Signal Transduction Responses to Enteropathogenic *Escherichia coli* and Shiga toxin-producing *Escherichia coli* Infections

by

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Dedicated to my dad (Stan) and mom (Mercy),
the rest of my family, Joannie, and all my friends

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List of Abbreviations

AMP	adenosine monophosphate
α-actinin	alpha-actinin
α-hemolysin	alpha-hemolysin
ABC	ATP binding cassette
AE	attaching and effacing
astA	EAST-1 gene
bfp	bundle-forming pilus gene
Ca^{2+}	calcium
CDT	cytolethal distending toxin
ces	chaperone for EPEC secretion gene
CFTR	cystic fibrosis transmembrane conductance regulator
CFU	colony forming unit
Cl^-	chloride
CNF	cytotoxic necrotizing factor
COX	cyclooxygenase
DAEC	diffusely adherent *E. coli*
DAG	diacylglycerol
dsbA	disulfide bond A gene
E. coli	*Escherichia coli*
eae	*E. coli* attaching and effacing gene
EAEC/EAggEC	enteroaggregative *E. coli*

EAF	EPEC adherence factor plasmid
EAST-1	EAEC heat-stable enterotoxin
EHEC	enterohemorrhagic *E. coli*
EIEC	enteroinvasive *E. coli*
EPEC	enteropathogenic *E. coli*
ERIC	enterobacterial repeat intergenic consenses element
esc	*E. coli* secretion gene
espA	*E. coli* secreted protein A gene
espB	*E. coli* secreted protein B gene
espC	*E. coli* secreted protein C gene
espD	*E. coli* secreted protein D gene
espE	*E. coli* secreted protein E gene
espF	*E. coli* secreted protein F gene
espP	*E. coli* secreted protein P gene
ETEC	enterotoxigenic *E. coli*
F-actin	filamentous actin
FAS	fluorescent F-actin stain
Fe	iron
FITC	fluorescein isothiocyanate
Gb_3	globotriaosylceramide
GTPase	guanosine triphosphatase
H	flagellar antigen

HC	hemorrhagic colitis
HEp-2	human laryngeal epithelial cell line
HPLC	high-performance liquid chromatography
HUS	hemolytic uremic syndrome
IBD	inflammatory bowel disease
IgA	immunoglobulin A
IL	interleukin
IP_3	inositol-1,4,5-trisphosphate
Ipa	invasion plasmid antigen
LA	localized adherence
LCT	large Clostridial toxin
LEE	locus of enterocyte effacement
LO	lipoxygenase
LT	heat-labile toxin
MLC	myosin light chain
MLCK	myosin light chain kinase
mRNA	messenger RNA
$NaHCO_3$	sodium bicarbonate
NFκB	nuclear factor kappa B
O	somatic antigen
OMP	outer membrane protein
ORF	open reading frame

P	phosphate
PAI	pathogenicity island
PBS	phosphate-buffered saline
perA	plasmid encoded regulator A gene
perB	plasmid encoded regulator B gene
perC	plasmid encoded regulator C gene
PG	prostaglandin
PI3-K	phosphoinositide 3-kinase
PtdIns-4,5-P_2	phosphatidylinositiol-4,5-bisphosphate
PtdIns-3,4,5-P_3	phosphatidylinositiol-3,4,5-trisphosphate
PKC	protein kinase C
PLC	phospholipase C
PMN	polymorphonuclear cell
pssA	protease secreted by STEC A gene
PtdIns	phosphatidylinositol
RDEC	rabbit diarrheagenic *E. coli*
rRNA	ribosomal RNA
sep	secretion of EPEC protein gene
Sip	*Salmonella* invasion protein
ST	heat-stable toxin
STEC	Shiga toxin-producing *E. coli*
Stx	Shiga toxin

TEM	transmission electron microscopy
TIBA	Tir-intimin binding area
TLC	thin-layer chromatography
TNF-α	tumor necrosis factor-alpha
tRNA	transfer RNA
TTP	thrombotic thrombocytopenic purpura
VacA	vacuolating cytotoxin A
VTEC	Verocytotoxin-producing *E. coli*
Yop	*Yersinia* outer membrane protein
ZO	zonula occludens

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Abstract

Enteropathogenic *Escherichia coli* (EPEC) and Shiga toxin-producing *Escherichia coli* (STEC) induce morphological changes in infected epithelial cells. The resulting attaching and effacing lesion is characterized by intimate bacterial adherence to epithelial cells with microvillus destruction, cytoskeletal rearrangement and aggregation of host cytoskeletal proteins, including α-actinin. This study was undertaken to further characterize host cytosolic responses to bacterial infection by using a series of specific cell signaling inhibitors of phosphoinositide-specific phospholipase C, phosphoinositide 3-kinase, 5-lipoxygenase, and cyclooxygenase. Inhibitory effects of the enzyme inhibitors were measured by their ability to disrupt formation of attaching and effacing lesions by EPEC and STEC. HEp-2 cells were preincubated with inhibitors before infection with EPEC strain E2348/69 (serotype O127:H6), STEC strain CL56 (O157:H7), or a signaling-deficient EPEC mutant UMD864 (O127:H6). Attaching and effacing lesions were detected by immunofluorescence microscopy using a monoclonal antibody against α-actinin. While initial bacterial adherence to epithelial cells was not reduced, α-actinin accumulation in infected HEp-2 cells was blocked by enzyme inhibitors ET-18-OCH_3, wortmannin, LY294002, and NDGA, but not by NS-398. This study suggests that the cytoskeletal reorganizations induced during EPEC and STEC infections are dependent on phospholipase C, phosphoinositide 3-kinase, and 5-lipoxygenase, but cyclooxygenase-independent. Understanding the signal transduction responses to EPEC and STEC infections could provide the basis for the development of novel therapies for use in the prevention and treatment of these infections in humans.

Chapter 1

Introduction

A. *Escherichia coli*

Escherichia coli was first isolated in 1885 by the German bacteriologist, Theodor Escherich, as a normal inhabitant of the intestinal tract. Escherich named the organism *Bacterium coli*, reflecting the rod shape of the cell (*Bacterium* means rod-shaped) and its intestinal habitat (*coli* for colon). The genus name *Bacterium* subsequently was changed to *Escherichia* in honour of its discoverer.

E. coli is a Gram-negative facultative anaerobic bacillus belonging to the family Enterobacteriaceae. *E. coli* colonizes the intestinal tract of the bottle-fed newborn within hours of life and the breast-fed infant after weaning (Elegbe & Ojofeitimi, 1984) (Tullus *et al.*, 1988). Thereafter, *E. coli* and the host derive mutual benefit (Nataro & Kaper, 1998). Although most *E. coli* strains exist as symbionts in the human gut, several distinct groups possess specific virulence determinants that make the organism capable of causing human illnesses (Giron *et al.*, 1991). These pathogenic strains include several categories that cause diarrhea by different mechanisms, affect specific age groups in diverse geographic locations, and lead to a variety of clinical presentations.

There are currently at least six distinct categories of *E. coli* that cause diarrhea (Nataro & Kaper, 1998). These include enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), enteroaggregative *E. coli* (EAggEC or EAEC), and diffusely adherent *E. coli* (DAEC) (**Table 1**). Despite differences in the epidemiology, pathogenesis, and clinical syndromes associated with *E. coli* in each of these categories, certain common themes are apparent. Strains belonging to most of these categories produce specific fimbriae associated with attachment to enterocytes. Strains belonging to several of these categories produce

Table 1. Categories of diarrheagenic *Escherichia coli*.

Category	Epidemiology	Clinical syndrome
ETEC	Travelers	Watery diarrhea, acute
EIEC	Occasional epidemics, children	Dysentery
EPEC	Infants in developing countries	Watery diarrhea, chronic
EHEC	All ages, outbreaks	Hemolytic uremic syndrome
EAEC	Children	Watery diarrhea, persistent
DAEC	Children	Watery diarrhea, persistent

specific toxins that can result in net intestinal fluid loss. Finally, virulence factors (toxins, invasion and adherence genes, and type III secretion apparatus and effector molecules) in most of the diarrheagenic *E. coli* are associated with the presence of specific transmissible elements including plasmids, phages, and pathogenicity islands.

B. Pathogenicity Island

Pathogenic microorganisms elaborate a diverse array of secreted virulence proteins which facilitate their colonization and persistence in a variety of eukaryotic hosts and host tissues (Finlay & Falkow, 1997). In recent years, it has emerged as a common theme among bacterial pathogens that virulence genes, clustered in distinct stretches of the bacterial chromosome, confer features associated with virulence upon the host bacterium. These clusters are known as pathogenicity islands (PAI) (Lee, 1996), and these pieces of DNA are often missing in avirulent bacteria.

Pathogenicity islands often exceed 30 kb in length and can contain multiple operons encoding several virulence phenotypes. Many pathogenicity islands differ from the bulk of the bacterial genome in G+C content and codon usage, and their borders are often marked by repeated sequences or insertion elements, suggesting a recombination event could have delivered them into the chromosome (Mecsas & Strauss, 1996). Transduction, transformation, and conjugation are well-characterized mechanisms for DNA transfer among bacteria.

Pathogenicity islands are horizontally acquired and often inserted at tRNA loci (Lee, 1996), although the significance of this observation remains unclear. Perhaps the conserved portion of tRNA genes is a useful landmark for mobile genetic elements that

inhabit a variety of prokaryotic hosts. In addition, the regions of dyad symmetry characteristic of tRNA genes could serve as binding sites for enzymes involved in recombination (Mecsas & Strauss, 1996).

Specific deletion of large virulence regions has been observed in some bacterial species. For example, PAI-I and PAI-II in uropathogenic *E. coli* can be lost from the chromosome by recombination events and a 102 kb region of the *Yersinia pestis* chromosome can be lost by spontaneous deletion. The biological significance of this instability is not fully understood, but it is possible that deletion benefits the organism, serving to modulate bacterial virulence and genome size during infection. Indeed, expression of particular genes at inappropriate times can be detrimental to bacterial pathogens (Akerley *et al.*, 1998).

It has been recently proposed that the deletion of commonly inherited genes that inhibit virulence is a complement to the acquisition of genes that augment virulence (Maurelli *et al.*, 1998). The formation of "blackholes" or genome deletion sheds genes that are detrimental to the new pathogenic lifestyle, thereby leading to enhanced virulence status of the pathogen. This proposal has been supported by the observation that deletion of the lysine decarboxylase activity (Maurelli *et al.*, 1998) and protease activity (Nakata *et al.*, 1993) in *Shigella* species enables the bacterial strains to augment virulence.

C. Type III Secretion

Proteins secreted by Gram-negative bacteria have to transfer across both the inner and outer membranes in addition to the intervening periplasmic space. Four pathways of

protein secretion have been described in Gram-negative bacteria (**Figure 1**) (Finlay & Falkow, 1997).

Type I secretion requires three secretory proteins: an inner membrane transport ATPase (termed ABC transporter), an outer membrane protein, and a membrane-fusion protein. Proteins secreted by type I system cross directly from the cytoplasm to the cell surface. The secreted proteins are not subject to proteolytic cleavage, and the secretion signal is located within the carboxy-terminal 60 amino acids of the secreted proteins. Type I secretion is exemplified by the *E. coli* α-hemolysin secretion system.

Type II-secreted proteins use the general secretory pathway to reach the periplasm. While some of the proteins transverse the outer membrane through distinct channels, some remain in the periplasmic space. A signature of *sec*-dependent protein export is the presence of a short amino-terminal signal sequence in the exported protein. The signal sequence aids protein export and is cleaved off by a periplasmic signal peptidase when the exported protein reaches the periplasm. Type II secretion is the primary pathway for the secretion of extracellular degradative enzymes by Gram-negative bacteria.

Type IV-secreted proteins, as in type II secretion, are exported from the cytoplasm to the periplasm via the *sec* pathway. Type IV secretion pathway comprises so-called autotransporters, including VacA of *Helicobacter pylori*, IgA protease from *Neisseria gonorrheae*, EspC from EPEC, and SepA for *Shigella flexneri*. These autotransporters form a pore in the outer membrane through which they pass, and autoproteolytic cleavage releases the proteins into the extracellular medium.

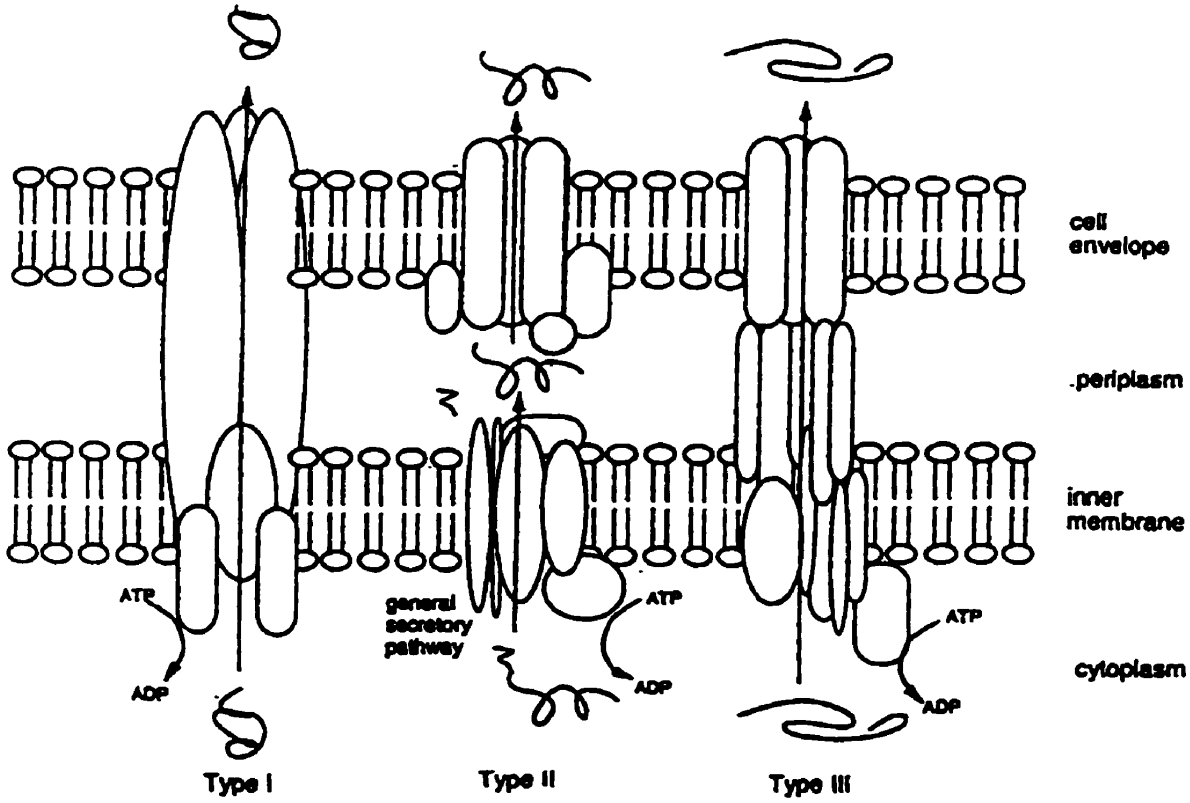


Figure 1. A schematic diagram of type I, type II, and type III secretion systems in Gram-negative bacteria. Taken from Mecsas & Strauss, 1996.

Interaction of bacterial pathogens with host cells is particularly characterized by factors that are either located on the bacterial surface or secreted into the extracellular space. Type III secretion system appears to be a dedicated machinery specifically translocating pathogenicity proteins into the cytosol of eukaryotic cells (Hueck, 1998). It is believed that the acquisition of this secretion system enhances virulence of the strains that possess it (Groisman & Ochmann, 1996). Many of the proteins injected into the cytosol by the type III system are virulence factors, such as the invasion plasmid antigens (Ipas) of *Shigella* species, *Salmonella* invasion proteins (Sips/Ssps), and the *Yersinia* outer membrane proteins (Yops).

Type III secretion, like type I, is independent of the *sec* system and thus does not involve cleavage of the leader peptide of the secreted protein. It has been proposed that the secretion signal resides in the 5' region of the mRNA which encodes the secreted protein (Anderson & Schneewind, 1997). Type III-secreted proteins require specific chaperones to protect the secreted factors from premature interaction with other components of the secretion system, and secretion occurs in a continuous process, through both inner and outer membranes of the bacterium, without the detectable presence of periplasmic intermediates. In contrast to the secretion process in other systems, type III secretion is triggered when a pathogen comes in close contact with host cells or host factors. Therefore, type III secretion apparatus is also known as contact-induced secretion system (Lee, 1997). However, a variety of environmental factors also induce the production of components of the type III secretion machinery. Such environmental signals include ambient temperature, extracellular calcium (Ca^{2+}) concentration, osmolarity, pH, and oxygen tension of the growth medium (Galan, 1996).

The synthesis of components of the secretion machinery and the production and secretion of substrates in *Salmonella typhimurium* do not require contact with a eukaryotic host cell (Daefler, 1999).

In most cases, the G+C contents of type III secretion genes are in the 40 to 45% range (Hueck, 1998) and are lower than the G+C contents of the surrounding genomes. Since the low G+C *Chlamydia* species carry a recently discovered type III secretion system (Hsia *et al.*, 1997), chlamydiae may be the evolutionary ancestors of type III secretion. This observation, however, needs further investigation.

Chapter 2

Enteropathogenic *Escherichia coli*

A. Introduction

Enteropathogenic *Escherichia coli*, also known as EPEC, is an important category of diarrheagenic *E. coli* that causes sickness in both humans and animals (Donnenerg & Kaper, 1992). EPEC, the first *E. coli* to be associated with diarrhea, is a small bowel pathogen that predominately infects the proximal small intestine, either in the duodenum or proximal jejunum, although there may be some colonic involvement due to the overgrowth of the organism from the small bowel (Rothbaum *et al.*, 1983). Worldwide, EPEC is a leading cause of bacteria-mediated diarrhea in children (Nataro & Kaper, 1998). The disease is characterized often by chronic and persistent watery diarrhea of varying severity, while vomiting and fever generally accompany fluid loss (Frankel *et al.*, 1998b).

Population genetic surveys, with multilocus enzyme electrophoresis, have shown that classical EPEC strains have diverged into two major or related clones, designated EPEC clone 1 and EPEC clone 2 (Whittam & McGraw, 1996). Within each group, a variety of somatic (O) antigens are present, while the flagellar (H) antigens are conserved; EPEC clone 1 typically expresses flagellar antigen H6, whereas EPEC clone 2 expresses flagellar antigen H2 or are non-motile (H-).

B. Attaching and Effacing Lesions

Although EPEC has long been recognized as an important paediatric pathogen, the pathogenesis of the diarrheal disease produced remains uncertain. The hallmark of infections due to classical EPEC is the distinctive attaching and effacing (AE) histopathology (**Figure 2**).

Figure 2. A transmission electron micrograph showing the attaching and effacing lesions on HEp-2 cells following infection with EPEC strain E2348/69 for 3 hrs at 37°C.

The first observations of AE lesions in humans were made in 1980, in biopsies of intestinal tissue from EPEC-infected children. An AE lesion is characterized by intimate adherence of the bacterium to epithelial cells with localized destruction and vesiculation of brush-border microvilli, cytoskeletal rearrangement and aggregation of host cell cytoskeletal proteins, consisting of filamentous actin (F-actin). Accumulation of F-actin in adhesion pedestals underneath the plasma membrane at sites of bacterial attachment can be demonstrated by the fluorescent-actin staining (FAS) test (Knutton *et al.*, 1989). In this test, fluorescein isothiocyanate (FITC)-labeled phalloidin, a mushroom-derived toxin, binds specifically to F-actin in cultured epithelial cells directly beneath adherent bacteria. Thus, F-actin accumulation can be detected by immunofluorescence microscopy. Before the development of this test, the AE lesion histopathology could be detected only by using transmission electron microscopy (TEM).

In addition to polymerized actin, the composition of the AE lesion includes other cytoskeletal proteins beneath the adherent organisms such as the actin-binding and crosslinking protein α-actinin, talin, ezrin, and myosin light chain (Finlay *et al.*, 1992) (Knutton *et al.*, 1989) (Manjarrez-Hernandez *et al.*, 1996). Although the identity of some cytoskeletal proteins in the pedestal is known, the hierarchy and organization of these cytoskeletal components are not well characterized (Goosney *et al.*, 1999a).

Associated with these cytoskeletal rearrangement events are major perturbations of the plasma membrane, where the plasma membrane swells and cups around the adherent bacteria to generate pedestal-like structures (Knutton *et al.*, 1989). The formation of a pedestal is a dynamic process: pedestals can bend and undulate, alternatively growing longer and shorter while remaining tethered in place on the cell

surface (Sanger *et al.*, 1996). These pedestals may develop into more extended pseudopods that can elevate the attached bacteria more than 10 μm above the surface of the host cell plasma membrane (Rosenshine *et al.*, 1996) (Goosney *et al.*, 1999b). Some of the attached EPEC can move along the surface of the cultured epithelial cell, reaching the speeds up to 0.07 μm/s, in a process driven by polymerization of actin at the base of the pedestal. The signals and mechanisms responsible for cytoskeletal disruption are currently unknown. Rac, Rho, and Cdc42-dependent pathways do not appear to be involved as suggested by inhibition of these small GTP-binding proteins does not inhibit formation of actin pedestals (Ben-Ami *et al.*, 1998).

Studies in animals and humans indicate that the ability of EPEC to cause diarrhea correlates with the formation of AE lesions (Robins-Browne, 1987). AE lesion formation is essential for full EPEC pathogenicity (Donnenberg *et al.*, 1993).

C. Locus of Enterocyte Effacement

Attaching and effacing lesion formation is a complex process, with at least 12 bacterial genes implicated by mutation or by similarity to genes of other pathogens as being necessary for the phenotype. All genes necessary for AE formation are encoded on a 35 kb chromosomal pathogenicity island called the locus of enterocyte effacement (LEE) (**Figure 3**) (McDaniel & Kaper, 1997), which is not present in *E. coli* strains found in the normal flora. The LEE pathogenicity island not only is necessary for the AE phenotype, but also is sufficient as a cloned pathogenicity island confers the AE phenotype on *E. coli* laboratory strain K-12 (McDaniel & Kaper, 1997).

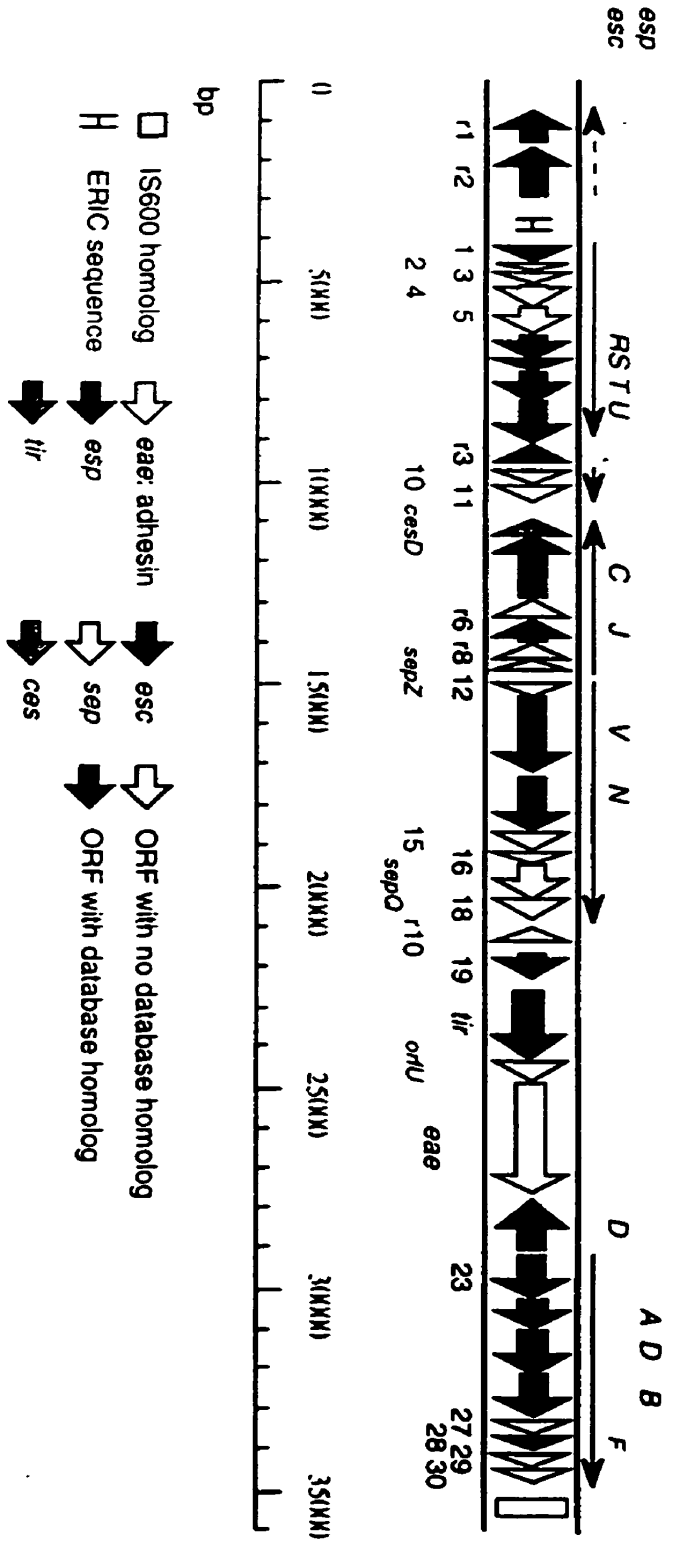


Figure 3. The locus of enterocyte effacement of EPEC strain E2348/69. Adapted from Elliott *et al.*, 1998.

The complete region of LEE is 35,624 bp with an average G+C content of 38.4 % (Elliot *et al.*, 1998), far below that of *E. coli* chromosome (50.8%) (Blattner *et al.*, 1997). Also, the glycine and cytosine content of LEE is strikingly different from the amino acid usage in the rest of the *E. coli* genome. These observations suggest that the LEE pathogenicity island has been acquired into the *E. coli* chromosomes from another species. The LEE contains 41 predicted open reading frames (ORFs) arranged in at least five polycistronic operons.

Analysis of EPEC mutants has enabled the sorting of these genes into at least three groups based on the role they play in the AE process. The central region of this pathogenicity island encodes the adhesin intimin (*eae*) and translocated intimin receptor (*tir*). Downstream of *eae* and *tir* (i.e. the right end of LEE) are genes encoding EPEC secreted proteins (*esp*), which are exported via a type III secretion apparatus (*esc*, *sep*) encoded by genes upstream (the left side of LEE). Moreover, there are genes of unknown function.

I. The Central Region of the LEE

The first gene to be associated with AE activity is the *eae* (formerly *eaeA*) gene (Jerse *et al.*, 1990) encoding intimin, a 94 kDa outer membrane protein, which mediates intimate binding of EPEC to the host. There are at least four antigenically distinct intimin subtypes: intimin α, intimin β, intimin γ, and intimin δ (Adu-Bobie *et al.*, 1998). The different intimin types likely possess different receptor-targeting specificities.

Intimin is an adhesin molecule that shares homology with invasin, a protein that promotes eukaryotic cell invasion by *Yersiniae* species. Studies of the intimin/invasin

family of proteins showed that their cell-binding activity is localized to the C-terminal 280 amino acids (Int280/Inv280). A specific cysteine residue (Cys937) in EPEC intimin is essential for binding activity (Frankel *et al.*, 1998a).

The intimin sequences of EPEC strain E2348/69 and STEC O157:H7 strain EDL933 have only 49% identity in the C-terminal region. It has been hypothesized that this sequence difference could explain why EPEC is a small bowel pathogen whereas STEC is a large bowel pathogen in humans (Yu & Kaper, 1992). Different intimin types might determine the tissue tropism, but other factors may also be responsible for tissue specificity because intimin-mediated intimate attachment occurs after initial bacterial adhesion and protein translocation.

Not only is intimin central to intestinal colonization through intimin-mediated intimate contact with the human intestinal mucosa (Hicks *et al.*, 1998), it is essential in organizing and focusing the polymerization of cytoskeletal components beneath adherent bacteria. Mutants defective in *eae* are unable to sharply focus cytoskeletal components under adherent bacteria (Donnenberg *et al.*, 1990a), thus preventing the concentration of polymerized actin necessary for a positive FAS test. The signal transduction process triggered by the mutant, however, remains intact (Rosenshine *et al.*, 1992a).

The role of intimin in human disease was demonstrated by studies in volunteers, who ingested an isogenic *eae* null mutant of E2348/69 (Donnenberg *et al.*, 1993). Diarrhea was observed in 11 of 11 volunteers ingesting the wild-type E2348/69 compared to 4 of 11 volunteers taking the isogenic mutant. These results suggest that intimin is essential for full virulence of EPEC but additional factors are clearly required for full disease manifestation.

The *tir* gene product of EPEC (homologue of *espE* of STEC) is delivered into the host cell cytoplasm and inserted into the plasma membrane (Kenny *et al.*, 1997b) (Deibel *et al.*, 1998). At least three possible functions of Tir have been identified. Tir is translocated via the type III secretion apparatus into the host cell where it serves as a cell surface receptor for the bacterial adhesin intimin. The intimin binding domain of Tir has been localized to a central portion (residues 277-322), designated TIBA (Tir-intimin binding area) (Kenny, 1999) or Tir-M (residues 255-362) (Hartland *et al.*, 1999). β_1-chain integrins, which were initially proposed to function as host cell receptors for intimin (Frankel *et al.*, 1996), are now confirmed to be not essential at any step in AE lesion formation, including intimin-mediated bacterial adherence to host target cells (Liu *et al.*, 1999).

The second function of Tir is to nucleate actin after intimin binding, possibly acting as a bridge between the bacteria and the host cytoskeleton. Tir cannot induce pedestal formation in host cells in the absence of intimin (Kenny & Finlay, 1997).

The third function of Tir is to transmit additional signals to host cells once Tir-intimin interaction occurs. These signal transduction events will be further discussed in Chapter 4.

II. The 3' Region of the LEE

EspA and EspB are two of the secreted proteins of EPEC (Jarvis *et al.*, 1995) (Kenny & Finlay, 1995). Proteins homologous to EspA and EspB of EPEC have also been identified in culture supernatants of STEC (Ebel *et al.*, 1996). However, STEC

strains of multiple serotypes isolated from calves with diarrhea do not consistently test positive for the presence of the *espB* gene (Wieler *et al.*, 1996).

EspA and EspB are needed to form AE lesions and pedestal-like structures *in vivo* (Abe *et al.*, 1998). However, EspA is not, as was previously thought, a protein involved directly in the activation of host cell signaling. Rather, EspA appears to be a structural protein that is a major component of a large filamentous organelle (Knutton *et al.*, 1998) that is transiently present on the bacterial surface which interacts with the host cell during the early stage of AE lesion formation. The EspA-containing appendages (Kenny *et al.*, 1996) are an important part of the bacterial machinery which functions as a molecular syringe to inject effector proteins into infected host cells. In STEC, EspA also contributes to the ability of the bacteria to adhere to their target cells, as demonstrated by the observation that the disruption of *espA* gene alone completely abolishes the ability of STEC to adhere to HeLa cells (Ebel *et al.*, 1998).

EspB (previously known as EaeB) (Foubister *et al.*, 1994a) is not a component of the EspA filament structure. Secretion of EspB is contact dependent; EspB is translocated into the host cell, where it is distributed to both membrane and cytosol after bacterial attachment (Wolff *et al.*, 1998). EspB has been proposed to form an eukaryotic membrane pore (Frankel *et al.*, 1998b), to which the type III secretion apparatus is connected.

EspD is required for AE activity as an *espD*-deficient mutant fails to induce cytoskeletal alternations (Lai *et al.*, 1997). Secretion of EspD increases upon contact with host cells and EspD is inserted into cell membrane at sites of bacterial contact but it is not translocated into the cytoplasm (Wachter *et al.*, 1999). Although the function of EspD is

currently uncertain, it may be a component of the EspA filaments, because an *espD* mutant secretes only low levels of EspA and produces barely detectable filaments (Knutton *et al.*, 1998).

Similar to other effector proteins, synthesis of EspF in EPEC is regulated by environmental signals and its secretion is mediated by the type III secretion apparatus. However, the precise role of EspF in EPEC pathogenesis is currently unknown (McNamara & Donnenberg, 1998). A *espF*-deletion mutant retains the ability to invade host epithelial cells, to induce Tir phosphorylation, and to rearrange host actin filaments beneath adherent bacteria. This suggests that LEE encodes functions other than those which contribute to AE histopathology. A EspF homologue is also found in STEC (Perna *et al.*, 1998).

Another secreted protein of EPEC is called EspC. STEC also has a EspC homologue: referred to as EspP in STEC O157:H7 (Brunder *et al.*, 1997) and PssA (protease secreted by STEC) in STEC O26:H11 (Djafari *et al.*, 1997). Unlike other Esps, EspC and its homologues are not encoded in the LEE of EPEC or STEC and are secreted by the *sec*-dependent type IV secretion pathway (Hueck, 1998). EspC in EPEC is not necessary for mediating EPEC-induced signal transduction and does not seem to play a role in adherence or invasion of tissue culture cells since an *espC*-deletion mutant is indistinguishable from its isogenic parent for events crucial in AE lesion formation (Stein *et al.*, 1996). Therefore, the precise role of EspC in disease pathogenesis is currently unclear.

III. The 5' Region of the LEE

Genes encoding the type III secretion system were initially named *sep* (for secretion of EPEC proteins) (Jarvis *et al.*, 1995) but were recently renamed *esc* to conform to the nomenclature of homologous type III system gene in *Yersiniae* species (Elliott *et al.*, 1998). At least 10 *esc* genes which encode proteins homologous to type III system proteins in other pathogens have been discovered (Elliott *et al.*, 1998). At least two additional LEE genes are involved in type III secretion. These two genes have no homology to other recognized systems and so they retain the *sep* gene designation. Components of the type III secretion machinery of *S. typhimurium* (and presumably other type III secretion systems) are shown to be organizing in a supramolecular structure that spans both the inner and outer membranes and there are 10 to 100 needle complexes per bacterial cell (Kubori *et al.*, 1998).

Another gene involved in EPEC type III secretion is *cesD* (for chaperone for EPEC secretion). Secretion of EspD into the culture supernatant is abolished by mutation of the *cesD* gene within the LEE region (Wainwright & Kaper, 1998). The encoded protein is a specific chaperone that interacts with EspD but not EspB or EspA for proper secretion, although levels of secreted EspB are reduced in a *cesD* mutant.

The type III secretion system is a complex machinery not only secretes target proteins across the bacterial envelope into culture supernatants but also translocates at least some bacterial proteins directly into the cytosol of the infected host cell. Mutation of genes encoding the type III secretion system or the genes encoding the secreted proteins abolishes subsequent signaling events.

EPEC protein secretion is subject to environmental regulation, and maximal secretion occurs under conditions comparable to those in the gastrointestinal tract (Kenny *et al.*, 1997a). Protein secretion is maximal at normal human body temperature (37°C) and is inhibited at both lower and higher temperatures. EPEC protein secretion is also modulated by external pH: highest levels of secretion occur between pH 6.4 and 7.6. Furthermore, maximal secretion requires the presence of sodium bicarbonate ($NaHCO_3$) and Ca^{2+} and is stimulated by millimolar concentrations of iron (Fe).

IV. The Non-coding Region of the LEE

Besides coding for functional proteins, the LEE pathogenicity island also contains non-coding regions. For example, there is a remnant transposase gene at the extreme right end of LEE, suggesting a potential mechanism for introduction of the LEE into the chromosome (Donnenberg *et al.*, 1997a), and a large enterobacterial repeat intergenic consenses (ERIC) element which has no known function but may influence gene regulation (Hulton *et al.*, 1991).

The LEE is unique to AE-causing pathogens, including *Hafnia alvei* (Albert *et al.*, 1992), the murine pathogen *Citrobacter rodentium* (formerly known as *Citrobacter freundii* biotype 4280) (Schauer & Falkow, 1993), RDEC-1 and RDEC-2 (rabbit-specific EPEC), some DAEC strains (Beinke *et al.*, 1998), and EHEC. Sequences throughout the length of LEE are highly conserved among phylogenetically distinct AE pathogens.

D. EPEC Adherence Factor Plasmid

Adherence of EPEC to HEp-2 cells was first described by Cravioto *et al.* (1979). The ability of EPEC strain E2348/69 (O127:H6) to produce a localized adherence (LA) pattern is dependent on the presence of a 60 MDa plasmid (Baldini *et al.*, 1983). A plasmid-cured EPEC strain is incapable of LA while a laboratory *E. coli* strain transformed with this plasmid acquires the ability to adhere to cells in a LA fashion. This plasmid was, therefore, termed the EPEC adherence factor (EAF) plasmid. A link between EAF plasmid and virulence was demonstrated in a volunteer trial when two of nine human volunteers fed a plasmid-cured strain developed diarrhea in comparison to nine of ten fed the wild-type parent strain (Levine *et al.*, 1985).

The identity of the EAF product as a fimbrial structure mediating LA was initially reported by Giron and Ho (1991). These fimbriae aggregate into rope-like bundles; thus, they are named bundle-forming pili. The bundle-forming pilus (BFP) mediates inter-bacterial adherence and may also mediate bacterial adherence to epithelial cells. However, binding of BFP to host cells has not been demonstrated and a specific receptor for BFP on host cells has not been identified (Donnenberg, 1999).

The BFP, a member of the type-IV fimbrial family, is encoded by 14 tandemly arrayed genes in the ***bfp*** gene cluster (**Figure 4**) (Stone *et al.*, 1996), which encompasses 11.5 kb of the EAF plasmid. These 14 genes alone are sufficient for the biogenesis of BFP in a heterologous avirulent *E. coli* host (Donnenberg *et al.*, 1997b). In addition to these plasmid-encoded genes, the formation of a functional BFP structure requires the presence of the chromosomal *dsbA* gene, which encodes a periplasmic oxidoreductase

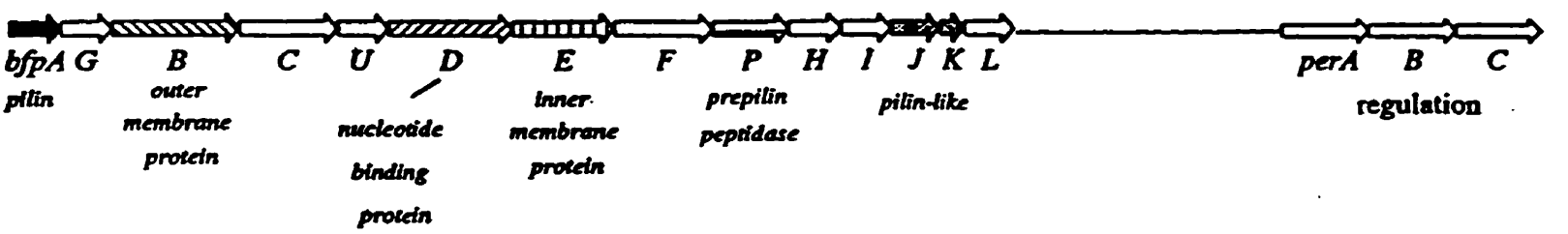


Figure 4. The *bfp* gene cluster on the EPEC adherence factor plasmid. Taken from Nataro & Kaper, 1998.

necessary for disulphide bond formation (Donnenberg *et al.*, 1997b). In the absence of the DsbA enzyme, both prebundlin (BfpA) and bundlin are rapidly degraded.

In summary, EPEC pathogenesis involves a carefully orchestrated cascade of events involving both activation and depression of a number of bacterial genes. Downstream of the *bfp* gene cluster on the EAF plasmid is a cluster of three genes (*perA*, *perB*, and *perC*) (Gomez-Duarte & Kaper, 1995) encoding a plasmid-encoded regulator (Per). The *trans*-acting Per serves as a global regulator affecting transcription of both chromosomal and plasmid-encoded genes necessary for the pathogenesis of EPEC (Donnenberg, 1999).

The Per regulator increases the transcription of *eae* and *espB*. The *per* locus also plays a role in both EspB secretion (Kenny *et al.*, 1997a) and BFP expression (Puente *et al.*, 1996). Besides being a positive regulator, *per* genes also down-regulate intimin expression following AE lesion formation by EPEC (Knutton *et al.*, 1997). Therefore, Per allows EPEC to respond to different environmental conditions and different phases of growth.

E. Model of Pathogenesis

Pathogenesis of disease caused by EPEC is a multistep process, involving a complex interaction between a range of bacterial and host factors. Multiple stages are involved in producing the characteristic attaching and effacing histopathology. A four-stage model of EPEC pathogenesis (Hicks *et al.*, 1998), which is a modification to the traditional three-stage EPEC infection model (Donnenberg & Kaper, 1992) has been proposed (**Figure 5**). It should be noted that the precise temporal sequence of these stages is not certain. Indeed, the different stages may occur simultaneously.

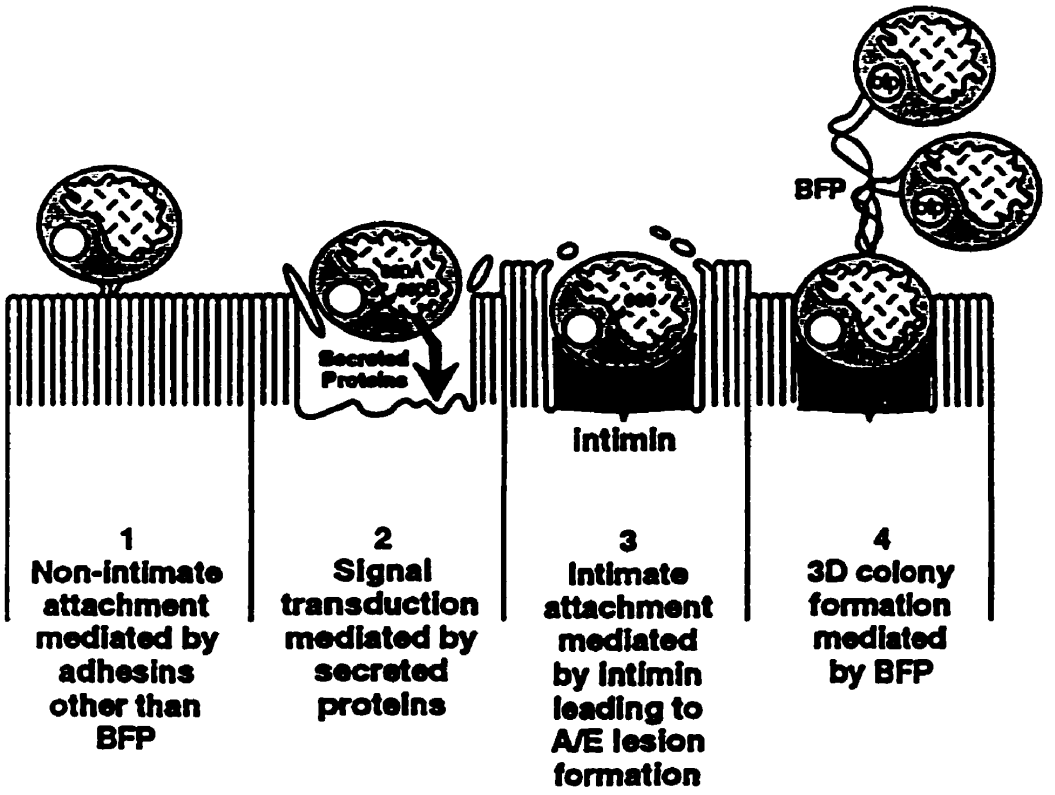


Figure 5. The proposed four-stage model of EPEC pathogenesis. Adapted from Hicks *et al.*, 1998.

It had been suggested from cell culture work that BFP is responsible for non-intimate, initial bacterial attachment to host cells (Donnenberg & Kaper, 1992). However, organ culture of human intestine with EPEC lacking BFP expression still show AE lesion formation (Hicks *et al.*, 1998). Thus, BFP likely is not required for the initial attachment of EPEC to mucosal surfaces. Under the current revised model, stage 1 involves the non-intimate adhesion of bacteria to the enterocyte cell surface via an adhesin(s) other than BFP.

Stage 2 of the infection involves a type III secretion apparatus which mediates the translocation of Esps (Hueck, 1998). Once within the cytoplasm, these effector molecules serve different functions depending on the strategy used by the respective pathogen.

Stage 3 involves intimate attachment to the enterocyte cell surface via the outer membrane protein intimin, producing rearrangement of host cytoskeletal proteins and result in the formation of AE lesions.

Stage 4 involves three-dimensional expansion of the discrete microcolony, forming a localized adherence cluster after intimate attachment of EPEC to the mucosal surface. BFP is responsible for interbacterial attachment and complex microcolony formation (Hicks *et al.*, 1998).

F. Toxins

EPEC does not produce classic enterotoxins. In the early 1970s, with the development of laboratory tests to assess heat-labile and heat-stable enterotoxin production, the classic EPEC strains were found to lack those particular properties. Also,

the EPEC chromosome does not contain the gene encoding the Shiga toxin - in contrast to Shiga toxin-producing *E. coli* (Chapter 3). However, EPEC does produce other toxins, including cytolethal distending toxin, cytotoxic necrotizing factor, and enteroaggregative *E. coli* heat-stable enterotoxin.

I. Cytolethal Distending Toxin

Cytolethal distending toxin (CDT) is a heat-labile protein cytotoxin produced by a number of diarrheal disease-causing enteropathogens, including EPEC (Johnson & Lior, 1988). The toxin was termed a cytolethal distending toxin to reflect the progressive cell distention and cytotoxicity evident in sensitive tissue culture cells. CDT is encoded by three genes, *cdtA*, *cdtB*, and *cdtC*, which are arranged in an operon. All three polypeptides are necessary for toxicity (Okuda *et al.*, 1997).

The action of CDT is irreversible. CDT causes an accumulation of actin stress fibers (Aragon *et al.*, 1997) and inhibits cell division by blocking the cell cycle in the G_2 stage (Comayras *et al.*, 1997). CDT may be an important factor in intestinal pathogenesis, as suggested by the report that *Shigella dysenteriae* CDT (and presumably other CDTs) induces tissue damage and watery diarrhea in a mouse model (Okuda *et al.*, 1997). It is speculated that CDT is able to inhibit the growth of actively dividing crypt cells, thus preventing the rapid renewal of the epithelium and leading to intestinal lesions. This may also promote the formation of the characteristic AE lesions during EPEC pathogenesis.

II. Cytotoxic Necrotizing Factor

Two types of cytotoxic necrotizing factors have been described: CNF1, produced by *E. coli* strains isolated from both humans and animals, and CNF2, produced by *E. coli* strains isolated from ruminants, including cows and sheep. CNFs are potent toxins that cause tissue damage, contribute to pathogenesis of colibacillosis and death of the animal host (De Rycke & Plassiart, 1990).

CNF inhibits cell mitosis by irreversibly blocking the G_2/M transition phase, leading to the formation of large multinucleated cells (De Rycke *et al.*, 1997). CNF can also affect assembly of actin stress fibers by modifying the structure of the Rho protein in such a way that the modified GTPase is functionally hyperactive as compared with its native form (Lerm *et al.*, 1999). The capacity of CNF to mediate polymerization of F-actin suggests that CNF can trigger the actin-dependent endocytosis of noninvasive bacteria into host cells through phagosome-like organelles (Falzano *et al.*, 1993). This could partly explain the internalization of EPEC during infection. Moreover, CNF1 has been shown to efface intestinal cell microvilli (Hofman *et al.*, 1998) and *in vivo*, CNF1 toxicity is been linked to pathological states, including diarrhea (Sears & Kaper, 1996).

III. Enteroaggregative *E. coli* Heat-Stable Enterotoxin

EAggEC strains produce a low-molecular-weight heat-stable toxin called EAST-1. However, it was found that EAST-1 production is not restricted only to EAggEC. The gene encoding EAST-1, *astA*, is broadly distributed among diarrheagenic *E. coli*, including EPEC and STEC, and may represent an additional determinant in the pathogenesis of *E. coli* diarrhea (Savarino *et al.*, 1996). Indeed, EAST-producing *E. coli*

strains are associated with diarrheal diseases (Vila *et al.*, 1998). EAST-1 toxin is similar to the heat-stable enterotoxin of ETEC (STa) in that it acts through the guanylate receptor, as demonstrated by EAST-1 clones causing net increases in short-circuit current in rabbit mucosa mounted into the Ussing chamber model (Savarino *et al.*, 1991).

It was reported that EPEC-infected cell line shows an increase in responsiveness to STa, suggesting that EPEC could sensitize epithelial cells to the effects of EAST-1. This may have clinical significance in cases of dual infections with ETEC and EPEC (Crane & Oh, 1997).

G. Internalization

EPEC organisms are generally considered to be non-invasive by the Sereny test (Levine *et al.*, 1978), which assesses the ability of a living culture to cause an ulcerative keratoconjunctivitis after instillation onto the cornea of a guinea pig. However, epithelial cell internalization may be an overlooked property of EPEC of potential relevance to disease pathogenesis.

EPEC strains are capable of entering a variety of epithelial cell lines (Andrade *et al.*, 1989) (Donnenberg *et al.*, 1989) (Francis *et al.*, 1991) (Miliotis *et al.*, 1989). Some atypical EPEC strains (*eae*$^+$ and EAF$^-$) are also invasive (Pelayo *et al.*, 1999). Genetic analysis indicates that there is significant overlap between the genes responsible for the invasion process and genes involved in producing AE lesions (Donnenberg *et al.*, 1990a) (Stein & Finlay, 1997).

The clinical significance of internalization in the pathogenesis of EPEC infection remains unclear. Unlike true intracellular pathogens, such as EIEC and *S. dysenteriae*,

EPEC strains do not multiply intracellularly or escape from a phagocytic vacúole and thus do not appear to be specifically adapted for intracellular survival (Nataro & Kaper, 1998).

The Rho family of small GTPases (Rho, Rac, and Cdc42) may be involved in EPEC internalization because inactivation of Rho proteins using *Clostridium difficile* ToxB inhibits EPEC invasion (Ben-Ami *et al.*, 1998). The role of microfilaments and microtubules in invasion by EPEC also has been suggested because microfilament inhibitors reduce invasion by both EIEC and EPEC, while microtubule inhibitors reduced invasion by EPEC only (Donnenberg *et al.*, 1990b). These results suggest that EPEC and EIEC differ in their mechanisms of epithelial cell invasion.

Chapter 3

Shiga toxin-producing *Escherichia coli*

A. Introduction

Shiga toxin-producing *E. coli* (STEC), also known as enterohemorrhagic *E. coli* (EHEC) or verocytotoxin-producing *E. coli* (VTEC), was first identified as a human pathogen in the early 1980s (Riley *et al.*, 1983). STEC is a noninvasive (McKee & O'Brien, 1995) large bowel pathogen which attaches primarily to the colon and perhaps the distal small intestine. At present, the processes involved in establishment and maintenance of gut colonization by STEC are poorly understood.

Estimates of the infectious dose for some STEC strains (O111:H- and O157:H7) are in the order of 1 to 100 CFU; which is many orders of magnitude lower than that of ETEC and EPEC strains. The low infectious dose of STEC favours the development of epidemic outbreaks (Cassels & Wolf, 1995). Infection with STEC is associated with both outbreaks and sporadic cases of hemorrhagic colitis (HC), a distinctive gastrointestinal illness characterized by severe crampy abdominal pain, watery diarrhea followed by grossly bloody diarrhea, and little or no fever. Bloody diarrhea can be followed by life-threatening systemic complications such as the hemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP). HUS, defined by a triad of acute renal failure, thrombocytopenia, and microangiopathic hemolytic anemia, affects mostly young children and represents the major cause of acute renal failure in children (Karmali *et al.*, 1985), while TTP is a disease with more neurologic manifestations generally affecting adults (Griffen & Tauxe, 1991).

Although as many as 57 different *E. coli* serotypes have been associated with HC, O157:H7 is by far the most frequent, accounting up to 75% of cases (Whittam *et al.*,

1998). However, STEC of serotypes other than O157:H7 can also play an important role in outbreaks as well as cases of sporadic human disease (Goldwater & Bettelheim, 1998).

STEC has been isolated from the feces of a wide variety of animals including sheep, goats, pigs, cats, dogs, chickens, gulls, and cattle (Armstrong *et al.*, 1996). There is a clear association of STEC infection with the consumption of fecally contaminated, undercooked ground beef as several of the major outbreaks reported involve fast-food restaurants that serve hamburgers (Bell *et al.*, 1994). Unpasteurized milk, turkey roll, apple cider, mayonnaise and water-borne outbreaks also have been reported (Marks & Roberts, 1993) (Swerdlow *et al.*, 1992). Transmission can be further amplified by secondary spread via person-to-person, as shown both in a nursing home outbreak (Carter *et al.*, 1987) and in day-care facilities (Belongia *et al.*, 1993).

The burden of STEC related illnesses is considerable. At present, there is no vaccine for STEC and the only established treatment is supportive therapy. The use of antimicrobial drugs and antimotility agents currently is not recommended because they neither shorten the course of illness nor prevent the development of sequelae. Furthermore, antimicrobial therapy may be harmful rather than helpful. Laboratory studies have shown that exposure of STEC O157:H7 to antibiotics *in vitro* increases the release of free toxin from the periplasm (Matsushiro *et al.*, 1999), which could be associated with an increased risk of progression to HUS.

A potential therapy now undergoing evaluation in clinical trials is Synsorb-Pk. The drug is designed to bind Shiga toxin in the lumen of gastrointestinal tract of patients with bloody diarrhea, thereby preventing the development of HUS. Initial phase I trials

were promising in terms of safety profile (Armstrong *et al.*, 1995), and phase II trials to assess therapeutic efficacy are in progress (Trachtman & Christen, 1999).

B. Acid Resistance

The ability of bacteria to act as food-borne pathogens depends their ability to survive the acidic environment of food and the gastric stomach to colonize the intestinal tract of humans. Enteric microorganisms have developed several mechanisms for surviving transient periods of extreme acid stress (Foster & Moreno, 1999).

E. coli in general has multiple genetic systems to respond to environmental stresses and confer resistance to low and lethal pH levels (Gorden & Small, 1993). STEC at stationary phase is more resistant to acid than exponentially growing cells and may not need prior exposure to low pH to exhibit acid resistance (Waterman & Small, 1996). Many STEC strains are capable of surviving a pH of at less than 2.5 for more than 2 hours (Benjamin & Datta, 1996). STEC O157:H7 is extremely acid-tolerant and the unusual acid tolerance likely serves as an adaptation for the organism to survive the rumen of cows (Armstrong *et al.*, 1996). Grain-fed cattle have lower colonic pH and more acid-tolerant *E. coli* strains than hay-fed cattle (Diez-Gonzalez *et al.*, 1998).

Three acid resistance systems, including an acid-induced oxidative system, an acid-induced arginine-dependent system, and a glutamate-dependent system, have been identified (Lin *et al.*, 1996). A distinct phenotype has also been described and is mediated by *rpoS*, which encodes a stationary-phase sigma factor. This factor, required for oxidative acid resistance but is only partially involved with the arginine- and glutamate-dependent systems, regulates acid resistance genes expression (Gorden &

Small, 1993). Once induced, the acid resistance systems of STEC remain active for prolonged periods of cold storage at 4°C (Lin *et al.*, 1996). The acid tolerance feature of STEC is considered to be a virulence factor as indicated by an outbreak associated with contaminated salami (Tilden *et al.*, 1996).

C. Locus of Enterocyte Effacement

The mechanisms by which STEC produces diarrheal disease remain to be elucidated. In many aspects, the pathogenesis of STEC resembles that of EPEC. It has been known for more than a decade that certain STEC strains are capable of causing AE lesions on enterocytes (Sherman *et al.*, 1988). The mechanism whereby STEC generates AE lesions is less well characterized but is essentially analogous to that for EPEC. STEC strains displaying AE phenotype have a LEE homologue (**Figure 6**) (McDaniel *et al.*, 1995). However, how much of the similarities and differences in the pathogenesis can be attributable to sequence variations in the LEE is unknown.

The complete sequence of the 43.36 kb LEE for STEC O157:H7 strain EDL933 was generated (Perna *et al.*, 1998). The overall G+C content of 40.91% is also far below the *E. coli* K-12 average. The genes found in the LEE of EPEC E2348/69 are also present in the O157:H7 LEE in the same order, and the average nucleotide identity between these strains is 93.9%. However, there is also a considerable variation among genes in the level of sequence divergence. These genes include *espA* (15% difference), *espB* (26% difference), *espD* (20% difference), *espF* (25% difference), *eae* (13% difference), and *tir* (34% difference), which encode proteins known to interact directly with host.

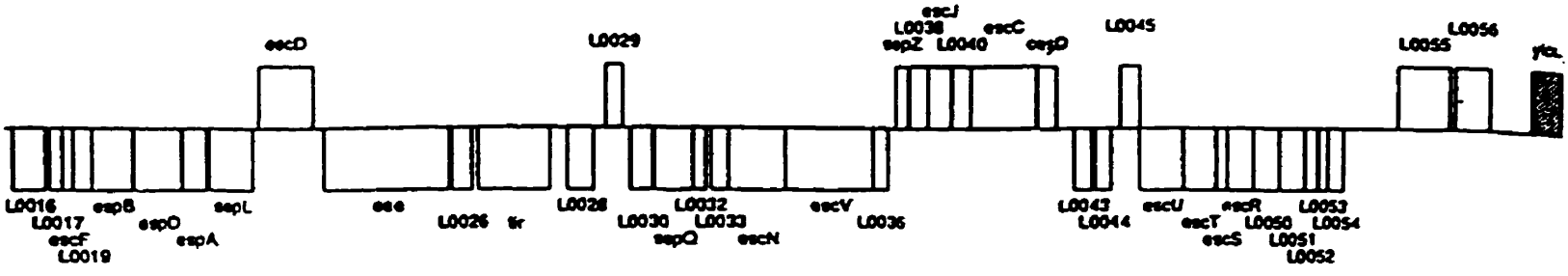


Figure 6. The locus of enterocyte effacement of STEC strain EDL933. Adapted from Perna *et al.*, 1998.

There is structural, and possibly functional, heterogeneity in secreted proteins between EPEC and STEC. The variability between the LEE in EPEC and STEC may be due to natural selection for adaptation to either host specificity or for evasion of the host immune system.

The LEE of EPEC strain E2348/69 and STEC O157:H7 strain EDL933 are both inserted into the *E. coli* K-12 chromosome at 82 min., just downstream of the *selC* locus encoding the tRNA for selenocysteine (McDaniel *et al.*, 1995). Benkel *et al.* (1997) identified the chromosomal site of insertion in an *E. coli* O26:H- strain as being at 94 min. on the *E. coli* chromosome at the tRNA encoding the *pheU* gene. A third chromosomal insertion site for LEE has also been detected (Sperandio *et al.*, 1998). These findings suggest that LEE has been inserted at multiple times and at multiple sites in the genome during the evolution of EPEC and STEC.

D. Shiga Toxin

STEC is lysogenized with one or more bacteriophages which encode the structural genes for the Shiga toxins (Stxs) (Melton-Celsa & O'Brien, 1998). There are two main types of Stxs, Stx1 and Stx2, which are most frequently associated with human disease. Stx1 is a homogenous family of toxins identical to the Stx of *S. dysenteriae* and Stx2 is more a heterogeneous family of toxins more distantly related to this Stx toxin. Three other members of the Stx family have also been described: Stx2c and Stx2d, which are linked to human disease, and Stx2e, which is associated with edema disease of swine.

Stxs are active on a variety of cell types, including intestinal epithelial cells and endothelial cells which possess the globotriaosylceramide (Gb_3) receptor. Stxs damage

vascular endothelial cells in certain organs and the systemic absorption of toxins is presumed to be a critical factor in the development of HUS and TTP. The essential role of Stxs in the pathogenesis of disease caused by STEC is supported by the evidence that only Stx-producing bacteria cause HUS (O'Brien & Holmes, 1987).

Members of the Stx family are compound toxins, comprising a single catalytic A subunit noncovalently associated with a pentamer of B subunits which mediates the binding of the toxin to its glycolipid receptor on the surface of target cells. Following binding, the toxin is internalized by a process of clathrin-dependent receptor-mediated endocytosis (Sandvig & van Deurs, 1996). The endosomal vesicles containing toxin-receptor complexes undergo retrograde transport via the Golgi apparatus to the endoplasmic reticulum, where the A subunit cleaves an *N*-glycoside bond at a specific adenine base (position 4324) of the 28S ribosomal RNA (rRNA) (Melton-Celsa & O'Brien, 1998). This cleavage prevents elongation factor 1-dependent binding of the aminoacyl-tRNA to the 60S rRNA (Paton & Paton, 1998), thereby inhibiting the peptide chain elongation step of protein synthesis and, ultimately, causing cell death.

E. STEC Plasmid

Virtually all STEC O157:H7 isolates carry a highly conserved plasmid of approximately 90 kDa, which is designated pO157 (**Figure 7**) (Tosh *et al.*, 1990) and lack the EAF plasmid present in EPEC. The genome consists of 92,077 bp forming a circular duplex (Burland *et al.*, 1998). Genes for an enterohemolysin (Schmidt *et al.*, 1996), catalase peroxidase (Brunder *et al.*, 1996), a secreted serine protease (Brunder et al., 1997), and a type II secretion system (Schmidt *et al.*, 1997) have been characterized on

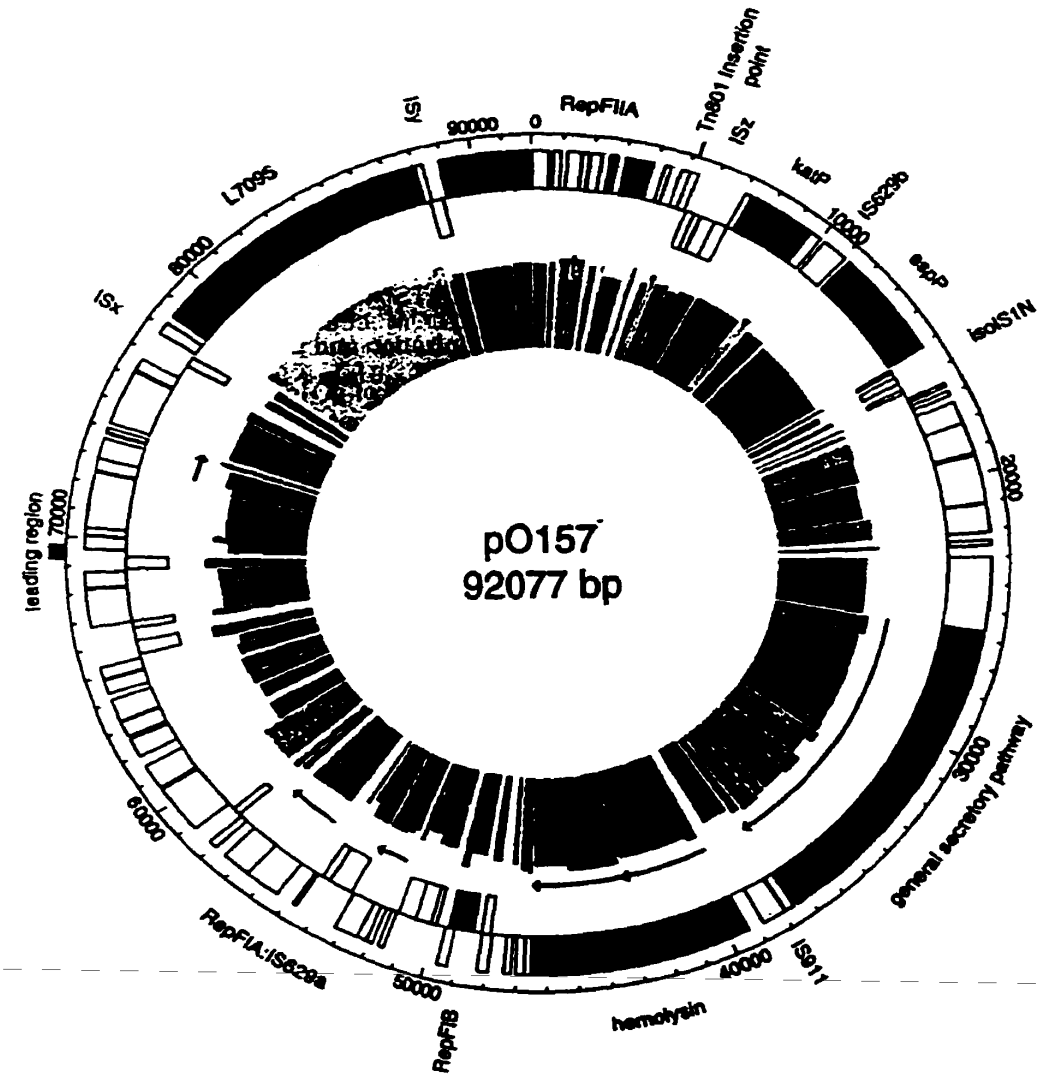


Figure 7. The map of STEC plasmid pO157. Adapted from Burland *et al.*, 1998.

the STEC plasmid. An ORF shows strong sequence similarity to a family of toxins referred to as large Clostridial toxins (LCT) also has been identified (Burland *et al.*, 1998), although its function has not been characterized. Among the 100 ORFs identified in pO157, 22 are referred to as FUN genes because they have no known function or homologies to sequence databases (Hinton, 1997).

The role of the pO157 plasmid in pathogenicity is not clearly defined (Nataro & Kaper, 1998) because there are conflicting results from studies in a variety of animal models. Investigators have reported that loss of the pO157 plasmid either decreases adhesion (Tosh *et al.*, 1990), enhances adherence (Junkins & Doyle, 1989), or has no effect on bacterial binding to host epithelial cells (Fratamico *et al.*, 1993). Also, it was demonstrated that the typical mucosal lesions of bacterial attachment observed and the development of diarrhea are not influenced by the presence or absence of the pO157 plasmid (Tzipori *et al.*, 1987). However, epidemiological evidence suggests a stronger correlation of the presence of the plasmid with the development of HUS rather than diarrhea (Nataro & Kaper, 1998).

The pO157 is present in most, but not all, STEC strains isolated from humans (Levine *et al.*, 1987). In addition to the 94 kb pO157 plasmid, a number of other smaller plasmids, ranging in size from 2 kb to 87 kb, are present in strains of STEC O157:H7 (Willshaw *et al.*, 1992). However, there is no correlation with possession of any of these smaller plasmids and clinical disease.

Although BFP is an established virulence factor in EPEC, BFP is not present in STEC (Burland *et al.*, 1998). STEC also does not possess *per* gene homologues. Thus, the regulation of expression of LEE-encoded proteins in STEC is, at present, unknown.

The absence of BFP may reflect the evolutionary origin of STEC strains from atypical EAF-minus EPEC. Alternatively, STEC could possess an, as yet, undescribed pilus that also functions in promoting bacterial colonization.

Chapter 4

Cellular Responses to EPEC and STEC Infection

Pathogenic bacteria cause disease by disturbing normal host cell activity, resulting in altered function of target tissues. This can be achieved by the production of cell-associated or secreted toxins or through the expression of adhesins and invasins on the surface of bacteria. Recent studies indicate that the initial targets for many of these virulence determinants are host cell surface receptors that have specific functions in sensing and responding to the extracellular environment (Rosenshine *et al.*, 1992b). Coincident with AE lesion formation, EPEC triggers a variety of host signal transduction pathways (**Figure 8**) (Kaper, 1998) which are considered below:

A. Tyrosine Phosphorylation

Following contact with host cells, EPEC induces the phosphorylation of Tir that is activated to bind intimin (Rosenshine *et al.*, 1992a). In EPEC, tyrosine phosphorylation of Tir is essential for F-actin aggregating activity (Kenny, 1999) and EPEC invasion (Rosenshine *et al.*, 1992b). Intimin-Tir interactions appear to trigger signal events including tyrosine phosphorylation and dephosphorylation of several proteins. However, it is not yet clear whether these events are directly related to intimin-Tir interaction or the consequence of enhanced translocation of effector molecules resulting from intimate bacterial attachment.

In contrast, STEC appears to have evolved an alternative tyrosine phosphorylation-independent mechanism to carry out similar functions. STEC O157:H7 does not induce tyrosine phosphorylation of the receptor protein EspE in HEp-2 cells and T84 cells (Ismaili *et al.*, 1995a) (DeVinney *et al.*, 1999). While EPEC tyrosine 474 on Tir is critical in the phosphorylation event, the corresponding residue in STEC is

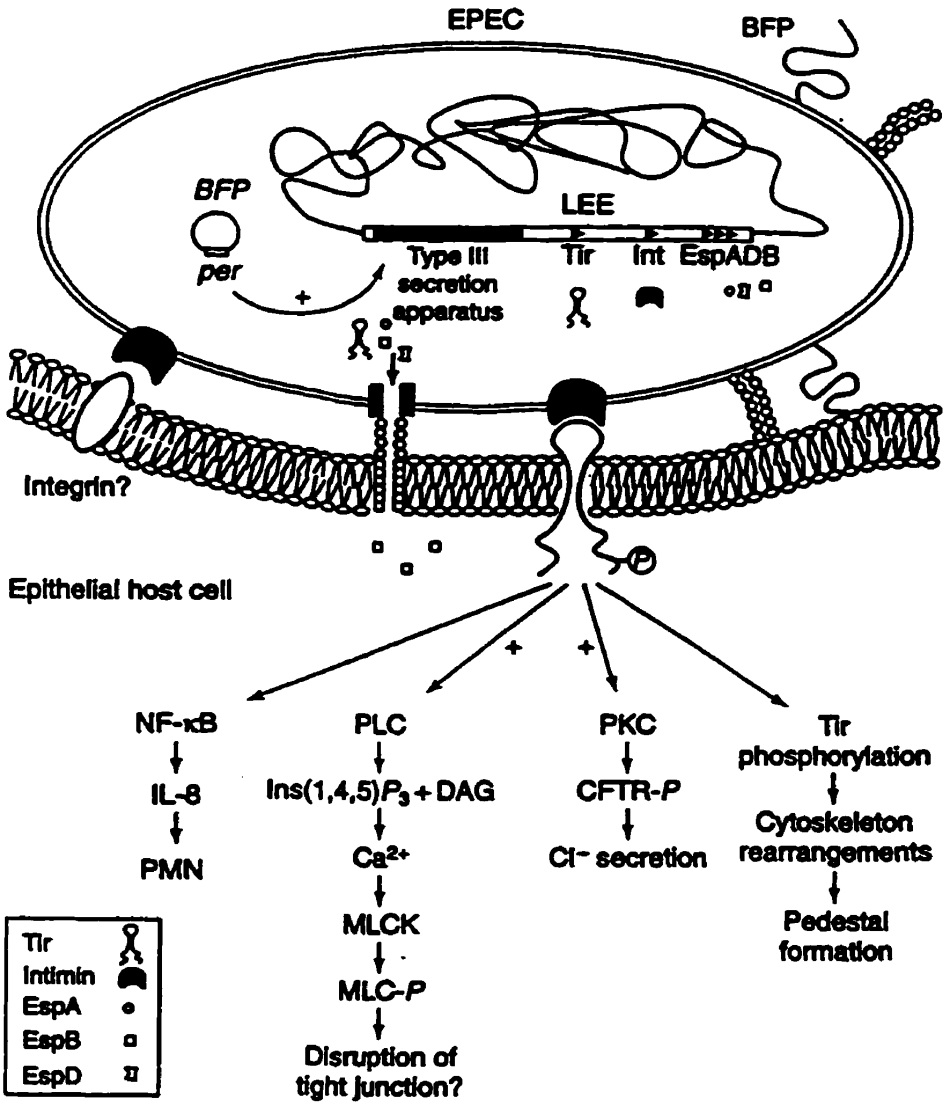


Figure 8. Host signaling events triggered during EPEC infection. Taken from Goosney *et al.*, 1999a. Refer to text for details.

substituted by a serine (Kenny, 1999). This finding likely explains why EspE of STEC is not tyrosine phosphorylated. The inability of STEC O157:H7 to induce tyrosine phosphorylation of translocated EspE also may account for the low efficiency of infection and actin pedestal formation that is characteristic of O157:H7 in tissue culture experiments (Deibel *et al.*, 1998).

Differences in tyrosine phosphorylation, however, are not a consistent difference between EPEC and STEC because STEC of serotype O26:H111, whose EspE contains the same number and sequence distribution of tyrosine residues as EPEC Tir, can induce tyrosine phosphorylation of EspE similar to that seen with EPEC (Deibel *et al.*, 1998). Therefore, the definitive role of protein tyrosine kinase signaling in mediating cytoskeletal rearrangements remains controversial.

B. Phosphatidylinositol Pathway

Binding of EPEC to host cells triggers tyrosine phosphorylation of phospholipase C-γ1 (PLC-γ1) (Kenny & Finlay, 1997), leading to production of inositol-1,4,5-trisphosphate (IP_3) (Foubister *et al.*, 1994b) and subsequent release of free Ca^{2+} from intracytoplasmic IP_3-sensitive stores (Baldwin *et al.*, 1991). The increase of Ca^{2+} is hypothesized to activate Ca^{2+}-dependent actin-severing protein gelsolin and actin-bundling protein villin, resulting in disassembly of the microvillar actin filament. As free Ca^{2+} becomes sequestered by intracellular stores, gelsolin dissociates from the actin fragments generating a nucleation site for renewed actin polymerization (Matsudaira & Burgrss, 1992). Furthermore, increases in cytosolic free Ca^{2+} can inhibit Na^+ and Cl^-

absorption and stimulate Cl^- secretion in enterocytes (Field *et al.*, 1989), thereby leading to a net loss of water and ions, which ultimately contributes to diarrhea.

New data, however, have raised some questions about the precise role for Ca^{2+} signaling in AE lesion formation (Bain *et al.*, 1998). Localized increases in Ca^{2+} at sites of bacterial attachment were not demonstrated and significant alternations in Ca^{2+} levels in infected compared with uninfected cells were not observed. Furthermore, buffering of intracellular Ca^{2+} did not prevent the formation of AE lesions. Thus, AE lesion formation may well occur in the absence of a detectable Ca^{2+} signal.

C. Serine/Threonine Phosphorylation

In addition to production of IP_3, the action of PLC on phosphatidylinositol-4,5-bisphosphate (PIP_2) generates the second messenger diacylglycerol (DAG) (Berridge, 1987), which remains membrane associated and is involved in the activation of protein kinase C (PKC). An increase in membrane-associated PKC activity is accompanied by a decrease in cytosolic PKC activity (Crane & Oh, 1997). PKC is implicated in the phosphorylation of myosin light chain (MLC) because activators of PKC cause an increase in phosphorylation levels similar to those observed during EPEC infection (Baldwin *et al.*, 1990). The involvement of an additional kinase in the phosphorylation of MLC in response to EPEC-mediated events has also been suggested (Manjarrez-Hernandez *et al.*, 1996).

Recent studies have shown that EPEC infection also induces Cl^- secretion in tissue culture cells (Collington *et al.*, 1998). PKC activity could contribute to the activation of Cl^- secretion through opening of the cystic fibrosis transmembrane

conductance regulator (CFTR), which is the major chloride channel in the apical brush border of enterocytes, thereby providing a possible mechanism of EPEC-induced diarrhea (Kaper, 1998).

Myosin light chain kinase (MLCK) activation leads to phosphorylation of MLC. Reversible serine/threonine phosphorylation of MLC is related to changes in actin organization. The phosphorylation state of MLC is important in the rearrangement of cytoskeletal elements which occurs during EPEC-induced AE lesion formation (Manjarrez-Hernandez *et al.*, 1996). A physiological consequence of EPEC-induced MLC phosphorylation could be to increase the permeability of tight junctions by contracting the tight junction-associated actomyosin cytoskeleton. This could then contribute to diarrhea by affecting transport processes and passive water absorption. Indeed, paracellular permeability is increased by overexpression of myosin light chain kinase (Spitz *et al.*, 1995) and prevented in EPEC-infected cells by inhibition of MLC phosphorylation (Yuhan *et al.*, 1997).

D. Nuclear Signaling Events

Further signaling events triggered by EPEC infection include activation of nuclear factor kappa B (NF-κB) in intestinal cells, perhaps by PKC, IκB kinase, or other kinases, leading to increased transcription of the interleukin-8 (IL-8) gene. Epithelium-derived IL-8 is a potent chemoattractant involved in the transmigration of polymorphonuclear cells (PMNs) in the physiological direction (basolateral to apical) (Savkovic *et al.*, 1997). Once PMNs have migrated to the intestinal lumen, they release 5'-adenosine

monophosphate (5'AMP), which is converted to adenosine. Adenosine can then bind to an adenosine receptor and stimulate Cl^- secretion (Madara *et al.*, 1993).

E. Phosphoinositide 3-Kinase

Although phosphatidylinositol (PtdIns) represents only a small percentage of total cellular phospholipids, it plays an important role in signal transduction as precursors of several second messenger molecules (Fruman *et al.*, 1998). To date, the following phosphoinositides have been identified in eukaryotic cells: PtdIns-3-phosphate (PtdIns-3-P), PtdIns-4-P, PtdIns-5-P, PtdIns-3,4-bisphosphate (PtdIns-3,4-P_2), PtdIns-3,5-P_2, PtdIns-4,5-P_2, and PtdIns-3,4,5-trisphosphate (PtdIns-3,4,5-P_3).

Phosphoinositide kinases can be classified into three general families: phosphoinositide 3-kinases (PI3-Ks), PI4-Ks, and PI5-Ks (Fruman *et al.*, 1998). The evolutionary conservation of these enzymes provides evidence of their importance in the physiology of all eukaryotic cells. Although sequence homology supports the separate classification of the kinases, certain phosphoinositide kinases have different or broader activities.

PI3-K is a heterodimeric protein consisting of an 85 kDa regulatory subunit (p85) and a 110 kDa catalytic subunit (p110) (Stephens *et al.*, 1993). The enzyme phosphorylates the hydroxyl group at position 3 on the inositol ring of PtdIns, leading to the formation of lipid second messengers crucial in the transduction of a variety of signals. PI3-K activity is implicated in an array of cellular processes, including survival (Anderson, 1997), protein and membrane trafficking (De Camilli *et al.*, 1996), and response to stress (Dove *et al.*, 1997).

PI3-K is also implicated in the regulation of actin cytoskeleton architecture (Burgering & Coffer, 1995). PI3-K is reported to play a role in cytoskeletal remodeling during *Cryptosporidium parvum* infection (Forney *et al.*, 1999), membrane ruffling (Wennstrom *et al.*, 1994), and rapid actin rearrangement in activated platelets (Schafer *et al.*, 1996). Moreover, actin-binding proteins profilin and gelsolin stimulate PI3-K activity, thereby controlling the generation of 3-OH phosphorylated phosphoinositides (Singh *et al.*, 1996) which, in turn, regulate actin polymerization.

There are also several lines of evidence supporting a role of PI3-K in bacterial invasion. Inhibition of PI3-K activity, by inhibitors such as wortmannin and LY294002, reduces entry of *Yersinia pseudotuberculosis* into HEp-2 cells (Mecsas *et al.*, 1998), inhibits the invasion of Caco-2 cells by *Campylobacter jejuni* (Wooldridge *et al.*, 1996), and blocks the ability of *Listeria monocytogenes* to invade Caco-2, HeLa, and Vero cells (Ireton *et al.*, 1996). Furthermore, entry of *L. monocytogenes* into the Chinese hamster ovary cells overexpressing a dominant negative form of p85 is reduced by 90% when compared to the parental cell line (Ireton *et al.*, 1996).

F. Arachidonic Acid Metabolism

Arachidonic acid, a 20-carbon fatty acid, is a common constituent of phospholipids in cell membranes (Sigal, 1991). Free arachidonic acid is released from plasma membranes by the action of phospholipases in response to immunological and nonimmunological stimuli (for example, by immune complexes and calcium ionophores). Arachidonic acid is then rapidly metabolized to oxygenated products by one of the two distinct enzymatic pathways: lipoxygenase (LO) and cyclooxygenase (COX). The

metabolism of arachidonic acid by these enzymes results in a wide range of oxidized products with potent biological activities (Sigal, 1991). Both LO and COX products of arachidonate are abundant in the human gut. Biological effects include modulation of fluid secretion and electrolyte secretion (Rask-Madsen, 1986).

The 5-lipoxygenase enzyme catalyzes the first step in the generation of the leukotrienes. In additional to its role as a critical mediator in inflammatory diseases such as arthritis and asthma, 5-lipoxygenase products also play a role in the cellular F-actin architecture. This is supported by the observation that inhibition of 5-lipoxygenase enzyme activity prevents cortical actin polymerization in epidermoid cells (Peppelenbosch *et al.*, 1993). Experiments with inhibitors further suggest a role of 5-lipoxygenase in regulating the entry of bacteria into host epithelial cells. For instance, preincubation of Henle-407 cells with the lipoxygenase inhibitor 5,8,11-eicosatriynoic acid or 8,11,14-eicosatrienoic acid prevents *S. typhimurium* entry (Pace *et al.*, 1993) and another lipoxygenase inhibitor NDGA blocks entry of *L. monocytogenes* and *Y. pseudotuberculosis* into HEp-2 cells (Mecsas *et al.*, 1998).

While leukotrienes are formed in the LO pathway by the enzyme 5-lipoxygenase, prostaglandins (PGs) are products of the COX pathway of arachidonic acid metabolism. The predominant form of PGs in humans seems to be PGE_2. In cholera, the cholera toxin stimulates the synthesis of PGE_2 (Peterson *et al.*, 1999) which, in turn, stimulates adenylate cyclase activity and prolongs the secretory response in diarrhea. Moreover, tumor necrosis factor-alpha (TNF-α) acts through the PGE_2 pathway, changing the Cl^- and K^+ transport towards secretion in inflammed bowel (Schmitz *et al.*, 1996). Besides being implicated in disease pathogenesis, cyclooxygenase also regulates cytoskeleton

assembly. This notion is supported by the observation that inhibition of the enzyme activity of cyclooxygenase by enzyme inhibitor indomethacin abolishes the breakdown of cytoplasmic actin filaments (Peppelenbosch *et al.*, 1993).

Recent studies have shown that cyclooxygenase exists in two isoenzymes (Kawai, 1998). COX-1 is constitutively expressed in most cell types, whereas COX-2 is inducibly expressed in response to a variety of proinflammatory agents. The current belief is that PGs produced by COX-1 are involved in the maintenance of regular cellular physiology whereas those generated by COX-2 are implicated in inflammatory processes.

Chapter 5

Study Objectives

Study Objectives

Multiple signal transduction pathways converge to induce rearrangements of the actin cytoskeleton. Recent advances have begun to clarify the molecular events leading to the formation of attaching and effacing lesions during EPEC and STEC infections. However, many questions remain unanswered and more remain unasked.

The overall objective of this research was to further characterize potential host cytosolic responses to EPEC and STEC infections by using a series of specific cell signaling inhibitors.

The specific aims of this study were to:

1. Test the effect of the phospholipase C inhibitor ET-18-OCH_3 on the formation of attaching and effacing lesions during EPEC and STEC infections.

2. Test the effect of phosphoinositide 3-kinase C inhibitors, wortmannin and LY294002, on the formation of attaching and effacing lesions during EPEC and STEC infections.

3. Test the effect of the 5-lipoxygnease inhibitor NDGA on the formation of attaching and effacing lesions during EPEC and STEC infections.

4. Test the effect of the cyclooxygenase-2 inhibitor NS-398 on the formation of attaching and effacing lesions during EPEC and STEC infections.

Chapter 6

Inhibition of Attaching and Effacing Lesion Formation

Materials and Methods

Bacteria and growth conditions. The bacterial strains employed in this study are listed in **Table 2**. EPEC strain E2348/69 (serotype O127:H6) was kindly provided by Dr. E. Boedeker (University of Maryland, Baltimore, MD). STEC strain CL56 (O157:H7) was donated by Dr. M. Karmali (Hospital for Sick Children, Toronto, ON). UMD864, kindly supplied by Dr. J. B. Kaper (University of Maryland, Baltimore, MD), is an *espB* deletion mutant of E2348/69. It was used as a negative control because of its incapability of triggering host signal transduction events (Lai *et al.*, 1997). Bacteria were grown for 3 hrs in static, non-aerated Penassay broth (Difco Laboratories, Detroit, MI) at 37°C to provide a logarithmic growth culture, since bacteria at this stage induce more rapid formation of attaching and effacing lesions compared with organisms grown to stationary phase (Rosenshine *et al.*, 1996).

Eukaryotic cell cultures. The human laryngeal epithelial cell line HEp-2 (American Type Culture Collection, Manassas, VA) was cultured in Minimal Essential Medium (Gibco Laboratories, Grand Island, NY) supplemented with 15% heat-inactivated fetal calf serum (Cansera International Inc., Rexdale, ON), 0.5% glutamine (Gibco), 0.1% sodium bicarbonate (Gibco) and 2% penicillin-streptomycin (Gibco) as monolayers in 25-cm^2 tissue culture flasks (Corning Glass Works, Corning, NY) at 37°C in 5% CO_2.

Immunofluorescence detection of α-actinin. Localization of α-actinin was detected in infected epithelial cells, as described previously (Ismaili *et al.*, 1995b). Briefly, HEp-2

Table 2. Characteristics of *Escherichia coli* strains employed in this study.

Bacterial strain	Serotype	Characteristic	Reference
E2348/69	O127:H6	wild-type EPEC	Knutton *et al.*, 1989
CL56	O157:H7	wild-type STEC	Karmali *et al.*, 1985
UMD864	O127:H6	*espB* deletion mutant of strain E2348/69	Lai *et al.*, 1997

cells were seeded onto 2-well chamber slides (Nunc Inc., Naperville, IL) and cultured overnight to obtain a subconfluent growth. Before bacterial infection the tissue culture medium was replaced with medium without antibiotics. The monolayers were then infected with $1x10^9$ E2348/69, CL56, or UMD864, at a multiplicity of infection of 100:1 at 37°C in 5% CO_2 for 3 hrs. Non-adherent organisms were removed by washing the tissue culture cells six times with phosphate-buffered saline (PBS), pH 7.4 (Gibco). The monolayers were then fixed in 100% cold methanol at room temperature for 10 min. After washing three times with PBS, the fixed monolayers were incubated with a 1 in 100 dilution of murine monoclonal IgG anti-α-actinin (Sigma, Oakville, ON) as primary antibody for 1 hr at 37°C with gentle shaking. Rewashed monolayers were then incubated with a 1 in 100 dilution of FITC-conjugated rabbit anti-murine IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) as secondary antibody, with protection from light, for 1 hr at 37°C with continuous gentle agitation. The slides were then mounted with *SlowFade*™ Antifade Kits (Molecular Probes, Eugene, OR) and examined by alternating phase-contrast and fluorescence microscopy (Leitz Dialux 22, Leica Canada Inc., Willowdale, ON). Phase-contrast microscopy was used to quantitate the number of adherent bacteria on 100 randomly selected HEp-2 cells. The number of attaching and effacing lesions, as measured by the number of foci of α-actinin accumulation in the same epithelial cells, was enumerated by immunofluorescence microscopy.

Inhibitor assays. Signal transduction inhibitors (Calbiochem, San Diego, CA) utilized in these studies are listed in **Table 3**. HEp-2 cells were preincubated with 1-octadecyl-2-

Table 3. Inhibitors used to investigate the pathways leading to the formation of attaching and effacing lesions.

Inhibitor	Vehicle	Target enzyme	Reference
ET-18-OCH_3	EtOH	phosphoinositide-specific phospholipase C	Powis *et al.*, 1992
wortmannin	H_2O	phosphoinositide 3-kinase	Ui *et al.*, 1995
LY294002	DMSO	phosphoinositide 3-kinase	Vlahos *et al.*, 1994
NDGA	DMSO	5-lipoxygenase	Peppelenbosch *et al.*, 1995
NS-398	EtOH	cyclooxygenase-2	Futaki *et al.*, 1994

methyl-*rac*-glycero-3-phosphocholine (ET-18-OCH_3), wortmannin, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002), nordihydroguaretic acid (NDGA), or N-(2-cyclohexyloxy-4-nitrophenyl)-methanesulfonamide (NS-398) for 30 min to 3 hrs, as recommended in previous studies. After the removal of inhibitor, tissue culture cells were rinsed thoroughly with Minimal Essential Medium prior to infection with $1x10^9$ bacteria for 3 hrs at 37°C. The inhibitory effects of cell signaling inhibitors were determined by their ability to disrupt formation of attaching and effacing lesions, as indicated by α-actinin accumulation in infected epithelial cells.

Extraction of diacylglycerol. Diacylgylcerol was extracted by the Bligh-Dyer procedure (Bligh & Dyer, 1959). Briefly, HEp-2 cells were fixed with 1 ml 100% methanol for 10 min. Cells were scraped off with a Teflon spatula to a glass tube. Chamber slides were washed once with 1 ml methanol which was also transferred to the glass tube. Chloroform and water were added to obtain a chloroform/methanol/water ratio of 1:2:0.8 (v/v/v). The monophase was mixed by vortexing and left at room temperature for 1 hr. The cellular debris was pelleted by centrifugation and the supernatant was transferred to another tube. The pellet was re-extracted with 3.8 ml chloroform/methanol/water (1:2:0.8, v/v/v) for another hour. After centrifugation, the two supernatants were combined. To break phases, 2 ml chloroform and 2 ml 1 M sodium chloride were added, and phases were separated by brief centrifugation. The upper phase was aspirated and the lower phase was washed once with 4 ml of 1 M sodium chloride/methanol (9:1, v/v). The lipid extract was evaporated to dryness under nitrogen and stored at -20°C.

Detection of diacyglycerol. Diacylglycerol was analyzed by the method outlined by Ontko & Wang (1989). The lipid extract was dissolved in 40 µl chloroform and applied to thin-layer chromatography (TLC) plates coated with silica gel G (Macherey-Nagel, Germany). A diacylglycerol standard 1-stearoyl-2-arachidonoyl-*sn*-glycerol (Sigma) was also applied as an internal assay control. The plates were developed with a solvent system of 75 ml hexane, 25 ml diethyl ether, and 1 ml glacial acetic acid. After migration was complete, the plates were dried in air. Lipid fractions were visualized under UV illuminator by spraying the plates with dichlorofluorescein (Sigma).

Statistical analysis. Results are expressed as means ± standard deviation. Analysis of variance was used to test differences between multiple groups. A P value of < 0.05 was considered statistically significant.

Chapter 7

Results and Discussion

A. Results

ET-18-OCH_3 inhibits α-actinin accumulation in EPEC- and STEC-infected HEp-2 cells. Treatment of cell monolayers with varying concentrations of the phosphoinositide-specific phospholipase C inhibitor ET-18-OCH_3, along its ethanol vehicle, did not affect the ability of EPEC strain E2348/69, STEC strain CL56, or the signaling-deficient mutant UMD864 to adhere to HEp-2 monolayers. **Table 4** indicates that the number of adherent bacteria on 100 randomly selected epithelial cells remained comparable under each of the different treatment conditions. Furthermore, microcolony formation and localized adherence of EPEC was still detectable (**Figure 9, Panel A**).

When HEp-2 cells were infected with E2348/69 for 3 hr at 37°C, the accumulation of α-actinin, demonstrated by bright foci of fluorescence, corresponded to sites of bacterial adhesion (**Figure 9, Panel B**). However, when tissue culture cells were pretreated with 80 μM ET-18-OCH_3 before infection, α-actinin accumulation was reduced (**Figure 9, Panels C & D**). The vehicle alone did not affect the α-actinin reorganization. Similar inhibitory effects, following preincubation of HEp-2 cells with ET-18-OCH_3, were observed during infection with STEC O157:H7 strain CL56 (data not shown). No α-actinin response was detected using UMD864 as a negative control (**Figure 9, Panels E & F**).

Figure 10 summarizes a quantitation of the effects of the phosphoinositide-specific phospholipase C inhibitor ET-18-OCH_3 on α-actinin accumulation in both EPEC- and STEC-infected HEp-2 cells. A dose-dependent inhibition on the formation of attaching and effacing lesion on infected epithelial cells induced by attaching and effacing bacteria was demonstrated.

Table 4. Effects of the phospholipase C inhibitor ET-18-OCH_3 on initial bacterial adherence on HEp-2 cells. Results show bacterial attachment to 100 randomly selected HEp-2 cells expressed as mean ± standard deviation (n = 4-6 independent experiments). Variations are not statistical significant ($P > 0.05$).

ET-18-OCH_3 concentration (μM)	E2348/69	CL56	UMD864
0 (without vehicle)	184±44	108±36	159±33
0 (with vehicle)	194±18	138±7	144±17
50	191±56	145±27	162±19
80	182±32	124±14	151±67

Figure 9. Reduced attaching and effacing lesion formation with the phospholipase C inhibitor ET-18-OCH_3. Approximate magnification, x1250.

A. Phase-contrast micrograph demonstrating adherent EPEC strain E2348/69 (arrow) on HEp-2 monolayers after coincubation for 3 hrs at 37°C.

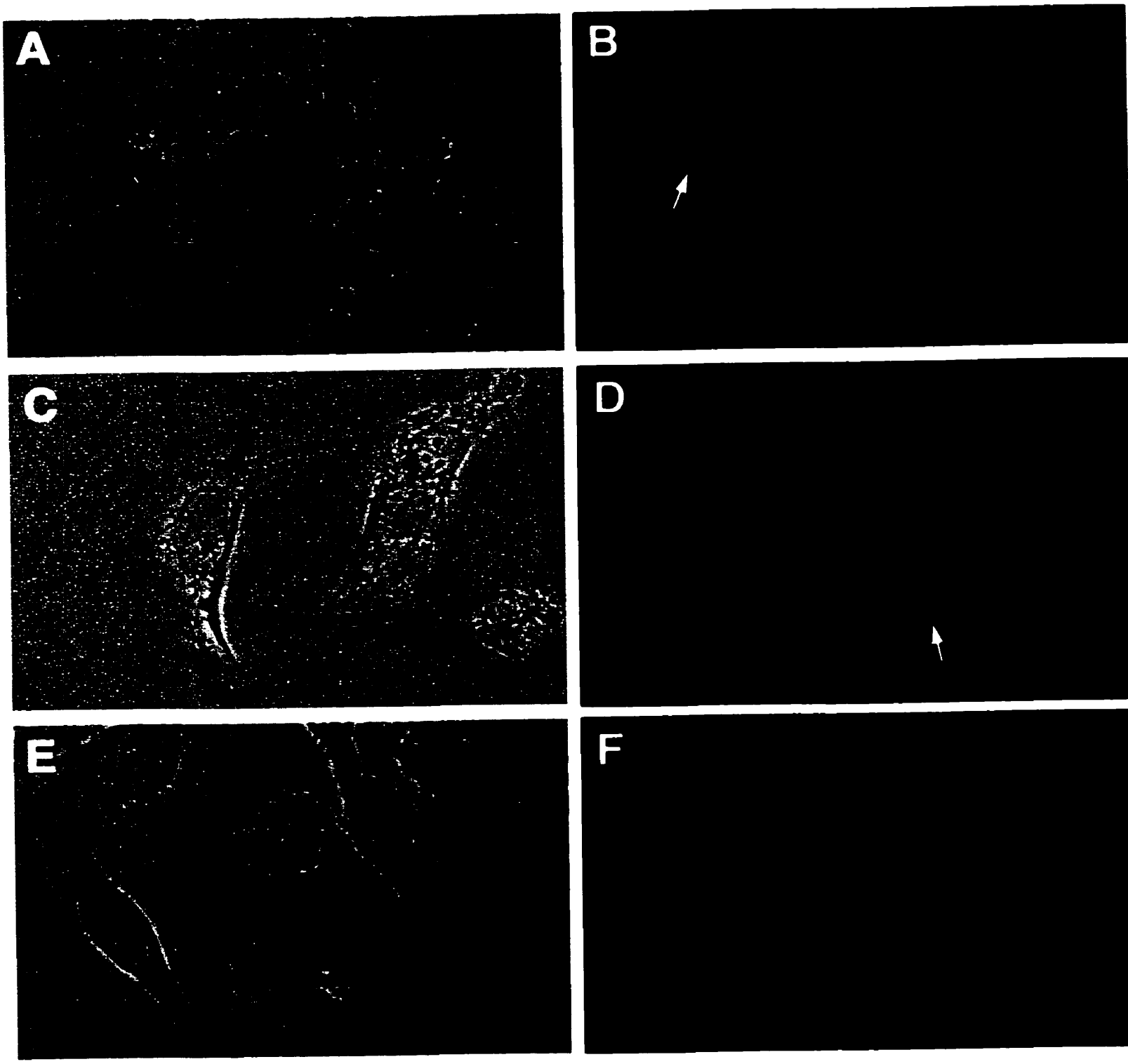
B. Corresponding fluorescence micrograph showing α-actinin accumulation, demonstrated by bright foci of fluorescence (arrow), underneath adherent microcolonies of bacteria.

C. Phase-contrast micrograph showing adherent E2348/69 (arrow) on ET-18-OCH_3-pretreated epithelial cells.

D. α-actinin accumulation under adherent EPEC was not observed in the corresponding fluorescence micrograph (arrow).

E. Phase-contrast micrograph demonstrating UMD864 adhering on tissue culture cells.

F. A negative α-actinin response was detected when HEp-2 cells were infected with a signaling-deficient mutant.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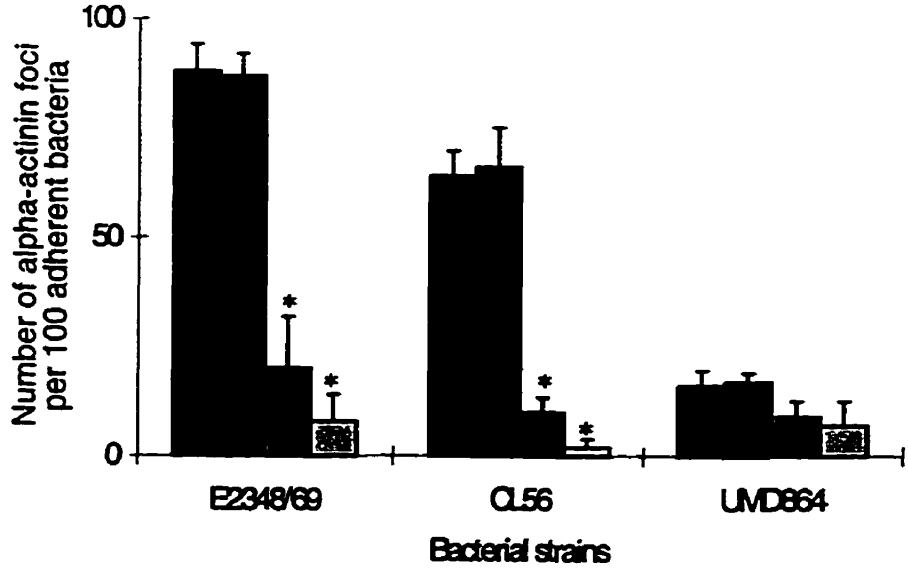


Figure 10. Quantitation of the inhibitory effect of the phospholipase C inhibitor ET-18-OCH_3 on attaching and effacing lesion formation. ET-18-OCH_3 blocked α-actinin accumulation in both EPEC- and STEC-infected HEp-2 cells in a dose-dependent manner. With increasing concentration of the inhibitor, fewer foci of bacterial attachment were accompanied with the presence of α-actinin accumulation. (*, ANOVA, $P < 0.05$; n = 4-6 independent experiments). Bars represent the infected control, the vehicle control, and inhibitor at concentrations of 50 μM and 80 μM, for E2348/69, CL56, and UMD864, respectively.

Wortmannin inhibits α-actinin condensation in EPEC- and STEC-infected HEp-2 cells. Wortmannin suspended in distilled water had no effect on bacterial adherence onto HEp-2 epithelial cells, as shown in **Table 5**. In the absence of the phosphoinositide 3-kinase inhibitor, formation of attaching and effacing lesions demonstrated by α-actinin accumulation was clearly observed (**Figure 11, Panels A & B**). However, fewer and less intense foci of α-actinin localization were detected in EPEC strain E2348/69-infected tissue culture cells after wortmannin treatment (**Figure 11, Panels C & D**). Preincubation of HEp-2 cells with wortmannin before infection with STEC strain CL56 resulted in a similar α-actinin response (data not shown). As shown in **Figure 12**, the presence of 10 nM wortmannin led to an 45% and 40% of reduction in α-actinin accumulation beneath adherent E2348/69 and CL56, respectively. A dose-dependent effect of wortmannin was not tested because of its nonspecific inhibitory effect at higher concentrations (Fruman *et al.*, 1998).

LY294002 inhibits α-actinin reorganization in EPEC- and STEC-infected HEp-2 cells. **Table 5** shows that bacterial adherence to tissue culture cells was not affected by pretreatment of epithelial cells with the phosphoinositide 3-kinase inhibitor LY294002 carried by DMSO as vehicle. Infection of HEp-2 cells with STEC strain CL56 resulted in recruitment of α-actinin protein at the site of bacterial attachment. In contrast, LY294002 inhibited α-actinin accumulation in CL56-infected cells, as demonstrated by the absence of foci of fluorescence in the infected epithelial cells beneath the adherent bacteria. DMSO alone did not affect the α-actinin response. Similar inhibitory effects on α-actinin

Table 5. Effects of phosphoinositide 3-kinase inhibitors on initial bacterial adherence on HEp-2 monolayers. Results show bacterial attachment to 100 randomly selected HEp-2 cells expressed as mean ± standard deviation (n = 4-6 independent experiments). Variations are not significant compared to cells infected in the absence of the inhibitors ($P > 0.05$).

Wortmannin concentration (nM)	E2348/69	CL56	UMD864
0 (without vehicle)	215±2	181±28	100±8
0 (with vehicle)	201±9	172±4	99±12
10	195±16	169±14	93

LY294002 concentration (μM)	E2348/69	CL56	UMD864
0 (without vehicle)	215±28	192±28	130±13
0 (with vehicle)	192±19	235±39	106±8
30	218±23	235±58	128±5
100	239±26	196±45	141±31
150	242±48	174±61	135±16

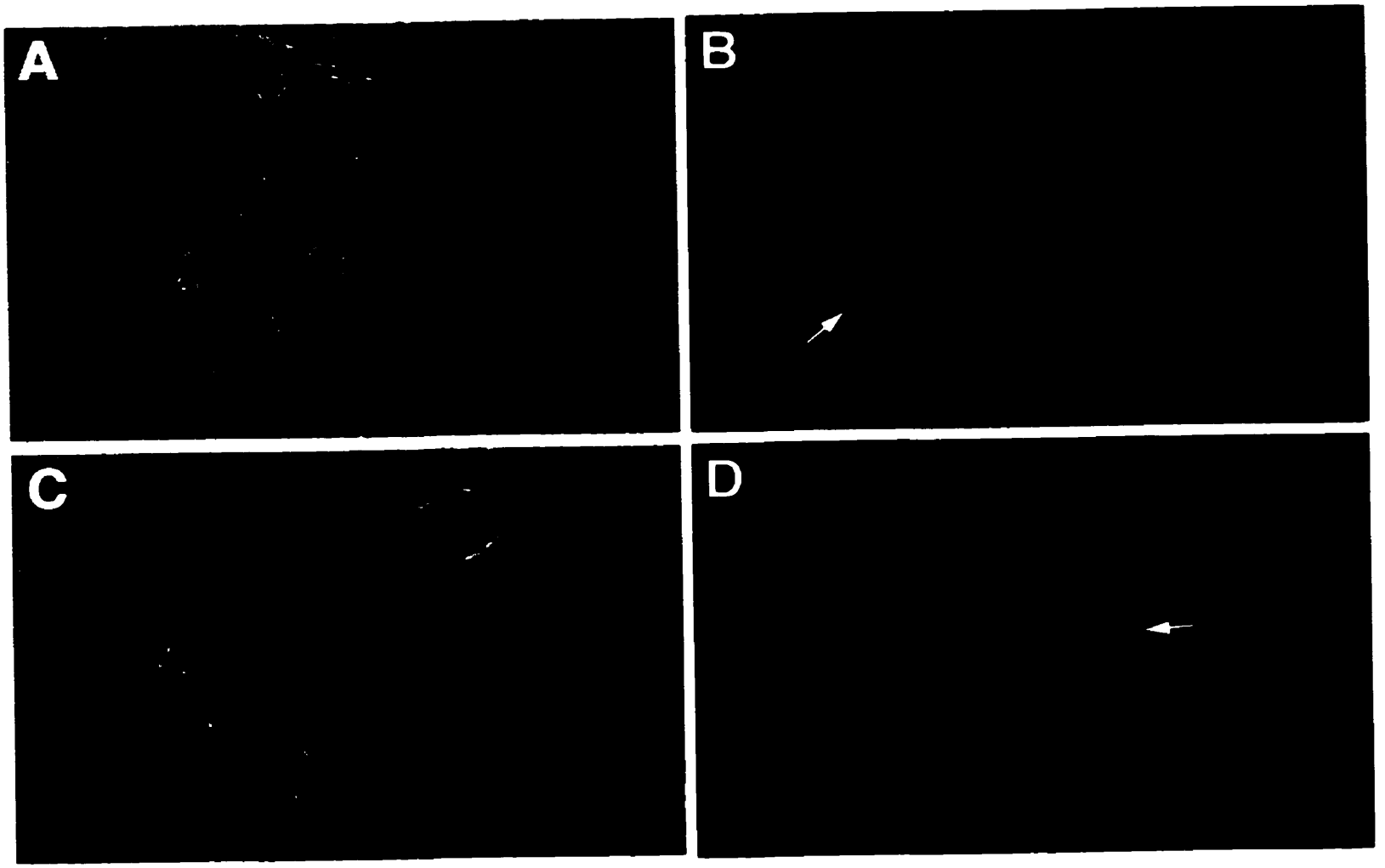
Figure 11. Reduced attaching and effacing lesion formation with the phosphoinositide 3-kinase inhibitor wortmannin (10 nM). Approximate magnification, x1250.

A. EPEC strain E2348/69 showing initial adherence to tissue culture HEp-2 cells by phase-contrast microscopy (arrow).

B. Intense foci of fluorescence demonstrate reaggregation of α-actinin (arrow) corresponding to areas of bacterial attachment.

C. Phase-contrast micrograph depicting adherent E2348/69 (arrow) on wortmannin-pretreated HEp-2 cells.

D. Fewer foci of α-actinin accumulation under attaching bacteria (arrow) were detected in the corresponding fluorescence micrograph.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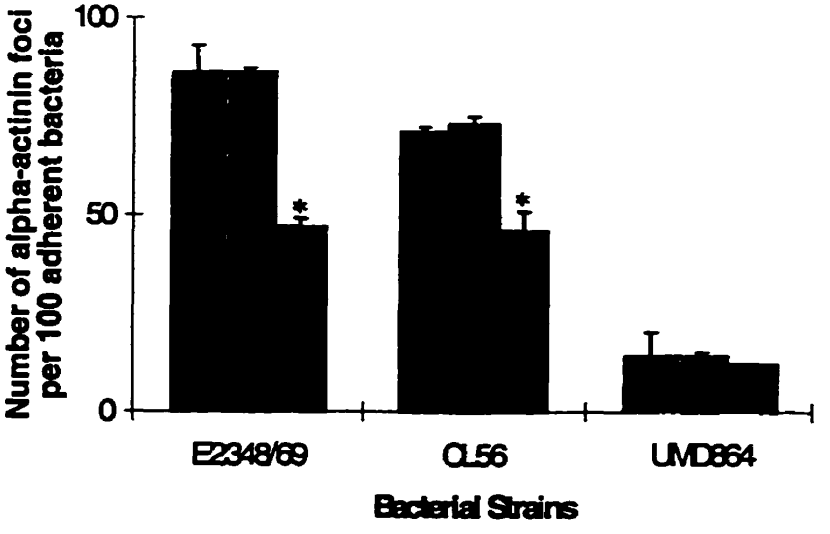


Figure 12. Quantitation of the inhibitory effect of the phosphoinositide 3-kinase inhibitor wortmannin on attaching and effacing lesion formation. At 10 nM of wortmannin, there was a decrease in ability of both EPEC and STEC to trigger α-actinin localization in infected epithelial cells. A dose-response relationship could not be established because of wortmannin's broad spectrum of inhibitory effects at higher concentrations (Fruman *et al.*, 1998). (*, ANOVA, $P < 0.05$; n = 4-6 independent experiments). Bars represent the infected control, the vehicle control, and inhibitor at concentration of 10 nM, for E2348/69, CL56, and UMD864, respectively.

localization were detected during infection with EPEC strain E2348/69. The inhibitory effect was concentration-dependent, as shown in **Figure 13.**

NDGA inhibits α-actinin rearrangement in EPEC- and STEC-infected HEp-2 cells. NDGA pretreatment of tissue culture cells did not reduce the capability of bacteria to adhere to HEp-2 cell monolayers (**Table 6**). STEC strain CL56 caused the formation of attaching and effacing lesions in the absence of the leukotriene inhibitor NDGA (**Figure 14, Panels A & B**). However, fewer adherent bacteria were accompanied by the foci of α -actinin accumulation when HEp-2 tissue culture cells were preincubated with the cell signaling inhibitor (**Figure 14, Panels C & D**). Similar inhibitory effects on α-actinin rearrangement were observed during EPEC infection (data not shown). As demonstrated in **Figure 15**, NDGA inhibited the recruitment of α-actinin in both EPEC- and STEC-infected HEp-2 cells in a dose-dependent pattern.

NS-398 does <u>not</u> inhibit α-actinin reorganization in EPEC- and STEC-infected HEp-2 monolayers. Pretreatment of HEp-2 tissue culture cells did not reduce the initial bacterial adherence on the monolayers (**Table 7**). EPEC strain E2348/69 triggered the formation of attaching and effacing lesions in the absence of the cyclooxygenase-2 inhibitor NS-398 (**Figure 16, Panels A & B**). This inhibitor did not affect the α-actinin rearrangement during EPEC and STEC infections, even at concentration up to 50 μM, despite the IC_{50} for NS-398 of 3.8 μM (Futaki *et al.*, 1994) (**Figure 16, Panels C & D**). The cyclooxygenase-2 inhibitor NS-398 did not inhibit the recruitment of α-actinin in both EPEC- and STEC-infected HEp-2 cells at various concentrations (**Figure 17**).

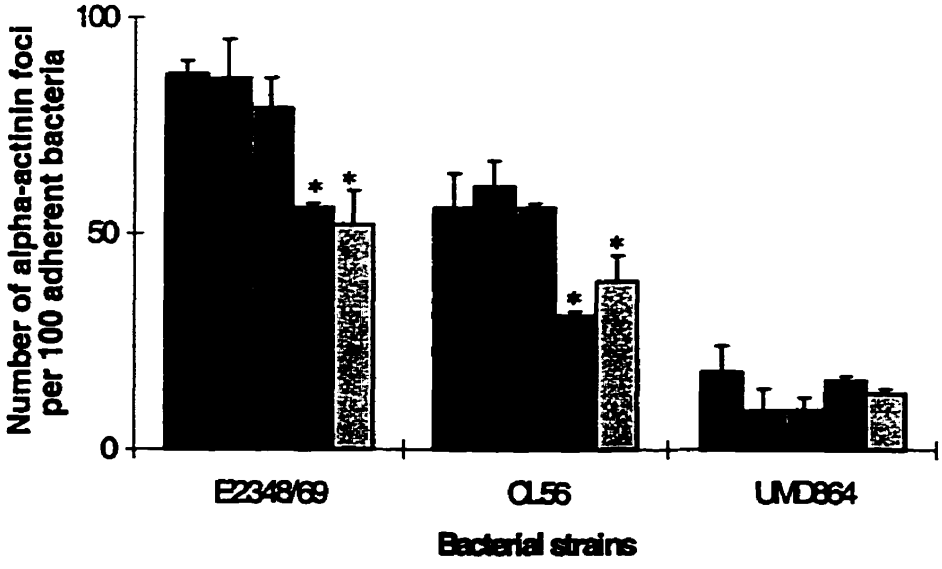


Figure 13. Quantitation of the inhibitory effect of the phosphoinositide 3-kinase inhibitor LY294002 on attaching and effacing lesion formation. α-actinin accumulation in both EPEC- and STEC-infected HEp-2 cells was blocked by the phosphoinositide 3-kinase inhibitor LY294002 in a dose-dependent manner. (*, ANOVA, $P < 0.05$; n = 4-6 independent experiments). Bars represent the infected control, the vehicle control, and inhibitor at concentrations of 30 μM, 100 μM, and 150 μM.

Table 6. Effects of the 5-lipoxygenase inhibitor NDGA on initial bacterial adherence on HEp-2 tissue culture cells. Results are expressed as bacterial attachment to 100 randomly selected HEp-2 cells ± standard deviation of the mean (n = 4-6 independent experiments). Variations are not statistical significant ($P > 0.05$).

NDGA concentration (μM)	E2348/69	CL56	UMD864
0 (without vehicle)	171±6	191±28	154±25
0 (with vehicle)	174±4	182±8	147±11
30	176±3	192±10	156±29
75	144±23	128±39	143±13

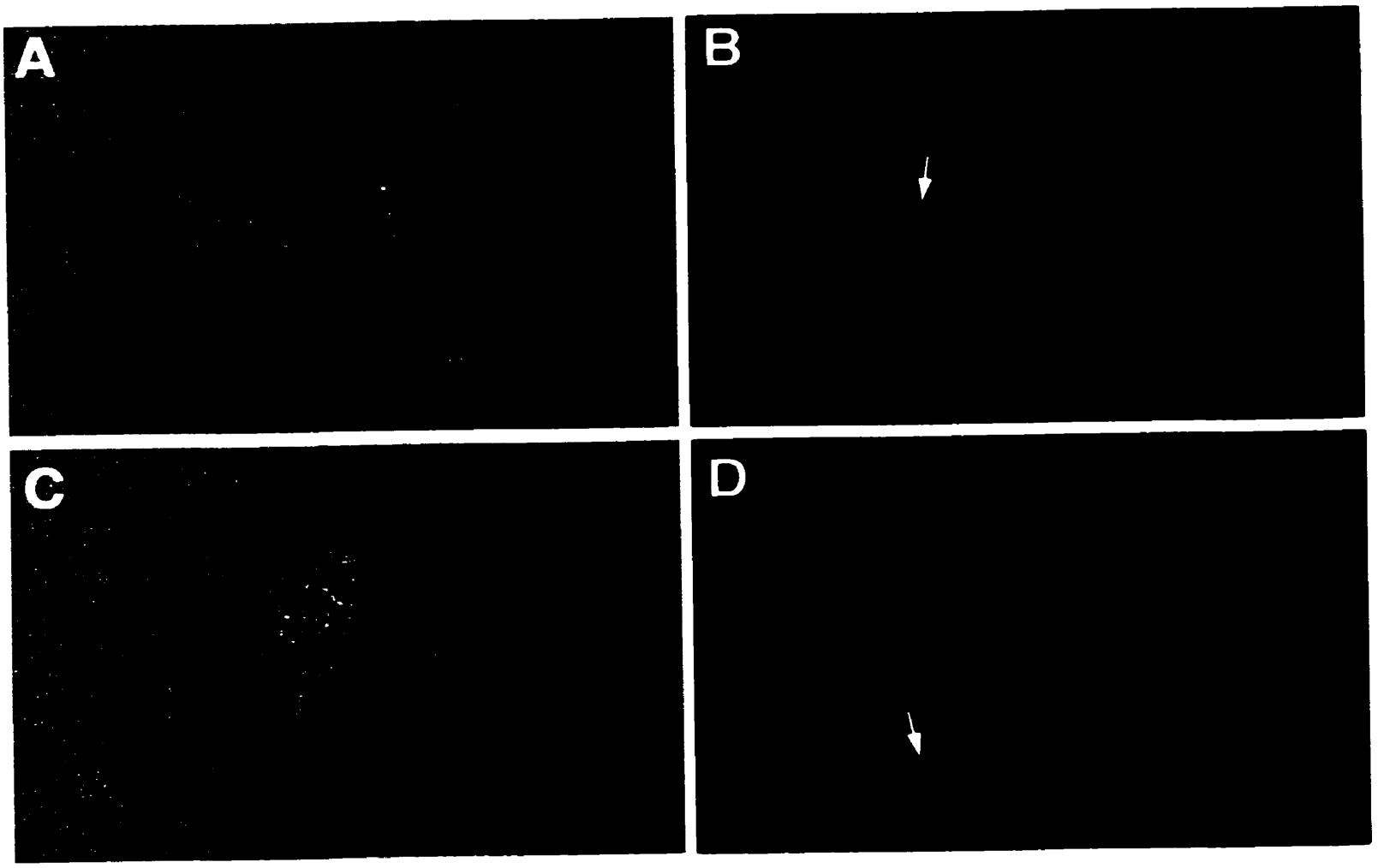
Figure 14. Reduced attaching and effacing lesion formation with the 5-lipoxygenase inhibitor NDGA. Approximate magnification, x1250.

A. Phase-contrast micrograph showing initial adherence of STEC strain O157:H7 CL56 (arrow) to HEp-2 cells following infection for 3 hrs at 37°C.

B. Bright foci of α-actinin fluorescence were detected in infected HEp-2 cells in regions subjacent to areas of bacterial adhesion (arrow).

C. Phase-contrast micrograph shows CL56 adhered (arrow) onto epithelial cells pretreated with 150 μM NDGA.

D. A negative α-actinin response was detected in the corresponding fluorescence micrograph (arrow).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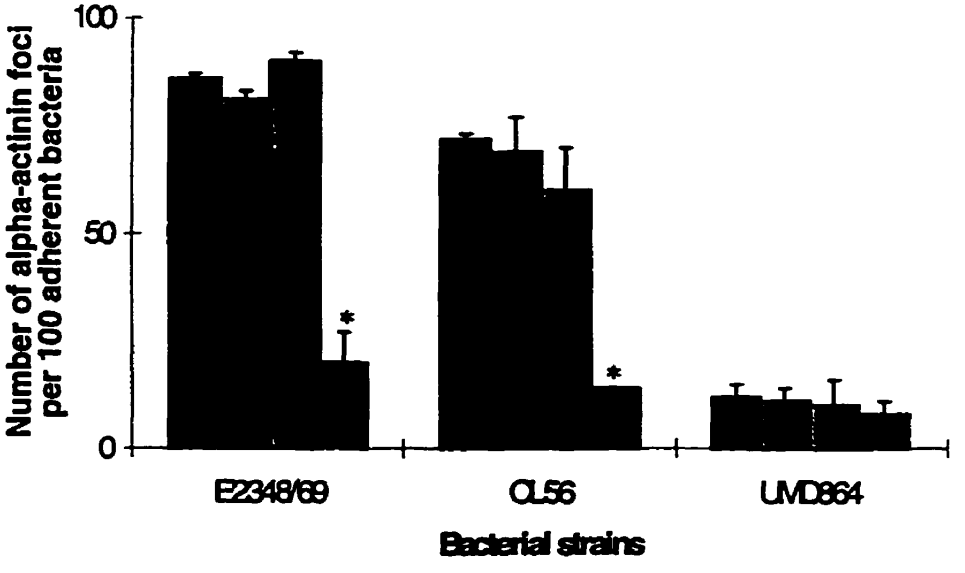

Figure 15. Quantitation of the inhibitory effect of the 5-lipoxygenase inhibitor NDGA on attaching and effacing lesion formation. A dose-dependent inhibitory effect of NDGA on the formation of attaching and effacing lesions triggered during EPEC and STEC infections was observed. (*, ANOVA, $P < 0.05$; n = 4-6 independent experiments). Bars represent the infected control, the vehicle control, and inhibitor at concentrations of 30 μM and 75 μM, for E2348/69, CL56, and UMD864, respectively.

Table 7. Effects of the cyclooxygenase-2 inhibitor NS-398 on initial bacterial adherence on HEp-2 epithelial cells. Results are expressed as bacterial attachment to 100 randomly selected HEp-2 cells ± standard deviation of the mean (n = 4-6 independent experiments). Variations are not statistical significant ($P > 0.05$).

NS-398 concentration (μM)	E2348/69	CL56	UMD864
0 (without vehicle)	140±7	102±8	110±7
0 (with vehicle)	144±10	110±6	99±10
10	169±20	99±11	100±6
20	114±4	100±12	96±5
50	116±7	105±3	101±12

Figure 16. Lack of an effect of the cyclooxygenase-2 inhibitor NS-398 on attaching and effacing lesion formation. Approximate magnification, x1250.

A. Phase-contrast micrograph demonstrating initial adherence of EPEC strain E2348/69 CL56 (arrow) to HEp-2 epithelial cells following infection for 3 hrs at 37°C.

B. Corresponding fluorescence micrograph showing α-actinin relocalization, illustrated by intense foci of fluorescence (arrow), underneath adherent microcolonies of bacteria.

C. Phase-contrast micrograph shows E2348/69 adhered (arrow) onto NS-398-pretreated HEp-2 monolayers.

D. A positive α-actinin response was detected in the corresponding fluorescence micrograph (arrow). The reorganization of α-actinin was not affected by the treatment of the inhibitor.

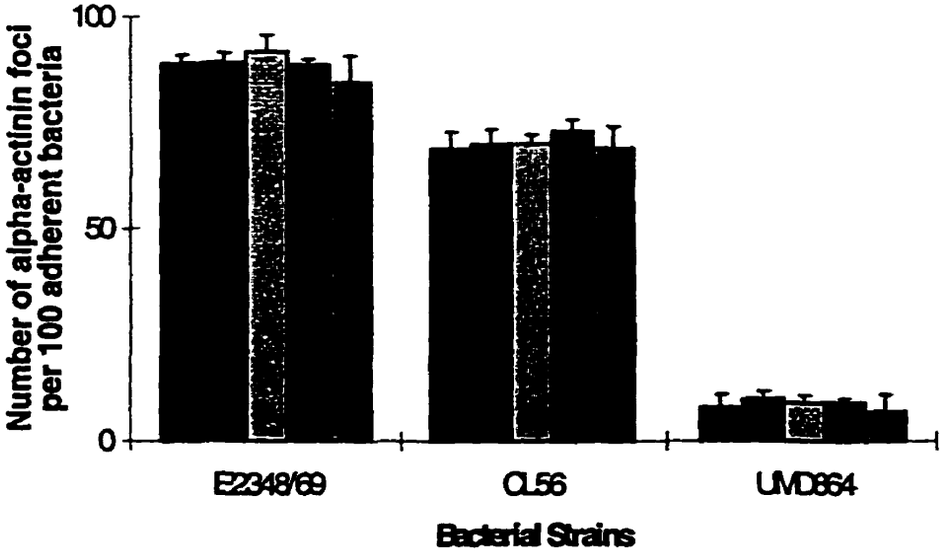

Figure 17. Quantitation of the effect of the cyclooxygenase-2 inhibitor NS-398 on attaching and effacing lesion formation. NS-398, tested at various concentrations, did not affect α-actinin condensation during EPEC and STEC infections. Bars represent the infected control, the vehicle control, and inhibitor at concentrations of 10 μM, 20 μM, and 50 μM, for E2348, CL56, and UMD864, respectively.

B. Discussion

Epithelial cells in tissue culture serve as a valuable model to evaluate the mechanisms by which enteric pathogens interact with eukaryotic cells *in vivo*. Epithelial cell lines such as HeLa (human cervix), HEp-2 (human larynx), Caco-2 (human colon), Henle 407 (human fetal small intestine) and T84 (human colon) each has been employed to study the mechanisms of adherence of pathogenic *E. coli* (McKee & O'Brien, 1995). Small intestinal cells of infants or animals infected by EPEC show ultrastructural alternations similar to those observed in infected HEp-2 cells. Thus, infection with HEp-2 cells by EPEC provides a useful and reliable model system for investigating the virulence properties of human enteropathogens (Nataro & Kaper, 1998).

EPEC and STEC are enteric pathogens that constitute a significant risk to human health. Both EPEC and STEC are capable of inducing cytoskeletal rearrangement leading to the formation of AE lesions on host epithelial surfaces during infection. The FAS test was developed, using FITC-labeled phalloidin to detect F-actin. However, phalloidin is a toxic substance and, therefore, poses a safety hazard (Law, 1994). Detection of α-actinin accumulation forms the basis for the development of a safe alternative test for observing an AE lesion (Ismaili *et al.*, 1995b).

Four potential signal transduction pathways were being investigated in this study. Firstly, elevations in inositol-1,4,5-trisphosphate are present within the cytoplasm of both EPEC- and STEC-infected cells (Foubister *et al.*, 1994b) (Ismaili *et al.*, 1995a). Since inositol-1,4,5-trisphosphate is one of the end products in the hydrolysis of phosphatidylinositol-4,5-bisphosphate by the enzyme phospholipase C, this led to the hypothesis that phospholipase C plays a central role in mediating the activation of the

signal transduction pathway thereby leading to AE lesion formation and diarrhea. To test the hypothesis, the phospholipase C inhibitor ET-18-OCH_3 was employed.

The ether lipid analogue ET-18-OCH_3 inhibits phosphoinositide-specific phospholipase C, although the precise mechanism of inhibition remains unknown (Powis *et al.*, 1992). Since ET-18-OCH_3 blocked α-actinin accumulation in infected HEp-2 cells in a dose-dependent manner, the present study suggests that phospholipase C is likely to be involved in the signal transduction pathway leading to the AE lesion formation.

To demonstrate the inhibitory effect of ET-18-OCH_3, diacylglycerol, one of the second messengers in the hydrolysis of phosphatidylinositol-4,5-bisphosphate by phospholipase C, was measured. The TLC assay, however, was not sensitive enough to detect subtle changes in levels of cytoplasmic diacylglycerol following EPEC and STEC infections. Other more sensitive assay systems, such as the *sn*-1,2-diacylglycerol assay reagents system (Amersham, Oakville, ON), which is a bioassay kit based upon the enzymatic conversion of diacylglycerol to phosphatidic acid using diacylglycerol kinase and ^{32}P-γ-ATP, and a high-performance liquid chromatography (HPLC) based assay (Eaton *et al.*, 1995) should be used in future studies.

Phosphoinositides are important regulators and signaling molecules of a variety of proteins linked to the actin cytoskeleton (Martin, 1998) (Schmidt & Hall, 1998). For instance, phosphoinositide 3-kinase is involved in the regulation of actin polymerization (Ma *et al.*, 1998). As a result, phosphoinositide 3-kinase inhibitors, wortmannin and LY294002, were utilized to determine if phosphoinositide 3-kinase is a component of the pathway that leads to AE lesion formation during EPEC and STEC infections.

The fungal metabolite wortmannin inhibits multiple signaling enzymes at high concentrations (Fruman *et al.*, 1998). However, at nanomolar concentrations wortmannin selectively targets phosphoinositide 3-kinase by irreversibly inhibiting the catalytic subunit of phosphoinositide 3-kinase (Wymann *et al.*, 1996). The inhibition of α-actinin accumulation by 10 nM wortmannin indicates that phosphoinositide 3-kinase also is likely to be involved in the pathway leading AE lesion formation. As a complementary assay, LY294002, a specific inhibitor of phosphoinositide 3-kinase which reversibly inhibits phosphoinositide 3-kinase by competing with ATP for its substrate binding site (Vlahos *et al.*, 1994), also inhibited the formation of AE lesions in EPEC- and STEC-infected epithelial cells. Taken together, these findings demonstrate that phosphoinositide 3-kinase plays an important role in AE lesion formation following infection of epithelial cells with EPEC and STEC.

Components of both lipoxygenase and cyclooxygenase pathways are abundant in the human gut and their role in the reorganization of cytoskeleton has been suggested previously (Peppelenbosch *et al.*, 1993). Therefore, the specific 5-lipoxygenase inhibitor NDGA and the highly selective cyclooxygenase-2 inhibitor NS-398 were used in this study to define a role for these enzymes involved in arachidonic acid metabolism in the formation in AE lesion resulting from EPEC and STEC infections.

NDGA inhibits 5-lipoxygenase activity (Peppelenbosch *et al.*, 1995), an enzyme which generates leukotrienes from arachidonic acid. The concentration-dependent inhibition of α-actinin accumulation observed following infection of HEp-2 cells with EPEC and STEC in the presence of NDGA indicates that leukotrienes are involved in the signal transduction cascade leading to cytoskeletal reorganization in infected eukaryotic

cells. This is the first study showing that 5-lipoxygenase activity and its subsequent metabolites leukotrienes are involved in the signal transduction pathway leading to cytoskeletal rearrangement triggered during EPEC and STEC infections.

While cyclooxygenase-2 plays an important role in mediating pro-inflammatory responses, the enzyme was not involved in the formation of AE lesions during EPEC and STEC infections. The results suggest that pathways involved in the attaching and effacing histopathology observed during infection of host cells with EPEC and STEC are cyclooxygenase-2-independent. Preliminary data from study with another potent cyclooxygenase inhibitor indomethacin further suggest that both cyclooxygenase-1 and cyclooxygenase-2 are not required in the formation of AE lesions.

Initial adherence of bacteria to epithelial cells lining the gastrointestinal tract is a critical step in the pathogenesis of gastrointestinal infections (Beachey, 1981) and is necessary for subsequent signal transduction and intimate attachment (Hicks *et al.*, 1998). The number of bacteria adherent to HEp-2 cells did not differ following pretreatment of cells with each of the inhibitors of cell signaling. This finding indicates that none of the inhibitors, nor the vehicles in which they were suspended, had an effect on the first stage of EPEC and STEC infections. Therefore, the inhibitory effects of the signaling inhibitors on their protein targets are responsible for the observed reduction in formation of AE lesions.

Toxicity of each inhibitor, along with its vehicle, was studied to ensure the viability of tissue culture cells. The observation that both the number of HEp-2 cells and the morphology of the eukaryotic cells were not affected by preincubation with any of the inhibitors or the vehicle alone indicate that there were no marked toxic effects on HEp-2

epithelial cells. The viability of HEp2 cells following treatment with an inhibitor can also be assessed by using trypan blue exclusion, uptake of propidium iodide, release of lactate dehydrogenase, or by employing a live-dead assay such as the commercial EukoLight Viability/Cytotoxic kit (Molecular Probes).

Based on these findings multiple signal transduction events likely are involved in the attaching and effacing lesions formed in response to EPEC and STEC infections. As shown in **Figure 18**, a working model could begin with activation of the inositide signaling pathway and culminate in cytoskeletal rearrangements in infected host epithelial cells. Such cytoskeletal rearrangements are likely to have biological relevance because studies in both animals and humans indicate that the ability of EPEC to cause diarrhea correlates with the formation of attaching and effacing lesions (Robins-Browne, 1987).

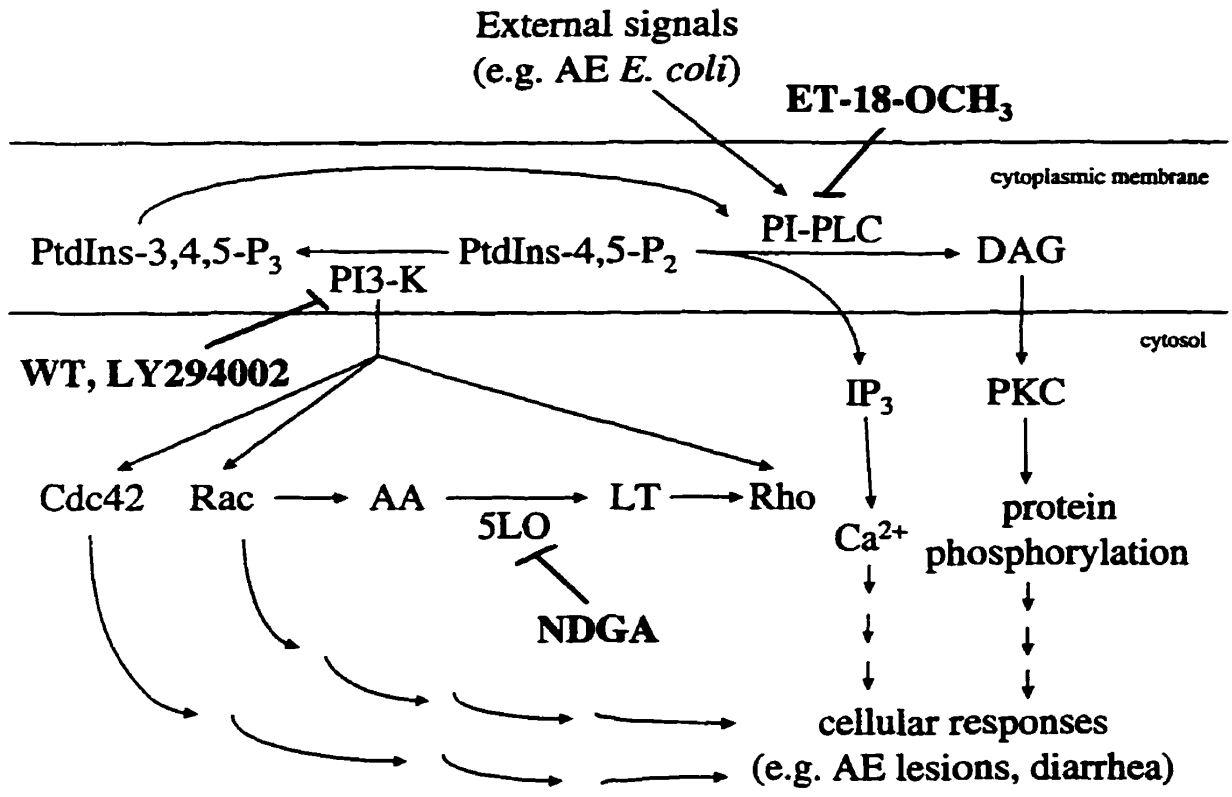

Figure 18. Proposed model of cytosolic signal transduction responses to EPEC and STEC infections. Attaching and effacing *E. coli* act as an external signal to activate several intracellular signaling events. Divergent pathways converge to ultimately give rise to the formation of the attaching and effacing lesion. (AE *E. coli* = attaching and effacing *Escherichia coli*; PtdIns-3,4,5-P_3 = phosphatidylinositol-3,4,5-trisphosphate; PI3-K = phosphoinositide 3-kinase; PtdIns-4,5-P_2 = phosphatidylinositol-4,5-bisphosphate; PI-PLC = phosphoinositide-specific phospholipase C; DAG = diacylglycerol; IP_3 = inositol trisphosphate; Ca^{2+} = calcium; PKC = protein kinase C; Cdc42, Rac, Rho = members of small G protein family; AA = arachidonic acid; LT = leukotriene).

Chapter 8

Conclusions and Future Directions

A. Conclusions

In summary, while initial adherence of EPEC strain E2348/69 and STEC strain CL56 to HEp-2 epithelial cells was not affected, α-actinin accumulation in infected tissue culture cells was effectively blocked by enzyme inhibitors ET-18-OCH_3, wortmannin, LY294002, and NDGA, but not by NS-398. This study suggests that the cytoskeletal effects induced in host tissue cells during EPEC and STEC infections are dependent on phospholipase C, phosphoinositide 3-kinase, and 5-lipoxygenase, but cyclooxygenase-independent. Understanding the signal transduction responses to EPEC and STEC infections could provide the basis for the development of novel therapies for use in the prevention and treatment of these infections in humans.

B. Future Directions

I) To confirm the inhibitory effect of inhibitors

Biochemical analysis of the end products of a reaction which the enzyme mediates will assure the inhibitory effect of the cell signaling inhibitor. Future studies should measure the cytoplasmic concentrations of inositol trisphosphate and diacylglycerol, phosphatidylinositol trisphosphate, leukotrienes, and prostaglandins before and after pretreatment of HEp-2 epithelial cells with ET-18-OCH_3, wortmannin and LY294002, NDGA, and NS-398, respectively.

II) To define the role of a particular enzyme

Studies with the use of a cell signaling inhibitor only suggest the involvement of the enzyme being investigated because of the potential for a broader spectrum of the

inhibitory effects of the inhibitor (**Table 8**). Further experiments are necessary before a definitive role of the enzyme can be identified.

While inhibitor provides a means to block the action of the enzyme, experiments using cells transiently expressing dominant negative mutants of the enzyme (or overexpressing the activated protein) can also serve as a complementary approach. Antisense nucleotide sequences against the mRNA encoding the enzyme may also be used if the enzyme is induced during the course of infection.

III) To study pathways leading to changes in transepithelial resistance

Although the mechanisms by which EPEC and STEC produce diarrhea are not completely clear, increased intestinal permeability as a result of breakdown in tight junctions has been postulated (Sears & Kaper, 1996). Indeed, the tight junction-associated protein ZO-1 is disrupted following both EPEC and STEC infections (Philpott *et al.*, 1996) (Philpott *et al.*, 1998).

The tight junction or zonula occludens (ZO) serves two primary functions in epithelial cells (Stevenson & Keon, 1998). It forms a regulated barrier in the spaces between the plasma membranes of adjacent cells and prevents the free passage of molecules as small as ions through the paracellular pathway, thereby ensuring the maintenance of compositionally distinct body compartments. The tight junction also acts as a boundary within the plasma membrane itself and maintains the unique protein and lipid composition between the apical and basolateral cell surface domains.

Ten proteins have now been specifically localized to the tight junction, and actin is strongly implicated in the regulation of junction permeability (Citi & Cordenonsi,

Table 8. Potential enzyme inhibitors for phospholipase C (A), phosphoinositide 3-kinase (B), 5-lipoxygenase (C), and cyclooxygenase (D).

A.

Phospholipase C inhibitors	Other enzyme targets tested
ET-18-OCH_3	
manoalide	phospholipase A_2 (20 - 200 nM)
neomycin sulphate	phospholipase D (65 μM)
U73122	ET-1 (800 nM)

B.

Phosphoinositide 3-kinase inhibitors	Other enzyme targets tested
wortmannin	MAP kinase (200 - 300 nM) myosin light chain kinase (200 nM) phosphoinositide 4-kinase (1 μM)
LY294002	
quercetin	phospholipase A_2 (2 μM)

C.

5-lipoxygenase inhibitors	Other enzyme targets tested
NDGA	cyclooxygenase (100 μM) 12-lipoxygenase (30 μM) 15-lipoxygenase (30 μM)
DEAE	phospholipase A_2 (16 μM)
ETYA	cyclooxygenase (8 μM) 12-lipoxygenase (300 nM) 15-lipoxygenase (200 nM)
ETI	12-lipoxygenase (20 μM) cyclooxygenase (50 μM)
curcumin	cyclooxygenase (52 μM) nitric oxide synthase (6 μM)

D.

Cyclooxygenase inhibitors	Other enzyme targets tested
NS-398	
indomethacin	phospholipase A_2 (145 μM)
ETYA	5-lipoxygenase (10 μM) 12-lipoxygenase (300 nM) 15-lipoxygenase (200 nM)
ETI	5-lipoxygenase (20 μM) 12-lipoxygenase (20 μM)
curcumin	5-lipoxygenase (8 μM) nitric oxide synthase (6 μM)

* Number in brackets represents concentration of the inhibitor to inhibit 50% of the enzyme activity or IC_{50}.

1998). **Figure 19** shows a schematic representation of the known binding interactions of tight junction proteins. The membrane domain of tight junction is the occludin protein, which is intimately associated with the cytoplasmic plaque proteins ZO-1, ZO-2, and ZO-3. The C-terminus of ZO-1 then provides a link between the transmembrane protein occludin and the actin cytoskeleton (Fanning *et al.*,1998).

Signaling molecules, such as protein kinase C and myosin light chain kinase, are known to play a role in the intracellular signal transaction pathways which lead to an increase in transepithelial permeability (Philpott *et al.*, 1998). Therefore, additional studies will use these enzyme inhibitors to examine the roles of phosphoinositide-specific phospholipase C, phosphoinositide 3-kinase, and 5-lipoxygenase as mediators of EPEC- and STEC-induced changes in tight junction permeability.

IV) To further define the role of enzymes with an animal model

The HEp-2 cell line was used in this study because it mimics changes observed in intestinal epithelia during infection *in vivo*. Although this cell line is widely employed as a model system for investigating the virulence properties of human enteropathogens, however, it may not truly represent the *in vivo* environment.

HEp-2 epithelial cells originate from a human cancer of the larynx, but EPEC and STEC are enteric pathogens that primarily attach to the small intestine and colonic epithelial surfaces, respectively. Epithelial cells along the gastrointestinal tract to which pathogenic *E. coli* bind are non-dividing and fully differentiated, whereas HEp-2 epithelial cells are immortalized (always dividing) and undifferentiated.

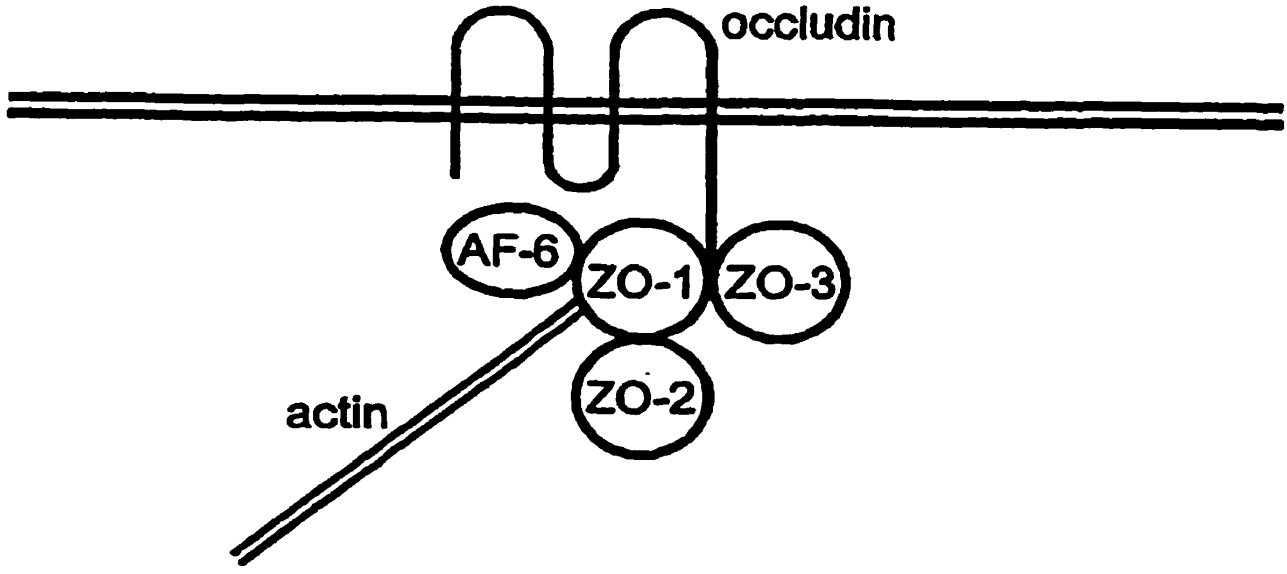

Figure 19. Schematic diagram showing the known binding interactions of tight junction proteins. Taken from Stevenson & Keon, 1998.

These subtle variance in experimental conditions can sometimes lead to varying results. For example, *in vitro* experiments with human epithelial cell lines implicated the EAF plasmid-encoded bundle-forming pili in initial binding of EPEC (Giron and Ho, 1991). However, in human intestine in organ culture, bundle-forming pili do not appear to be involved in the initial stages of EPEC non-intimate adhesion (Hicks *et al.*, 1998).

In vitro experiments serve only as a model for initial investigations. Future studies *in vivo* are needed to investigate signal transduction events between enteropathogens and the host. Future studies should employ a relevant animal model to study the roles of phospholipase C, phosphoinositide 3-kinase, 5-lipoxygenase, and cyclooxygenase in the formation of AE lesions during EPEC and STEC infections.

Chapter 9

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