However, EDL933-stimulated cells induced levels of IL-8 protein that were comparable to control cells (< 60 pg/ml IL-8) at all time points, except at 4 h (73 ± 23 pg/ml IL-8) (Fig. 3.3). Although EDL933 may have increased IL-8 protein at later time points, samples were not taken after 4 h post-infection because HCT-8 monolayers were observed to have become detached from the substratum. These results indicate that there are differences in the kinetics of chemokine induction elicited by different STEC isolates, and that 4 h is the most appropriate time for sampling IL-8 in these assays.

3.2.2.3 Effect of 98NK2 and EDL933 dosage on chemokine production by HCT-8 cells

Given the vast differences in chemokine expression elicited by 98NK2 and EDL933, it was important to ensure that these results were not due to slight differences in the initial doses administered (approximately 3×10^7 CFU) for these strains. Dose-response experiments

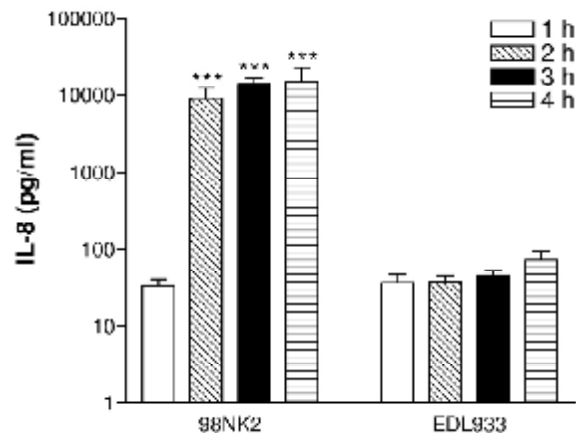


Figure 3.3 Induction of IL-8 protein in HCT-8 cells infected with 98NK2 and EDL933. HCT-8 cells were stimulated with 3×10^7 CFU/ml of the LEE-negative STEC strain 98NK2 or the LEE-positive STEC strain EDL933. At 1, 2, 3 or 4 h, supernatants were collected and assayed for IL-8 by ELISA (Section 2.20). Data are shown as the means \pm standard error of the means (SEM) from two experiments. The significance of differences between IL-8 secretion by infected versus uninfected 4 h control cells (< 60 pg/ml IL-8) is indicated as follows: ***, $P < 0.001$ (determined by ANOVA and a post-hoc Tukey test).

indicated that infecting HCT-8 cells with two-fold higher (6×10^7 CFU) or two-fold lower (1.5×10^7 CFU) doses of either 98NK2 or EDL933 did not significantly affect the degree of IL-8 or MIP-2 α mRNA upregulation (Fig. 3.4A). However, diminished responses were observed at doses $< 1.5 \times 10^7$ CFU (data not shown). Similarly, with IL-8 protein there was little difference with the 2-fold higher or 2-fold lower dose of either 98NK2 or EDL933 ($P > 0.05$ compared to the standard dose of 98NK2 or EDL933) (Fig. 3.4B). These data indicate that the differences observed in chemokine production between 98NK2 and EDL933 are not attributable to differences in the initial dose.

3.2.2.4 Viability of HCT-8 cells after incubation with 98NK2 or EDL933

To ensure that HCT-8 cells remained viable throughout the assay, cells were infected with 98NK2 or EDL933 and viability was measured by trypan blue exclusion. After 4 h

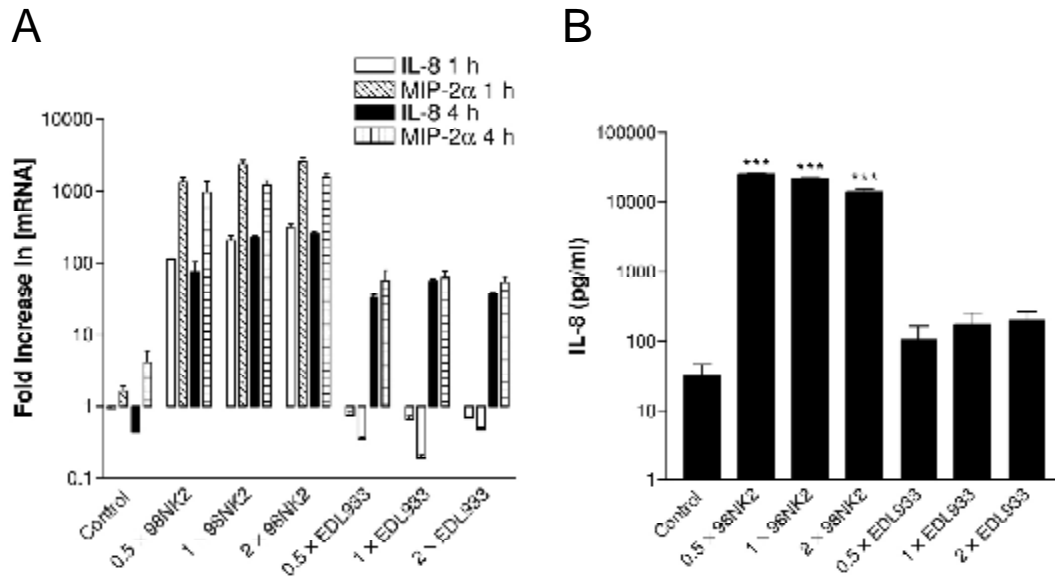


Figure 3.4 Dose-dependent induction of IL-8 and MIP-2 α mRNA and IL-8 protein in HCT-8 cells infected with 98NK2 or EDL933. HCT-8 cells were stimulated with the standard dose of either 98NK2 or EDL933 (3×10^7 CFU) or two-fold higher or lower doses of 98NK2 or EDL933. (A) At 1 or 4 h, total RNA was extracted from cells (Section 2.12.1) and IL-8 and MIP-2 α mRNA was quantitated by real-time RT-PCR (Section 2.13.2). Results are expressed as the fold increase in [mRNA] relative to levels at 0 h, and data are shown as the means \pm SD for triplicate assays. (B) At 4 h, supernatants were collected and assayed for IL-8 by ELISA (Section 2.20). Data are shown as the means \pm SEM from two experiments. The significance of differences between IL-8 secretion by infected versus uninfected cells is indicated as follows: ***, $P < 0.001$.

incubation with 98NK2 or EDL933, HCT-8 cells were incubated for 5 min with 0.4% (w/v) trypan blue and viable cells (those excluding the dye) were counted. This showed that after 4 h incubation with either strain, approximately 90–95% of HCT-8 cells were viable. However, after 5 h incubation, a significant number of cells were detached from the substratum. These data suggest that at the 4 h time point used, the majority of HCT-8 cells remained viable and, therefore, viability was not responsible for the differences observed between 98NK2 and EDL933.

3.2.3 Chemokine induction by a range of STEC isolates

3.2.3.1 Chemokine mRNA induction in response to a range of STEC isolates by HCT-8 cells

MIP-2 α and IL-8 responses were examined in HCT-8 cells infected with a range of LEE-positive and LEE-negative STEC strains with diverse characteristics, as listed in Table 3.1. After 1 or 4 h incubation with the various STEC strains, total cellular RNA was extracted from HCT-8 cells (Section 2.12.1) and real-time RT-PCR performed (Section 2.13.2). Results of these studies are expressed as the amount of IL-8 or MIP-2 α mRNA present at 1 or 4 h, compared to a 0 h control (Fig. 3.5). Significant differences ($P < 0.0001$) were seen between the chemokine responses elicited by the various strains. In particular, all of the LEE-negative STEC strains tested (98NK2, 97MW1, B2F1, 94CR and 95HE4) induced higher levels of both IL-8 and MIP-2 α mRNA than any of the LEE-positive strains (EDL933, 95NR1 and 95SF2) and EPEC 87A, with the exception of 95ZG1 (Fig. 3.5). All of the

Table 3.1 *E. coli* strains used in Section 3.2.3 and their relevant characteristics.

Strain	Serotype	Source ^a	Characteristic ^b			
			<i>eae</i>	<i>stx</i> ₁	<i>stx</i> ₂	<i>ehxA</i>
94CR	O48:H11	HUS	–	+	+	+
95HE4	O91	Diarrhoea	–	+	–	+
95NR1	O111:H [–]	HUS	+	+	+	+
95SF2	O157:H [–]	HUS	+	–	+	+
95ZG1	O26	Diarrhoea	+	+	–	+
97MW1	O113:H21	Bloody diarrhoea, MHA-T	–	–	+	+
98NK2	O113:H21	HUS	–	–	+	+
B2F1	O91:H21	HUS	–	–	+	+
EDL933	O157:H7	Bloody diarrhoea	+	+	+	+
EPEC 87A	O111	Diarrhoea	+	–	–	–

^a STEC strains were originally isolated from the faeces of patients with diarrhoea, bloody diarrhoea, HUS or microangiopathic haemolytic anaemia and thrombocytopenia (MHA-T).

^b Determined by multiplex PCR (Paton and Paton, 1998a); *eae* and *ehxA* are markers for the LEE pathogenicity island and the large STEC virulence plasmid, respectively.

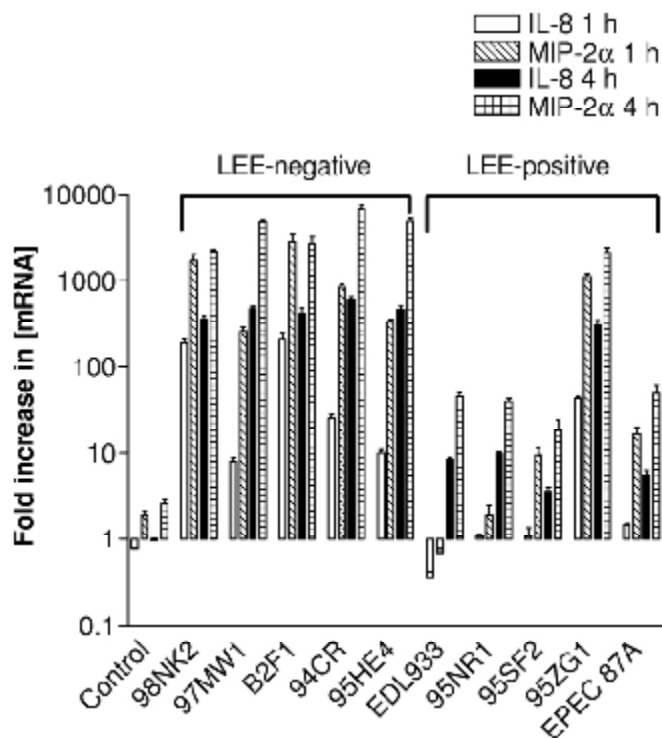


Figure 3.5 IL-8 and MIP-2a induction in HCT-8 cells infected with LEE-negative and LEE-positive STEC strains or with EPEC 87A. HCT-8 cells were infected with 3×10^7 CFU/ml of the indicated *E. coli* strain (Table 3.1). At 1 or 4 h, total RNA was extracted from cells (Section 2.12.1) and IL-8 and MIP-2 α mRNA was quantitated by real-time RT-PCR (Section 2.13.2). Results are expressed as the fold increase in [mRNA] relative to levels at 0 h, and data are shown as the means \pm SD for triplicate assays.

LEE-negative STEC strains tested induced significant upregulation of IL-8 and MIP-2 α mRNA at both 1 and 4 h after infection. STEC strain 95ZG1 was the only LEE-positive strain tested that was capable of eliciting significant IL-8 and MIP-2 α responses at 1 h. At 4 h after infection, significant IL-8 and MIP-2 α responses were seen for all the LEE-positive strains relative to uninfected control cells, but these were markedly lower than those observed for LEE-negative strains. Collectively, IL-8 and MIP-2 α responses at 1 h for cells infected with LEE-negative strains were on average 14- and 6-fold greater, respectively, than those elicited by infection with LEE-positive strains ($P < 0.05$ and $P < 0.01$, respectively). At 4 h, the responses were 6- and 8-fold greater, respectively ($P < 0.01$ in both cases).

3.2.3.2 STEC infection induces IL-8 secretion by HCT-8 cells

To confirm that upregulation of IL-8 mRNA resulted in increased secretion of the chemokine itself, levels of IL-8 in STEC-infected HCT-8 culture supernatants were measured by ELISA, as described in Section 2.20. At 1 h there was no detectable increase in IL-8 secretion compared to the uninfected control cultures for any of the strains tested (data not shown). However, at 4 h the levels of IL-8 protein in culture supernatants of cells infected with the LEE-negative STEC strains were significantly (approximately 100-fold) greater than that observed in uninfected HCT-8 culture supernatants, or those from cells infected with the LEE-positive STEC strains EDL933, 95NR1 and 95SF2 ($P < 0.001$) (Fig. 3.6). However, 95ZG1 induced similar levels of IL-8 secretion to the LEE-negative STEC strains, while EPEC 87A induced an intermediate response ($P < 0.05$) (Fig. 3.6). All of these findings are in accordance with that predicted from the mRNA induction studies above (Section 3.1.3.1).

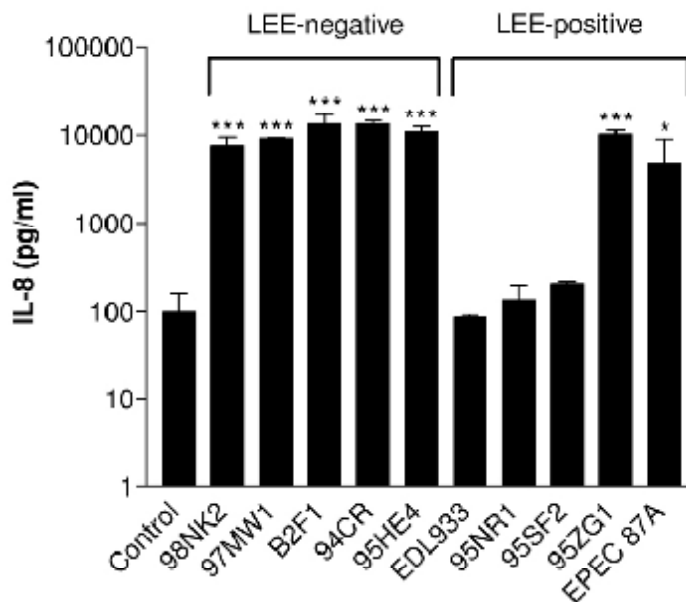


Figure 3.6 IL-8 protein induction in HCT-8 cells infected with LEE-negative and LEE-positive STEC strains or with EPEC 87A. HCT-8 cells were infected with 3×10^7 CFU/ml of the indicated *E. coli* strain (Table 3.1). At 4 h, culture supernatants were collected and assayed for IL-8 protein by ELISA (Section 2.20). Data are shown as the means \pm SEM from two experiments. The significance of differences between IL-8 secretion by infected versus uninfected cells is indicated as follows: ***, $P < 0.001$; *, $P < 0.05$.

3.2.3.3 Growth of STEC in RPMI 1640 medium

As discussed in Section 3.2.2.3, the initial dose of 98NK2 and EDL933 did not significantly affect the responses observed in either mRNA or IL-8 protein induction. However, it was also necessary to check that differences in the growth of STEC strains during the assay was not influencing the results obtained. Therefore, growth of several STEC strains in RPMI 1640 medium was analysed over 4 h (the time of the assay). The strains studied included the high chemokine-inducing STEC strains 98NK2, 97MW1 and B2F1, and the low chemokine-inducing STEC strain EDL933. After a 1, 2 or 4 h incubation, the STEC strains were diluted and plated on LB agar for CFU determination. As can be seen in Fig. 3.7, there were no significant differences in the growth of 98NK2, EDL933, B2F1, or 97MW1 in RPMI 1640 medium during the time course of the experiment ($P > 0.05$; Student's t test). These results show that the reduced ability of EDL933 to induce chemokine mRNA and IL-8 protein

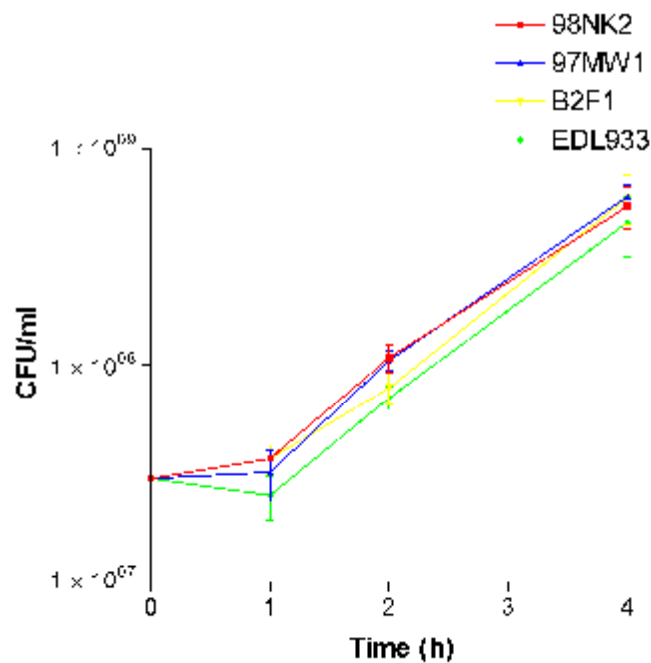


Figure 3.7 Growth of STEC strains in RPMI 1640 medium. STEC strains 98NK2, 97MW1, B2F1 and EDL933 were suspended in RPMI 1640 at an initial density of approx 3×10^7 CFU/ml, incubated for 1, 2 or 4 h, and then diluted and plated on LB agar for CFU determination. Data are expressed as mean CFU/ml \pm SD for triplicate assays.

in HCT-8 cells is not due to lower bacterial numbers at any time point, and that the high chemokine-inducing strains do not have higher growth rates during the assay.

3.2.3.4 Adherence of STEC strains to HCT-8 cells

To eliminate the possibility that the differences in chemokine production observed in Sections 3.2.3.1 and 3.2.3.2 were due to differences in the ability of the STEC strains to adhere to HCT-8 cells, *in vitro* adherence assays were performed as described in Section 2.18. HCT-8 cells were infected with 1×10^4 CFU of each strain and after 3 h incubation, the total number of adherent bacteria per well was determined by plating on LB agar. The total adherence of the strains ranged between 5.85 and 16.60×10^4 CFU/well for each of the strains tested (Table 3.2). No significant differences in the adherence of 98NK2, 97MW1, B2F1 or the low chemokine-inducing strain EDL933 was observed ($P > 0.05$; Student's *t* test). Levels of adherence were similar to that reported previously for 98NK2 (Paton *et al.*, 1999; Paton *et al.*, 2001a; Srimanote *et al.*, 2002). These results indicate that the differences observed in chemokine induction by LEE-negative and LEE-positive STEC strains are not due to differences in their ability to adhere to HCT-8 cells.

Table 3.2 Adherence of 98NK2, 97MW1, B2F1 and EDL933 to HCT-8 cells and the amount of IL-8 produced.

Strain	No. of bacteria adhered to HCT-8 cells (CFU/well $\times 10^4$) ^a
98NK2	8.43 (± 1.57)
97MW1	16.60 (± 3.71)
B2F1	6.33 (± 1.68)
EDL933	5.85 (± 0.88)

^a Quantitative adherence assays were carried out as described in Section 2.18. Data shown are the means (\pm SEM) CFU/well for quadruplicate assays of two separate experiments.

3.3 Discussion

Much of the intestinal pathology associated with STEC disease, as well as its life-threatening complications, results from microvascular angiopathy due to systemic absorption and dissemination of Stx. Unlike enteric pathogens such as *Salmonella*, *Shigella* and EIEC, STEC do not invade the gut mucosa to any significant extent, and their capacity to cause severe disease is heavily dependent upon translocation of Stx across the epithelial barrier. Recent evidence strongly implicates PMNs in both the penetration of Stx through the epithelium into underlying tissues (Hurley *et al.*, 2001) and in the transport of Stx to remote target tissues (te Loo *et al.*, 2000a; te Loo *et al.*, 2000b). Thus, recruitment of PMNs to the site of STEC colonisation may be a watershed event in the disease process. In this chapter, the capacity of various STEC strains to elicit CXC chemokine responses in a human colonic epithelial (HCT-8) cell line was examined, because these chemokines are potent PMN chemoattractants.

A real-time RT-PCR assay for the detection and quantitation of CXC chemokines from total cellular RNA was developed. The CXC chemokines have high homology at the DNA level, but the PCR primer oligonucleotides used were specific to the chemokines being assayed, since they are based on regions of non-homologous mRNA. The oligonucleotides are also suitable for use with SYBR green nucleic acid staining, which is a cost-effective way of examining the production of mRNA by real-time RT-PCR.

In general, infection of HCT-8 cells with two well-characterised STEC strains, the LEE-negative O113:H21 strain 98NK2 and the LEE-positive O157:H7 strain EDL933, induced different chemokine profiles. 98NK2 induced a much earlier and higher induction of a range of chemokine mRNAs, including IL-8, MIP-2 α and MIP-2 β at 1 and 4 h, and ENA-78 at 4 h. In contrast, EDL933 elicited no upregulation of any of the chemokine mRNAs at 1 h, and only a modest increase in IL-8, MIP-2 α and MIP-2 β mRNAs by 4 h post-infection. This result was confirmed by measuring IL-8 protein by ELISA, which showed a massive upregulation of IL-8 in 98NK2-infected HCT-8 cells as early as 2 h post-infection. This upregulation in response to 98NK2 was maintained throughout the 4 h assay. In comparison, EDL933 did not elicit any significant IL-8 induction over that of control cells,

even at 4 h post-infection. These results were also shown not to be attributable to differences in the initial doses of these strains, or to a loss of viability of the HCT-8 cells during the assay. These results, therefore, suggest that there may be interesting differences in the ability of distinct STEC strains to induce chemokine production in intestinal epithelial cells.

When a range of STEC isolates from clinical samples were tested for their capacity to induce chemokine production in HCT-8 cells, massive differences were observed between the strains. In particular, all of the LEE-negative STEC strains were able to induce a high and early production of IL-8 and MIP-2 α mRNA, and IL-8 protein. In comparison, the majority of the LEE-positive strains had responses similar to that observed previously with EDL933. One exception was the LEE-positive strain 95ZG1, which like the LEE-negative strains, was also capable of inducing a high and early chemokine mRNA, and IL-8 protein, response. Another LEE-positive isolate, EPEC strain 87A (which lacks Stx), was capable of eliciting an intermediate response, indicating that the factor(s) responsible for this phenomenon are not unique to STEC strains *per se*. These findings are consistent with a previous report that LEE-negative STEC strains were able to induce higher transmigration of PMNs across polarised T84 cells compared to LEE-positive strains (Hurley *et al.*, 2001). Additional experiments indicated that the various STEC strains did not grow at significantly different rates in the tissue culture medium during the assay, and that their ability to adhere to HCT-8 cells was not responsible for the differences observed.

Many STEC strains associated with serious disease in humans (e.g. those belonging to serogroups such as O157, O111 and O26) are LEE-positive. However, the presence of LEE is not essential for pathogenesis. Many LEE-negative strains are able to cause disease, and disease caused by certain LEE-negative STEC is often at the severe extreme of the clinical spectrum. Serotypes, including O113:H21, have been shown to cause HUS (Karmali *et al.*, 1985) including small outbreaks thereof (Paton and Paton, 1998b; Paton *et al.*, 1999). Differences in the ability of STEC to induce chemokine production may have implications for the ability of STEC to attract PMNs *in vivo*. This, in turn, may affect the translocation of Stx across intestinal epithelial cells and the transport and availability of Stx to target tissues expressing Gb₃. This may be particularly important in aiding certain LEE-negative STEC to

cause severe disease, in absence of LEE-mediated adherence. In Chapter 4, the STEC factor(s) responsible for induction of chemokine production by HCT-8 cells is examined.

CHAPTER 4: Determination of the STEC Factor(s) Responsible for Chemokine Induction in HCT-8 Cells

4.1 Introduction

Although STEC strains belonging to over 200 O:H serotypes all produce Stx, not all of these have been associated with human disease, indicating that other factors contribute to pathogenesis. Many STEC strains associated with serious disease in humans (e.g. those belonging to serogroups such as O157, O111 and O26) form A/E lesions on enterocytes, a property encoded on a chromosomal pathogenicity island termed the LEE (Section 1.5.1). Both LEE-positive and LEE-negative STEC strains also encode a variety of putative adherence and virulence factors, some of which are encoded on the megaplasmids carried by these strains (Section 1.5.2 and Section 1.6). Many of these putative adherence or virulence factors are unique to either LEE-positive or LEE-negative STEC, or to a particular serogroup/serotype of STEC, and may therefore play a role in the differences in chemokine expression by different STEC isolates observed in Section 3.2.3.1 and Section 3.2.3.2.

Intestinal epithelial cells play an important role in mediating inflammatory responses since they are the first cells to come into contact with intestinal pathogens. Bacterial components such as LPS (Bliss *et al.*, 1998; Philpott *et al.*, 2000) and flagella (Steiner *et al.*, 2000; Gewirtz *et al.*, 2001b) are important mediators of such responses in these pathogens. In STEC, Stx1 is capable of inducing IL-8, MGSA (Gro- α), MIP-2 α (Gro- β), MIP-2 β (Gro- γ) and ENA-78 mRNA, as well as IL-8 and MGSA protein expression in a dose-dependent manner, via a ribotoxic stress response pathway in HCT-8 cells (Thorpe *et al.*, 2001). The

production of these chemokines would be expected to lead to the recruitment of inflammatory cells, including PMNs, and lead to damage of the intestinal barrier and, therefore, increased Stx absorption. The apical application of STEC to polarised T84 cells can induce the migration of PMNs and the production of IL-8 (Hurley *et al.*, 2001). However, the effect is greater for LEE-negative than for LEE-positive STEC strains (Hurley *et al.*, 2001), and therefore most likely involves factors other than Stx production. In Sections 3.2.3.1 and 3.2.3.2, differences between LEE-negative and LEE-positive STEC strains in their ability to induce chemokine mRNA, and IL-8 protein, were observed. In this chapter, the relative contributions of Stx and other STEC factors, including the LEE pathogenicity island, the megaplasmid, LPS and flagella to this process were investigated.

4.2 Results

4.2.1 LEE-encoded STEC factors do not influence chemokine induction by HCT-8 cells or HEp-2 cells

One possible explanation for the difference in CXC chemokine induction between LEE-positive and LEE-negative STEC-infected HCT-8 cells might be that host cell perturbations (such as cytoskeletal rearrangements) caused by LEE-encoded products might interfere with cellular signalling pathways. To examine this, chemokine induction was investigated in cells infected with EDL933 and an otherwise isogenic derivative with an in-frame deletion mutation in the intimin (*eae*) gene (EDL933 Δ *eae*) that cannot form A/E lesions on enterocytes (Ogierman *et al.*, 2000). Derivatives of EDL933 carrying either normal or defective copies of *ler* on a multicopy plasmid were also tested. Over-expression of the defective *ler* gene (derived from STEC 95SF2) represses production of multiple LEE-encoded proteins and abrogates A/E lesion formation in EDL933. Over-expression of wild-type *Ler* (derived from EDL933) significantly upregulates production of LEE proteins and confers a hyper-adherent phenotype on EDL933 (Ogierman *et al.*, 2000).

There were no significant differences between the levels of IL-8 and MIP-2 α mRNA in HCT-8 cells infected with either EDL933 or any of the above derivatives at 1 or 4 h (Fig. 4.1A). 98NK2, which was used as a positive control for chemokine induction, elicited a 184- and 696-fold increase in IL-8 mRNA at 1 and 4 h, and an 818- and 570-fold increase in MIP-2 α mRNA at 1 and 4 h, respectively, compared to control cells (Fig. 4.1A). Similarly, there were no significant differences between levels of IL-8 protein in culture supernatants of cells infected for 4 h with EDL933 or any of the EDL933 derivatives, compared to control monolayers ($P > 0.05$; Fig. 4.1B). There were also no significant differences between IL-8 levels induced by EDL933 or any of the EDL933 derivatives ($P > 0.05$). However, IL-8 levels were approximately 100-fold higher in supernatants of HCT-8 cells infected with 98NK2 compared to cells infected with EDL933 or any of the EDL933 derivatives ($P < 0.001$; Fig. 4.1B).

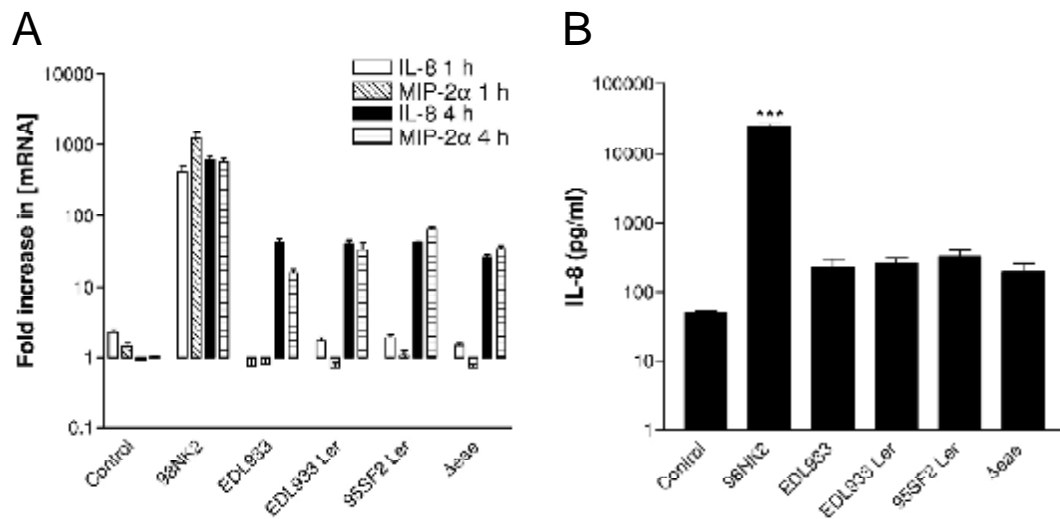


Figure 4.1 Stimulation of HCT-8 cells by EDL933 derivatives. Cells were infected with 3×10^7 CFU/ml of 98NK2, EDL933, EDL933 expressing wild-type or defective copies of the *ler* gene carried on a multicopy plasmid (designated EDL933 Ler and 95SF2 Ler, respectively), and EDL933 with a deletion mutation in *eae* (Δeae) (Ogierman *et al.*, 2000). **(A)** At 1 or 4 h, total RNA was extracted from cells (Section 2.12.1) and IL-8 and MIP-2 α mRNA was quantitated by real-time RT-PCR (Section 2.13.2). Results are expressed as the fold increase in [mRNA] relative to levels at 0 h, and data shown are the means \pm SD for triplicate assays. **(B)** At 4 h, supernatants were collected and assayed for IL-8 by ELISA (Section 2.20). Data shown are the means \pm SEM from two experiments. Significant differences relative to untreated control cells are indicated as follows: ***, $P < 0.001$.

Stimulation of IL-8 and MIP-2 α mRNA and IL-8 protein in HEp-2 cells was also examined, because A/E lesions are generally studied in this cell line (Knutton *et al.*, 1989). However, no induction of IL-8 or MIP-2 α mRNA was observed in HEp-2 cells infected with EDL933 or its derivatives, or with 98NK2 (data not shown). Similarly, no IL-8 protein was observed in culture supernatants infected with either EDL933 or its derivatives, or with the high IL-8-inducing STEC strain 98NK2 (data not shown). Thus, notwithstanding their susceptibility to A/E lesion formation, HEp-2 cells are unresponsive to STEC in terms of chemokine induction. Collectively, the above findings indicate that there is no apparent association (either positive or negative) between capacity of an STEC strain to form A/E lesions and chemokine induction.

4.2.2 Megaplasmid-encoded STEC factors do not influence chemokine production by HCT-8 cells

The majority of STEC strains carry megaplasms, which encode a variety of adherence factors and putative accessory virulence factors (Sections 1.5.2 and 1.6). Since there is marked heterogeneity in the genetic composition of LEE-positive and LEE-negative STEC megaplasms (Boerlin *et al.*, 1998; Gyles *et al.*, 1998; Brunder *et al.*, 1999), it was of interest to determine whether any of these factors were responsible for the differences observed in chemokine induction by LEE-positive and LEE-negative strains. Derivatives of both EDL933 and 98NK2 which had been cured of their respective megaplasms (EDL933-Cu and 98NK2-Cu, respectively) elicited IL-8 and MIP-2 α mRNA responses that were indistinguishable from that of their respective parent strains, indicating that genes encoded on these elements do not contribute to chemokine induction (Fig. 4.2A). Similarly, in the IL-8 protein assays, no significant differences were observed between cells infected with 98NK2 or 98NK2-Cu ($7,450 \pm 1,950$ and $7,250 \pm 850$ pg/ml IL-8, respectively; $P > 0.05$), or between EDL933 and EDL933-Cu (86 ± 6 and 101 ± 10 pg/ml IL-8, respectively; $P > 0.05$) (Fig. 4.2B). These results suggest that genes carried on either megaplasmid do not contribute directly to chemokine induction by 98NK2 or EDL933.

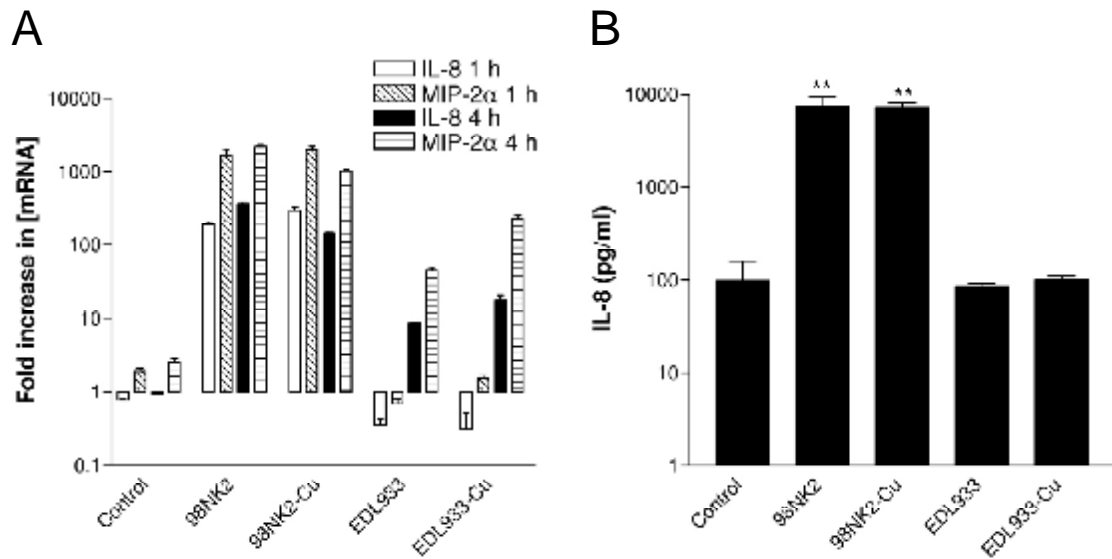


Figure 4.2 Induction of IL-8 and MIP-2a mRNA and IL-8 protein in HCT-8 cells infected with 98NK2 and EDL933 and their respective plasmid-cured derivatives. HCT-8 cells were stimulated with 3×10^7 CFU/ml 98NK2, 98NK2-Cu, EDL933 or EDL933-Cu. **(A)** At 1 or 4 h, total RNA was extracted from cells (Section 2.12.1) and IL-8 and MIP-2 α mRNA was quantitated by real-time RT-PCR (Section 2.13.2). Results are expressed as the fold increase in [mRNA] relative to levels at 0 h, and data shown are the means \pm SD for triplicate assays. **(B)** At 4 h, supernatants were collected and assayed for IL-8 by ELISA (Section 2.20). Data shown are the means \pm SEM from two experiments. Significant differences relative to untreated control cells are indicated as follows: **, $P < 0.01$.

4.2.3 Effect of Stx1 and Stx2 on chemokine production by HCT-8 cells

4.2.3.1 Stimulation of HCT-8 cells with purified Stx1 and Stx2

Thorpe *et al.* (2001) have previously demonstrated that purified Stx1 stimulates production of IL-8 and other CXC chemokines by HCT-8 cells. Stx2 was not tested in that study, and so it was conceivable that differences in chemokine induction elicited by LEE-positive and LEE-negative strains might be attributable to differences in the type or amount of Stx secreted during the 4 h assay. To examine this, chemokine induction was first compared in HCT-8 cells treated with 98NK2, EDL933, or purified Stx1 or Stx2 at doses ranging from 100 ng to 10 μ g per ml. Both toxins elicited IL-8 and MIP-2 α mRNA responses in a dose-dependent fashion, which at 4 h were comparable to that seen for cells infected with 98NK2

and markedly greater than that seen for cells infected with EDL933 (Fig. 4.3A). Dose-dependent stimulation of IL-8 secretion by Stx1 and Stx2 was also demonstrated by ELISA, and at the highest dose (10 μg per ml), the response elicited by Stx2 was significantly greater than that elicited by Stx1 ($P < 0.01$) (Fig. 4.3B). HCT-8 cells were stimulated with Stx1 or Stx2 ranging at 10 $\mu\text{g}/\text{ml}$, 1 $\mu\text{g}/\text{ml}$ and 100 ng/ml. At these doses, Stx1 elicited 615 ± 85 , 365 ± 85 and 175 ± 15 pg/ml IL-8, respectively, and Stx2 elicited $5,250 \pm 950$, $1,350 \pm 200$ and 146 ± 49 pg/ml IL-8, respectively.

The concentration of Stx produced by the various STEC strains used in this study during the 4 h assay was estimated by assaying the bacterial culture supernatants for Vero cell cytotoxicity, using the purified toxins as standards (Section 2.19). Total levels of Stx ranged from 29 to 150 ng/ml (Table 4.1). However, there was no correlation between total Stx

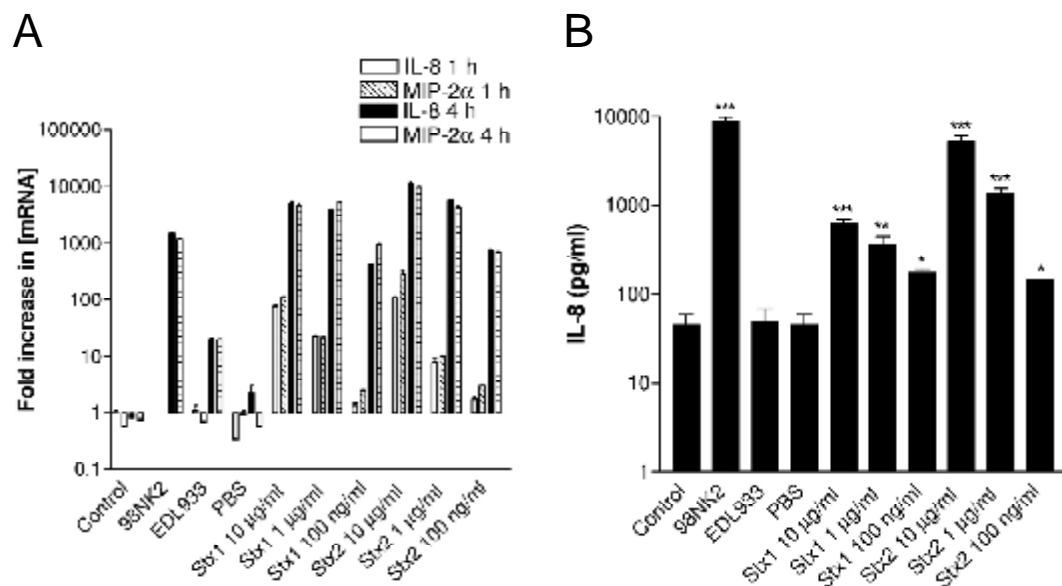


Figure 4.3 Induction of IL-8 and MIP-2 α mRNA and IL-8 protein in HCT-8 cells treated with purified Stx1 or Stx2. HCT-8 cells were treated with purified Stx1 or Stx2 at the indicated concentrations, or with 3×10^7 CFU/ml 98NK2 or EDL933. (A) At 1 or 4 h, total RNA was extracted from cells (Section 2.12.1) and IL-8 and MIP-2 α mRNA was quantitated by real-time RT-PCR (Section 2.13.2). Results are expressed as the fold increase in [mRNA] relative to levels at 0 h, and data shown are the means \pm SD for triplicate assays. (B) At 4 h, supernatants were collected and assayed for IL-8 by ELISA (Section 2.20). Data shown are the means \pm SEM from two experiments. Significant differences relative to untreated control cells are indicated as follows: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Table 4.1 *E. coli* strains used in Section 3.2.3 and the amount of Stx and IL-8 produced.

Strain/purified toxin	Stx1	Stx2	Amount of Stx produced by STEC (ng \pm SEM) ^a or amount of purified Stx ^c	IL-8 (pg/ml \pm SEM) ^b
94CR	+	+	150 (\pm 50) ^a	13,250 (\pm 1,250)
95HE4	+	-	59 (\pm 22) ^a	10,950 (\pm 1,550)
95NR1	+	+	38 (\pm 13) ^a	136 (\pm 62)
95SF2	-	+	29 (\pm 11) ^a	205 (\pm 10)
95ZG1	+	-	29 (\pm 11) ^a	10,150 (\pm 1,350)
97MW1	-	+	58 (\pm 22) ^a	9,100 (\pm 100)
98NK2	-	+	38 (\pm 13) ^a	7,450 (\pm 1,950)
B2F1	-	+	58 (\pm 22) ^a	13,250 (\pm 4,250)
EDL933	+	+	75 (\pm 25) ^a	86 (\pm 6)
EPEC 87A	-	-	0 (\pm 0) ^a	4,740 (\pm 4,260)
10 μ g/ml Stx1	+	-	10,000 ^c	615 (\pm 85)
1 μ g/ml Stx1	+	-	1,000 ^c	365 (\pm 85)
100 ng/ml Stx1	+	-	100 ^c	175 (\pm 15)
10 μ g/ml Stx2	-	+	10,000 ^c	5,250 (\pm 950)
1 μ g/ml Stx2	-	+	1,000 ^c	1,350 (\pm 200)
100 ng/ml Stx2	-	+	100 ^c	146 (\pm 49)

^a Determined by Vero cell cytotoxicity assay (Section 2.19) using purified Stx1 and Stx2 as standards^c. Data shown are the means (\pm SEM).

^b Determined by IL-8 ELISA (Section 2.20) from 4 h supernatants of HCT-8 monolayers infected with *E. coli* or treated with purified Stx1 or Stx2. Data shown are the means (\pm SEM).

^c Amount of purified toxin added to HCT-8 monolayers for standardisation in cytotoxicity assays^a or for determination of IL-8 by ELISA^b.

activity in culture supernatants for a given STEC strain and the level of chemokine mRNA or IL-8 protein induced. Moreover, the total amount of Stx in culture supernatants of LEE-negative STEC strains appeared insufficient to account for the level of chemokine induction observed. For example, the 4 h 98NK2 culture supernatant contained approximately 38 ng/ml of Stx2, but infection of HCT-8 cells with 98NK2 elicited 100 times more IL-8 secretion than treatment with 100 ng/ml of Stx2 (Fig. 4.3B).

In another experiment, infection of HCT-8 cells with *E. coli* K-12 clones expressing either Stx1 or Stx2 did not elicit levels of either IL-8 or MIP-2 α mRNA, or IL-8 protein, that were significantly different to those elicited by treatment with the host *E. coli* K-12 strain ($P > 0.05$) (Fig. 4.4). Furthermore, infection of HCT-8 cells with the non-toxigenic LEE

positive EPEC strain 87A induced upregulation of IL-8 and MIP-2 α mRNA similar to that seen with the LEE-positive STEC strains (Section 3.2.3.1 and Fig. 3.5), indicating that production of Stx is not essential for these responses. Thus, STEC factors other than Stx appear to be responsible for a major portion of the chemokine induction observed in infected HCT-8 cultures.

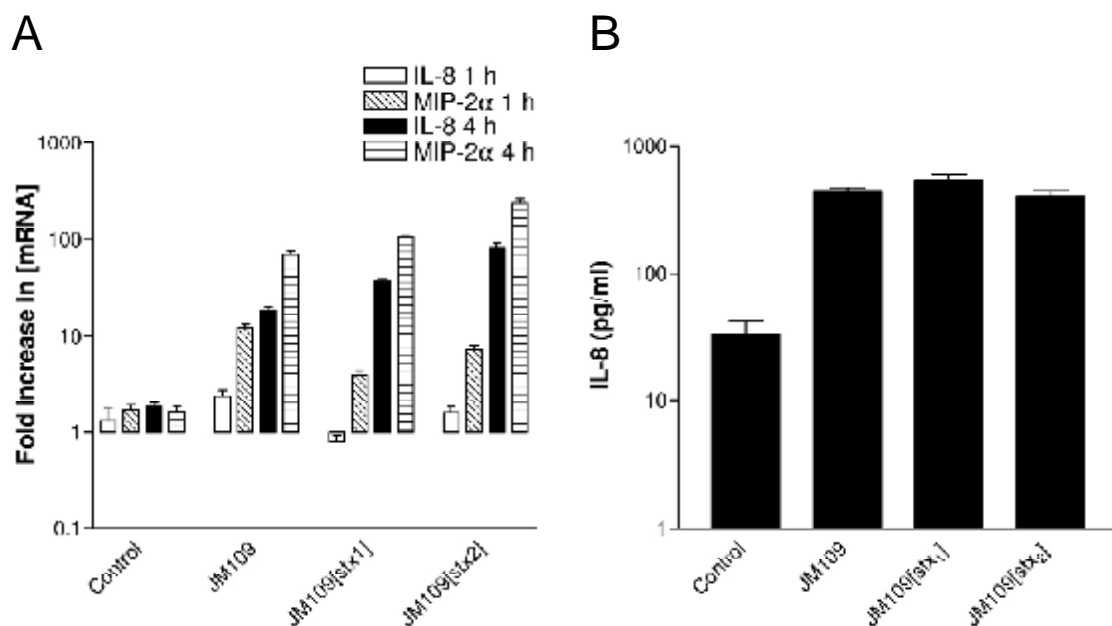


Figure 4.4 Induction of IL-8 and MIP-2a mRNA and IL-8 protein in HCT-8 cells treated with Stx1- or Stx2-producing *E. coli* clones. HCT-8 cells were treated with 3×10^7 CFU/ml *E. coli* JM109 or JM109 expressing either Stx1 or Stx2 (Table 2.1). (A) At 1 or 4 h, total RNA was extracted from cells (Section 2.12.1) and IL-8 and MIP-2 α mRNA was quantitated by real-time RT-PCR (Section 2.13.2). Results are expressed as the fold increase in [mRNA] relative to levels at 0 h, and data shown are the means \pm SD for triplicate assays. (B) At 4 h, supernatants were collected and assayed for IL-8 by ELISA (Section 2.20). Data shown are the means \pm SEM from two experiments.

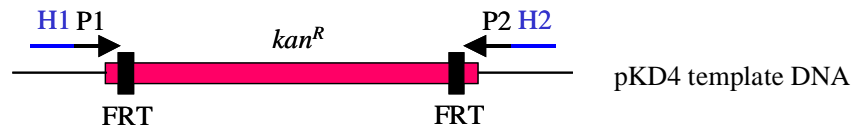
4.2.3.2 Effect of deletion of the *stx*₂ gene from 98NK2 on chemokine production by HCT-8 cells

4.2.3.2.1 Construction of 98NK2 *stx*₂ deletion mutant

To definitively determine the contribution of Stx2 (if any) to chemokine induction in 98NK2-infected HCT-8 cells, the lambda red recombinase system (Section 2.8) was used to construct a deletion mutation in the single *stx*₂ gene of 98NK2 (Fig. 4.5). 98NK2 was first electroporated with the temperature-sensitive helper plasmid pKD46 (Table 2.2), and transformants were selected on LB agar supplemented with Amp at 30°C. Presence of the plasmid in an Amp^R transformant was confirmed by PCR with the pKD46 specific primers, pKD46 Fwd and pKD46 Rev (Table 2.4), and was designated 98NK2[pKD46]. Primers TR27 and TR28, that contain regions homologous to nucleotides 89–129 and 835–875 of the 98NK2 *stx*₂ gene, respectively, followed by priming sequences specific for plasmid pKD4 (Table 2.4), were used to amplify the *kan* resistance gene of pKD4 (Table 2.2). The PCR product was purified (Section 2.9.3), electroporated into 98NK2[pKD46], and then plated on LB agar supplemented with Kan and incubated overnight at 37°C (to eliminate the temperature-sensitive Amp^R helper plasmid pKD46), as described in Section 2.8 and Fig. 4.5.

Kan^R, Amp^S transformants were screened for the deletion of *stx*₂ by PCR using the primers *stx*2F and *stx*2R, and with TR34 and k1 (Table 2.2). Amplification with *stx*2F and *stx*2R yielded no PCR product in the *stx*₂ deletion mutants compared to a 255-bp product in STEC 98NK2. On the other hand, PCR amplification with TR34 and k1 yielded a PCR product of 675-bp in the *stx*₂ deletion mutant, and no product for STEC 98NK2 (Fig. 4.6). A PCR positive transformant was selected, and sequencing confirmed the deletion of the major portion of the *stx*₂ gene (nucleotides 130–834, encoding amino acids 44–278 of the Stx2 A subunit), and the insertion of the kanamycin resistance gene (1,476-nucleotides). This transformant was designated 98NK2Δ*stx*₂. A Vero cell cytotoxicity assay (Section 2.19) was performed, and no Stx2 toxicity was observed after 3 days of incubation (data not shown).

Step 1: PCR amplify the FRT-flanked antibiotic resistance gene.



Step 2: Transform strain expressing λ Red recombinase.



Step 3: Select antibiotic-resistant transformants.



Figure 4.5 Lambda red recombinase mutagenesis of 98NK2 *stx*₂. **Step 1:** The plasmid pKD4 (Amp^R) carries an antibiotic resistance cassette, *kan*^R, which contains FRT (FLP recombinase recognition target) sites. Plasmid DNA was amplified by PCR using primers that contain 40 nucleotides homologous to the gene of interest (*stx*₂; H1 and H2) followed by the priming sequences specific for the template plasmid pKD4 (P1 and P2); primers TR27 and TR28. **Step 2:** The PCR product was electroporated into the host STEC strain, 98NK2, which has previously been transformed with the lambda red recombinase expression vector pKD46. Recombination occurs between the homologous sequences on the ends of the PCR product (H1 and H2) and the chromosome, and the antibiotic resistance cassette (*kan*) replaces the target gene (*stx*₂). **Step 3:** Transformants were selected on LB plates supplemented with 50 μ g/ml Kan, and for loss of the helper plasmid by growth at 37°C and sensitivity to Amp. Mutants were screened by PCR and sequencing for loss of the major portion of *stx*₂ and insertion of *kan*. This diagram is not to scale. Modified from Datsenko and Wanner (2000); see also Section 2.8.

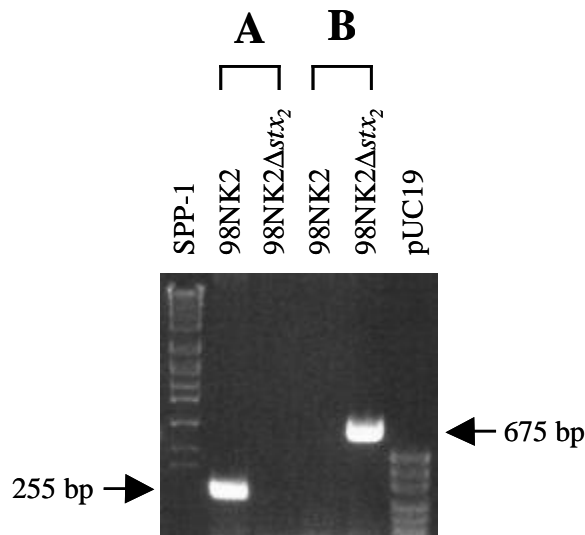


Figure 4.6 PCR analysis of 98NK2 *stx*₂ deletion mutant. PCR amplification of wild-type 98NK2 and 98NK2Δ*stx*₂ using (A) *stx*₂ specific primers (*stx*2F and *stx*2R) or (B) *stx*₂ and *kan* specific primers (TR34 and k1) (Table 2.4). The arrows indicate the 255-bp PCR product obtained using wild-type 98NK2 as template DNA and the primers *stx*2F and *stx*2R, and the 675-bp product obtained using 98NK2Δ*stx*₂ as template DNA and the primers TR34 and k1. The fragment sizes of the SPP-1 DNA markers are 8.51, 7.35, 6.11, 4.84, 3.59, 2.81, 1.95, 1.86, 1.51, 1.39, 1.16, 0.98, 0.72, 0.48 and 0.36 kb, and for pUC19 are 501, 404, 331, 242, 190, 147, 111, 110, 67, 34 and 26 bp.

4.2.3.2.2 Stimulation of HCT-8 cells with 98NK2Δ*stx*₂

The capacity of wild-type 98NK2 and 98NK2Δ*stx*₂ to elicit CXC chemokine responses in HCT-8 cells was then examined. Both IL-8 and MIP-2α mRNA responses in HCT-8 cells infected with strain 98NK2 or with 98NK2Δ*stx*₂ were similar (Fig. 4.7A). Likewise, IL-8 levels in 98NK2Δ*stx*₂-infected HCT-8 culture supernatants at 4 h were indistinguishable from that for cells infected with wild-type 98NK2 ($P > 0.05$) (Fig. 4.7B). Thus, although purified Stx2 is able to induce chemokine production, STEC factors other than Stx appear to be responsible for a major portion of the chemokine induction observed in infected HCT-8 cultures.

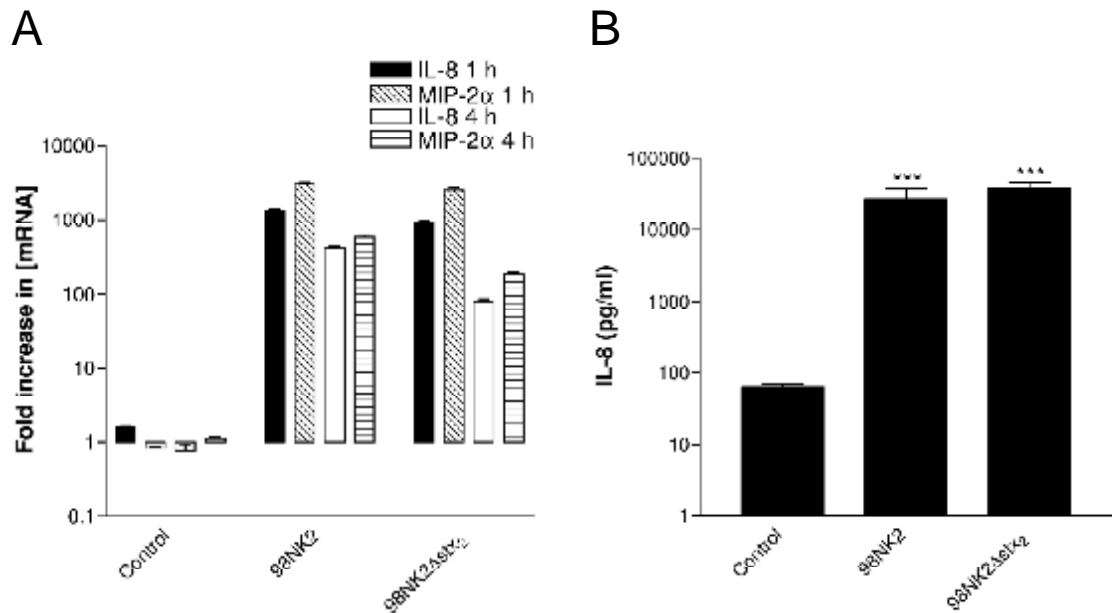


Figure 4.7 Induction of IL-8 and MIP-2 α mRNA and IL-8 protein in HCT-8 cells infected with 98NK2 or 98NK2 Δ stx₂. HCT-8 cells were stimulated with 3×10^7 CFU/ml 98NK2 or 98NK2 Δ stx₂. (A) At 1 or 4 h, total RNA was extracted from HCT-8 cells (Section 2.12.1) and IL-8 and MIP-2 α mRNA was quantitated by real-time RT-PCR (Section 2.13.2). Results are expressed as the fold increase in [mRNA] relative to levels at 0 h, and data shown are the means \pm SD for triplicate assays. (B) At 4 h, supernatants were collected and assayed for IL-8 by ELISA (Section 2.20). Data shown are the means \pm SEM from two experiments. Significant differences relative to untreated control cells are indicated as follows: ***, $P < 0.001$.

4.2.4 LPS does not influence chemokine production by HCT-8 cells

LPS is a well-known pro-inflammatory mediator of cytokine responses to a range of pathogens including *S. flexneri*, *Helicobacter pylori* and UPEC (Bliss *et al.*, 1998; Philpott *et al.*, 2000; Backhed *et al.*, 2001). To examine whether the differences observed in chemokine responses of HCT-8 cells to different STEC strains were due to the type of LPS (O serotype) expressed, crude LPS was extracted from 98NK2 (serogroup O113) and EDL933 (serogroup O157) as described in Section 2.23. LPS preparations were checked for purity by SDS-PAGE analysis (Section 2.14.2) and silver staining (Section 2.14.3) (data not shown).

HCT-8 monolayers were treated with O113 or O157 LPS at doses ranging from 10 ng/ml to 10 μ g/ml. As can be seen in Fig. 4.8A, stimulation of HCT-8 cells with O113 or

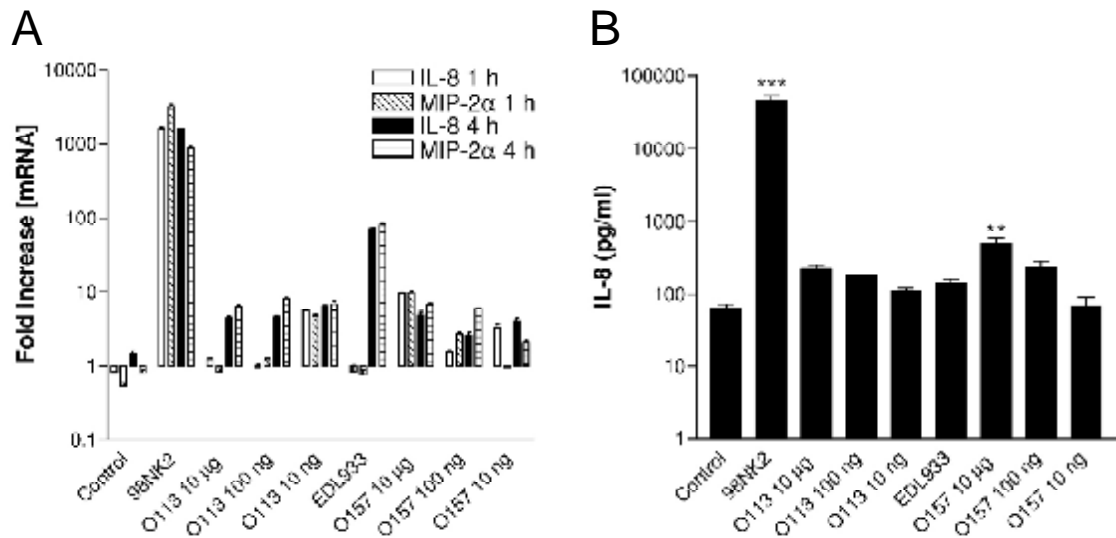


Figure 4.8 Induction of IL-8 and MIP-2 α mRNA and IL-8 protein in HCT-8 cells treated with purified O113 or O157 LPS. HCT-8 cells were treated with purified O113 or O157 LPS at the indicated concentrations, or with 3×10^7 CFU/ml 98NK2 (O113:H21) or EDL933 (O157:H7). **(A)** At 1 or 4 h, total RNA was extracted from cells (Section 2.12.1) and IL-8 and MIP-2 α mRNA was quantitated by real-time RT-PCR (Section 2.13.2). Results are expressed as the fold increase in [mRNA] relative to levels at 0 h, and data shown are the means \pm SD for triplicate assays. **(B)** At 4 h, supernatants were collected and assayed for IL-8 by ELISA (Section 2.20). Data shown are the means \pm SEM from two experiments. Significant differences relative to untreated control cells are indicated as follows: **, $P < 0.01$; ***, $P < 0.001$.

O157 LPS resulted in only very modest increases in both IL-8 and MIP-2 α mRNA compared to untreated cells. At both 1 and 4 h, 98NK2 produced a 2,037-fold and 1,063-fold increase in IL-8 and 5,442- and 1,109-fold increase in MIP-2 α mRNA, which is much greater than that observed with the O113 LPS-treated monolayers. EDL933 also resulted in a higher stimulation of IL-8 and MIP-2 α mRNA at 4 h compared to the control and O157 LPS-treated HCT-8 cells. Similarly, with IL-8 protein (Fig. 4.8B), 98NK2 elicited $46,000 \pm 7,000$ pg/ml IL-8 ($P < 0.001$ compared to untreated control cells), whereas stimulation with O113 LPS elicited only 225 ± 25 , 178 ± 3 and 113 ± 8 pg/ml IL-8 at 10 μ g/ml, 100 ng/ml and 10 ng/ml O113 LPS, respectively ($P > 0.05$ compared with untreated control). EDL933 elicited 143 ± 18 pg/ml IL-8 ($P > 0.05$ compared with untreated control) whereas purified O157 LPS elicited 495 ± 95 , 230 ± 50 and 68 ± 20 pg/ml IL-8 at 10 μ g/ml, 100 ng/ml and 10 ng/ml, respectively. Only at the very high dose of 10 μ g/ml O157 LPS was the level of IL-8

significantly higher than that for untreated HCT-8 cells ($P < 0.01$), but this was not significantly different to EDL933-treated cells ($P > 0.05$). Despite 98NK2 producing a higher chemokine induction than EDL933, stimulation with O113 LPS did not elicit IL-8 levels significantly higher than that in control cells, even at the maximum dose ($P > 0.05$).

In another experiment, *E. coli* K-12 clones expressing either O111 (Bastin *et al.*, 1991) or O113 LPS (Paton and Paton, 1999) (derived from strains eliciting low and high chemokine responses, respectively) elicited similar responses to those of cells infected with the *E. coli* K-12 host strain ($P > 0.05$) (Fig. 4.9). These data suggest that LPS is only able to induce significant chemokine production at very high doses. Therefore, this is not the factor responsible for the high responses observed in 98NK2 and other LEE-negative STEC-infected HCT-8 monolayers.

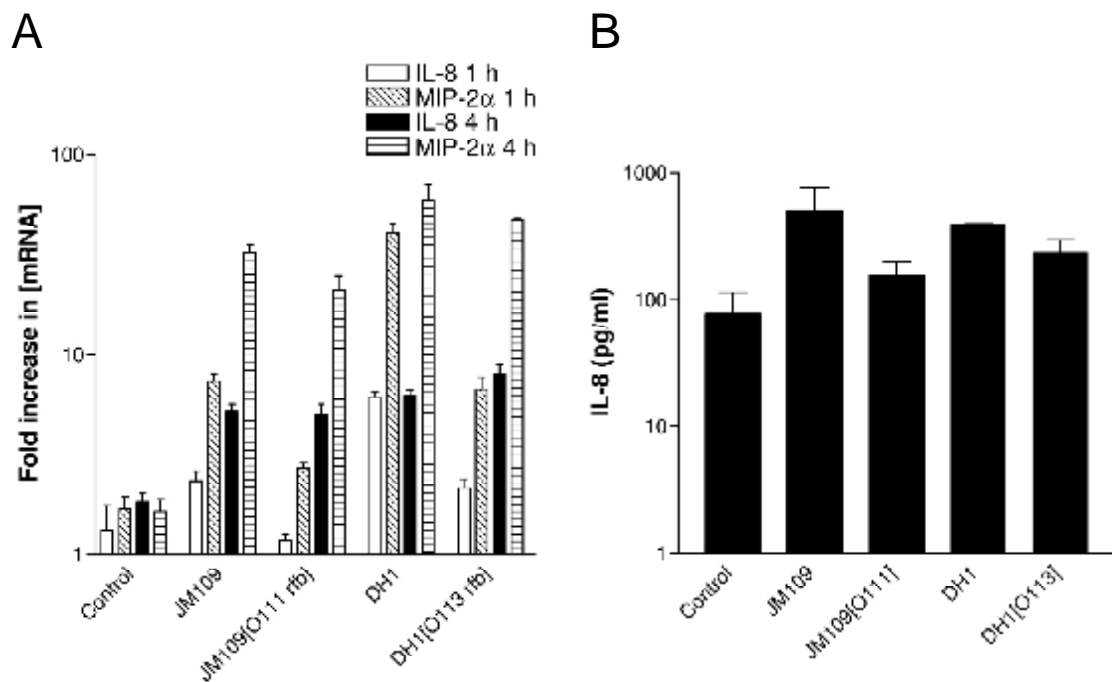


Figure 4.9 Induction of IL-8 and MIP-2α mRNA and IL-8 protein in HCT-8 cells treated with O111 *rfb* and O113 *rfb* clones. HCT-8 cells were stimulated with either 3×10^7 CFU/ml of the host strain JM109 or JM109[O111 *rfb*], or with the host strain DH1 or DH1[O113 *rfb*] (Table 2.1). (A) At 1 or 4 h, total RNA was extracted from cells (Section 2.12.1) and IL-8 and MIP-2α mRNA was quantitated by real-time RT-PCR (Section 2.13.2). Results are expressed as the fold increase in [mRNA] relative to levels at 0 h, and data shown are the means \pm SD for triplicate assays. (B) At 4 h, supernatants were collected and assayed for IL-8 by ELISA (Section 2.20). Data shown are the means \pm SEM from two experiments.

4.2.5 H21 flagella induces chemokine production by HCT-8 cells

4.2.5.1 Stimulation of HCT-8 cells with purified H21 flagella

Interestingly, it has previously been observed (Paton and Paton, 1998b) that many LEE-negative STEC strains associated with serious human disease (including three of the STEC strains tested in Section 3.2.3) belong to flagellar type H21. Purified flagellin (the major structural component of flagella) from several enteric pathogens has been shown to elicit inflammatory responses in epithelial cells, including EAaggEC, and *S. typhimurium* (Steiner *et al.*, 2000; Gewirtz *et al.*, 2001a; Gewirtz *et al.*, 2001b; Donnelly and Steiner, 2002). This raised the possibility that flagella was a major contributor to chemokine induction in STEC-infected cells, and that serotype H21 flagella may be particularly potent.

To examine this, H21 flagella were extracted from 98NK2 as described in Section 2.24. The major component of this preparation had a molecular size of 51-kDa when examined by SDS-PAGE, consistent with that expected for H21 flagellin (Fig. 4.10). The

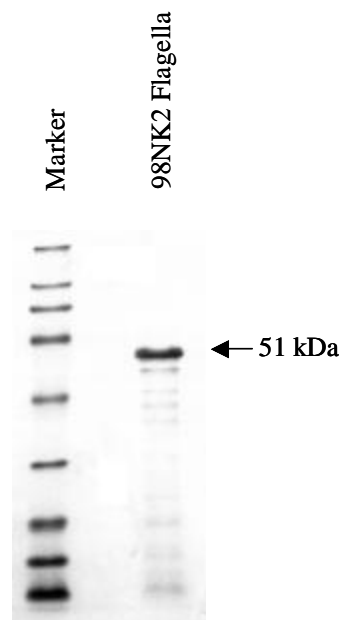


Figure 4.10 SDS-PAGE of flagella preparation from 98NK2. Flagella were extracted as described in Section 2.24 and electrophoresed on a 12% SDS-PAGE gel (Section 2.14.2). The ~ 51-kDa putative H21 FliC protein band is indicated by an arrow. The markers are BioRad broad range markers (sizes 200, 112.5, 97.4, 66.2, 45, 31, 21.5, 14.4 and 6.5 kDa).

capacity of the preparation to elicit chemokine responses in HCT-8 cells was then investigated using total flagella protein concentrations ranging from 0.01 ng to 1 μ g per ml. Western blot analysis using anti-H21 serum, and using the purified flagella as a standard, indicated that the total amount of H21 flagella present in 98NK2-infected HCT-8 cultures was approximately 8 ng and 60 ng at 1 and 4 h, respectively (data not shown). As shown in Fig. 4.11A, HCT-8 cells stimulated with 1 μ g/ml, 100 ng/ml and 10 ng/ml of H21 flagella produce IL-8 and MIP-2 α mRNA levels at 1 h and 4 h comparable to those elicited by infection with 98NK2 ($P < 0.001$ compared to untreated control cells). Even at H21 flagella concentrations as low as 1 ng/ml, the levels of IL-8 and MIP-2 α mRNA were similar to that observed in cell cultures infected with STEC strain EDL933 and greater than that observed for untreated control cells ($P < 0.05$) (Fig. 4.11A). However, significant chemokine induction was not observed at 0.1 and 0.01 ng H21 flagella per ml ($P > 0.05$).

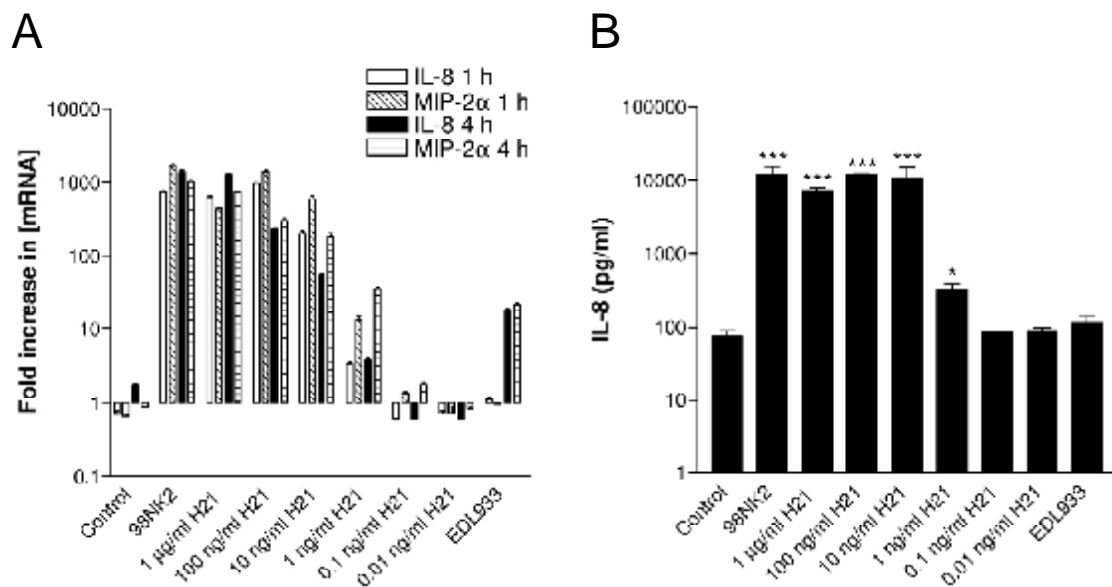


Figure 4.11 Induction of IL-8 and MIP-2a mRNA and IL-8 protein in HCT-8 cells treated with H21 flagella. HCT-8 cells were stimulated with H21 flagella at the indicated concentrations or with 3×10^7 CFU/ml 98NK2 or EDL933. (A) At 1 or 4 h, total RNA was extracted from cells (Section 2.12.1) and IL-8 and MIP-2 α mRNA was quantitated by real-time RT-PCR (Section 2.13.2). Results are expressed as the fold increase in [mRNA] relative to levels at 0 h, and data shown are the means \pm SD for triplicate assays. (B) At 4 h, supernatants were collected and assayed for IL-8 by ELISA (Section 2.20). Data shown are the means \pm SEM from two experiments. Significant differences relative to untreated control cells are indicated as follows: ***, $P < 0.001$; *, $P < 0.05$.

Similar results were obtained when IL-8 levels in culture supernatants were measured by ELISA (Fig. 4.11B). Stimulation of cells with H21 flagella at 1 $\mu\text{g/ml}$, 100 ng/ml and 10 ng/ml yielded $7,300 \pm 600$, $12,600 \pm 100$ and $11,100 \pm 4,400$ pg/ml IL-8, respectively, and these levels were significantly different from that in unstimulated control cell supernatants (< 100 pg/ml ; $P < 0.001$). At these H21 doses, the levels of IL-8 were similar to that elicited by infection with 98NK2 ($12,500 \pm 3,500$ pg/ml IL-8). Even at concentrations as low as 1 ng/ml , H21 flagella was capable of inducing 320 ± 70 pg/ml IL-8, a level comparable to that elicited by strain EDL933. These results implicate H21 flagella as the major contributing factor to the large chemokine mRNA and IL-8 protein responses elicited in 98NK2-infected HCT-8 monolayers.

4.2.5.2 Effect of deletion of *fliC* (and *stx*₂) from 98NK2 on chemokine production by HCT-8 cells

4.2.5.2.1 Construction of 98NK2 *fliC* deletion mutant

To determine the relative contribution of H21 flagellin to chemokine induction in 98NK2-infected HCT-8 cells, a 98NK2 derivative with a deletion mutation in the *fliC* gene (which encodes flagellin) was constructed using the lambda red recombinase system (Section 2.8). The primers TR30 and TR31 (Table 2.3), that have regions with homology to nucleotides 23–63 and 1,323–1,282 of the incomplete ORF of H21 *fliC* (GenBank accession number AF517663) and the priming sites for the *kan/cat* resistance genes of pKD4/pKD3, respectively, were used to amplify the *kan* resistance gene of pKD4 using high-fidelity PCR (Section 2.9.2). The PCR product was purified (Section 2.9.3), and electroporated into 98NK2[pKD46] as described in Section 2.8.

Kan^R and Amp^S transformants were screened by PCR using the primers F-FliC1 and R-FliC2, and F-FliC1 and k1 (Table 2.4). Amplification with F-FliC1 and R-FliC2, which amplifies the complete ORF of the *fliC* gene, yielded a 1,754-bp PCR product in the *fliC* deletion mutant compared to a 1,479-bp product in STEC 98NK2; whereas PCR amplification

with F-FliC1 and k1 yielded a PCR product of 624 bp in the *fliC* deletion mutant, and no product for STEC 98NK2 (Fig. 4.12). These results are consistent with replacement of the major portion of *fliC* with the *kan* cartridge. Sequencing then confirmed the deletion of nucleotides 124–1,341 of the *fliC* gene (representing amino acids 42–446), and insertion of the 1,476-bp kanamycin resistance gene. The mutant, designated 98NK2 Δ *fliC*, was also shown not to express flagellin by Western blotting using H21-antiserum (Fig. 4.13) and was non-motile on semi-soft agar (Fig. 4.14).

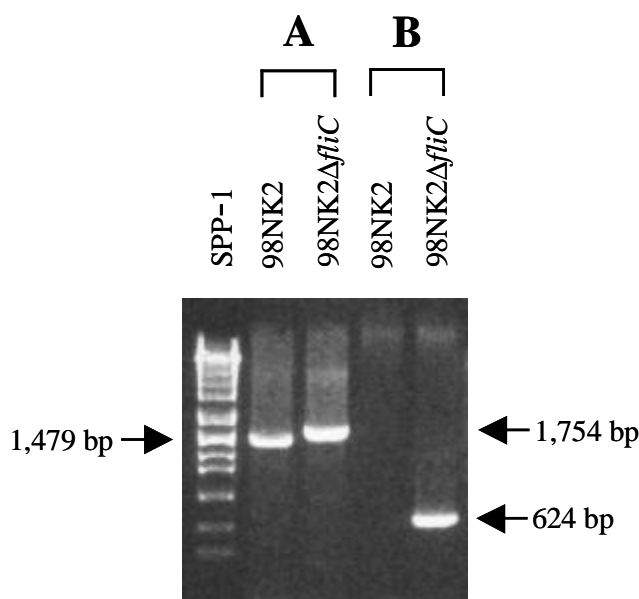


Figure 4.12 PCR analysis of 98NK2 *fliC* deletion mutant. PCR amplification of wild-type 98NK2 and 98NK2 Δ *fliC* using (A) *fliC* specific primers (F-FliC1 and R-FliC2) or (B) *fliC* and *kan* specific primers (F-FliC1 and k1) (Table 2.4). The arrows indicate the predicted mobilities of the 1,479-bp PCR product obtained using 98NK2 as template DNA or the 1,754-bp product using 98NK2 Δ *fliC* as template DNA and the primers F-FliC1 and R-FliC2, and the 624-bp product obtained using 98NK2 Δ *fliC* as template DNA and the primers F-FliC1 and k1. The fragment sizes of SPP-1 DNA markers are 8.51, 7.35, 6.11, 4.84, 3.59, 2.81, 1.95, 1.86, 1.51, 1.39, 1.16, 0.98, 0.72, 0.48 and 0.36 kb.

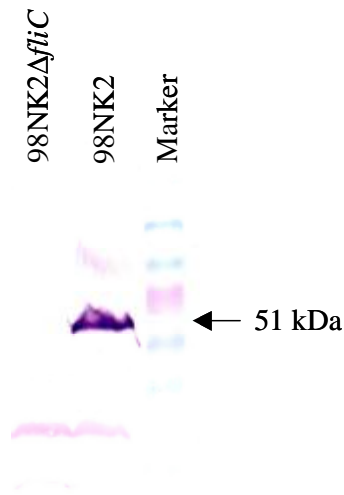


Figure 4.13 Western blot of whole cell lysates from 98NK2 and 98NK2Δ*fliC*. Whole cell lysates were prepared from overnight cultures of STEC (Section 2.14.1) then electrophoresed on a 12% SDS-PAGE gel (Section 2.14.2). SDS-PAGE gels were then subjected to Western blotting (Section 2.14.4) with H21 antiserum. The arrow indicates the 51-kDa band obtained with wild-type 98NK2, however no flagellin is detectable with 98NK2Δ*fliC*. The markers are BenchMark pre-stained protein ladder (sizes 172.0, 110.2, 79.0, 62.4 [pink], 48.0, 36.6, 24.5, 19.0, 13.5 and 5.3 kDa).

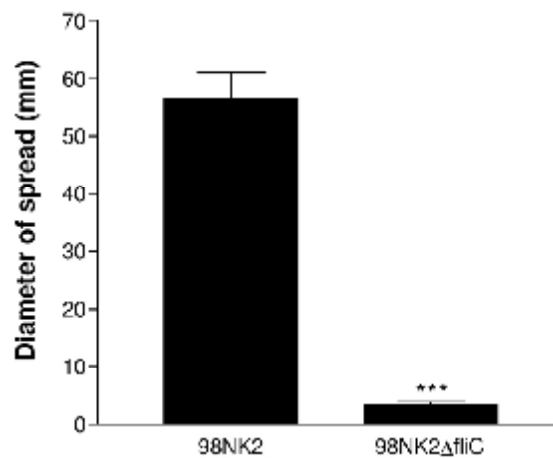


Figure 4.14 Motility of 98NK2 and 98NK2Δ*fliC* on semi-soft agar. STEC were stab-inoculated onto 0.3% (w/v) LB agar and incubated at 37°C for 18 h, after which the diameter of spread (mm) was measured. Data shown are the means \pm SD from duplicate samples. Significant differences relative to 98NK2 are indicated as follows: ***, $P < 0.0001$.

4.2.5.2.2 Construction of 98NK2 *stx*₂ and *fliC* double deletion mutant

To further examine the relative contribution of Stx₂ and FliC to chemokine induction, a double mutant derivative of 98NK2 lacking both *stx*₂ and *fliC* was constructed from the 98NK2Δ*stx*₂ deletion mutant described in Section 4.2.3.2.1, using the lambda red recombinase system (Section 2.8). The 98NK2Δ*stx*₂ strain (Kan^R) was electroporated with the temperature-sensitive helper plasmid pKD46 (Table 2.2), and transformants were selected on LB agar supplemented with Amp at 30°C. Presence of the plasmid in an Amp^R, Kan^R transformant was confirmed by PCR with the pKD46 specific primers pKD46 Fwd and pKD46 Rev (Table 2.4), and this was designated 98NK2Δ*stx*₂[pKD46]. The chloramphenicol (*cat*) resistance gene was amplified by PCR as described in Section 2.8, using pKD3 (Table 2.2) as the template DNA and the primers TR30 and TR31 (Table 2.4). The PCR product was purified (Section 2.9.3) and electroporated into 98NK2Δ*stx*₂[pKD46] as described in Section 2.8.

Resultant Kan^R, Cml^R and Amp^S transformants were screened with the primers *stx*2F and *stx*2R and TR34 and *k1* as described in Section 4.2.3.2.1, and with F-FliC1 and R-FliC2 primers, as described in Section 4.2.5.2.1 (Table 2.4). Amplification with *stx*2F and *stx*2R, and TR34 and *k1*, indicated that the *stx*₂ mutation was present in the double mutant (Fig. 4.15). Amplification with F-FliC1 and R-FliC2, which amplifies the complete ORF of the *fliC* gene, yielded a 1,291-bp PCR product in the *fliC* deletion mutants compared to a 1,479-bp product in STEC 98NK2; whereas PCR amplification with F-FliC1 and *c1* yielded a PCR product of 341-bp in the *fliC* deletion mutant and no product for STEC 98NK2 (Fig. 4.15). A PCR positive transformant was then sequenced to confirm deletion of the major portion of the *fliC* gene (nucleotides 124–1,341, encoding amino acids 42–446), and insertion of the chloramphenicol resistance gene (1,013 bp). One positive mutant was selected for further studies and designated 98NK2Δ*stx*₂/Δ*fliC*. 98NK2Δ*stx*₂/Δ*fliC* was shown in a Vero cell cytotoxicity assay (Section 2.19) not to have any Stx₂ toxicity after three days of incubation (result not shown), and Western blotting with H21 antiserum demonstrated no H21 flagellin expression (Fig. 4.16) and was non-motile on semi-soft agar (Fig. 4.17).

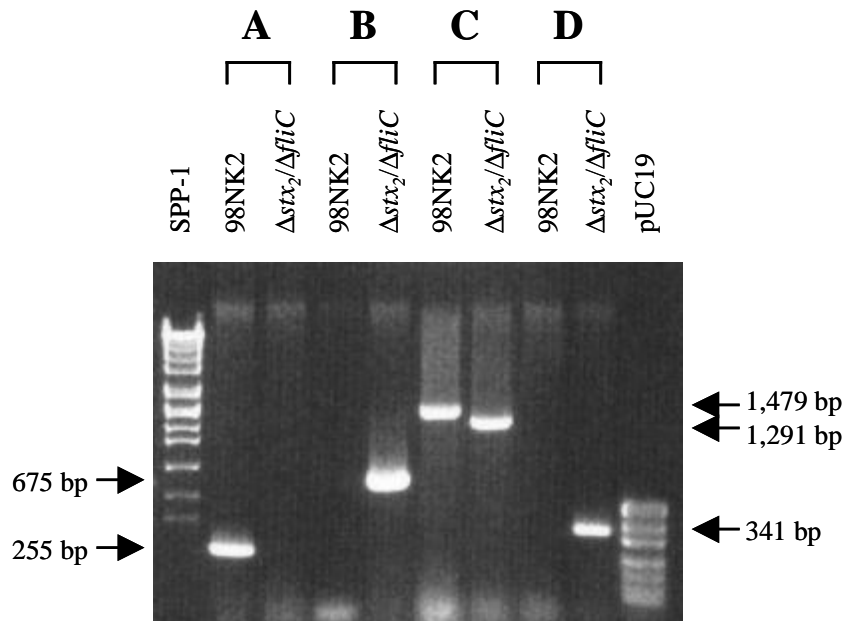


Figure 4.15 PCR analysis of 98NK2 Δ *stx*₂/ Δ *fliC* double mutant. PCR amplification of wild-type 98NK2 and 98NK2 Δ *stx*₂/ Δ *fliC* using (A) *stx*₂ specific primers (*stx*2F and *stx*2R), (B) *stx*₂ and *kan* specific primers (TR34 and *k1*), (C) *fliC* specific primers (F-FliC1 and R-FliC2) or (D) *fliC* and *cat* specific primers (F-FliC1 and *c1*) (Table 2.4). The arrows on the left indicate the 255-bp PCR product obtained using 98NK2 as template DNA and the primers *stx*2F and *stx*2R and the 675-bp product obtained using 98NK2 Δ *stx*₂ as template DNA and the primers TR34 and *k1*. The arrows on the right indicate the 1,479-bp product obtained using 98NK2 as template DNA or the 1,291-bp product using 98NK2 Δ *stx*₂/ Δ *fliC* as template DNA and F-FliC1 and R-FliC2 or the 341-bp product using 98NK2 Δ *stx*₂/ Δ *fliC* as template DNA and F-FliC1/*c1*. The fragment sizes of SPP-1 DNA markers are: 8.51, 7.35, 6.11, 4.84, 3.59, 2.81, 1.95, 1.86, 1.51, 1.39, 1.16, 0.98, 0.72, 0.48 and 0.36 kb, and pUC19 markers are: 501, 404, 331, 242, 190, 147, 111, 110, 67, 34 and 26 bp.

4.2.5.2.3 Stimulation of HCT-8 cells with 98NK2 Δ *stx*₂, 98NK2 Δ *fliC* and 98NK2 Δ *stx*₂/ Δ *fliC*

The capacity of the mutant and wild-type strains to elicit CXC chemokine responses in HCT-8 cells was then examined. IL-8 and MIP-2 α mRNA responses in HCT-8 cells infected with strain 98NK2 or with 98NK2 Δ *stx*₂ were similar. In contrast, chemokine mRNA responses of cells infected with 98NK2 Δ *fliC* were markedly depressed (Fig. 4.18A). The 98NK2 Δ *fliC* strain elicited 439- and 21-fold less IL-8 mRNA at 1 and 4 h, respectively,

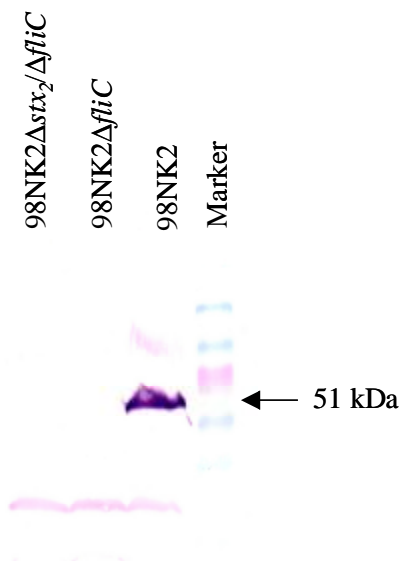


Figure 4.16 Western blot of whole cell lysates from 98NK2, 98NK2 Δ fliC and 98NK2 Δ stx₂/ Δ fliC. Whole cell lysates were prepared from overnight cultures of STEC (Section 2.14.1) then electrophoresed on a 12% SDS-PAGE gel (Section 2.14.2). SDS-PAGE gels were then subjected to Western blotting (Section 2.14.4) with H21 antiserum. The arrow indicates the 51-kDa band obtained with wild-type 98NK2, however no flagellin is detectable with the FliC mutants 98NK2 Δ fliC or 98NK2 Δ stx₂/ Δ fliC. The markers are BenchMark pre-stained protein ladder (sizes 172.0, 110.2, 79.0, 62.4 [pink], 48.0, 36.6, 24.5, 19.0, 13.5 and 5.3 kDa).

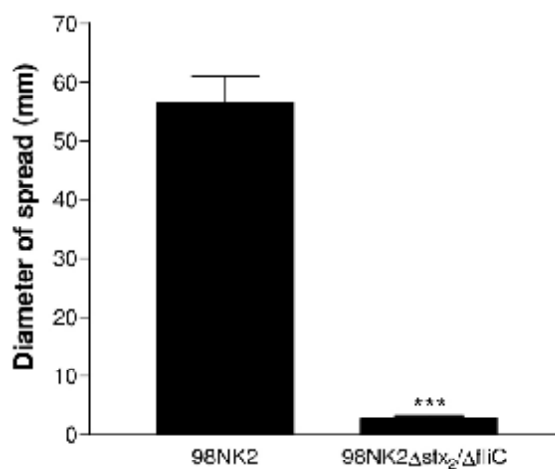


Figure 4.17 Motility of 98NK2 and 98NK2 Δ stx₂/ Δ fliC on semi-soft agar. STEC were stab-inoculated onto 0.3% (w/v) LB agar and incubated at 37°C for 18 h, after which the diameter of spread (mm) was measured. Data shown are the means \pm SD from duplicate samples. Significant differences relative to 98NK2 are indicated as follows: ***, $P < 0.0001$.

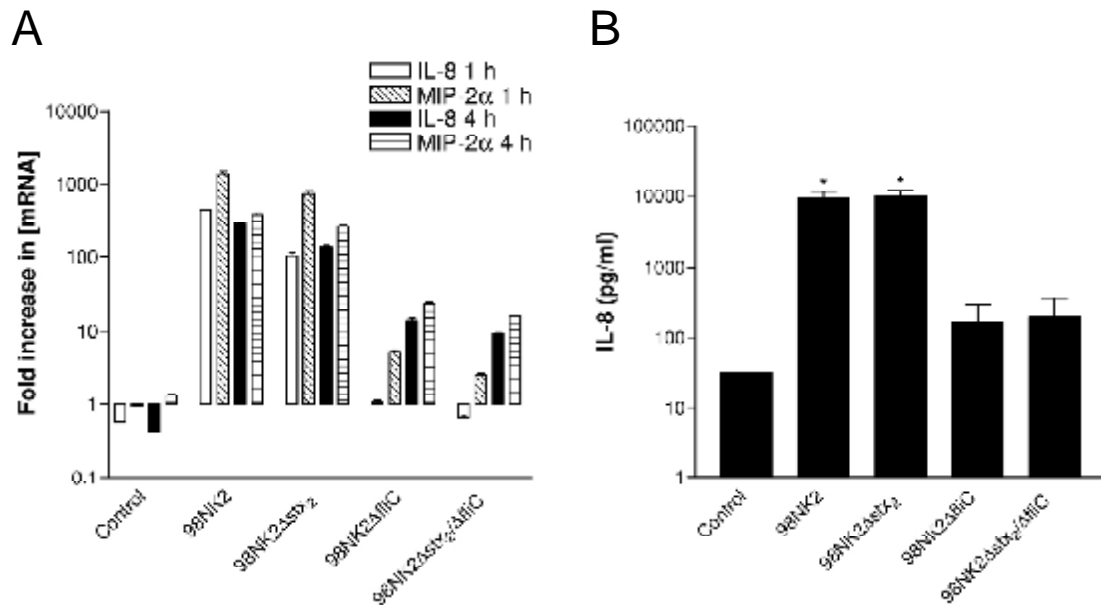


Figure 4.18 Induction of IL-8 and MIP-2a mRNA and IL-8 protein in HCT-8 cells infected with strain 98NK2, or *stx*₂, *fliC* or *stx*₂/*fliC* deletion mutants thereof. HCT-8 cells were stimulated with 3×10^7 CFU/ml 98NK2, 98NK2Δ*stx*₂, 98NK2Δ*fliC* or 98NK2Δ*stx*₂/Δ*fliC*. **(A)** At 1 or 4 h, total RNA was extracted from cells (Section 2.12.1) and IL-8 and MIP-2α mRNA was quantitated by real-time RT-PCR (Section 2.13.2). Results are expressed as the fold increase in [mRNA] relative to levels at 0 h, and data shown are the means \pm SD for triplicate assays. **(B)** At 4 h, supernatants were collected and assayed for IL-8 by ELISA (Section 2.20). Data shown are the means \pm SEM from two experiments. Significant differences relative to the Δ*fliC*, Δ*stx*₂/Δ*fliC* and untreated control cells are indicated as follows: *, $P < 0.05$.

and 273- and 18-fold less MIP-2α mRNA at 1 and 4 h, respectively, compared to wild-type 98NK2. Infection of HCT-8 cells with 98NK2Δ*stx*₂/Δ*fliC* resulted in mRNA levels similar to that observed with the single *fliC* deletion mutant. The double mutant elicited a 627- and 31-fold lower IL-8 mRNA response, and a 546- and 24-fold lower MIP-2α mRNA response, at 1 and 4 h respectively, compared to the wild-type 98NK2.

Similarly, with IL-8 protein, levels in infected HCT-8 culture supernatants at 4 h were indistinguishable between 98NK2 and 98NK2Δ*stx*₂ ($P > 0.05$), whereas for cells infected with 98NK2Δ*fliC* or 98NK2Δ*stx*₂/Δ*fliC*, IL-8 levels were more than 100-fold lower than that of either 98NK2- or 98NK2Δ*stx*₂- infected HCT-8 cells ($P < 0.05$) (Fig. 4.18B). At 4 h, 98NK2 and 98NK2Δ*stx*₂ elicited $9,250 \pm 2,250$ and $10,050 \pm 1,950$ pg/ml IL-8, whereas 98NK2Δ*fliC* and 98NK2Δ*stx*₂/Δ*fliC* elicited 161 ± 130 and 195 ± 165 pg/ml IL-8,

respectively. These results suggest that flagella are indeed responsible for the major portion of IL-8 and MIP-2 α mRNA, as well as IL-8 protein secretion in 98NK2.

4.2.5.2.4 Adherence of 98NK2D stx_2 , 98NK2D fliC and 98NK2D stx_2 /D fliC to HCT-8 and HEp-2 cells

It was recently reported that the EPEC H6 and H2 flagella serotypes, but not STEC H7 flagella, were able to mediate adherence to HeLa cells (Giron *et al.*, 2002). To investigate whether the reduction in IL-8 induction was mediated, in part, by an inability of the *fliC* mutant to adhere to HCT-8 cells, *in vitro* adherence assays were performed as described in Section 2.18. No significant differences in the ability of 98NK2 Δ *fliC* to adhere to HCT-8 cells compared to wild-type 98NK2 were observed ($P > 0.05$) (Table 4.2). Similarly, no significant differences could be found between the adherence of 98NK2 or 98NK2 Δ *fliC* to HEp-2 cells ($P > 0.05$) (Table 4.2). Levels of adherence were similar to that reported previously for 98NK2 (Paton *et al.*, 1999; Paton *et al.*, 2001a; Srimanote *et al.*, 2002). Thus, in contrast to the findings of Giron *et al.* (2002) for EPEC, there is no evidence that H21 flagella contribute to the adherence of STEC 98NK2 to epithelial cells.

Table 4.2 Adherence of 98NK2 and 98NK2D*fliC* to HCT-8 and HEp-2 cells.

Strain	No. of bacteria adhered to HCT-8 cells (CFU/well $\times 10^4$) ^a	No. of bacteria adhered to HEp-2 cells (CFU/well $\times 10^5$) ^a
98NK2	7.90 (± 1.00)	1.73 (± 0.34)
98NK2 Δ <i>fliC</i>	12.20 (± 2.24)	2.52 (± 0.60)

^a Quantitative adherence assays were carried out as described in Section 2.18. Data shown are the means (\pm SEM) CFU/well for quadruplicate assays.

4.2.6 Chemokine responses of HCT-8 cells to other flagellar types

Since H21 flagella appear to be central to the chemokine responses elicited by 98NK2, it was important to investigate whether other types of STEC flagella are similarly capable of eliciting such responses in HCT-8 cells.

4.2.6.1 Effect of purified flagella from other STEC strains on chemokine production by HCT-8 cells

4.2.6.1.1 Cloning and sequencing of 95ZG1 and 95HE4 *fliC* genes

Two of the high chemokine-inducing STEC strains from our laboratory tested in Sections 3.2.3.1 and 3.2.3.2, had not been H serotyped. In order to determine what type of flagellin was expressed by these strains, the *fliC* genes of 95ZG1 (serotype O26) and 95HE4 (serotype O91) were cloned into pGEM[®]-T Easy (Table 2.2) for sequence analysis (Section 2.10). 95ZG1 chromosomal DNA was amplified using the primers F-FliC1 and R-FliC2 (Table 2.4), which amplifies the entire ORF of the *fliC* gene in all serotypes, using high-fidelity PCR (Section 2.9.2). This resulted in the amplification of an approximately 1.5- to 1.6-kb fragment that was purified (Section 2.9.3) and directly ligated into pGEM[®]-T Easy (Section 2.9.4 and Table 2.2). An Amp^R transformant, containing the appropriate sized insert, was sequenced using M13 Fwd and Rev primers (Table 2.4). This confirmed that the cloned fragment was *fliC*, and the remainder of the insert was sequenced using custom-designed primers. The sequence (see Appendix I) was then analysed by BLASTN and this indicated that the *fliC* gene from 95ZG1 was identical to that for *E. coli* serotype H11 flagellin (GenBank accession number AY337465). This is consistent with previous studies that suggest that O26 STEC strains are highly clonal and usually express H11 flagellin (Zhang *et al.*, 2000a).

Chromosomal DNA from strain 95HE4 was amplified using the primers EcoH1 and EcoH2 using high-fidelity PCR (Table 2.4 and Section 2.9.2). The resultant 1.5-kb product, was purified (Section 2.9.3) and ligated into pGEM[®]-T Easy (Section 2.9.4 and Table 2.2).

Sequence analysis of this insert with M13 Fwd and Rev primers (Table 2.3), confirmed that the cloned fragment was *fliC*, and the remainder of the insert was sequenced using custom-designed primers. The sequence (Appendix II) was then analysed by BLASTN and this indicated that the *fliC* gene from 95HE4 was identical to that for *E. coli* H7 flagellin (GenBank accession number AF228496).

4.2.6.1.2 Stimulation of HCT-8 cells with 95HE4 H7 and 95ZG1 H11 flagella

In order to determine the effects of H7 and H11 flagella (from the LEE-negative O91:H7 strain 95HE4 and the LEE-positive O26:H11 strain 95ZG1) on chemokine production by HCT-8 cells, flagella were isolated from these strains (Section 2.24) and used to stimulate HCT-8 cells. The purified flagella had a molecular mass of approximately 60- and 51-kDa for 95HE4 and 95ZG1, respectively as judged by SDS-PAGE (result not shown). At 1 µg/ml, 100 ng/ml and 10 ng/ml, H7 flagella isolated from 95HE4 induced high IL-8 and MIP-2α mRNA levels comparable to that observed with 95HE4-infected HCT-8 cultures (Fig. 4.19A). However, the same concentrations of H11 flagella from 95ZG1 were less active, inducing mRNA levels similar to strain 95ZG1 at 1 µg/ml, while at 100 ng/ml and 10 ng/ml, the degree of induction was only slightly higher than in control cells ($P > 0.05$).

Measurement of IL-8 in the supernatants corroborated these findings (Fig. 4.19B). At 1 µg/ml, 100 ng/ml and 10 ng/ml, 95HE4 H7 flagella produced $11,500 \pm 500$, $23,000 \pm 2,000$ and $4,900 \pm 500$ pg/ml IL-8 compared with $24,000 \text{ pg/ml} \pm 2,000 \text{ pg/ml}$ IL-8 elicited by infection of HCT-8 cells with 95HE4. IL-8 levels induced by 95HE4, as well as all three concentrations of 95HE4 H7 flagella, were significantly different from that in the control supernatants ($P < 0.001$), and comparable to that observed previously for H21 flagella from 98NK2 (Fig. 4.11B). However, stimulation of HCT-8 cells with 95ZG1 H11 flagella at 1 µg/ml, 100 ng/ml and 10 ng/ml produced $1,290 \pm 460$, 106 ± 24 and 143 ± 3 pg/ml IL-8 respectively, compared to $1,330 \pm 570$ pg/ml IL-8 that was elicited by infection with 95ZG1. Only treatment with 95ZG1 and the highest concentration of 95ZG1 H11 flagella tested (1

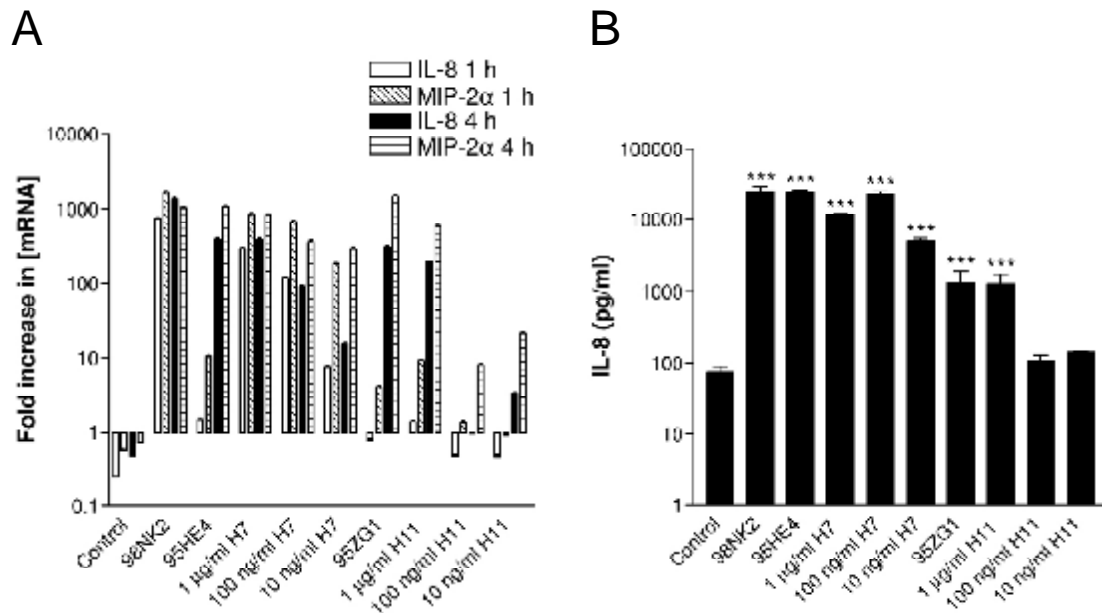


Figure 4.19 Induction of IL-8 and MIP-2a mRNA and IL-8 protein in HCT-8 cells treated with H7 and H11 flagella. HCT-8 cells were stimulated with H7 or H11 flagella at the indicated concentrations or with 3×10^7 CFU/ml 98NK2, 95HE4 or 95ZG1. (A) At 1 or 4 h, total RNA was extracted from cells (Section 2.12.1) and IL-8 and MIP-2 α mRNA was quantitated by real-time RT-PCR (Section 2.13.2). Results are expressed as the fold increase in [mRNA] relative to levels at 0 h, and data shown are the means \pm SD for triplicate assays. (B) At 4 h, supernatants were collected and assayed for IL-8 by ELISA (Section 2.20). Data shown are the means \pm SEM from two experiments. Significant differences relative to untreated control cells are indicated as follows: ***, $P < 0.001$.

$\mu\text{g/ml}$) resulted in significantly increased IL-8 secretion relative to control cells ($P < 0.001$). Thus, 95ZG1 H11 flagella (at ng/ml doses) are less potent at eliciting IL-8 secretion than either H7 or H21 flagella from 95HE4 and 98NK2, respectively (Fig. 4.11B and Fig. 4.19B).

The fact that the LEE-positive STEC strains 95NR1 (O111:H⁻) and 95SF2 (O157:H⁻) were poor inducers of CXC chemokine responses in HCT-8 cells is compatible with the fact that they are non-motile and do not produce flagella. On the other hand, the poor responses elicited by strain EDL933 (O157:H7) were unexpected, particularly since H7 flagella isolated from strain 95HE4 was a strong inducer. To confirm that this isolate of EDL933 still has an intact copy of the *fliC* gene, chromosomal DNA from EDL933 was amplified using the primers EcoH1 and EcoH2 (Table 2.4) using high-fidelity PCR (Section 2.9.2). The resultant

1.7-kb product, was purified (Section 2.9.3) and ligated into pGEM[®]-T Easy (Section 2.9.4 and Table 2.2).

Sequence analysis of this insert with M13 Fwd and Rev primers (Table 2.3), as well as custom-designed primers, confirmed that this isolate of EDL933 does contain an intact copy of the H7 *fliC* gene (Appendix III), as previously described (GenBank accession number AE005415). However, examination of whole cell lysates and flagella preparations from EDL933 by SDS-PAGE (Section 2.14.2) and Western blot analysis (Section 2.14.4) using H7-specific antiserum indicated that no intact flagella were present (Fig. 4.20) and that EDL933 was non-motile on semi-solid LB agar (Fig. 4.21), suggesting that this strain may have some other defect in flagella biosynthesis. However, a 60-kDa band was observed with

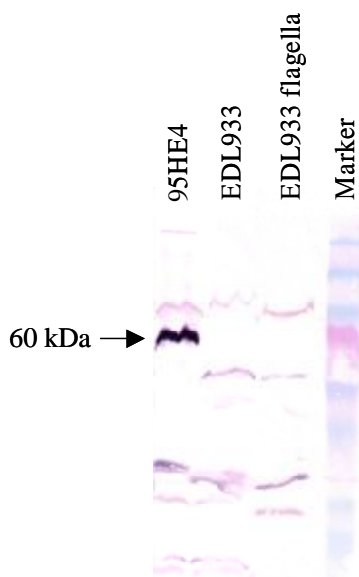


Figure 4.20 Western blot of flagella preparation from EDL933 and whole cell lysates from EDL933 and 95HE4. Flagella were prepared from EDL933 (Section 2.24) and whole cell lysates were prepared from overnight cultures of EDL933 and 95HE4 (Section 2.14.1) then electrophoresed on a 12% SDS-PAGE gel (Section 2.14.2). SDS-PAGE gels were then subjected to Western blotting (Section 2.14.4) with H7 antiserum. The arrow indicates the 60-kDa band obtained with 95HE4. However, no flagellin is detectable with EDL933 or the material isolated from the EDL933 flagella preparation (Section 2.24). The markers are BenchMark pre-stained protein ladder (sizes 172.0, 110.2, 79.0, 62.4 [pink], 48.0, 36.6, 24.5, 19.0, 13.5 and 5.3 kDa).

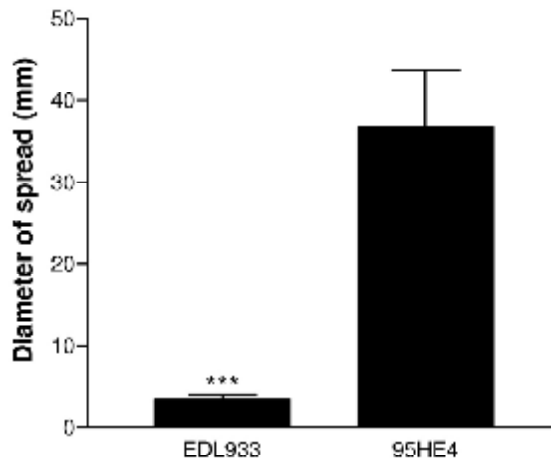


Figure 4.21 Motility of EDL933 and 95HE4 on semi-soft agar. STEC were stab-inoculated onto 0.3% (w/v) LB agar and incubated at 37°C for 18 h, after which the diameter of spread (mm) was measured. Data shown are the means \pm SD from duplicate samples. Significant differences relative to 95HE4 are indicated as follows: ***, $P < 0.001$.

whole cell lysates from 95HE4 (Fig. 4.20) and 95HE4 was motile on semi-soft LB agar (Fig. 4.21). Similarly, stimulation of HCT-8 cells with material obtained from an EDL933 flagella preparation (Section 2.24) induced minimal CXC chemokine responses similar to those elicited by infection with wild-type EDL933 (data not shown).

4.2.6.2 Stimulation of HCT-8 cells by other O157:H7 STEC strains

Since EDL933 was found not to express H7 flagellin, other LEE-positive O157:H7 STEC clinical isolates were examined to determine whether they are high or low chemokine inducers. The STEC strains 86-24, 90-103 and 94-6752 (Table 2.1), were used to stimulate HCT-8 monolayers and the IL-8 and MIP-2 α mRNA levels, as well as IL-8 protein were determined by real time RT-PCR and ELISA, respectively. All of these strains induced IL-8 and MIP-2 α mRNA responses that were comparable to the high chemokine-producing LEE-negative strain 98NK2 (Fig. 4.22A). Similarly, levels of IL-8 protein in 86-24, 90-103 and

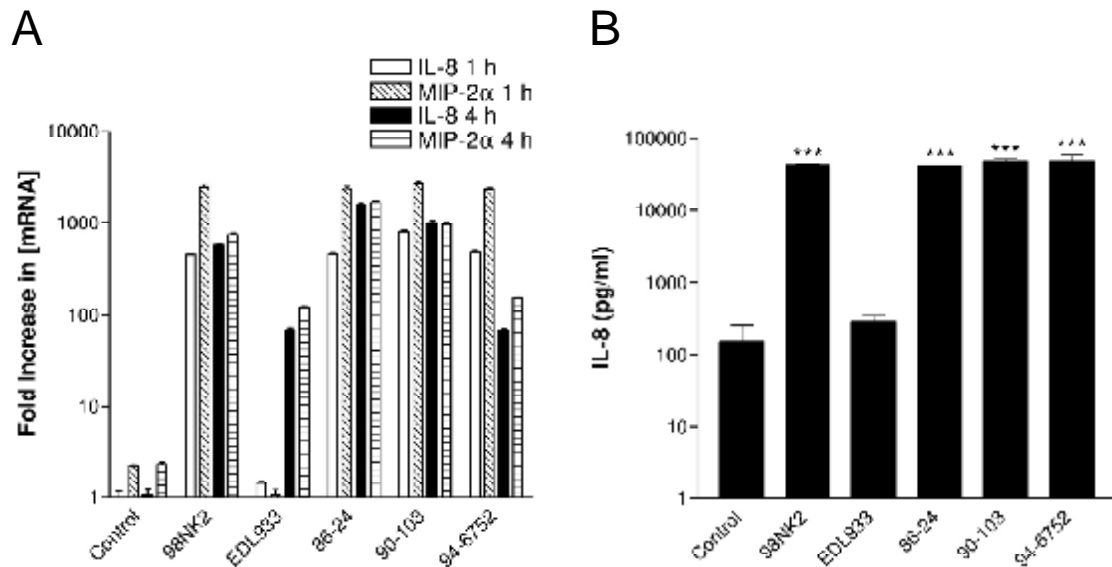


Figure 4.22 Induction of IL-8 and MIP-2a mRNA and IL-8 protein in HCT-8 cells infected with O157:H7 strains. HCT-8 cells were stimulated with 3×10^7 CFU/ml 98NK2 (O113:H21) or the O157:H7 strains EDL933, 86-24, 90-103 or 94-6752. **(A)** At 1 or 4 h, total RNA was extracted from cells (Section 2.12.1) and IL-8 and MIP-2 α mRNA was quantitated by real-time RT-PCR (Section 2.13.2). Results are expressed as the fold increase in [mRNA] relative to levels at 0 h, and data shown are the means \pm SD for triplicate assays. **(B)** At 4 h, supernatants were collected and assayed for IL-8 by ELISA (Section 2.20). Data shown are the means \pm SEM from two experiments. Significant differences relative to untreated control cells are indicated as follows: ***, $P < 0.001$.

94-6752 infected HCT-8 culture supernatants were significantly higher than that for both the control and EDL933-infected cells ($P < 0.001$) (Fig. 4.22B). Strain 86-24, 90-103 and 94-6752 elicited $42,000 \pm 500$, $48,500 \pm 3,500$ and $48,500 \pm 10,500$ pg/ml IL-8, respectively. This is comparable to the levels of IL-8 that were induced by 98NK2 ($43,250 \pm 250$ pg/ml IL-8). However, EDL933-infected monolayers secreted only 283 ± 58 pg/ml of IL-8. Western immunoblot analysis using H7 antiserum also confirmed the expression of H7 flagellin in 86-24, 90-103 and 94-6752 (data not shown). Thus, the capacity of O157:H7 STEC to induce high level CXC chemokine responses in HCT-8 cells correlates with the expression of H7 flagella.

4.2.6.3 Effect of *fliC* deletion mutations in other STEC strains on chemokine induction in HCT-8 cells

To confirm the role of *fliC* in chemokine responses, two other *fliC* deletion mutations were constructed in STEC strains 97MW1 (O113:H21) and 86-24 (O157:H7). Both 97MW1 and 86-24 were previously shown to be high chemokine-inducing strains (Sections 3.2.3 and 4.2.6.2, respectively).

4.2.6.3.1 Construction of 97MW1 *fliC* deletion mutant

Strain 97MW1 Δ *fliC*, was constructed in a manner identical to that described for 98NK2 Δ *fliC* in Section 4.2.5.2.1, since it also expresses H21 flagellin. Strain 97MW1 was electroporated with the helper-plasmid pKD46 and selected on LB agar supplemented with Amp at 30°C. An Amp^R transformant was checked by PCR with the pKD46 specific primers pKD46 Fwd and pKD46 Rev (Table 2.3), and designated 97MW1[pKD46]. The *kan* gene from pKD4 was then amplified using primers TR30 and TR31 as described in Section 4.2.5.2.1, and transformed into 97MW1[pKD46]. Kan^R transformants were screened by PCR (Section 2.9.1). Amplification of the 97MW1 Δ *fliC* with F-FliC1 and R-FliC2 resulted in a 1,754-bp product compared to a 1,476-bp product in STEC 97MW1, whereas PCR amplification with F-FliC1 and k1 yielded a PCR product of 621 bp in the 97MW1 Δ *fliC* mutant, and no product for STEC 97MW1 (Fig. 4.23). Sequencing confirmed the deletion of the major portion of the *fliC* gene (nucleotides 124–1,341, representing amino acids 42–446), and insertion of the kanamycin resistance gene (1,476 bp). Western blotting with H21 antiserum showed no H21 flagellin expression in 97MW1 Δ *fliC* (Fig. 4.24) and no motility on semi-soft agar (Fig. 4.25).

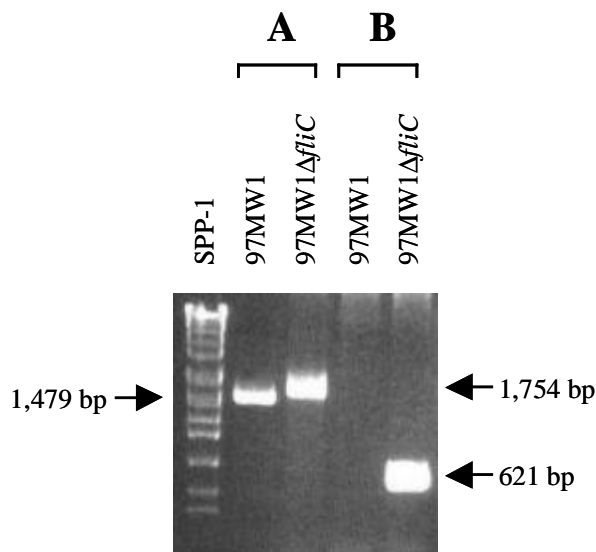


Figure 4.23 PCR analysis of 97MW1Δ*fliC* mutant. PCR amplification of wild-type 97MW1 and 97MW1Δ*fliC* using (A) *fliC* specific primers (F-FliC1 and R-FliC2) or (B) *fliC* and *kan* specific primers (F-FliC1 and k1) (Table 2.4). The arrows indicate the 1,479-bp PCR product obtained using wild-type 97MW1 as template DNA or the 1,754-bp PCR product obtained using 97MW1Δ*fliC* as template DNA and the primers F-FliC1 and R-FliC2; or the 621-bp product obtained using 97MW1Δ*fliC* as template DNA and the primers F-FliC1 and k1. The fragment sizes of the SPP-1 DNA markers are 8.51, 7.35, 6.11, 4.84, 3.59, 2.81, 1.95, 1.86, 1.51, 1.39, 1.16, 0.98, 0.72, 0.48 and 0.36 kb.

4.2.6.3.2 Construction of 86-24 *fliC* deletion mutant

Strain 86-24 expresses H7 flagellin and was constructed using the primers TR36 and TR37 (Table 2.4) which have regions with homology to nucleotides 82–123 and 1,621–1,662, respectively, of the H7 *fliC* gene of EDL933 (GenBank accession number AE005415), and the priming sites for sequences flanking the *kan* resistance gene of pKD4. The STEC strain 86-24 was electroporated with the temperature-sensitive helper plasmid pKD46 (Table 2.2), and selected on LB agar supplemented with Amp at 30°C. An Amp^R transformant was screened by PCR with the pKD46 specific primers pKD46 Fwd and pKD46 Rev (Table 2.3), and designated 86-24[pKD46]. The primers TR36 and TR37 (Table 2.4) were used to amplify

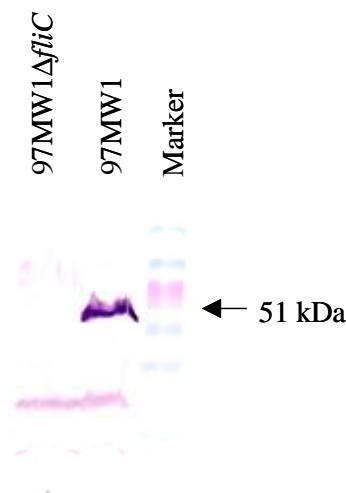


Figure 4.24 Western blot of whole cell lysates from 97MW1 and 97MW1 Δ *fliC*. Whole cell lysates were prepared from overnight cultures of STEC (Section 2.14.1) then electrophoresed on a 12% SDS-PAGE gel (Section 2.14.2). SDS-PAGE gels were then subjected to Western blotting (Section 2.12.4) with H21 antiserum. The arrow indicates the 51-kDa band obtained with wild-type 97MW1, however no flagellin is detectable with the *FliC* mutant 97MW1 Δ *fliC*. The markers are BenchMark pre-stained protein ladder (sizes 172.0, 110.2, 79.0, 62.4 [pink], 48.0, 36.6, 24.5, 19.0, 13.5 and 5.3 kDa).

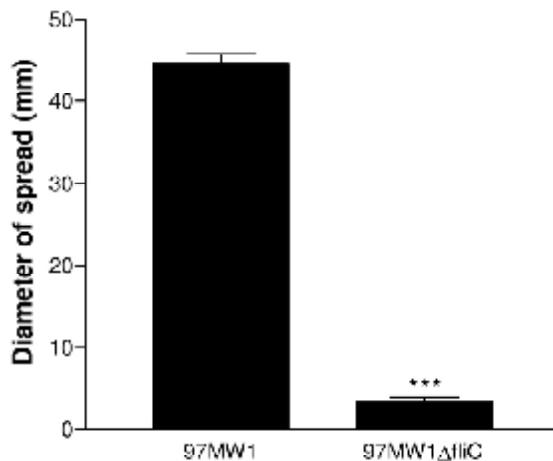


Figure 4.25 Motility of 97MW1 and 97MW1 Δ *fliC* on semi-soft agar. STEC were stab-inoculated onto 0.3% (w/v) LB agar and incubated at 37°C for 18 h, after which the diameter of spread (mm) was measured. Data shown are the means \pm SD from duplicate samples. Significant differences relative to 97MW1 are indicated as follows: ***, $P < 0.0001$.

the *kan* resistance gene of pKD4 using high-fidelity PCR (Section 2.9.2). The PCR product was purified (Section 2.9.3), then electroporated into 86-24[pKD46], as described in Section 2.8.

Kan^R and Amp^S transformants were checked by PCR using F-FliC1 and R-FliC2, and F-FliC1 and k1 (Table 2.4). Amplification with F-FliC1 and R-FliC2, which amplifies the complete ORF of the *fliC* gene, yielded a 1,719-bp PCR product in the *fliC* deletion mutants compared to a 1,758-bp product in STEC 86-24, whereas PCR amplification with F-FliC1 and k1 yielded a PCR product of 621-bp in the *fliC* deletion mutant and no product for STEC 86-24 (Fig. 4.26). Sequencing confirmed the deletion of the major portion of the *fliC* gene (nucleotides 124–1,620, encoding amino acids 42–540), and insertion of the kanamycin resistance gene (1,476 bp). The mutant, designated 86-24 Δ *fliC*, was also shown not to express H7 flagella by Western blotting using H7 antiserum (Fig. 4.27) and was non-motile on semi-soft agar (Fig. 4.28).

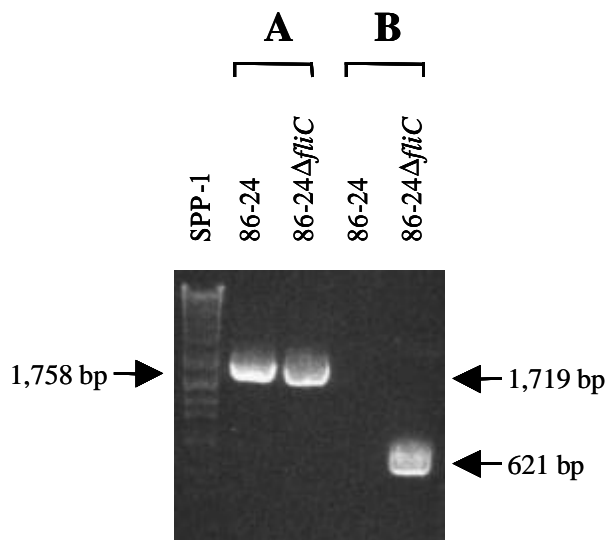


Figure 4.26 PCR analysis of 86-24 *fliC* mutant. PCR amplification of wild-type 86-24 and 86-24 Δ *fliC* using (A) *fliC* specific primers (F-FliC1 and R-FliC2) or (B) *fliC* and *kan* specific primers (F-FliC1 and k1) (Table 2.4). The arrows indicate the 1,758-bp PCR product obtained using wild-type 86-24 as template DNA, the 1,719-bp PCR product obtained using 86-24 Δ *fliC* as template DNA and the primers F-FliC1 and R-FliC2 or the 621-bp product obtained using 86-24 Δ *fliC* as template DNA and the primers F-FliC1 and k1. The fragment sizes of SPP-1 DNA markers are 8.51, 7.35, 6.11, 4.84, 3.59, 2.81, 1.95, 1.86, 1.51, 1.39, 1.16, 0.98, 0.72, 0.48 and 0.36 kb.

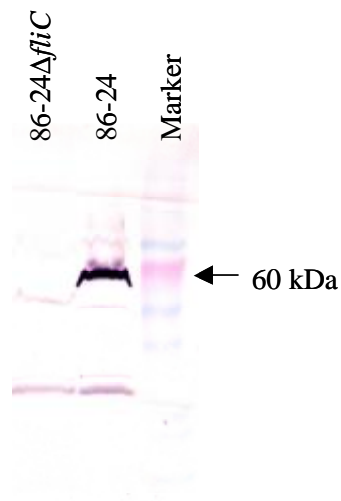


Figure 4.27 Western blot of whole cell lysates from 86-24 and 86-24ΔfliC. Whole cell lysates were prepared from overnight cultures of STEC (Section 2.14.1) then electrophoresed on a 12% SDS-PAGE gel (Section 2.14.2). SDS-PAGE gels were then subjected to Western blotting (Section 2.14.4) with H7 antiserum. The arrow indicates the 60-kDa band obtained with wild-type 86-24, however no flagellin is detectable with the FliC mutant 86-24ΔfliC. The markers are BenchMark pre-stained protein ladder (sizes 172.0, 110.2, 79.0, 62.4 [pink], 48.0, 36.6, 24.5, 19.0, 13.5 and 5.3 kDa).

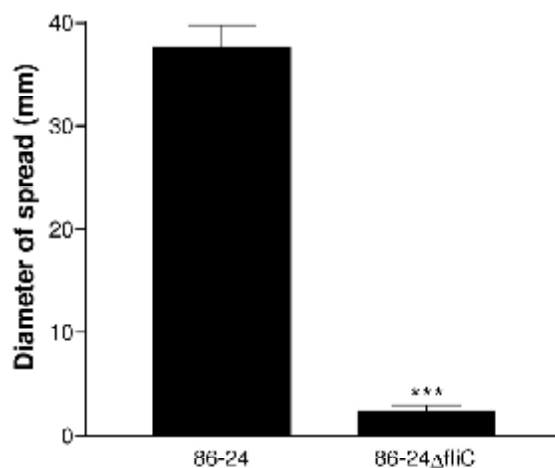


Figure 4.28 Motility of 86-24 and 86-24ΔfliC on semi-soft agar. STEC were stab-inoculated onto 0.3% (w/v) LB agar and incubated at 37°C for 18 h, after which the diameter of spread (mm) was measured. Data shown are the means \pm SD from duplicate samples. Significant differences relative to 86-24 are indicated as follows: ***, $P < 0.0001$.

4.2.6.3.3 Stimulation of HCT-8 cells with 98NK2, 97MW1 and 86-24 *fliC* deletion mutants

The effect of deletion of *fliC* in strains 86-24, 97MW1 and 98NK2, was then examined by infecting HCT-8 cells with the various mutant or wild-type strains. In each case, the chemokine mRNA responses of cells infected with the *fliC* deletion mutants were markedly depressed compared to their respective wild-type strains (Fig. 4.29A). The 98NK2 Δ *fliC* strain elicited 558-fold and 7-fold reduction in IL-8 mRNA at 1 and 4 h, respectively and a 1,476-fold and 13-fold decrease in MIP-2 α mRNA at 1 and 4 h, respectively, compared to wild-type 98NK2. The 97MW1 Δ *fliC* strain elicited an 8- and 13-fold lower IL-8 response at 1 and 4 h, respectively, and a 19- and 10-fold reduction in

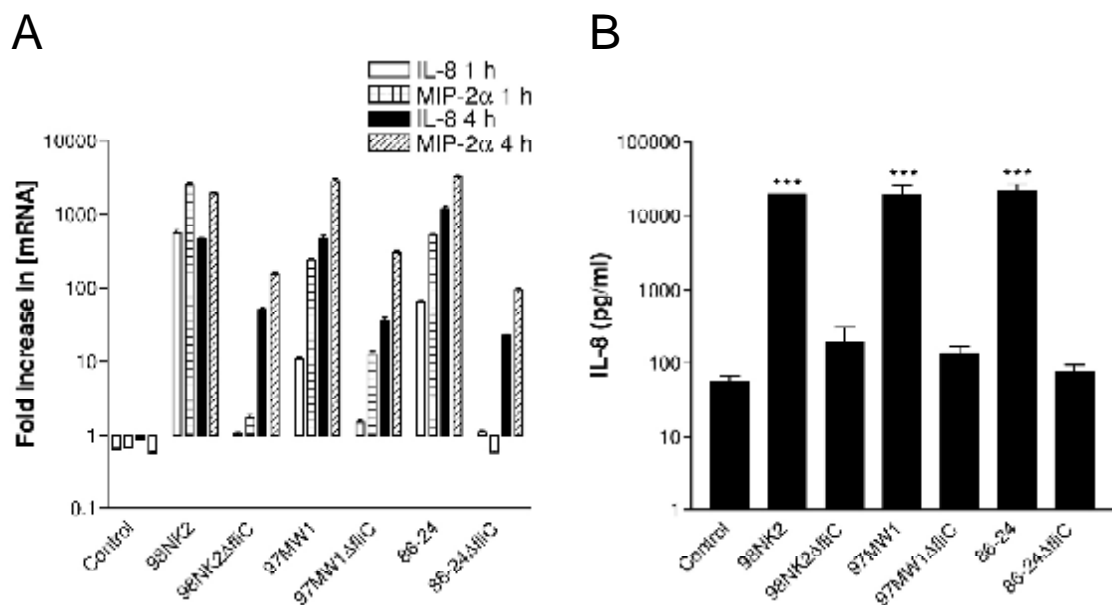


Figure 4.29 Induction of IL-8 and MIP-2a mRNA and IL-8 protein in HCT-8 cells infected with strains 98NK2, 97MW1 or 86-24 and their respective *fliC* deletion mutants. HCT-8 cells were stimulated with 3×10^7 CFU/ml 98NK2, 98NK2 Δ *fliC*, 97MW1, 97MW1 Δ *fliC*, 86-24 or 86-24 Δ *fliC*. (A) At 1 or 4 h, total RNA was extracted from cells (Section 2.12.1) and IL-8 and MIP-2 α mRNA was quantitated by real-time RT-PCR (Section 2.13.2). Results are expressed as the fold increase in [mRNA] relative to levels at 0 h, and data shown are the means \pm SD for triplicate assays. (B) At 4 h, supernatants were collected and assayed for IL-8 by ELISA (Section 2.20). Data shown are the means \pm SEM from two experiments. Significant differences relative to the respective Δ *fliC* strain and control cells are indicated as follows: ***, $P < 0.001$.

MIP-2 α mRNA response at 1 and 4 h, respectively compared to wild-type 97MW1. Likewise 86-24 Δ *fliC*, elicited a 59- and 53-fold reduction in IL-8 mRNA at 1 and 4 h, respectively, and an 875- and 713-fold decrease in MIP-2 α mRNA at 1 and 4 h, respectively, compared to wild-type 86-24.

Similarly, with IL-8 protein, levels in infected HCT-8 culture supernatants at 4 h were significantly different between all wild-type strains and their respective Δ *fliC* mutants (Fig. 4.29B). Strain 98NK2 elicited $19,950 \pm 50$ pg/ml IL-8 whereas 98NK2 Δ *fliC* elicited only 189 ± 116 pg/ml IL-8 ($P < 0.001$). Similarly, 97MW1 elicited $19,550 \pm 6,950$ pg/ml IL-8 compared to only 138 ± 30 pg/ml IL-8 for 97MW1 Δ *fliC* ($P < 0.001$) and 86-24 elicited $22,100 \pm 5,300$ compared to 77 ± 18 pg/ml IL-8 for 86-24 Δ *fliC* ($P < 0.001$). These results indicate that flagellin is an important determinant of IL-8 and MIP-2 α mRNA, and IL-8 protein responses in a range of STEC isolates, not just 98NK2.

4.2.7 Purified His₆-FliC induces chemokine production by HCT-8 cells

Since intact flagella are a potent chemokine inducer, it was of interest to determine whether purified flagellin alone is capable of eliciting this response in HCT-8 cells.

4.2.7.1 Cloning and purification of 98NK2 H21 His₆-FliC into pQE-30

In order to obtain highly pure FliC, STEC 98NK2 (O113:H21) FliC was expressed as a His₆-fusion protein using the vector pQE-30 (Table 2.2), in the *E. coli* K-12 expression strain M15 (Table 2.1). To achieve this, the 98NK2 H21 *fliC* gene (nucleotides 1–1,479) was amplified using high-fidelity PCR (Section 2.9.2) with the primers TR45 and TR46 (Table 2.4). These primers incorporate *Sph*I and *Sal*I sites, respectively, enabling the resultant product to be cloned between the *Sph*I and *Sal*I sites of pQE-30. This placed the His₆ tag, and four linker amino acids, at the N-terminus of the His₆-FliC fusion protein expressed by the construct. The recombinant pQE-30 plasmid was then transformed into the expression host, *E. coli* M15[pREP4] (Section 2.7.3). The recombinant pQE-30:*fliC* construct was also

sequenced in order to confirm the correct in-frame gene fusion. The His₆-FliC protein was induced and purified by Ni-NTA chromatography, as described in Section 2.25. The fraction containing FliC was dialysed, and analysed by SDS-PAGE (Section 2.14.2) (Fig 4.30A) and Western blotting (Section 2.14.4) using H21-antiserum before use (Fig. 4.30B). The purified recombinant flagellin exhibited a molecular size of approximately 51 kDa.

4.2.7.2 Stimulation of HCT-8 cells by H21 His₆-FliC

In order to determine whether His₆-FliC alone is able to induce chemokine production in HCT-8 cells, His₆-FliC was added to HCT-8 monolayers at concentrations ranging

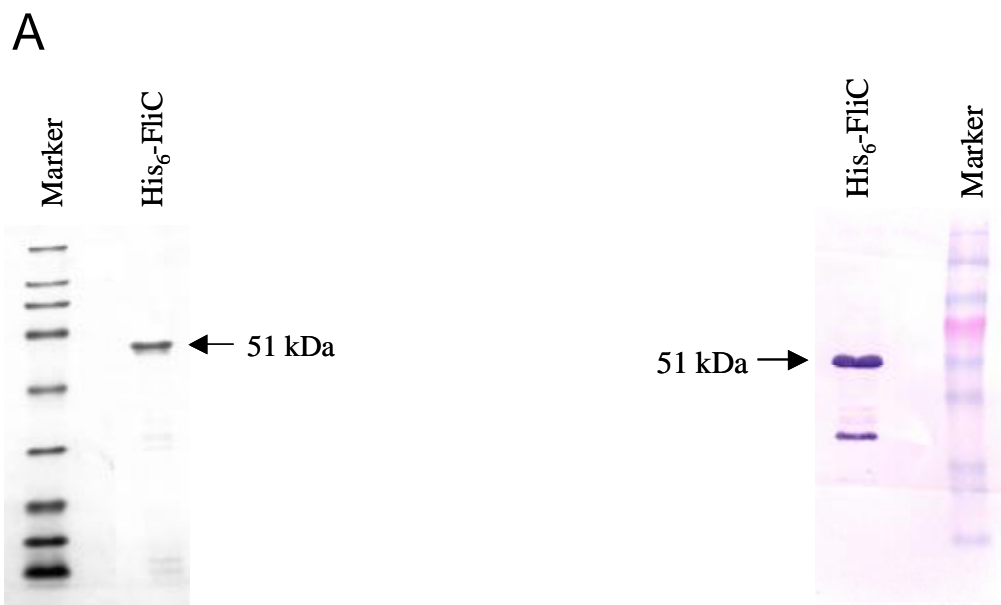


Figure 4.30 SDS-PAGE and Western blot analysis of His₆-FliC preparation. The *fliC* gene was cloned from STEC 98NK2 and FliC purified as described in Section 2.24. The His₆-FliC preparation was analysed by SDS-PAGE and (A) Coomassie blue staining (Section 2.12.2) or (B) Western blotting (Section 2.12.4) with H21 antiserum. The ~51-kDa His₆-FliC protein band is indicated by an arrow. The markers are (A) BioRad broad range molecular size markers (sizes 200, 112.5, 97.4, 66.2, 45, 31, 21.5, 14.4, and 6.5 kDa) and (B) BenchMark pre-stained protein ladder (sizes 172.0, 110.2, 79.0, 62.4 [pink], 48.0, 36.6, 24.5, 19.0, 13.5 and 5.3 kDa).

from 10 ng to 1 μg per ml. As shown in Fig. 4.31A, HCT-8 cells incubated with 1 $\mu\text{g}/\text{ml}$ or 100 ng/ml His₆-FliC produced IL-8 and MIP-2 α mRNA in a dose-dependent fashion. At 1 h, with 1 $\mu\text{g}/\text{ml}$ and 100 ng/ml His₆-FliC, the levels of IL-8 and MIP-2 α mRNA were comparable to that elicited by infection with 98NK2. However, treatment with 10 ng/ml His₆-FliC resulted in a slightly reduced IL-8 and MIP-2 α response compared to 98NK2. For IL-8 protein (Fig. 4.31B), stimulation of cells with His₆-FliC at 1 $\mu\text{g}/\text{ml}$, 100 ng/ml and 10 ng/ml yielded $3,650 \pm 250$, $4,900 \pm 10$ and $3,550 \pm 250$ pg/ml IL-8, respectively, and these levels were significantly different from that in unstimulated control cell supernatants (< 100 pg/ml IL-8; $P < 0.001$) (Fig. 4.31B). At these His₆-FliC doses, the levels of IL-8 were similar to that elicited by infection with 98NK2 ($6,100 \pm 200$ pg/ml IL-8; $P > 0.05$). These data indicate that

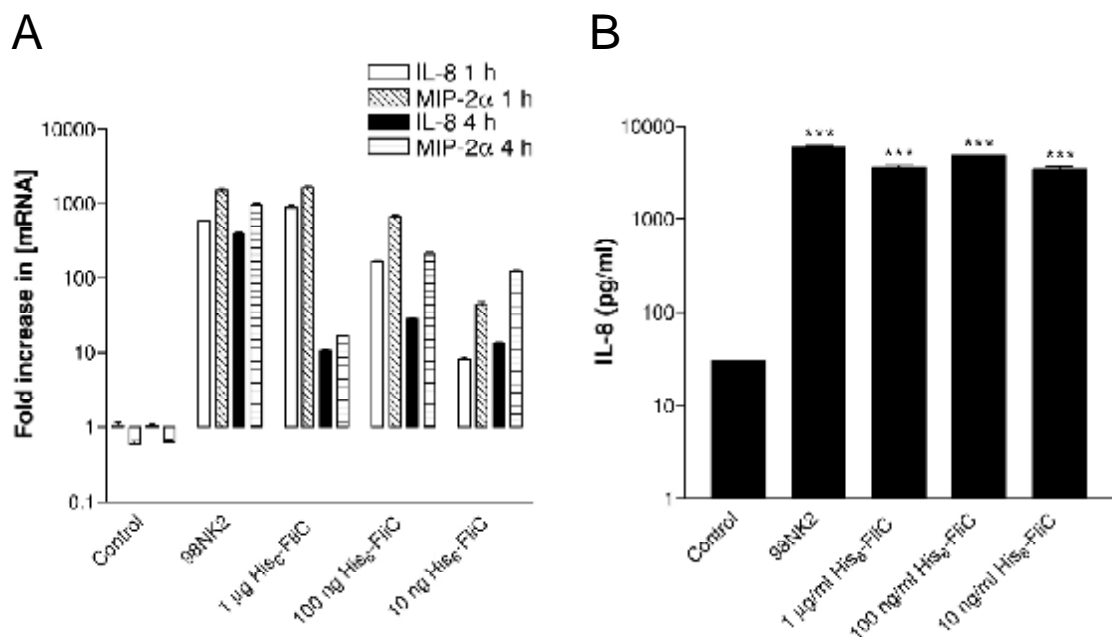


Figure 4.31 Induction of IL-8 and MIP-2 α mRNA and IL-8 protein in HCT-8 cells treated with His₆-FliC. HCT-8 cells were stimulated with His₆-FliC at the indicated concentrations or with 3×10^7 CFU/ml 98NK2. (A) At 1 or 4 h, total RNA was extracted from cells (Section 2.12.1) and IL-8 and MIP-2 α mRNA was quantitated by real-time RT-PCR (Section 2.13.2). Results are expressed as the fold increase in [mRNA] relative to levels at 0 h, and data shown are the means \pm SD for triplicate assays. (B) At 4 h, supernatants were collected and assayed for IL-8 by ELISA (Section 2.20). Data shown are the means \pm SEM from two experiments. Significant differences relative to untreated control cells are indicated as follows: ***, $P < 0.001$.

His₆-FliC alone is capable of inducing chemokine production.

Initial studies with 98NK2 (Section 3.2.2.1) showed an upregulation of IL-8, MIP-2 α and MIP-2 β mRNA at 1 and 4 h, and an upregulation of ENA-78 at 4 h only, in HCT-8 cells. It was therefore of interest to determine whether His₆-FliC is also able to induce chemokines other than IL-8 and MIP-2 α in a manner similar to that observed with 98NK2-infected HCT-8 cells. In addition to the upregulation of IL-8 and MIP-2 α mRNA, 100 ng/ml His₆-FliC induced a 324- and 256- fold induction of MIP-2 β at 1 and 4 h, respectively, and a 7-fold upregulation of ENA-78 at 4 h only (Fig. 4.32). However, no induction of GCP-2 or MGSA was observed at either time point. These data are similar to that observed with HCT-8 cells infected with wild-type 98NK2 (Fig. 3.2), and imply that the induction of all of these CXC chemokines in 98NK2 is mediated largely by flagellin (FliC).

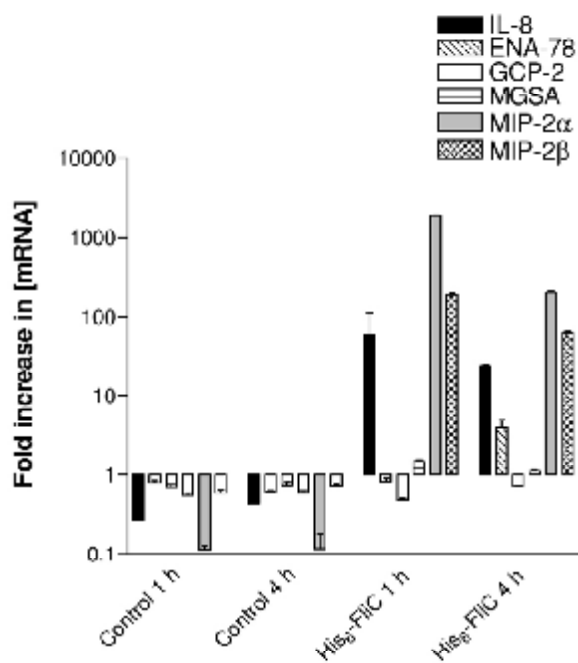


Figure 4.32 Induction of CXC chemokine mRNA in HCT-8 cells treated with His₆-FliC. HCT-8 cells were stimulated with 100 ng/ml His₆-FliC. At 1 or 4 h, total RNA was extracted from cells (Section 2.12.1) and chemokine mRNA was quantitated by real-time RT-PCR (Section 2.13.2). Results are expressed as the fold increase in [mRNA] relative to levels at 0 h, and data shown are the means \pm SD for triplicate assays.

4.3 Discussion

In the previous chapter the capacity of various STEC strains to elicit CXC chemokine responses in a human colonic epithelial cell line (HCT-8) was examined, because these chemokines are potent PMN chemoattractants. Infection of HCT-8 cells with LEE-negative STEC strains, in general, produced much higher and earlier induction of IL-8 and MIP-2 α mRNA, and IL-8 protein, than the LEE-positive STEC strains. In this chapter the STEC factor(s) responsible for these differences in HCT-8 cell responses were determined.

Since there were significant differences in the responses of LEE-negative and LEE-positive STEC isolates, the role of the LEE locus in chemokine production was investigated. However, analysis of an in-frame *eae* deletion mutant of EDL933, or EDL933 derivatives over-expressing wild-type or defective copies of the LEE regulatory gene *ler*, demonstrated that the marked difference in chemokine induction elicited by LEE-positive versus LEE-negative STEC was not directly related to their capacity to form A/E lesions, or the level of expression of intimin and other LEE-encoded genes. Experiments with plasmid-cured derivatives of EDL933 and 98NK2 also demonstrated that genes encoded on the large virulence plasmids of these STEC have no impact upon chemokine induction. Differences in LPS O antigen serogroup also do not account for the observed difference, since purified O113 and O157 LPS did not significantly induce chemokine production in HCT-8 cells. Similarly, *E. coli* K-12 clones expressing either O111 (Bastin *et al.*, 1991) or O113 LPS (Paton and Paton, 1999), derived from strains exhibiting low and high chemokine responses, respectively, elicited similar responses to their respective *E. coli* K-12 host strain.

Stx1 and Stx2 have previously been shown to induce CXC chemokine production in intestinal epithelial cells as well as in endothelial cells (Thorpe *et al.*, 1999; Yamasaki *et al.*, 1999; Thorpe *et al.*, 2001; Zoja *et al.*, 2002). However, the differences in responses elicited by the various STEC strains examined in this study were unrelated to the type or amount of Stx produced. Moreover, IL-8 and MIP-2 α mRNA levels and IL-8 protein levels elicited by infection of HCT-8 cells with 98NK2 were significantly higher than those induced by treatment of cells with purified Stx2 at a concentration comparable to that present in 98NK2-infected cultures (98NK2 produces Stx2 only; Paton *et al.*, 1999). Deletion mutagenesis of

the single *stx₂* gene in 98NK2 also had a negligible impact upon IL-8 and MIP-2 α responses in HCT-8 cells infected with the mutant, compared with those infected with the wild-type strain. A recent study has also shown that an O157:H7 STEC strain and an otherwise isogenic *stx*-negative derivative induced similar levels of IL-8 secretion by Caco-2 cells (Berin *et al.*, 2002).

Flagellin from a variety of pathogens, including *Salmonella* (Gewirtz *et al.*, 2000; Gewirtz *et al.*, 2001a), EAaggEC (Donnelly and Steiner, 2002; Steiner *et al.*, 2000) and EPEC (Zhou *et al.*, 2003), are known to induce inflammatory responses in epithelial cells, and Berin *et al.* (2002) demonstrated that much of the IL-8 response elicited by the O157:H7 STEC strain studied was attributable to the presence of H7 flagellin. Interestingly, four of the LEE-negative strains used in this study, all of which were isolated from patients with serious disease, belonged to flagellar type H21. Furthermore, treatment of HCT-8 cells with low doses of H21 flagellin elicited CXC chemokine responses comparable to those achieved by infection with the STEC strains themselves. Semi-quantitative Western blot analysis confirmed that the concentrations of purified H21 flagellin used in these experiments were comparable to the total amounts of flagellin present in the STEC-infected cell cultures. Deletion mutagenesis of the *fliC* gene in strain 98NK2 also resulted in a massive reduction in CXC chemokine responses of infected HCT-8 cells. However, stimulation of HCT-8 cells with a 98NK2 double mutant lacking both *stx₂* and *fliC*, had the same response as cells stimulated with the single *fliC* deletion mutant. Thus, these findings unequivocally demonstrate that the bulk of the CXC chemokine responses elicited by infection of intestinal epithelial cells with strain 98NK2 can be attributed to its H21 flagella.

Interestingly, two of the non-H21 STEC strains examined in this study (the LEE-negative O91:H7 strain 95HE4 and the LEE-positive O26:H11 strain 95ZG1) elicited significant CXC chemokine responses. Flagella isolated from these strains also elicited similar responses from HCT-8 cells, and the H7 flagella preparation from 95HE4 was as potent as H21 flagella. However, flagella prepared from strain 95ZG1 was a weaker chemokine inducer. The N- and C-terminal portions of flagellin are highly conserved, while the central domain is hypervariable. Thus, comparison of deduced amino acid sequences of *fliC* genes from these and other STEC strains may enable localisation of critical pro-

inflammatory domains. Interestingly, a previous study also found that LEE-negative STEC strains are capable of inducing higher IL-8 responses in T84 cells than the LEE-positive STEC strains that were tested, but the LEE-negative strains studied were all reported to be H⁻ (Hurley *et al.*, 2001). It is, therefore, possible that additional LEE-negative STEC factors are also capable of inducing CXC chemokine responses. However, an alternative explanation could be that a non-typeable flagellin was actually produced by these STEC strains under the conditions used in the assay.

Three of the four LEE-positive strains tested in this study elicited poor CXC chemokine responses, and this correlated with an apparent inability to produce flagella. These STEC strains were the O111:H⁻ strain 95NR1, and the O157:H⁻ strain 95SF2, both of which were isolated from patients with HUS, as well as the prototype O157:H7 STEC strain EDL933. The poor capacity of EDL933 to elicit CXC chemokine responses appears to be due to a defect in H7 flagellin expression, in spite of its having an intact *fliC* gene (Appendix III; GenBank accession number NC_002655). This defect is unlikely to be due to a recently introduced mutation, as its plasmid-cured derivative strain EDL933-Cu, which was constructed in 1987 (Tzipori *et al.*, 1987), also elicits similarly weak CXC chemokine responses. Three other O157:H7 clinical isolates in our collection were subsequently tested, but these induced CXC chemokine responses in HCT-8 cells similar to those seen for strain 98NK2. Likewise, deletion mutagenesis of the *fliC* gene from O113:H21 strain 97MW1 and O157:H7 strain 86-24 largely abrogated any IL-8 response elicited by these strains, suggesting that flagella are responsible for the major portion of IL-8 and MIP-2 α mRNA, and IL-8 protein responses, from a diverse range of STEC isolates, including both LEE-positive and LEE-negative strains.

The His₆-tagged flagellin from 98NK2 was then tested for its ability to induce chemokines in HCT-8 cells. His₆-FliC was capable of inducing a range of chemokines (IL-8, MIP-2 α , MIP-2 β and ENA-78) to a similar extent as was demonstrated for infection of HCT-8 cells with wild-type 98NK2 in Section 3.2.2.1. This result confirms the capacity of flagella, and flagellin (FliC), to induce the majority of the CXC chemokine responses observed in STEC-infected HCT-8 cells. In the next chapter, the pathways of inflammation in response to H21 flagellin are investigated.

The work described in this chapter demonstrates that flagellin appears to be the most important determinant affecting the high and early chemokine production seen in HCT-8 cells infected with the STEC strains used in this study. H21 is a particularly potent type, as is H7, but the relative potency of flagellins produced by other common STEC serotypes remains to be determined. Deletion of *fliC* from STEC strains 98NK2, 97MW1 and 86-24 completely abrogated the high and early chemokine responses observed in cells infected with their respective wild-type strains. Also purified His₆-FliC, as opposed to whole flagella, is capable of inducing these chemokine responses. Given the important role postulated for PMNs in the translocation of Stx across the intestinal epithelium and in transport of Stx to remote tissues, chemokine induction by flagellin may contribute significantly to the pathogenesis of STEC disease. Moreover, the increased intestinal inflammation may cause significant local damage. These effects may be particularly important for LEE-negative STEC strains, as they might compensate in part for their inability to produce the A/E lesions characteristic of LEE-positive STEC strains.

CHAPTER 5: The Role of Toll-like Receptor 5 and the Mitogen Activating Protein Kinases in the Inflammatory Response to H21 Flagellin

5.1 Introduction

In Chapter 4, it was shown that flagellin was the most significant mediator of CXC chemokine induction in HCT-8 cells in response to infection with STEC. Recognition of flagellin is part of the innate immune response for pathogen detection and is mediated by Toll-like receptor 5 (TLR5) (Hayashi *et al.*, 2001; Vasselon and Detmers, 2002). The Toll-like receptors are a family of evolutionarily conserved receptors found in all vertebrates (including birds, reptiles and mammals) (Aderem and Ulevitch, 2000; Armant and Fenton, 2002). TLRs function in innate immunity via recognition of conserved pathogen-associated molecular patterns (PAMPs) that are expressed on infectious agents, but not on host cells (Aderem and Ulevitch, 2000; Armant and Fenton, 2002).

The human TLR family comprises at least ten characterised homologues of the *Drosophila* Toll protein, which are important in the innate recognition of pathogens, and accordingly, the human Toll homologues were named TLR1 to TLR10 (Aderem and Ulevitch, 2000; Armant and Fenton, 2002). A wide variety of bacterial components are responsible for eliciting innate immune responses, including LPS, peptidoglycan, lipoproteins, lipoteichoic acid, flagellin and unmethylated CpG DNA among others, and are recognised by pattern recognition receptors (PRRs), which include the TLRs (Aderem and Ulevitch, 2000; Armant and Fenton, 2002). The PRRs recognising these PAMPs are

summarised in Table 5.1. TLRs are also responsible for the detection of yeast and viral pathogens (Aderem and Ulevitch, 2000; Armant and Fenton, 2002). The TLRs are transmembrane receptors that are distinguished by their extracellular N-terminal leucine rich repeat (LRR) domain, which is responsible for ligand binding (Aderem and Ulevitch, 2000; Armant and Fenton, 2002). The intracellular C-terminal domain is highly conserved amongst all TLRs and has homology to the IL-1 receptor, and is therefore named the Toll/interleukin-1 receptor (TIR) domain (Aderem and Ulevitch, 2000; Armant and Fenton, 2002).

All TLRs signal through the adaptor protein myeloid differentiation primary response gene 88 (MyD88), and result in the activation of both the mitogen activated protein kinases (MAPK) and NF- κ B (Fig. 5.1) (Aderem and Ulevitch, 2000; Anderson, 2000; Akira *et al.*,

Table 5.1 Pathogen associated molecular patterns (PAMPs) and their Toll-like receptor(s). Modified from Aderem and Ulevitch (2000) and Armant and Fenton (2002).

PAMP	Origin of PAMP	Toll-like receptor(s)
ds RNA	Viruses	TLR3
Flagellin	Gram-negative and -positive bacteria	TLR5
F protein	Respiratory syncytial virus (RSV)	TLR4
Heat shock proteins	Prokaryotes and eukaryotes	TLR4
Lipoarabinomannan	Mycobacteria	TLR2
Lipopolysaccharide	Gram-negative bacteria	TLR4, TLR2
Lipopeptide	Gram-positive bacteria	TLR1
Lipoproteins	Eubacteria	TLR2
Lipoteichoic acid	Gram-positive bacteria	TLR2, TLR4
Peptidoglycan	Most bacteria	TLR2
Unmethylated CpG DNA	Many microbial pathogens	TLR9
Zymosan	Yeast	TLR2

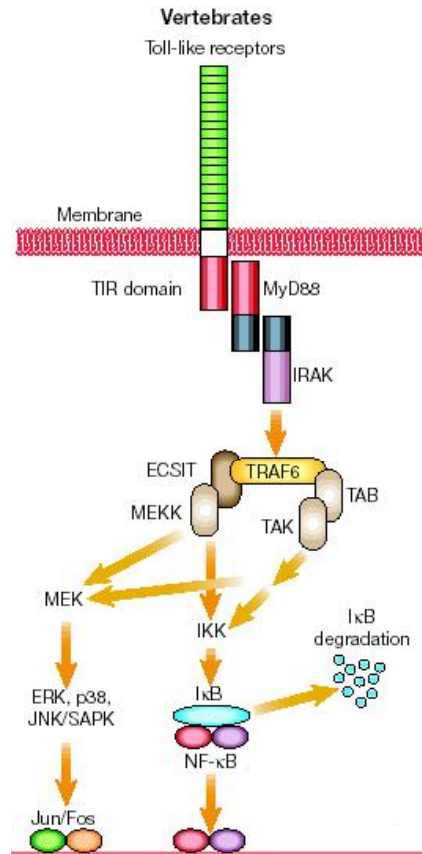


Figure 5.1 Signalling pathways of Toll-like receptors in vertebrates. The TIR domain of all TLRs signal through the adaptor protein MyD88, which activates the serine kinase IRAK, and another adaptor protein, TRAF6. Transforming growth factor- β -activated kinase-1 (TAK-1) and MAPK kinase kinase-1 (MEKK-1) are then involved in the activation of the transcription factor NF- κ B through the activation of I κ B kinases, and the activation of the AP-1 transcription family members Jun and Fos through the MAPKs (ERK, p38 and JNK). Modified from Aderem and Ulevitch (2000).

2001; Armant and Fenton, 2002). TIR binds to a homologous domain in the adaptor protein MyD88, which contains a death domain, and then interacts with the death domain in the IL-1 receptor-associated kinase (IRAK) (Aderem and Ulevitch, 2000; Armant and Fenton, 2002). This interaction leads to the subsequent activation of the TNF-receptor-associated factor 6 (TRAF6) and the downstream activation of the MAPK and NF- κ B pathways (Aderem and Ulevitch, 2000; Armant and Fenton, 2002). The MAPKs include the extracellular regulated kinases (ERK)-1 and -2, and the stress activated protein kinases (SAPKs), p38 and c-Jun N-terminal kinases (JNK)-1 and -2 (Aderem and Ulevitch, 2000; Akira *et al.*, 2001). Activation of either the MAPK or NF- κ B pathway results in the upregulation of several

cytokines, including IL-8 (Yang *et al.*, 1997; Aderem and Ulevitch, 2000; Davis, 2000; Akira *et al.*, 2001), and may also lead to the upregulation of several receptors including ICAM-1 and VCAM-1 on endothelial cells (Maaser *et al.*, 2001), which are important for the recruitment and migration of PMNs (Nasreen *et al.*, 2001).

Several bacterial pathogens including STEC, EIEC and EPEC, have been shown to lead to the activation of MAPKs and/or the transcription factors NF- κ B and AP-1 *in vitro* (Savkovic *et al.*, 1997; Elewaut *et al.*, 1999; de Grado *et al.*, 2001; Dahan *et al.*, 2002). Stx1 and Stx2 are also capable of inducing MAPK and/or NF- κ B activation *in vitro* (Sakiri *et al.*, 1998; Thorpe *et al.*, 1999; Ikeda *et al.*, 2000; Cameron *et al.*, 2002; Foster and Tesh, 2002; Zoja *et al.*, 2002; Smith *et al.*, 2003b). Although the Stxs are potent protein synthesis inhibitors (Section 1.4.1), both Stx1 and Stx2 have been shown to upregulate the secretion of IL-8 mRNA and protein in HCT-8 cells via the ribotoxic stress response and by inducing *c-jun* mRNA and p38 MAPK (Thorpe *et al.*, 1999). Stx1 has also been shown to superinduce the mRNAs for other CXC chemokines including ENA-78, MGSA, MIP-2 α and MIP-2 β in HCT-8 cells (Thorpe *et al.*, 2001). The A subunit of Stx is required for this effect which is thought to be mediated, at least in part, through enhanced chemokine mRNA stability (Thorpe *et al.*, 1999; Thorpe *et al.*, 2001). These data suggest that the NF- κ B and MAPK pathways may play an important role in the inflammatory responses to pathogens and/or Stx and may be important in the upregulation of these signalling pathways.

Flagellin purified from EPEC and *Salmonella* spp. has also been shown to upregulate the MAPK or NF- κ B pathways (Eaves-Pyles *et al.*, 2001a; Eaves-Pyles *et al.*, 2001b; Gewirtz *et al.*, 2001a; Zeng *et al.*, 2003; Zhou *et al.*, 2003). The p38, ERK-1 and -2 and JNK-1 and -2 pathways have been shown to be upregulated in response to the flagellin from either *Salmonella* or EPEC (Yu *et al.*, 2003; Zhou *et al.*, 2003). Recently a role for flagellin has also been implicated in the pro-inflammatory responses of STEC (Berin *et al.*, 2002). Therefore, flagellin may play an important role in the upregulation of chemokines including IL-8 via the MAPK pathway. In this chapter the role of TLR5 and the MAPK pathway in the inflammatory responses to the H21 flagellin from 98NK2 was investigated.

5.2 Results

5.2.1 Superinduction of chemokine responses to flagellin with Stx2

The ability of Stx2 to superinduce chemokine responses in the presence of flagellin was examined in HCT-8 cells. HCT-8 cells were incubated with 100 ng/ml of either His₆-FliC or heat-inactivated (HI) His₆-FliC for 1 or 4 h, in the presence or absence of 1 μg/ml Stx2. Stx2 was added to the cells because the level of Stx produced by STEC *in vitro* (approximately 29 to 150 ng/ml) (Section 4.2.3.1) has been reported not to resemble the levels found in the intestine and/or stools during infection, which may be up to mg/ml range (Thorpe *et al.*, 2001). Thus, levels of Stx2 produced *in vivo* may be important mediators of chemokine induction. At 1 h no observable differences were observed in the IL-8 or MIP-2α mRNA levels under any of the conditions in the presence of Stx2 alone (Fig. 5.2). His₆-FliC induced

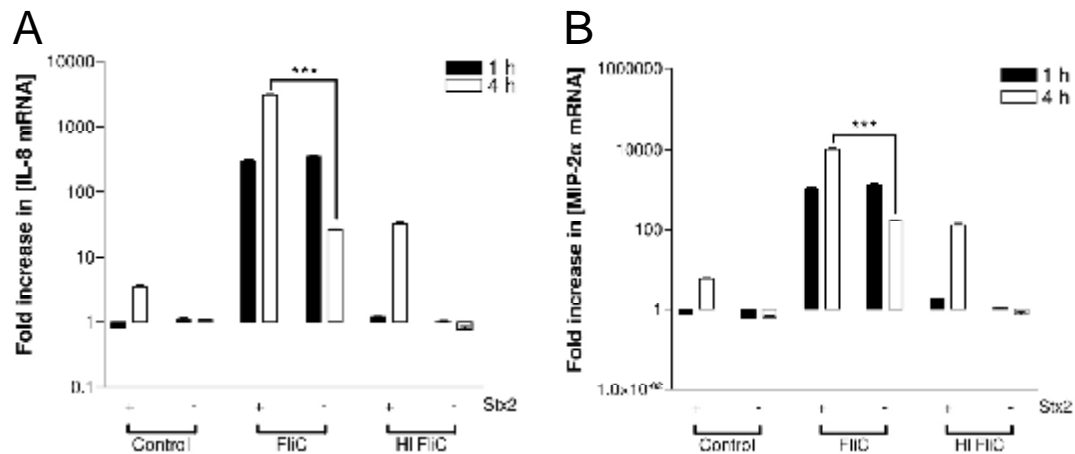


Figure 5.2 Superinduction of flagellin-mediated IL-8 and MIP-2a mRNA in HCT-8 cells by Stx2. HCT-8 cells were treated with 100 ng/ml His₆-FliC (FliC) or HI His₆-FliC (HI FliC), with or without 1 μg/ml Stx2, for 1 or 4 h, then total RNA was extracted (Section 2.12.1) and (A) IL-8 and (B) MIP-2α mRNA was quantitated by real-time RT-PCR (Section 2.13.2). Results are expressed as the fold increase in [mRNA] relative to levels at 0 h, and data shown are the means ± SD for triplicate assays. Significant differences relative to and His₆-FliC-treated and His₆-FliC + Stx2-treated cells are indicated as follows: ***, $P < 0.0001$ (Student's *t* test).

a 345- and 1,361-fold upregulation of IL-8 and MIP-2 α , respectively at 1 h, but there was no significant additional effect of the addition of Stx2, which induced a 344- and 1,043-fold upregulation of IL-8 and MIP-2 α , respectively (similar to His₆-FliC alone).

However, at 4 h, superinduction of IL-8 and MIP-2 α mRNA was observed in His₆-FliC-treated cells in the presence of Stx2 (Fig. 5.2). At this time, Stx2 alone induced a modest 4- and 6-fold upregulation of IL-8 and MIP-2 α , respectively, and His₆-FliC alone induced a 26- and 165-fold upregulation of IL-8 and MIP-2 α , respectively. However, in the presence of Stx2 and His₆-FliC, IL-8 and MIP-2 α mRNA was upregulated by 3,074- and 10,226-fold, respectively ($P < 0.0001$ compared to His₆-FliC alone). With HI His₆-FliC, there was no significant effect of the addition of Stx2 at either 1 or 4 h ($P > 0.05$). Therefore, Stx2 is able to superinduce both IL-8 and MIP-2 α mRNA in the presence of flagellin.

5.2.2 Activation of p38 MAPK in response to 98NK2 and flagellin

The ability of His₆-FliC, wild-type 98NK2 and 98NK2 Δ *fliC* to activate p38 in response to H21 flagellin was determined by p38 immunoprecipitation kinase assays. Initially, HCT-8 cells were stimulated with 98NK2, 98NK2 Δ *fliC* or 100 ng/ml His₆-FliC for 1 or 4 h. After stimulation, whole cell extracts (Section 2.21) were assayed for p38 MAPK activity (Section 2.22) by detection of p38-induced phosphorylation of ATF-2 at Thr71 by Western blotting (Section 2.14.4). As shown in Fig. 5.3A, p38 activation can be observed 1 h after stimulation with 98NK2 or His₆-FliC, but no activation is seen with control cells or cells infected with the 98NK2 Δ *fliC* strain. At 4 h post-infection, some p38 activity is still evident in 98NK2- and His₆-FliC-treated cells, but at this time point some activation of p38 can also be seen with the 98NK2 Δ *fliC* mutant, possibly due to the production of Stx2, which can also activate p38 at the concentrations present *in vitro* at 4 h (Fig. 5.3B) (Smith *et al.*, 2003b). These data clearly demonstrate the activation of p38 MAPK in response to H21 flagellin by HCT-8 cells.

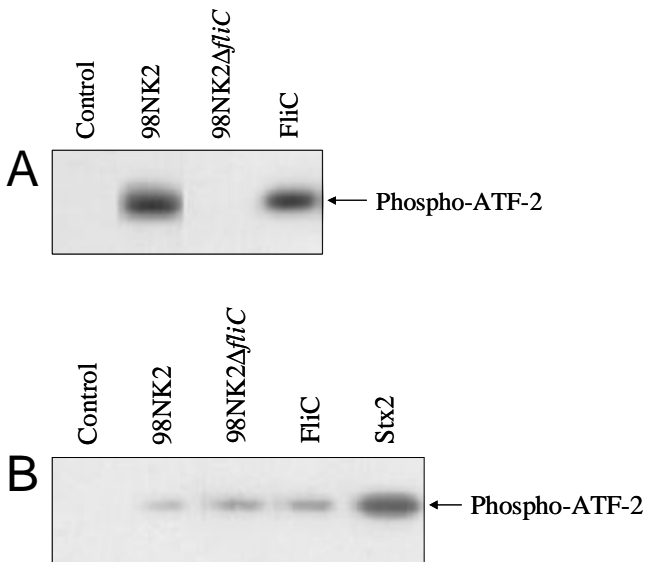


Figure 5.3 Activation of p38 MAPK by flagellin and 98NK2. HCT-8 cells were stimulated with 3×10^7 CFU/ml of 98NK2 or 98NK2 Δ fliC, 100 ng/ml His₆-FliC (FliC) or 1 μ g/ml Stx2 (4 h only). At (A) 1 h or (B) 4 h, whole-cell extracts (Section 2.21) were prepared and 200 μ g of protein extract was used to perform an IP kinase assay for p38 MAPK (Section 2.22). Phosphorylation of ATF-2 at Thr71 was then measured by Western blot using Phospho-ATF-2 (Thr71) antibody (Section 2.14.4).

5.2.3 Effect of p38 MAPK inhibition on IL-8 mRNA and protein

Previously, it has been shown that the activation of chemokine mRNA, including IL-8, by p38 MAPK occurs via a post-transcriptional mechanism, possibly involving the regulation of the poly-(A) tail length and increased stabilisation of mRNA (Josse *et al.*, 2001; Clark *et al.*, 2003). As shown in Fig. 5.4, there is little difference between the IL-8 and MIP-2 α mRNA levels in HCT-8 cells stimulated with His₆-FliC in the presence of the p38 inhibitor SB203580 (SB) (Cuenda *et al.*, 1995) or the carrier DMSO, at either 1 or 4 h. At 1 h, His₆-FliC induced a 108-fold increase in IL-8 mRNA in the presence of DMSO, whereas in the presence of SB, stimulation was only 50-fold. However, at 4 h, this trend was reversed; His₆-FliC treatment resulted in a 6- and 13-fold stimulation of IL-8 mRNA in the presence of DMSO and SB, respectively (Fig. 5.4). Thus, there appears to be no consistent effect of SB on IL-8 mRNA induction.

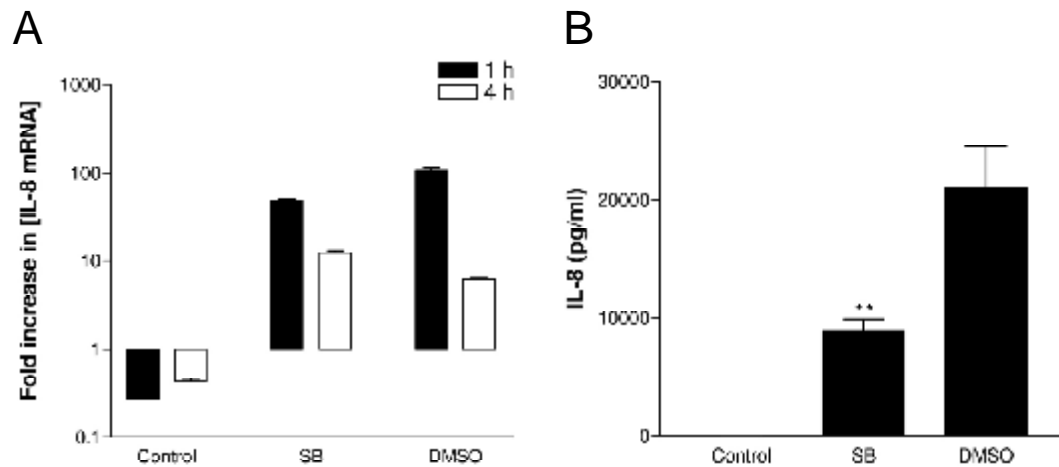


Figure 5.4 Effect of p38 MAPK inhibition on IL-8 mRNA and protein induction by flagellin. HCT-8 cells were stimulated with 100 ng/ml His₆-FliC in the presence of either 10 μ M SB203580 (SB; p38 inhibitor) or an equal volume of carrier (DMSO). **(A)** At 1 or 4 h, total RNA was extracted from cells (Section 2.12.1) and IL-8 mRNA was quantitated by real-time RT-PCR (Section 2.13.2). Results are expressed as the fold increase in [IL-8 mRNA] relative to levels at 0 h, and data are shown as the means \pm SD for triplicate assays. **(B)** At 4h, supernatants were collected and assayed for IL-8 by ELISA (Section 2.20). Data shown are the means \pm SEM from two experiments. Significant differences relative to DMSO- and His₆-FliC-treated cells are indicated as follows: **, $P < 0.015$ (Student's t test).

However, with IL-8 protein, DMSO- and His₆-FliC-stimulated HCT-8 cells secreted 20,990 \pm 3,492 pg/ml IL-8 whereas His₆-FliC- and SB-treated cells secreted only 8,891 \pm 996 pg/ml IL-8 (a 57% reduction in IL-8 secretion compared to DMSO-treated cells; $P < 0.015$). Thus, levels of IL-8 mRNA do not appear to correlate with the differences observed in IL-8 protein secretion in the presence of the inhibitor SB, suggesting that the reduction in IL-8 protein occurs through a post-transcriptional mechanism.

5.2.4 Effect of MAPK inhibition on IL-8 secretion by HCT-8 cells in response to flagellin

It has recently been demonstrated that ERK-1 and -2 and JNK-1 and -2 are phosphorylated in response to H6 flagellin from EPEC E2348/69 and also that inhibition of p38 and ERK-1 and -2 blocks the IL-8 response to flagellin in T84 cells (Zhou *et al.*, 2003).

ERK-1 and -2 and p38 are also phosphorylated in response to *Salmonella* flagellin, but JNK-1 and -2 are not essential for IL-8 induction (Yu *et al.*, 2003). The p38 and ERK-1 and -2 pathways have also been shown to play a role in the response to infection with wild-type STEC O157:H7 in Caco-2 cells (Berin *et al.*, 2002). The role of all three of the MAPK pathways in the upregulation of IL-8 in response to H21 flagellin from 98NK2 was therefore examined in HCT-8 cells.

To determine the role of the MAPKs in IL-8 induction in response to flagellin, the MAPK inhibitors SB (p38 inhibitor), PD98059 (PD) (mitogen activated protein kinase kinase [MEK]-1 and -2 inhibitor) and SP600125 (SP) (JNK-1 and -2 inhibitor) were used (Alessi *et al.*, 1995; Cuenda *et al.*, 1995; Bennett *et al.*, 2001). MEK-1 and -2 is the upstream kinase that activates ERK-1 and -2 (Fig. 5.1). HCT-8 cells were pre-incubated for 60–90 min with either 10 μ M SB, 50 μ M PD or 10 μ M SP alone, or with combinations of two or more inhibitors, or with DMSO (vehicle only control), before the addition of 100 ng/ml His₆-FliC. A negative control containing all three inhibitors (and no His₆-FliC) was also used to determine the effects of the inhibitors on HCT-8 cells. The inhibitors were also added individually to non-stimulated cells and showed no adverse effects on cell viability and no IL-8 induction in HCT-8 cells (< 60 pg/ml IL-8; result not shown).

As shown in Fig. 5.5, HCT-8 cells stimulated with 100 ng/ml His₆-FliC in the presence DMSO (without inhibitors) secreted high levels of IL-8 protein (26,800 \pm 900 pg/ml IL-8). In comparison, stimulation of HCT-8 cells with His₆-FliC in the presence of either SB, SP or PD resulted in a reduced secretion of IL-8 (10,500 \pm 700 pg/ml [61% reduction; $P < 0.005$], 13,350 \pm 2,650 pg/ml [50% reduction; $P < 0.05$] and 16,200 \pm 2,100 pg/ml IL-8 [40% reduction; $P < 0.05$], respectively). Similarly, incubation with His₆-FliC and a combination of any of two inhibitors further decreased the amount of IL-8 secreted in HCT-8 supernatants (range 2,935 \pm 1,365 pg/ml IL-8 for SB + SP to 6,800 \pm 1,000 pg/ml IL-8 for SP + PD), which is a reduction of between 75 and 89% compared to DMSO- and His₆-FliC-treated cells ($P < 0.005$) (Fig. 5.5). The combination of all three inhibitors had the greatest inhibition on His₆-FliC-induced IL-8 protein, which was only 2% of that secreted by DMSO- and His₆-FliC-treated cells (535 \pm 45 pg/ml IL-8; $P < 0.001$) (Fig. 5.5). These data strongly implicate

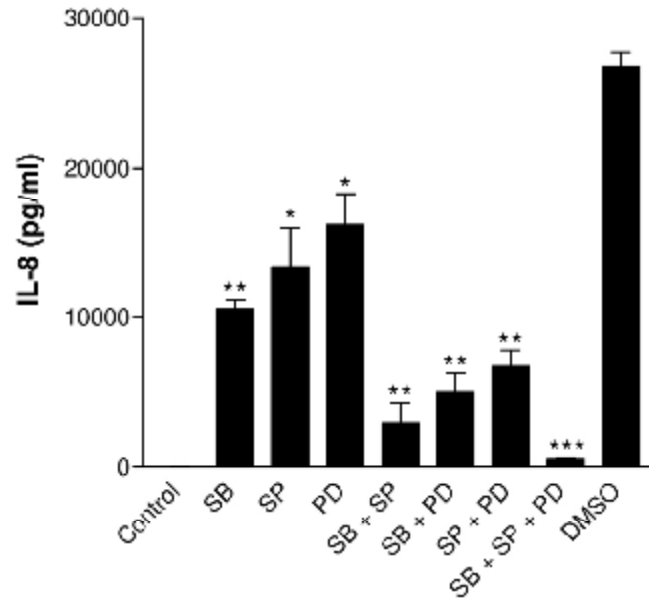


Figure 5.5 Inhibition of the MAPKs (p38, JNK-1 and -2 and ERK-1 and -2) reduces IL-8 secretion by HCT-8 cells in response to flagellin. HCT-8 cells were pre-incubated for 60 min with one or more of either 10 μ M SB203580 (SB) (p38 inhibitor), 50 μ M PD98059 (PD) (ERK-1 and -2 inhibitor), 10 μ M SP600125 (SP) (JNK-1 and -2 inhibitor) or with DMSO (positive control), before the addition of 100 ng/ml His₆-FliC. Control monolayers were incubated with all three inhibitors but without His₆-FliC (negative control). After 4 h, supernatants were collected and assayed for IL-8 by ELISA (Section 2.20). Data are the means \pm SEM from two experiments. Significant differences relative to DMSO- and His₆-FliC-treated cells are indicated as follows: *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.001$ (Student's t test).

the p38, ERK-1 and -2 and the JNK-1 and -2 pathways in the IL-8 response to flagellin by HCT-8 cells. This is the first time that the roles of all three MAPK pathways have been studied in cellular responses to flagellin stimulation.

5.2.5 Role of TLR5 in induction of p38 MAPK in response to flagellin

TLR5 is considered to be the primary TLR for flagellin recognition (Hayashi *et al.*, 2001). Since p38 is activated in response to H21 flagellin from STEC strains, the role of TLR5 in the activation of p38 was investigated. Initially, p38 activation in transfected MDCK cells stably expressing TLR5 or dominant-negative (DN) TLR5 (Table 2.3) was

investigated in response to His₆-FliC. TNF- α was used as a positive control for p38 stimulation. After 30 min incubation, p38 activity in cell extracts (Section 2.21) was analysed by detection of p38-induced phosphorylation of ATF-2 at Thr71 by Western blotting (Sections 2.22 and 2.14.4). As shown in Fig. 5.6, p38 MAPK activity was observed in TLR5-expressing MDCK cells in the presence of His₆-FliC. However p38 was not activated in DN TLR5 MDCK cells stimulated with His₆-FliC.

Activation of p38 in HeLa cells stably expressing either TLR5 or DN TLR5 (Table 2.3) were then investigated in response to 98NK2, 98NK2 Δ *fliC* and 1 μ g/ml of either His₆-FliC or HI His₆-FliC. After 30 min incubation, p38 activity in cell extracts (Section 2.21) was analysed by detection of p38-induced phosphorylation of ATF-2 at Thr71 by Western blotting (Sections 2.22 and 2.14.4). The results in Fig. 5.7 show an upregulation of p38 MAPK in TLR5-expressing HeLa cells stimulated with either 98NK2 or His₆-FliC, but not in 98NK2 Δ *fliC*- or HI His₆-FliC-treated cells. However, some activation of p38 was observed in the DN TLR5 expressing HeLa cells in response to His₆-FliC, perhaps due to basal level expression of native TLR5 by this cell line (Fig. 5.7). These data confirm the previous results with MDCK cells and show that H21 flagellin does indeed activate p38 MAPK through TLR5.

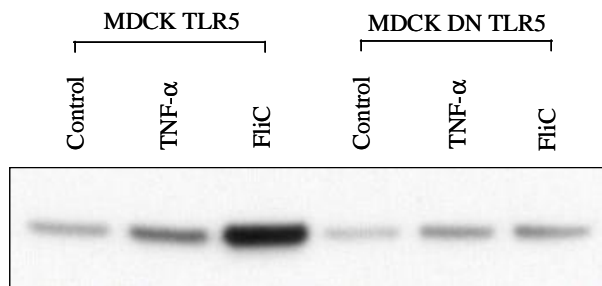


Figure 5.6 TLR5 mediates flagellin-induced p38 MAPK activation in MDCK cells. MDCK cells stably transfected with either TLR5 or DN TLR5 expressing plasmids were stimulated with 1 μ g/ml His₆-FliC (FliC) or 25 ng/ml TNF- α . After 30 min, whole-cell extracts were prepared (Section 2.21) and 200 μ g of protein extract was used to perform an IP kinase assay for p38 MAPK (Section 2.22). Phosphorylation of ATF-2 at Thr71 was then measured by Western blot using Phospho-ATF-2 (Thr71) antibody; Section 2.14.4).

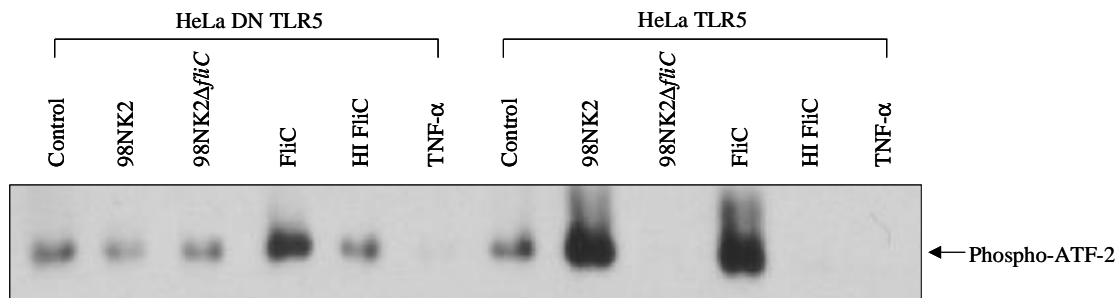


Figure 5.7 TLR5 mediates flagellin-induced p38 MAPK activation in HeLa cells. HeLa cells stably transfected with either DN TLR5 or TLR5 expressing plasmids were stimulated with 3×10^7 CFU/ml 98NK2 or 98NK2 Δ *fliC*, 1 μ g/ml His₆-FliC (FliC) or HI His₆-FliC (HI FliC), or 25 ng/ml TNF- α . After 30 min, whole-cell extracts were prepared (Section 2.21) and 200 μ g of protein extract was used to perform an IP kinase assay for p38 MAPK (Section 2.22). Phosphorylation of ATF-2 at Thr71 was then measured by Western blot using Phospho-ATF-2 (Thr71) antibody; Section 2.14.4).

5.2.6 Apical and basolateral secretion of IL-8 protein in polarised HCT-8 monolayers

In T84 cells, TLR5 is exclusively expressed on the basolateral surface of polarised monolayers (Gewirtz *et al.*, 2001a). In contrast, polarised human colon carcinoma epithelial (HCA-7) monolayers can respond to STEC applied to either the apical or the basolateral surface, suggesting that TLR5 is distributed on both surfaces, but secrete IL-8 only from the basolateral surface (Berin *et al.*, 2002). Polarised Caco-2 cells express TLR5 both apically and basolaterally *in vitro* (Sierro *et al.*, 2001). To date, no studies have determined the distribution of TLR5 on HCT-8 cells. To determine the directional secretion of IL-8 in response to wild-type STEC or flagellin, polarised HCT-8 monolayers grown on Transwell inserts (Section 2.16), were stimulated with either 100 ng/ml His₆-FliC or HI His₆-FliC, or with 98NK2 or 982NK2 Δ *fliC*, on either the apical (mucosal) or basolateral (serosal) surface. HCT-8 monolayers were found to attain a high TEER, which is a measure of passive ion permeability, and were only used when they obtained a minimum TEER of 1,000 Ω cm⁻² (Section 2.16.1).

When either His₆-FliC or wild-type 98NK2 was added to the apical surface of polarised HCT-8 monolayers, IL-8 was secreted both apically and basolaterally, but was slightly higher from the apical surface (Fig. 5.8A). The apical application of 98NK2 induced $8,046 \pm 2,327$ and $6,196 \pm 1,165$ pg/monolayer IL-8 apically and basolaterally, respectively. His₆-FliC stimulated $8,215 \pm 2,663$ and $7,190 \pm 1,136$ pg/monolayer IL-8 apically or basolaterally, respectively. All of these were significantly higher than that secreted by control cells (< 700 pg/monolayer IL-8; $P < 0.02$). The apical application of either HI His₆-FliC or 98NK2 Δ fliC did not induce the secretion of IL-8 over that observed in control monolayers (Fig. 5.8A).

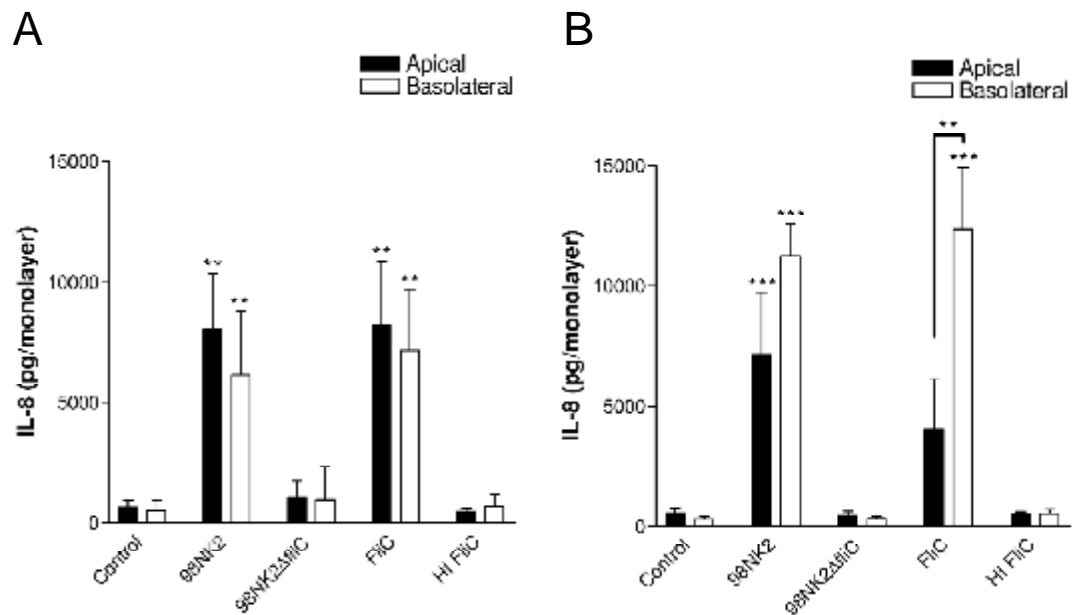


Figure 5.8 Apical or basolateral secretion of IL-8 in response to 98NK2 or flagellin. HCT-8 cells were grown on Transwell filters until they reached a minimum TEER of $1,000 \Omega \text{ cm}^{-2}$ before use (Section 2.16.1). Polarised HCT-8 monolayers were then stimulated with either 3×10^7 CFU/ml 98NK2 or 98NK2 Δ fliC or with 100 ng/ml of His₆-FliC (FliC) or HI His₆-FliC (HI FliC) applied either (A) apically or (B) basolaterally to the monolayer. At 4 h, supernatants were collected and assayed for IL-8 by ELISA (Section 2.20). Data are shown as the means \pm SEM from at least 5 wells. Data have been corrected for differences in the volumes of the upper and lower chambers of the Transwell, respectively, and are expressed as the total IL-8 (pg) secreted per monolayer. The significance of differences between IL-8 secretion relative to that of control cells is indicated as follows: **, $P < 0.02$; ***, $P < 0.005$ (Student's t test).

When 98NK2 or His₆-FliC was added to the basolateral surface of polarised HCT-8 monolayers, IL-8 was secreted both apically and basolaterally, but was generally higher from the basolateral surface ($P < 0.01$ for His₆-FliC-treated monolayers) (Fig. 5.8B). 98NK2 applied to the basolateral surface of polarised HCT-8 monolayers secreted $7,168 \pm 1,152$ and $11,240 \pm 1,341$ pg/monolayer IL-8 in the apical or basolateral direction, respectively ($P > 0.05$), whereas His₆-FliC stimulated $4,055 \pm 866$ and $12,350 \pm 2,363$ pg/monolayer IL-8 in the apical or basolateral direction, respectively ($P < 0.01$). However, monolayers treated with 98NK2 Δ fliC or HI His₆-FliC all secreted amounts of IL-8 similar to control monolayers (less than 600 pg/monolayer IL-8) in either direction. When either 98NK2 or His₆-FliC was applied to the basolateral surface of polarised HCT-8 monolayers there was a slightly higher secretion of IL-8 than when they were applied apically. Collectively these data suggest that polarised HCT-8 monolayers can secrete IL-8 in response to the application of flagellin (or wild-type STEC) to both the apical and basolateral surface. Unlike T84 and HCA-7 cells, this results in the secretion of IL-8 on both surfaces. However, secretion was generally higher on the surface of stimulation.

5.3 Discussion

In the previous chapter, the factor(s) responsible for CXC chemokine responses of HCT-8 cells to STEC were examined, and the responses were found to be mediated largely by flagellin. Wild-type STEC strains expressing flagellin, but not Δ fliC mutants thereof, were able to induce the production of chemokine mRNA and IL-8 protein in HCT-8 cells. Similarly, purified flagella and purified His₆-tagged H21 flagellin (FliC) from 98NK2 was also able to induce chemokine production. The innate recognition of flagellin is mediated by TLR5 (Hayashi *et al.*, 2001), and results in the activation of the NF- κ B and MAPK pathways, resulting in the upregulation of several cytokines, including IL-8 (Yang *et al.*, 1997; Aderem and Ulevitch, 2000; Davis, 2000; Akira *et al.*, 2001). Therefore, both TLR5 and the MAPK pathways are likely to be important in the inflammatory response to flagellin and the

upregulation of chemokines. In this chapter, the ability of H21 flagellin from 98NK2 to activate TLR5 and the MAPK pathways in intestinal epithelial cells was examined.

Phosphorylation of p38 in response to *Salmonella* flagellin and wild-type O157:H7 has been observed previously (Berin *et al.*, 2002; Yu *et al.*, 2003), but not in response to STEC flagellin. Both wild-type 98NK2 and purified His₆-FliC, but not 98NK2 Δ fliC or HI His₆-FliC, were able to induce p38 at 1 and 4 h. These data indicate that H21 flagellin alone is capable of activating p38 MAPK. In the presence of the specific p38 inhibitor (SB203580) a significant (61%) reduction was observed in levels of IL-8 protein, suggesting that the p38 MAPK pathway plays an important role in IL-8 induction in response to H21 flagellin from 98NK2. However, only very small differences were observed in mRNA concentration, suggesting that this may occur via a post-transcriptional mechanism, possibly by the regulation of poly-(A) tail length resulting in the increased stabilisation of mRNA (Josse *et al.*, 2001; Clark *et al.*, 2003). The data presented are consistent with previous studies that have shown that SB203580 is able to significantly decrease the amount of IL-8 protein secreted in response to both O157:H7 STEC strains (Berin *et al.*, 2002), and to H6 flagellin from EPEC E2348/69 (Zhou *et al.*, 2003), and that SB203580 affects flagellin-induced IL-8 induction by *Salmonella* via a post-transcriptional mechanism (Yu *et al.*, 2003).

The p38 and ERK-1 and -2 MAPKs are activated (phosphorylated) in response to *Salmonella* flagellin, but the major response was p38-mediated (Yu *et al.*, 2003). Another study demonstrated activation of ERK-1 and -2 and JNK-1 and -2 in response to H6 flagellin from EPEC E2348/69 (Zhou *et al.*, 2003). However, no study has looked directly at the role of the MAPK pathways in response to STEC flagellin, or of all three pathways simultaneously. The data presented here show that all three MAPK pathways are involved in the IL-8 response to H21 flagellin and that inhibition of any of the pathways resulted in a decrease in IL-8 secretion of between 40 and 61%. Furthermore, the inhibition of all three pathways resulted in a massive 98% reduction in IL-8 secretion compared to flagellin-treated cells. These data clearly demonstrate that the p38, ERK-1 and -2 and JNK-1 and -2 pathways are all involved in the IL-8 induction in response to H21 flagellin from STEC in HCT-8 cells.

Despite being protein synthesis inhibitors, Stx1 and Stx2 are able to superinduce the production of IL-8 mRNA and protein via the ribotoxic stress response, resulting in the

activation of *c-jun* mRNA and p38 MAPK (Iordanov *et al.*, 1997; Thorpe *et al.*, 1999). Stx1 is also able to superinduce other chemokine mRNAs in the presence of TNF- α , probably by enhanced stabilisation of chemokine mRNA (Thorpe *et al.*, 2001). Other protein synthesis inhibitors, including ricin (Iordanov *et al.*, 1997) and cycloheximide (Roger *et al.*, 1998), have been shown to induce JNK or IL-8 mRNA, respectively, by enhancing mRNA stabilisation. In the present study, Stx2 was also capable of superinducing both IL-8 and MIP-2 α mRNA in the presence of purified flagellin from 98NK2, at physiological levels. This may occur by a mechanism similar to that previously observed with Stx1, in an A-subunit dependent manner (Thorpe *et al.*, 1999; Thorpe *et al.*, 2001). This superinduction may be important during human infection, because levels of Stx present in the gut lumen may reach much higher levels than that produced *in vitro* by STEC strains. The mechanism(s) by which Stx and H21 flagellin synergise to induce large amounts of chemokine mRNA is unknown. Interestingly, there is significant redundancy in the MAPK activation profiles of these two stimuli: Stx can also induce all three MAPK pathways in intestinal epithelial cells, including ERKs (Smith *et al.*, 2003b; W. Smith and C. Thorpe, personal communication).

Flagellin is a PAMP that is recognised by TLR5 (Hayashi *et al.*, 2001). TLR5 is constitutively expressed on both human colonic microvascular endothelial cells (Maaser *et al.*, 2004) and colonic epithelial cells (Cario and Podolsky, 2000) *in vivo*. The innate recognition of *Salmonella* and EAEC O42 flagellins is thought to be mediated by binding of the conserved N- and C-terminal regions (Donnelly and Steiner, 2002; Murthy *et al.*, 2004) to the N-terminal extracellular domain of TLR5 (amino acids 386–407) (Mizel *et al.*, 2003). This latter region contains a putative leucine-rich repeat (Mizel *et al.*, 2003) and recognises and binds to the flagellin monomer but not to flagellar filaments (Smith *et al.*, 2003a). In *Salmonella*, mutations in either the conserved N-terminus (amino acids 95–108) or C-terminus (amino acids 441–449) of the flagellin completely abolished its pro-inflammatory activity (Murthy *et al.*, 2004). However, differences in the pro-inflammatory action of flagellin may exist since *H. pylori* flagellins have been shown to have little or no pro-inflammatory activity, despite having extensive amino acid homology to other pro-inflammatory flagellin types at the N- and C-terminus (Lee *et al.*, 2003). In this study, H21 flagellin induced p38 MAPK activation in a TLR5-dependent manner. In both HeLa and

MDCK cells stably transfected with a plasmid encoding TLR5, p38 MAPK activity was upregulated in the presence of purified H21 flagellin; this did not occur in cells stably transfected with a DN TLR5-expressing plasmid.

In T84 cells, TLR5 is exclusively expressed on the basolateral surface of polarised monolayers (Gewirtz *et al.*, 2001a). In HCA-7 cells TLR5 may be expressed on both the apical and basolateral surface, since application of flagellin to either surface resulted in the secretion of IL-8 (Berin *et al.*, 2002). Both polarised T84 and HCA-7 monolayers are able to secrete IL-8, predominantly in the basolateral direction, in response to the application of flagellin (Gewirtz *et al.*, 2001a; Berin *et al.*, 2002). In the case of *Salmonella* flagellin, it was shown that only flagellin that is translocated across polarised T84 monolayers, and/or is in contact with the basolateral surface, is able to induce an inflammatory response (Gewirtz *et al.*, 2001a). In contrast, Zhou *et al.* (2003) found that in T84 cells His₆-tagged H6 flagellin from EPEC E2348/69 was able to induce IL-8 secretion when applied to either the apical or the basolateral surface; in either case secretion was generally higher from the basolateral surface. Since T84 cells were not believed to express TLR5 on the apical surface, Zhou *et al.* (2003) suggested that another receptor might be involved in triggering IL-8 secretion from the apical surface in response to EPEC H6 flagellin. Alternatively, it is possible that there is a low level of TLR5 expression on the apical surface of T84 cells. The data obtained in this study from polarised HCT-8 monolayers showed that IL-8 was secreted in response to H21 flagellin when it was applied either to the apical or basolateral surface, suggesting that TLR5 may be constitutively expressed on both surfaces. Alternatively, STEC flagellin may be efficiently translocated across the cells.

Polarised Caco-2 cells were also found to express TLR5 apically and basolaterally (Sierro *et al.*, 2001). TLR5 has also been observed on the apical surface of human enterocytes (Cario and Podolsky, 2000) and, in a recent study, TLR5 was expressed on both the apical and basolateral surface of normal human intestinal epithelium (Schmausser *et al.*, 2004). Although the basolateral secretion of IL-8 would be more effective in mediating an inflammatory response (including the recruitment of PMNs), STEC strains do not significantly invade the intestinal epithelium. Therefore, STEC initially are only able to stimulate the apical surface of intestinal epithelia *in vivo*. Thus, the ability of flagellin to

stimulate IL-8 secretion from both the apical and basolateral surfaces may be physiologically relevant.

A large proportion of commensal bacteria possess flagella, but the mechanism(s) by which intestinal epithelial cells discriminate between pathogens and commensal bacteria are poorly understood. Recent data suggest that TLR5 may be expressed apically in normal human intestinal epithelium, and therefore translocation of flagellin to the basolateral surface would not be required for the upregulation of pro-inflammatory chemokines (Schmausser *et al.*, 2004). Thus, commensal organisms may have evolved mechanisms to avoid the activation of a pro-inflammatory response through TLR5. One hypothesis is that commensal bacteria may be localised to the mucus layer of the intestinal surface, and therefore would not come into direct contact with TLR5 on the epithelial cells (Tlaskalova-Hogenova *et al.*, 2004). Also, some flagellins, like those from *H. pylori* (Lee *et al.*, 2003), may not evoke an inflammatory response. Another possibility is that commensal organisms have acquired anti-inflammatory strategies, including blocking I κ B degradation, which prevents the nuclear translocation of active NF- κ B (Neish *et al.*, 2000). Commensal bacteria may also induce production of anti-inflammatory proteins and/or cytokines (e.g. IL-2 and IL-10) which can down-regulate inflammatory signals (Henderson *et al.*, 1996; Schiffrin and Blum, 2002; Tlaskalova-Hogenova *et al.*, 2004). The administration of probiotic bacteria to humans has also been shown to down-regulate inflammation in the intestine, suggesting that these bacteria do possess anti-inflammatory properties (Isolauri *et al.*, 2002).

However, the ability of STEC strains to induce IL-8 secretion may be crucial to the development of HUS, given the proposed importance of PMNs in breaking down intestinal barriers and in the binding and transporting of Stx to target tissues expressing Gb₃ (te Loo *et al.*, 2000a; te Loo *et al.*, 2000b; Hurley *et al.*, 2001). The break down of intestinal barriers may also result in the translocation of flagellin, which may further increase inflammatory responses, since TLR5 is expressed constitutively on intestinal microvascular endothelial cells (Maaser *et al.*, 2004). This may also lead to the upregulation of VCAM and ICAM (Maaser *et al.*, 2001) and, therefore, increased recruitment of PMNs, further enhancing the severity of STEC infection. Stx and flagellin may act in concert, perhaps via enhanced MAPK activation, to cause increased inflammation when both are present. The potent inflammatory

capacity of flagellin from LEE-negative STEC strain 98NK2 may be important in enhancing the pathogenicity of this, as well as other LEE-negative STEC isolates during infection, because increased local damage to the intestinal epithelium could occur despite the absence of LEE-mediated A/E lesion formation. Similarly, flagellin may enhance the ability of other highly virulent STEC serotypes, e.g. O157:H7, to cause serious disease.

CHAPTER 6: Examination of the Role of H21 Flagellin in Epithelial PMN Transmigration and Virulence for Streptomycin-Treated Mice

6.1 Introduction

The previous chapters have shown a role for STEC flagellin in both chemokine responses and in MAPK activation in intestinal epithelial cells. Although CXC chemokines, including IL-8, may be important in PMN recruitment and migration, no direct role for flagellin in PMN recruitment has yet been shown. Likewise, in spite of the proposed function of PMNs in the pathogenesis of serious STEC disease including HUS, no role has yet been demonstrated for flagellin in STEC virulence.

Several enteric pathogens, including EPEC, *Salmonella* spp. and *Shigella* spp., are able to induce PMN transmigration across polarised intestinal epithelial (T84) cells (McCormick *et al.*, 1993; Savkovic *et al.*, 1996; McCormick *et al.*, 1998b) and chemokines, including IL-8, are thought to be important in this process (Eckmann *et al.*, 1993a; Eckmann *et al.*, 1993b; McCormick *et al.*, 1993; McCormick *et al.*, 1995; Savkovic *et al.*, 1997; Yang *et al.*, 1997). In addition to high levels of PMNs in the blood of STEC patients (Kelly *et al.*, 1987; Richardson *et al.*, 1988; Griffin *et al.*, 1990; Kelly *et al.*, 1990; Inward *et al.*, 1997a; Slutsker *et al.*, 1997), STEC have also been shown to induce PMN transmigration across polarised intestinal epithelia *in vitro* (Hurley *et al.*, 2001) and in several animal models (Francis *et al.*, 1986; McKee *et al.*, 1995; Dean-Nystrom *et al.*, 1997; Dean-Nystrom *et al.*, 1998; Isogai *et al.*, 1998; Heczko *et al.*, 2000; Kang *et al.*, 2001) (Section 1.7). Hurley *et al.* (2001) have previously shown that the transmigration of PMNs induced by LEE-negative

STEC isolates was on average higher than that induced by LEE-positive STEC isolates, and generally correlated with the level of IL-8 secretion elicited by these strains (Hurley *et al.*, 2001).

The streptomycin-treated mouse model for STEC disease has been described briefly in Section 1.4.2 and is a model for Stx-mediated systemic disease. In this model, mice display no glomerular damage or thrombotic microangiopathy, but have extensive acute tubular necrosis (Wadolowski *et al.*, 1990a; Wadolowski *et al.*, 1990b), which is also seen in patients with HUS (Richardson *et al.*, 1988). This model is therefore useful for studying systemic Stx-mediated pathology. Mice do not have an IL-8 homologue, but they do have other CXC chemokine homologues. They have macrophage inflammatory peptide-2 (MIP-2) and KC, which are homologous to the human CXC chemokines MIP-2 α , MIP-2 β and MGSA (Oquendo *et al.*, 1989; Tekamp-Olson *et al.*, 1990), and LPS-induced CXC chemokine (LIX), which is homologous to human GCP-2 and ENA-78 (Smith *et al.*, 2002). Mice also do not have a homologue for the IL-8 receptor CXCR1, but they do have a homologue to the human CXCR2 receptor, murine CXCR2 (mCXCR2 or murine IL-8 receptor homologue [mIL-8Rh]) (Bozic *et al.*, 1994), which is a functional receptor for all of the CXC chemokines, including IL-8 (Baggiolini *et al.*, 1997; Gale and McColl, 1999). Given the proposed importance of PMNs in the transport and binding of Stx it is possible that in this model of STEC disease, virulence of the 98NK2 Δ *fliC* strain may be decreased due to a reduced inflammatory response, including reduced recruitment of PMNs, and thus lower uptake and transport of Stx. In this chapter, the role of flagellin in PMN transmigration *in vitro* and in the virulence of STEC for streptomycin-treated mice will be investigated.

6.2 Results

6.2.1 Role of flagellin in PMN transmigration *in vitro*

Although a role for flagellin in pro-inflammatory responses has been demonstrated *in vitro*, no role in PMN transmigration has yet been observed. The ability of 98NK2 and

98NK2 Δ *fliC* to induce PMNs to transmigrate from the basolateral to the apical surface was therefore determined in T84 and HCT-8 monolayers (Section 2.17). Inverted monolayers were grown on Transwell inserts (Section 2.16) and used once they attained a minimum TEER of 1,000 Ω cm² (Section 2.16.1). T84 or HCT-8 monolayers were infected apically with 2×10^8 CFU/monolayer STEC for 1 h, washed to remove non-adherent bacteria, and then PMNs (1×10^6 per monolayer) were added to the basolateral surface and allowed to transmigrate for 2 h in the physiologically relevant direction (i.e. basolateral [serosal] to apical [mucosal]) (Hurley and McCormick, 2003), as described in Section 2.17. The number of PMNs that transmigrated to the apical compartment was then determined by MPO assays (Section 2.17.3).

No significant differences were observed between the numbers of PMNs that transmigrated across T84 cells in response to 98NK2 or 98NK2 Δ *fliC* (Fig 6.1). In T84 cells, 98NK2 Δ *fliC* induced slightly higher PMN transmigration than 98NK2 ($27.2 \pm 6.3 \times 10^4$ PMNs and $20.7 \pm 2.0 \times 10^4$ PMNs, respectively) (both $P < 0.001$ compared to DH5 α - or HBSS-treated monolayers). However, non-pathogenic *E. coli* DH5 α did not induce

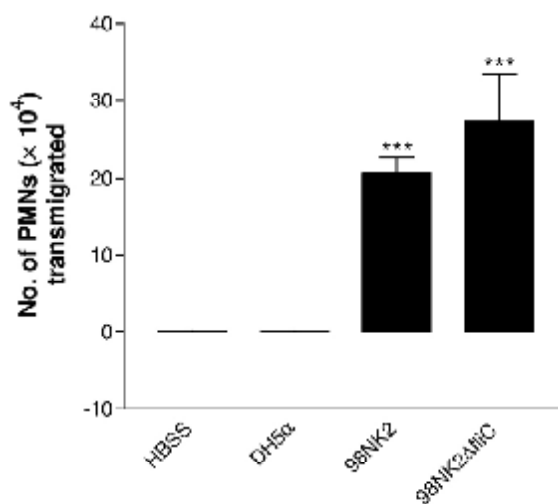


Figure 6.1 PMN transmigration induced by 98NK2 and 98NK2 Δ *fliC* in T84 cells. Polarised inverted T84 monolayers (Section 2.16) were infected with 2×10^8 DH5 α , 98NK2 or 98NK2 Δ *fliC* as described in Section 2.17.2 and PMNs (1×10^6) (Section 2.17.1) were added to the basolateral chamber and allowed to transmigrate for 2 h. The number of PMNs that transmigrated from the basolateral to the apical compartment was determined by MPO assays (Section 2.17.3). Data are the means \pm SD from six wells. Significant differences relative to control HBSS- and DH5 α -treated cells are indicated as follows: ***, $P < 0.001$.

significant migration of PMNs ($P > 0.05$ compared to untreated [HBSS] monolayers). These data indicate that 98NK2 and 98NK2 Δ *fliC* are both able to induce PMN transmigration in T84 cells and therefore that although flagellin does induce IL-8 secretion, this factor is not essential for PMN transmigration *in vitro* in T84 cell monolayers.

Similarly, no significant differences were observed between the numbers of PMNs that transmigrated across HCT-8 monolayers in response to 98NK2 and 98NK2 Δ *fliC* (Fig. 6.2). In these cells, 98NK2 induced slightly higher PMN transmigration than 98NK2 Δ *fliC* ($9.0 \pm 1.0 \times 10^4$ PMNs and $7.2 \pm 0.5 \times 10^4$ PMNs, respectively) (both $P < 0.001$ compared to DH5 α - or HBSS-treated monolayers). DH5 α induced $0.6 \pm 0.1 \times 10^4$ PMNs to transmigrate ($P > 0.05$ compared to untreated [HBSS] control monolayers). Collectively, these data suggest that there were no significant effects on the levels of PMNs that transmigrated in response to infection with either 98NK2 or 98NK2 Δ *fliC* in either T84 or HCT-8 cells.

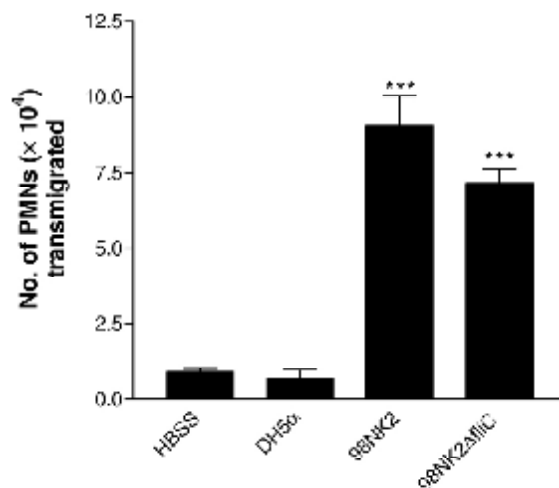


Figure 6.2 PMN transmigration induced by 98NK2 and 98NK2 Δ *fliC* in HCT-8 cells. Polarised inverted HCT-8 monolayers (Section 2.16) were infected with 2×10^8 DH5 α , 98NK2 or 98NK2 Δ *fliC* as described in Section 2.17.2 and PMNs (1×10^6) (Section 2.17.1) were added to the basolateral chamber and allowed to transmigrate for 2 h. The number of PMNs that transmigrated from the basolateral to the apical compartment was determined by MPO assays (Section 2.17.3). Data are the means \pm SD from six wells. Significant differences relative to control HBSS- and DH5 α -treated cells are indicated as follows: ***, $P < 0.001$.

6.2.2 Role of flagellin in virulence in streptomycin-treated mice

Although a role for H21 flagellin in pro-inflammatory responses has been previously determined (Section 4.2.5 and Chapter 5), a role for flagellin in virulence *in vivo* has not been established. The virulence of 98NK2 for streptomycin-treated BALB/c mice has previously been described (Paton *et al.*, 2001b). This strain was found to be more virulent than most O157:H7 strains and resulted in the deaths of approximately 60% of mice challenged (with a median survival time of 6 days) (Paton *et al.*, 2001b). Thus, virulence studies were performed in streptomycin-treated BALB/c mice with 98NK2 and 98NK2 Δ *fliC*, respectively.

The streptomycin-resistant derivative of 98NK2 (98NK2^{SR}) has been described previously (Table 2.1) (Paton *et al.*, 2001b). Spontaneous streptomycin-resistant derivatives of 98NK2 Δ *fliC* were selected by exposing bacterial lawns to paper discs containing streptomycin. Growth around the zone of inhibition was then re-inoculated into LB broth, and re-plated onto fresh LB plates, until no zone of inhibition was observed around a paper disc containing 50 μ g Str. The Str^R derivative was designated 98NK2 Δ *fliC*^{SR}. Western blotting of whole cell lysates with H21 antiserum showed no H21 flagellin expression in 98NK2 Δ *fliC*^{SR}, but a 51-kDa band representing H21 flagellin in 98NK2^{SR} (Fig. 6.3). Motility assays on semi-soft agar also demonstrated that 98NK2 Δ *fliC*^{SR} was non-motile (Fig. 6.4).

Groups of 16 BALB/c mice were initially pre-treated with streptomycin, which inhibits the normal mouse microflora, thereby enabling streptomycin-resistant STEC to colonise the mouse gastrointestinal tract (Section 2.26). BALB/c mice were fed approximately 10^8 CFU of 98NK2^{SR} or 98NK2 Δ *fliC*^{SR}, respectively. After 24 h, faecal pellets were collected, and plated onto LB agar containing Str for CFU determination. Twenty-four h after challenge, faecal pellets contained approximately 5.9 ± 1.3 and $6.8 \pm 1.5 \times 10^9$ CFU/g, for 98NK2^{SR}- and 98NK2 Δ *fliC*^{SR}-treated mice, respectively (Fig. 6.5). Colonisation of 98NK2^{SR} and 98NK2 Δ *fliC*^{SR} gradually declined to approximately 9.3 ± 3.1 and $7.0 \pm 3.0 \times 10^8$ CFU/g, respectively, on day 12 (Fig. 6.5). There were no significant differences between the levels of colonisation in BALB/c mice challenged with either 98NK2 or 98NK2 Δ *fliC* at any time point ($P > 0.05$).

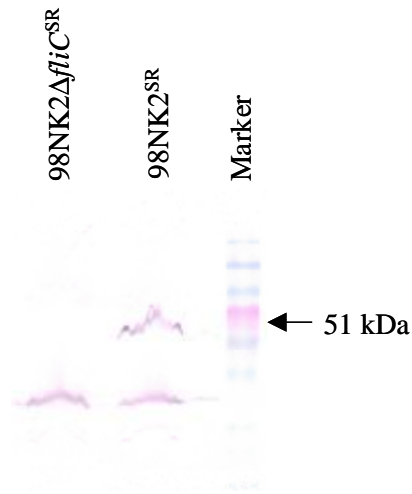


Figure 6.3 Western blot of whole cell lysates from 98NK2^{SR} and 98NK2Δ*fliC*^{SR}. Whole cell lysates were prepared from overnight cultures of STEC (Section 2.14.1) then electrophoresed on a 12% SDS-PAGE gel (Section 2.14.2). SDS-PAGE gels were then subjected to Western blotting (Section 2.14.4) with H21 antiserum. The arrow indicates the 51-kDa band obtained with 98NK2^{SR}. The markers are BenchMark pre-stained protein ladder (sizes 172.0, 110.2, 79.0, 62.4 [pink], 48.0, 36.6, 24.5, 19.0, 13.5 and 5.3 kDa).

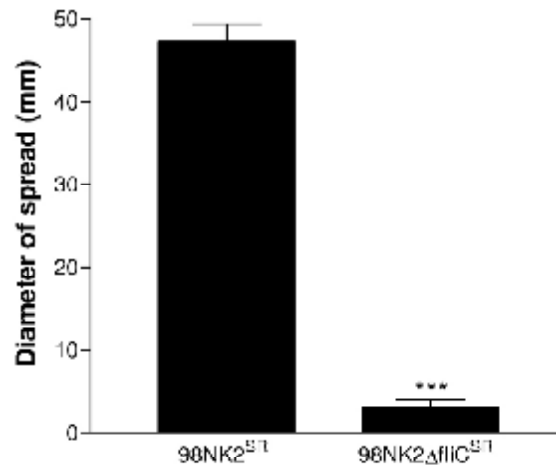


Figure 6.4 Motility of 98NK2^{SR} and 98NK2Δ*fliC*^{SR} on semi-soft agar. STEC were stab-inoculated onto 0.3% (w/v) LB agar and incubated at 37°C for 18 h, after which the diameter of spread (mm) was measured. Data shown are the means ± SD from duplicate samples. Significant differences relative to 98NK2^{SR} are indicated as follows: ***, $P < 0.0001$.

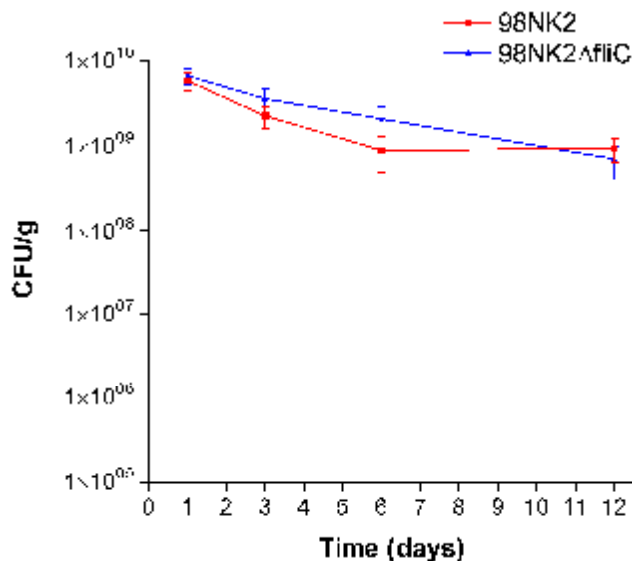


Figure 6.5 Colonisation of mice with 98NK2^{SR} and 98NK2Δ*fliC*^{SR}. Streptomycin pre-treated BALB/c mice were orally inoculated with 10⁸ CFU of 98NK2^{SR} or 98NK2Δ*fliC*^{SR} as described in Section 2.26. At days 1, 3, 6 and 12 after challenge, mouse faecal pellets were collected, weighed, and dilutions of homogenates were plated on LB agar supplemented with 50 μg/ml Str. Each point represents the mean ± SEM CFU/g faeces obtained from at least two mice.

All mice challenged with 98NK2^{SR} showed signs of sickness five days after challenge, including ruffled fur, lethargy, and reduced food and water intake. In mice that subsequently succumbed, this was followed by hind limb paralysis, ataxia and death. Interestingly, only one mouse challenged with 98NK2Δ*fliC*^{SR} showed signs of hind limb paralysis and ataxia in the 4 h prior to death; the remaining mice appeared apparently healthy. As shown in Fig. 6.6, 9 of 16 mice (56%) challenged with 98NK2^{SR} died during the course of the experiment (median survival time 7.6 days), but only 3 of 16 mice (19%) challenged with 98NK2Δ*fliC*^{SR} died (median survival time > 14 days). This difference in survival rate was statistically significant (χ^2 test; $P < 0.025$), implicating flagellin as being important in virulence of 98NK2 in the streptomycin-treated mouse model.

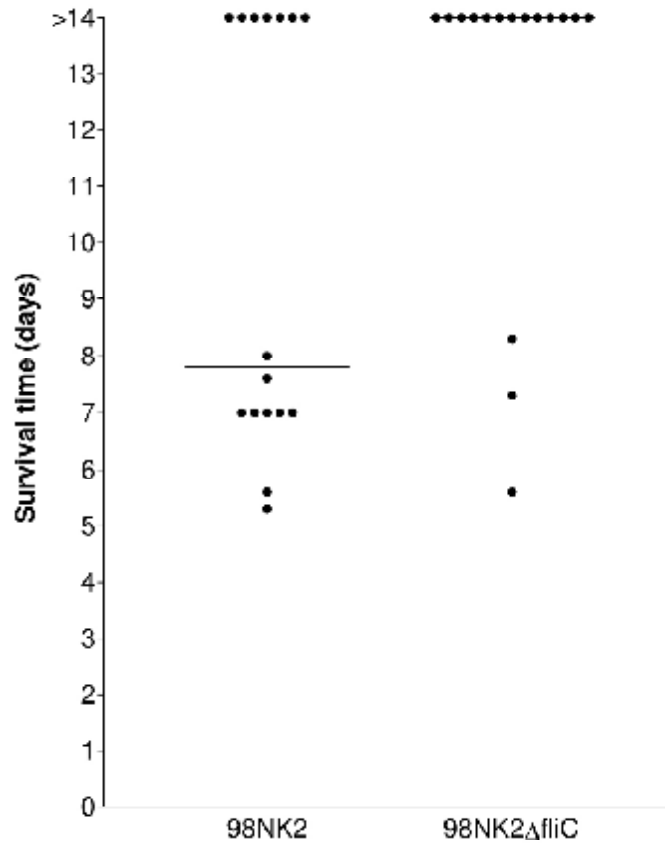


Figure 6.6 Survival of mice challenged with 98NK2^{SR} and 98NK2 Δ fliC^{SR}. Groups of 16 streptomycin pre-treated BALB/c mice were orally inoculated with 10^8 CFU of 98NK2^{SR} or 98NK2 Δ fliC^{SR} as described in Section 2.26, and their survival time recorded. The median survival time for each group is indicated by a horizontal bar.

6.3 Discussion

Since H21 flagellin was previously shown to be responsible for inducing pro-inflammatory responses, including the upregulation of CXC chemokines and MAPKs (Section 4.2.5, Section 4.2.7 and Chapter 5), the role of flagellin in virulence and/or PMN transmigration was examined. The LEE-negative STEC strain 98NK2 was able to induce relatively high levels of PMN-transmigration compared to that reported previously for several LEE-positive STEC isolates (Hurley *et al.*, 2001). However, the deletion of *fliC* from 98NK2 had no significant effect on PMN-transmigration in T84 or HCT-8 monolayers, despite this

mutation completely abrogating the IL-8 response of HCT-8 cells to this strain (Section 4.2.5.2.3).

Infection of epithelial cells with *Salmonella* leads to the secretion of a range of pro-inflammatory cytokines and chemokines, including IL-8 (Eckmann *et al.*, 1993b; Jung *et al.*, 1995). Invasion by *Salmonella* spp. results in the secretion of IL-8 and the subsequent migration of PMNs (Eckmann *et al.*, 1993b). However, in response to *Salmonella* infection, IL-8 is not wholly responsible for the transmigration of PMNs across polarised epithelial monolayers. Neutralising antibodies against IL-8 were not able to effectively inhibit PMN transmigration in response to the apical colonisation of T84 cells with *S. typhimurium* (McCormick *et al.*, 1993). In addition to IL-8, the small molecular weight eicosanoid, hepoxilin A3 (HepA3) (Mrsny *et al.*, 2004), previously referred to as pathogen-elicited epithelial chemoattractant (PEEC), was required for PMN transmigration in T84 cells *in vitro* (McCormick *et al.*, 1993; McCormick *et al.*, 1998a). HepA3 is secreted apically from T84 cells and is responsible for inducing the final stage of PMN transmigration, i.e. across the tight junction barriers to the apical surface of the cells (Mrsny *et al.*, 2004). Thus, it is now thought that IL-8 is responsible for attracting PMNs through the lamina propria to the subepithelial compartment, but that HepA3 is responsible for directing PMN migration across the epithelial cell barrier (McCormick *et al.*, 1995; McCormick *et al.*, 1998a; Mrsny *et al.*, 2004). HepA3 is also thought to be secreted from other intestinal epithelial cells, including HCT-8 cells (B. Hurley, personal communication).

It is uncertain whether HepA3 is secreted in response to infection with STEC. Hurley *et al.* (2001) studied both IL-8 secretion and PMN migration in response to STEC strains in T84 cells and found that PMN transmigration generally correlated with IL-8 levels (Hurley *et al.*, 2001). Both IL-8 secretion and PMN transmigration was higher in cells infected with LEE-negative STEC than in LEE-positive STEC-infected T84 cells (Hurley *et al.*, 2001). However, there were two outliers to this trend; one LEE-negative strain induced high PMN-transmigration but low IL-8 production, while another LEE-negative STEC strain showed high IL-8 production and low PMN-transmigration (Hurley *et al.*, 2001). Thus, HepA3 or other unidentified factors may also be important for migration of PMNs across T84 cells in response to STEC infection (Hurley *et al.*, 2001). These findings are consistent with the data

presented in this chapter, since although deletion of *fliC* completely abrogated IL-8 secretion from HCT-8 cells (Section 4.2.5.2.3), there were no significant differences in PMN transmigration induced by this and the wild-type strain in either T84 or HCT-8 cells. Although IL-8 appears not to be important for the transmigration of PMNs *in vitro*, it is clearly, important in the recruitment and migration of PMNs to the site of infection *in vivo* (Godaly *et al.*, 2000; Godaly *et al.*, 2001), and so it can be concluded that flagellin may still be important in virulence.

Interestingly, in the streptomycin-treated mouse model of STEC disease, 98NK2 Δ *fliC* was less able to cause serious disease in mice, as measured by survival rate. At the end of the experiment, only 44% of mice challenged with 98NK2 survived, compared with 81% for mice challenged with 98NK2 Δ *fliC*. There were no significant differences between the levels of colonisation of these strains that would otherwise account for the reduced virulence of 98NK2 Δ *fliC* *in vivo*. Similarly, *in vitro* studies in Section 4.2.5.2.4 also showed no reduction in adherence of 98NK2 Δ *fliC* to either HCT-8 or HEP-2 cells. These data suggest that 98NK2 Δ *fliC* may indeed have an effect on the absorption and/or translocation of Stx, since in this model, deaths are believed to be due solely to the systemic effects of Stx (Wadolkowski *et al.*, 1990a; Wadolkowski *et al.*, 1990b). Although PMN recruitment in mice was not investigated in this study, it is likely that the increased survival of mice challenged with the Δ *fliC* strain was due either to a decrease in inflammation, a decrease in Stx uptake or translocation, or to changes in receptor expression affecting the uptake or binding of Stx.

Although mutagenesis of the flagellin gene decreased the virulence of 98NK2 in the mouse model, the mutant strain was nevertheless responsible for deaths of 19% of mice implying significant uptake of Stx in these animals. However, an augmented inflammatory response *in vivo* from the combined effect of Stx2 and flagellin, may explain why the 98NK2 strain was more virulent than 98NK2 Δ *fliC*. The increase in inflammation induced by STEC strains producing flagellin may help to explain why some LEE-negative STEC strains (including O48:H21, O91:H21 and O113:H21), are able to cause severe disease in humans, including HUS, despite the absence of LEE-mediated adherence (Lindgren *et al.*, 1993; Paton *et al.*, 1999; Elliott *et al.*, 2001). This may also explain why motile LEE-positive STEC strains belonging to serotype O157:H7 are the most common cause of HUS world wide

(Mead and Griffin, 1998; Banatvala *et al.*, 2001). Nevertheless, STEC strains that do not express flagella (e.g. O111:H⁻ and O157:H⁻) are also able to cause serious disease in humans, including HUS (Henning *et al.*, 1998; Elliott *et al.*, 2001; Karch and Bielaszewska, 2001). Thus, flagellin is not essential for causing severe disease, but may enhance inflammation and Stx-mediated pathology. Future studies are required to confirm the direct role of flagellin-induced inflammation in STEC virulence *in vivo*.

CHAPTER 7: Final Discussion

STEC are important human pathogens responsible for significant morbidity and mortality worldwide. The pathogenesis of STEC disease is multi-factorial and not fully understood. STEC infection may result in diarrhoea, HC, or the life-threatening conditions TTP and HUS. In order to cause severe disease, STEC must first colonise and adhere to the intestine. STEC infection then results in the absorption of Stx systemically, followed by the onset of diarrhoea and intestinal pathology. Stx is transported to target tissues and interacts with host cells expressing the receptor Gb₃, followed by internalisation, inhibition of protein synthesis and cell death. However, free Stx has not been detected in the serum of patients with STEC infections or HUS (Acheson *et al.*, 1998; Paton and Paton, 1998b; te Loo *et al.*, 2000b). Thus, the mechanisms by which Stx gains access to target tissues expressing Gb₃ are important in the pathogenesis of serious STEC disease.

Since 1989, elevated PMN levels in the blood have been recognised as a prognostic marker for the development of more severe STEC disease, including HUS (Walters *et al.*, 1989). However, at the time that the work described in this thesis commenced, the precise role of PMNs in the pathogenesis of HUS was only just beginning to be elucidated (Section 1.7). *In vitro* data suggested that locally in the gut, PMNs may break down epithelial tight junction barriers, resulting in increased absorption of Stx from the lumen into underlying tissues and the blood stream (Hurley *et al.*, 2001). Stx was also shown to bind to PMNs when incubated with whole blood *in vitro*, via a unique receptor with 100-fold lower affinity than Gb₃, and Stx was released when in contact with cells expressing the high-affinity receptor,

Gb₃ (te Loo *et al.*, 2000a). In HUS patients, up to 90% of PMNs were shown to be positive for Stx, suggesting that this process is relevant during STEC infections, and may explain why free Stx has not been detected in the serum of patients (te Loo *et al.*, 2000b). These findings therefore provide a mechanism whereby PMNs may locally increase intestinal permeability and the absorption of Stx into underlying tissues, and also bind and transport Stx systemically to target tissues expressing Gb₃, thereby increasing the probability of developing HUS. Since PMNs are crucial to the development of serious STEC disease, the mechanism(s) responsible for PMN recruitment are also fundamental to the pathogenicity of these organisms. As intestinal epithelial cells are the first host cell contact of STEC, this interaction is likely to be important in pathogenesis. The induction of CXC chemokines (which are potent PMN chemoattractants) in intestinal epithelial cells may also be crucial to the pathogenesis of serious STEC disease, through the recruitment of PMNs.

Analysis of chemokine profiles elicited by a range of STEC isolates (Section 3.2.3.1) demonstrated that infection with LEE-negative rather than LEE-positive STEC strains elicited a higher and earlier chemokine mRNA response by HCT-8 cells. Infection of HCT-8 cells with these isolates elicited a range of CXC chemokine mRNAs, including IL-8, ENA-78, MIP-2 α and MIP-2 β , all of which are potent PMN chemoattractants *in vivo* (Baggiolini *et al.*, 1997; Gale and McColl, 1999). Similarly, induction of IL-8 protein was generally much higher in HCT-8 cells infected with LEE-negative STEC isolates rather than LEE-positive isolates, with the exception of 95ZG1 (Section 3.2.3.2). This phenomenon was also described by Hurley *et al.* (2001), who showed that infection with LEE-negative isolates, in general, resulted in higher IL-8 secretion and PMN transmigration, than infection with LEE-positive strains. These data suggested that significant differences exist in the mechanisms of inflammation and PMN recruitment between LEE-positive and LEE-negative STEC strains.

Analysis of the factor(s) responsible for these effects was undertaken in Chapter 4, with a focus on the known adherence and virulence factors of STEC strains. No role in chemokine induction was observed for the pO157 or pO113 megaplasms of EDL933 and 98NK2, respectively. This, therefore, eliminated a number of characterised or putative adherence or virulence factors encoded by these plasmids as contributors to chemokine induction. Similarly, no role for factors associated with A/E lesion formation or effect of LPS

type was observed. Stx has previously been shown to induce a range of chemokine mRNAs and proteins, including IL-8, in intestinal epithelial (HCT-8) cells (Thorpe *et al.*, 1999; Thorpe *et al.*, 2001). In Section 4.2.3, purified Stx1 or Stx2 were able to induce these responses, but the type and amount of Stx produced by the STEC strains *in vitro* could not account for the levels of chemokine induction observed in cells infected with the wild-type strains. Similarly, deletion of the single *stx*₂ gene from 98NK2 had no effect on chemokine induction compared to the wild-type strain. In addition, *E. coli* K-12 clones expressing Stx1 or Stx2 did not exhibit any significant differences in chemokine induction in HCT-8 cells compared to the host *E. coli* K-12 strain. These data collectively suggest that Stx was not responsible for the majority of chemokine production observed after infection with wild-type STEC.

Purified flagella from several enteric pathogens have been shown to elicit inflammatory responses in epithelial cells, including EAggEC and *S. typhimurium* (Steiner *et al.*, 2000; Gewirtz *et al.*, 2001a; Gewirtz *et al.*, 2001b; Donnelly and Steiner, 2002). Interestingly, many LEE-negative STEC strains associated with serious human disease (including three of the STEC strains tested in Section 3.2.3) belong to flagellar type H21 (Paton and Paton, 1998b). Purified H21 flagella from 98NK2 (O113:H21), was found to induce chemokine production by HCT-8 cells at levels comparable to that induced by infection with the wild-type strain itself (Sections 4.2.5.1). Even doses as small as 10 ng of H21 flagella induced chemokine levels comparable to that induced by infection with wild-type 98NK2. Purified His₆-H21 FliC from 98NK2 was also able to induce chemokine responses similar to that induced by infection with 98NK2 or by stimulation with purified flagella, suggesting that flagellin itself is responsible for these effects (Section 4.2.7).

Similarly, flagella isolated from other high chemokine-inducing strains, 95HE4 (O91:H7) and 95ZG1 (O26:H11), were also able to induce chemokine production (Section 4.2.6.1.2). All of the high chemokine-inducing STEC strains studied in Section 3.2.3 produced flagella, which explained the high and early chemokine responses of these strains; this included the LEE-positive strain 95ZG1. However, only two of the three LEE-positive strains that elicited poor chemokine responses were known to lack flagella (O111:H⁻ strain 95NR1 and the O157:H⁻ strain 95SF2). However, the poor capacity of EDL933 (an O157:H7

reference STEC strain) to induce these responses was found to be due to an unexpected lack of expression of H7 flagella (Section 4.2.6.1.2). A number of additional LEE-positive O157:H7 strains were then tested and shown also to be capable of inducing chemokine responses comparable to that observed for the LEE-negative strains, further demonstrating that this phenomenon is not restricted to LEE-negative STEC (Section 4.2.6.2). Mutation of the *fliC* gene from the LEE-negative O113:H21 strains 98NK2 and 97MW1, and the LEE-positive O157:H7 strain 86-24 (Sections 4.2.5 and 4.2.6) confirmed the role of flagellin in inducing this high and early chemokine response. The complete abrogation of IL-8 responses in cells infected with 98NK2 Δ *fliC* was also found not to be due to reduced adherence to either HCT-8 or HEp-2 cells *in vitro*. The possible importance of flagellin in the LEE-negative STEC strains is emphasised by its presence in all of the LEE-negative isolates studied from Section 3.2.3 that were isolated from cases of severe clinical disease. The presence of flagellin in these LEE-negative STEC strains may therefore enhance the virulence of these isolates in the absence of LEE-mediated intimate adherence.

As discussed in Chapter 5, flagellin was able to induce the secretion of IL-8 in a p38-, ERK- and JNK-dependent manner. This upregulation of p38 MAPK also occurred in a TLR5-dependent manner in stably transfected TLR5-expressing MDCK and HeLa cells, but not in DN TLR5-expressing cells. Polarised HCT-8 monolayers were able to secrete IL-8 in response to purified H21 flagellin when applied either apically or basolaterally, implying that TLR5 is distributed evenly on both surfaces. This may be important *in vivo* since detection of pathogens by TLR5 may occur before the translocation of flagellin to the basolateral compartment. IL-8 and MIP-2 α mRNA were also superinduced in the presence of Stx2 and H21 flagellin suggesting that *in vivo* inflammation may increase in the presence of both flagellin and Stx. This may be particularly important during STEC infection since levels of Stx produced by STEC strains *in vivo* are thought to be much higher than that produced *in vitro* (Thorpe *et al.*, 2001). These data suggest that TLR5 and the p38, ERK and JNK MAPK pathways all play an important role in the response of intestinal epithelial cells to H21 flagellin from STEC, and that the combined effects of Stx and flagellin on host intestinal epithelial cells may result in an augmented inflammatory response during STEC infections.

Although no role for flagellin in transepithelial PMN migration was observed *in vitro*, it is likely that the reduction of IL-8 secretion induced by 98NK2 Δ *fliC* would result in reduced recruitment of PMNs *in vivo*, since IL-8 is essential for the recruitment and migration of PMNs to the site of infection (Godaly et al., 2000; Godaly et al., 2001). *In vitro*, HepA3 is responsible for inducing the transmigration of PMNs across the epithelial cell tight junction barrier (McCormick *et al.*, 1995; McCormick *et al.*, 1998a; Mrsny *et al.*, 2004). Thus, since the Δ *fliC* mutation had no effect on the level of PMN transmigration compared to the wild-type strain, this mutation is unlikely to have an effect on the production of HepA3 in infected T84 or HCT-8 cells. Interestingly, flagellin was found to play a significant role in enhancing the virulence of 98NK2 in the streptomycin-treated mouse model. Despite the fact that 98NK2 Δ *fliC* was able to colonise the mouse intestine at levels comparable to the wild-type strain, virulence was reduced in mice receiving the Δ *fliC* mutant (from a survival rate of 44% for mice challenged with 98NK2 to 81% in mice receiving 98NK2 Δ *fliC*). Since the streptomycin-treated mouse model is a model for Stx-mediated pathology, these results suggest that the pro-inflammatory responses induced by flagellin may affect the systemic availability of Stx *in vivo*. Elucidation of the mechanism(s) by which this occurs *in vivo* was beyond the scope of this thesis, but may be a result of reduced translocation of Stx and/or a reduced recruitment of PMNs, resulting in reduced binding and transport of Stx to target tissues expressing Gb₃. The presence of PMNs may also affect virulence by augmentation of Stx2 production. H₂O₂ released by activated PMNs is known to induce Stx-encoding phages in STEC strains (Wagner *et al.*, 2001), and may also result in localised tissue damage and increased absorption of Stx (Forsyth *et al.*, 1989; Milford *et al.*, 1991; Fitzpatrick *et al.*, 1992a). Alternatively, reduced virulence may be the result of differences in receptor expression in target tissues resulting in reduced Stx binding, since cytokines such as IL-1 and TNF- α are known to upregulate Gb₃ expression on endothelial cells (Section 1.4.2) (van de Kar *et al.*, 1992; Hughes *et al.*, 2000). The mechanism(s) by which flagella reduce virulence in the mouse model may be the focus of future studies into the pathogenesis of STEC disease.

This study has demonstrated a significant role for flagellin in the induction of inflammatory responses in epithelial cells *in vitro* and in the virulence of 98NK2 in streptomycin-treated mice. These data suggest that STEC have evolved mechanisms to

exploit the normal innate immune response of the host in order to enhance inflammation, and thereby, generation of Stx-mediated pathology. The induction of CXC chemokines, including IL-8, and the activation of the MAPK pathways, may result in the recruitment of PMNs to the intestine, thereby resulting in the break down of intestinal barriers, and the binding and transport of Stx to target tissues. In addition, this response may be further enhanced in the presence of both flagellin and physiological levels of Stx, thereby augmenting inflammation and increasing Stx translocation. The break down of intestinal barriers, in addition to aiding Stx translocation, may also result in the translocation of flagellin itself, and thus further enhance inflammation, since TLR5 is expressed on intestinal microvascular endothelial cells (Maaser *et al.*, 2004). Pro-inflammatory responses may also lead to the upregulation of receptors, including VCAM and ICAM (Maaser *et al.*, 2001) resulting in increased recruitment of PMNs, which would further potentiate the severity of infection.

The presence of flagellin in the majority of LEE-negative STEC isolates, but only 2 of 5 LEE-positive isolates from Section 2.3.2, suggests that flagellin may be more important for virulence of LEE-negative isolates, since increased local damage to the intestinal epithelium could occur despite the absence of LEE-mediated adherence. Similarly, flagellin may enhance the ability of other highly virulent STEC serotypes (e.g. O157:H7) to cause HUS. Delineating the mechanisms of pro-inflammatory responses mediated by STEC flagellin may aid in our understanding of the pathogenesis of serious STEC disease and provide novel therapeutic targets to reduce the probability of progression of STEC infection. However, H⁺ STEC isolates are also able to cause serious disease, including HUS. Thus, flagellin, is not essential for pathogenesis, but may nevertheless enhance virulence through the augmentation of inflammatory responses.

This study has provided a better understanding of the mechanisms of pathogenesis of STEC disease and may have implications for treatment of serious STEC infections. To date, serious STEC disease has no effective treatment(s), other than the hospitalisation and close monitoring of patients. Anti-microbial and anti-motility agents are contraindicated, and treatment is directed largely at supportive therapy. Prevention of serious STEC disease would require preventing or limiting the uptake of Stx into the circulation, thereby reducing the chance of developing HUS. Several toxin-binding therapeutic strategies are being developed

for STEC infections, including a recombinant *E. coli* strain expressing a Gb₃ mimic on its surface (Paton *et al.*, 2000). Oral administration of this strain provides 100% protection to mice challenged with an otherwise fatal dose of STEC (Paton *et al.*, 2000; Paton *et al.*, 2001b). Injectable toxin-binding compounds are also under development (Kitov *et al.*, 2000; Mulvey *et al.*, 2003). The data from this study also suggest that the administration of anti-inflammatory agents targeting the chemokine or MAPK pathways may reduce the severity of STEC infection and, in combination with Stx-binding compounds, may further reduce the systemic complications associated with severe STEC disease.

Clearly, further studies are required in order to complete our understanding of the role of flagellin in pathogenesis, particularly its role in recruitment of PMNs *in vivo*. STEC are significant causes of morbidity and mortality worldwide, and a full understanding of pathogenic mechanisms is essential for the development of the most effective therapeutic and preventative strategies.

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Appendix I

Nucleotide and deduced amino acids of a 1,467-bp region of *fliC* from STEC strain 95ZG1 (O26) cloned into pGEM-T easy (Section 4.2.6.11).

The amino acid sequence represents amino acids 1–489 of the FliC ORF from *E. coli* O157:H11 strain 111 (GenBank accession number AY337465). The amino acid translation is represented by a single letter code below the second nucleotide of each codon. Termination codons are indicated by an asterisk (*).

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1      ATGGCACAAAGTCATTAATACCCAACAGCCTGTCGCTGTTGACCAGAATAACCTGAACAAA
1      M A Q V I N T Q Q P V A V D Q N N L N K

61     TCTCAGTCTTCTCTGAGCTCCGCCATTGAACGTCTCTCTTCTGGCCTGCGTATTAACAGT
21     S Q S S L S S A I E R L S S G L R I N S

121    GCTAAAGATGACGCAGCAGGTCAGGCGATTGCTAACCGTTTTACAGCAAATATTTAAAGGT
41     A K D D A A G Q A I A N R F T A N I K G

181    CTGACTCAGGCTTCCCGTAACGCGAATGATGGTATTTCTGTTGCGCAGACCACTGAAGGT
61     L T Q A S R N A N D G I S V A Q T T E G

241    GCGCTGAATGAAATTAACAACAACCTGCAGCGTGTACGTGAACTGACTGTTTCAGGCAACT
81     A L N E I N N N L Q R V R E L T V Q A T

301    AACGGTACTAACTCTGACAGCGATCTTTCTTCTATCCAGGCTGAAATTACTCAACGTCTG
101    N G T N S D S D L S S I Q A E I T Q R L

361    GAAGAAATTGACCGTGTATCTGAGCAAACCTCAGTTTAACGGCGTGAAAGTCCTTGCTGAA
121    E E I D R V S E Q T Q F N G V K V L A E

421    AATAATGAAATGAAAATTCAGGTTGGTGCTAATGATGGTGAAACCATCACTATCAATCTG
141    N N E M K I Q V G A N D G E T I T I N L

481    GCAAAAATTGATGCGAAAACCTCTCGGCCCTGGACGGTTTTAATATCGATGGCGCGCAGAAA
161    A K I D A K T L G L D G F N I D G A Q K

541    GCAACTGGCAGTGACCTGATTTCTAAATTTAAAGCGACAGGTACTGATAACTATGATGTT
181    A T G S D L I S K F K A T G T D N Y D V

601    GGCGGTGATGCTTATACTGTAAACGTAGATAGCGGAGCTGTTAAAGATACTACAGGGAAT
201    G G D A Y T V N V D S G A V K D T T G N

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661 GATATTTTTGTTAGTGCAGCAGATGGTTCCTGACAACCTAAATCTGACACAAACATAGCT
221 D I F V S A A D G S L T T K S D T N I A

721 GGTACAGGGATTGATGCTACAGCACTCGCAGCAGCGGCTAAGAATAAAGCACAGAATGAT
241 G T G I D A T A L A A A A K N K A Q N D

781 AAATTCACGTTTAAATGGAGTTGAATTCACAACAACAACCTGCAGCGGATGGCAATGGGAAT
261 K F T F N G V E F T T T T A A D G N G N

841 GGTGTATATTCTGCAGAAATTGATGGTAAGTCAGTGACATTTACTGTGACAGATGCTGAC
281 G V Y S A E I D G K S V T F T V T D A D

901 AAAAAAGCTTCTTTGATTACGAGTGAGACAGTTTACAAAAATAGCGCTGGCCTTTATACG
301 K K A S L I T S E T V Y K N S A G L Y T

961 ACAACCAAAGTTGATAACAAGGCTGCCACACTTCCGATCTTGATCTCAATGCAGCTAAG
321 T T K V D N K A A T L S D L D L N A A K

1021 AAAACAGGAAGCACGTTAGTTGTTAACGGTGCAACTTACGATGTTAGTGCAGATGGTAAA
341 K T G S T L V V N G A T Y D V S A D G K

1081 ACGATAACGGAGACTGCTTCTGGTAACAATAAAGTCATGTATCTGAGCAAATCAGAAGGT
361 T I T E T A S G N N K V M Y L S K S E G

1141 GGTAGCCCGATTCTGGTAAACGAAGATGCAGCAAAATCGTTGCAATCTACCACCAACCCG
381 G S P I L V N E D A A K S L Q S T T N P

1201 CTCGAAACTATCGACAAAAGCATTGGCTAAAGTTGACAATCTGCGTTCTGACCTCGGTGCA
401 L E T I D K A L A K V D N L R S D L G A

1261 GTACAAAACCGTTTCGACTCTGCTATACCAACCTTGGCAACACCGTAAACAACCTGTCT
421 V Q N R F D S A I T N L G N T V N N L S

1321 TCTGCCCGTAGCCGTATCGAAGATGCTGACTACGCGACCGAAGTGTCTAACATGTCTCGT
441 S A R S R I E D A D Y A T E V S N M S R

1381 GCGCAGATCCTGCAACAAGCGGGTACCTCTGTTCTGGCGCAGGCTAACCAGACCACGCAG
461 A Q I L Q Q A G T S V L A Q A N Q T T Q

1441 AACGTACTGTCTCTGCTGCAGGGTTAG
481 N V L S L L Q G *

Appendix II

Nucleotide and deduced amino acids of a 1,728-bp region of *fliC* from STEC strain 95HE4 (O91) cloned into pGEM-T easy (Section 4.2.6.11).

The amino acid sequence represents amino acids 6–582 of the FliC ORF from *E. coli* O53:H7 strain 14097 (GenBank accession number AF228496). The amino acid translation is represented by a single letter code below the second nucleotide of each codon.

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1      AATACCAACAGCCTCTCGCTGATCACTCAAAAATAATATCAACAAGAACCAGTCTGCGCTG
1      N T N S L S L I T Q N N I N K N Q S A L

61     TCGAGTTCTATCGAGCGTCTGTCTTCTGGCTTGCCTATTAACAGCGCGAAGGATGACGCC
21     S S S I E R L S S G L R I N S A K D D A

121    GCAGGTCAGGCGATTGCTAACCGTTTTACTTCTAACATTAAAGGCCTGACTCAGGCTGCA
41     A G Q A I A N R F T S N I K G L T Q A A

181    CGTAACGCCAACGACGGTATTTCTGTTGCGCAGACCACCGAAGGCGCGTGTCCGAAATC
61     R N A N D G I S V A Q T T E G A L S E I

241    AACAACTTACAGCGTATTCGTGAACTGACGGTTCAGGCCACTACAGGGACTAACTCC
81     N N N L Q R I R E L T V Q A T T G T N S

301    GATTCTGACCTGGACTCCATCCAGGACGAAATCAAATCTCGTCTTGATGAAATTGACCGC
101    D S D L D S I Q D E I K S R L D E I D R

361    GTATCCGGCCAGACCCAGTTCAACGGCGTGAACGTGCTGGCGAAAGACGGTTCAATGAAA
121    V S G Q T Q F N G V N V L A K D G S M K

421    ATTCAGGTTGGTGCGAATGACGGCGAAACCATCACGATCGACCTGAAAAAATCGATTCT
141    I Q V G A N D G E T I T I D L K K I D S

481    GATACTCTGGGTCTGAATGGCTTTAACGTAAATGGTAAAGGTACTATTACCAACAAAGCT
161    D T L G L N G F N V N G K G T I T N K A

541    GCAACGGTAAGTGATTTAACTTCTGCTGGCGCGAAGTTAAACACCACGACAGGTCTTTAT
181    A T V S D L T S A G A K L N T T T G L Y

601    GATCTGAAAACCGAAAATACCTTGTTAACTACCGATGCTGCATTTCGATAAATTAGGGAAT
201    D L K T E N T L L T T D A A F D K L G N

661    GCGGATAAAGTCACAGTTGGCGGCGTAGATTATACTTACAACGCTAAAATCTGGTGATTTT
221    G D K V T V G G V D Y T Y N A K S G D F

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721 ACTACCACTAAATCTACTGCTGGTACGGGTGTAGACGCCGCGGCGCAGGCTGCTGATTCA
241 T T T K S T A G T G V D A A A Q A A D S

781 GCTTCAAAACGTGATGCGTTAGCTGCCACCCTTCATGCTGATGTGGGTAAATCTGTTAAT
261 A S K R D A L A A T L H A D V G K S V N

841 GGTCTTACACCACAAAAGATGGTACTGTTTCTTTCGAAACGGATTACAGCAGGTAATATC
281 G S Y T T K D G T V S F E T D S A G N I

901 ACCATCGGTGGAAGCCAGGCATACGTAGACGATGCAGGCAACTTGACGACTAACAACGCT
301 T I G G S Q A Y V D D A G N L T T N N A

961 GGTAGCGCAGCTAAAGCTGATATGAAAGCGCTGCTCAAAGCAGCGAGCGAAGGTAGTGAC
321 G S A A K A D M K A L L K A A S E G S D

1021 GGTGCCTCTCTGACATTCAATGGCACAGAATATACCATCGAAAAGCAACTCCTGCGACA
341 G A S L T F N G T E Y T I A K A T P A T

1081 ACCACTCCAGTAGCTCCGTTAATCCCTGGTGGGATTACTTATCAGGCTACAGTGAGTAAA
361 T T P V A P L I P G G I T Y Q A T V S K

1141 GATGTAGTATTGAGCGAAACCAAAGCGGCTGCCGCGACATCTTCAATTACCTTTAATTCC
381 D V V L S E T K A A A A T S S I T F N S

1201 GGTGTACTGAGCAAACTATTGGGTTTACCGCGGGTGAATCCAGTGATGCTGCGAAGTCT
401 G V L S K T I G F T A G E S S D A A K S

1261 TATGTGGATGATAAAGGTGGTATCACTAACGTTGCCGACTATACAGTCTCTTACAGCGTT
421 Y V D D K G G I T N V A D Y T V S Y S V

1321 AGCAAGGATAACGGCTCTGTGACTGTTGCCGGGTATGCTTCAGCGACTGATACCAATAAA
441 S K D N G S V T V A G Y A S A T D T N K

1381 GATTATGCTCCAGCAATTGGCACTGCTGTAAATGTGAACTCCGCGGGTAAAATCACTACT
461 D Y A P A I G T A V N V N S A G K I T T

1441 GAGACTACCAGTGCTGGTTCTGCAACGACCAACCCGCTTGCTGCCCTGGACGACGCAATC
481 E T T S A G S A T T N P L A A L D D A I

1501 AGCTCCATCGACAAATTCGTTCTTCCCTGGGTGCTATCCAGAACCGTCTGGATTCCGCG
501 S S I D K F R S S L G A I Q N R L D S A

1561 GTCACCAACCTGAACAACCACTACCAACCTGTCCGAAGCGCAGTCCCGTATTCAGGAC
521 V T N L N N T T T N L S E A Q S R I Q D

1621 GCCGACTATGCGACCGAAGTGTCCAACATGTCGAAAGCGCAGATCATCCAGCAGGCCGGT
541 A D Y A T E V S N M S K A Q I I Q Q A G

1681 AACTCCGTGCTGGCAAAAAGCTAACCAGGTACCGCAGCAGGTTCTGTCT
561 N S V L A K A N Q V P Q Q V L S

Appendix III

Nucleotide and deduced amino acids of a 1,734-bp region of *fliC* from STEC strain EDL933 (O157:H7) cloned into pGEM-T easy (Section 4.2.6.11).

The amino acid sequence represents amino acids 5–583 of the FliC ORF from *E. coli* O157:H7 strain 51 (GenBank accession number AY337468). The amino acid translation is represented by a single letter code below the second nucleotide of each codon.

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1      ATTAATACCAACAGCCTCTCGCTGATCACTCAAATAATATCAACAAGAACCAGTCTGCG
1      I N T N S L S L I T Q N N I N K N Q S A

61     CTGTCGAGTTCTATCGAGCGTCTGTCTTCTGGCTTGGCTATTAACAGCGCGAAGGATGAC
21     L S S S I E R L S S G L R I N S A K D D

121    GCCGCAGGTCAGGCGATTGCTAACCGTTTTACTTCTAACATTAAAGGCCTGACTCAGGCG
41     A A G Q A I A N R F T S N I K G L T Q A

181    GCCCGTAACGCCAACGACGGTATTTCTGTTGCGCAGACCACCGAAGGCGCGCTGTCCGAA
61     A R N A N D G I S V A Q T T E G A L S E

241    ATCAACAACAACCTTACAGCGTATTCGTGAACTGACGGTTCAGGCCACTACAGGGACTAAC
81     I N N N L Q R I R E L T V Q A T T G T N

301    TCCGATTCTGACCTGGACTCCATCCAGGACGAAATCAAATCTCGTCTTGATGAAATTGAC
101    S D S D L D S I Q D E I K S R L D E I D

361    CGCGTATCCGGCCAGACCCAGTTCAACGGCGTGAACGTGCTGGCGAAAGACGGTTCAATG
121    R V S G Q T Q F N G V N V L A K D G S M

421    AAAATTCAGGTTGGTGCGAATGACGGCGAAACCATCACGATCGACCTGAAAAAATCGAT
141    K I Q V G A N D G E T I T I D L K K I D

481    TCTGATACTCTGGGTCTGAATGGCTTTAACGTAATGGTAAAGGTACTATTACCAACAAA
161    S D T L G L N G F N V N G K G T I T N K

541    GCTGCAACGGTAAGTGATTTAACTTCTGCTGGCGGAAGTTAAACACCACGACAGGTCTT
181    A A T V S D L T S A G A K L N T T T G L

601    TATGATCTGAAAACCGAAAATACCTTGTTAACTACCGATGCTGCATTCGATAAAATTAGGG
201    Y D L K T E N T L L T T D A A F D K L G

661    AATGGCGATAAAGTCACAGTTGGCGGCGTAGATTATACTTACAACGCTAAATCTGGTGAT
221    N G D K V T V G G V D Y T Y N A K S G D

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721 TTTACTACCACTAAATCTACTGCTGGTACGGGTGTAGACGCCGCGCGCAGGCTGCTGAT
241 F T T T K S T A G T G V D A A A Q A A D

781 TCAGCTTCAAACGTGATGCGTTAGCTGCCACCCTTCATGCTGATGTGGGTAAATCTGTT
261 S A S K R D A L A A T L H A D V G K S V

841 AATGGTTCTTACACCACAAAAGATGGTACTGTTTCTTTTCGAAACGGATTTCAGCAGGTAAT
281 N G S Y T T K D G T V S F E T D S A G N

901 ATCACCATCGGTGGAAGCCAGGCATACGTAGACGATGCAGGCAACTTGACGACTAACAAC
301 I T I G G S Q A Y V D D A G N L T T N N

961 GCTGGTAGCGCAGCTAAAAGCTGATATGAAAGCGCTGCTCAAAGCAGCGAGCGAAGGTAGT
321 A G S A A K A D M K A L L K A A S E G S

1021 GACGGTGCCTCTCTGACATTCAATGGCACAGAATATACCATCGCAAAGCAACTCCTGCG
341 D G A S L T F N G T E Y T I A K A T P A

1081 ACAACCACTCCAGTAGCTCCGTTAATCCCCGGTGGGATTACTTATCAGGCTACAGTGAGT
361 T T T P V A P L I P G G I T Y Q A T V S

1141 AAAGATGTAGTATTGAGCGAAACCAAAGCGGCTGCCGCGACATCTTCAATTACCTTTAAT
381 K D V V L S E T K A A A A T S S I T F N

1201 CCCGGTGTACTGAGCAAAACTATTGGGTTTACCGGGTGAATCCAGTGATGCTGCGAAG
401 P G V L S K T I G F T A G E S S D A A K

1261 TCTTATGTGGATGATAAAGGTGGTATCACTAACGTTGCCGACTATACAGTCTCTTACAGC
421 S Y V D D K G G I T N V A D Y T V S Y S

1321 GTTAAACAAGGATAACGGCTCTGTGACTGTTGCCGGGTATGCTTCAGCGACTGATACCAAT
441 V N K D N G S V T V A G Y A S A T D T N

1381 AAAGATTATGCTCCAGCAATTGGTACTGCTGTAAATGTGAACTCCGCGGGTAAAATCACT
461 K D Y A P A I G T A V N V N S A G K I T

1441 ACTGAGACTACCAGTGCTGGTTCTGCAACGACCAACCCGCTTGCTGCCCTGGACGACGCA
481 T E T T S A G S A T T N P L A A L D D A

1501 ATCAGCTCCATCGACAAAATTCGGTTCTTCCCTGGGTGCTATCCAGAACCGTCTGGATTCC
501 I S S I D K F R S S L G A I Q N R L D S

1561 GCAGTCACCAACCTGAACAACCACTACCAACCTGTCCGAAGCGCAGTCCCGTATTTCAG
521 A V T N L N N T T T N L S E A Q S R I Q

1621 GACGCCGACTATGCGACCGAAGTGTCCAACATGTGAAAGCGCAGATCATTCAGCAGGCC
541 D A D Y A T E V S N M S K A Q I I Q Q A

1681 GGTAACCTCCGTGCTGGCAAAAGCTAACCAAGGTACCGCAGCAGGTTCTGTCTCTA
561 G N S V L A K A N Q V P Q Q V L S L

Appendix IV

Publications

Rogers, T. J., Paton, A. W., McColl, S. R. and Paton, J. C. (2003). Enhanced CXC chemokine responses of human colonic epithelial cells to locus of enterocyte effacement-negative Shiga-toxigenic *Escherichia coli*. *Infect. Immun.* **71**: 5623-5632.

Rogers, T. J., Paton, A. W., McColl, S. R., and Paton, J. C. Enhanced CXC chemokine responses of human colonic epithelial cells to LEE-negative STEC are largely attributable to certain types of flagellin. Abstract O40, p. 40. 5th International Symposium on Shiga toxin (Verocytotoxin)- producing *Escherichia coli* infections, 8-11 June 2003, Edinburgh, Scotland.

Rogers, T. J., Smith, W. E., Paton, A. W., Paton, J. C., Yu, Y, Gewirtz, A. T. and Thorpe, C. M. (2004). The role of Toll-like receptor 5 and the mitogen activating protein kinases in the inflammatory response to H21 flagellin of Shiga-toxigenic *Escherichia coli*. Manuscript submitted to *Infection and Immunity*.

Rogers, T. J., Hurley, B. P., Paton, A. W. and Paton, J. C. (2004). Role of flagellin of O113:H21 Shiga-toxigenic *Escherichia coli* in epithelial cell PMN transmigration and virulence in mice. Manuscript in preparation.