ADHERENCE OF ENTEROHEMORRHAGIC *ESCHERICHIA COLI* TO HUMAN EPITHELIAL CELLS: THE ROLE OF INTIMIN

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Abstract

Title of Dissertation:

Adherence of enterohemorrhagic *Escherichia coli* to human epithelial cells: the role of intimin

Marian L. McKee, Doctor of Philosophy, 1995

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Enterohemorrhagic *E.coli* (EHEC) is the leading cause of infectious bloody diarrhea in the United States as well as acute renal failure among U.S. and Canadian children. EHEC colonize the large bowel and produce Shiga-like toxins (SLTs) that are considered essential for EHEC virulence. EHEC cause intestinal attaching and effacing (A/E) lesions in experimental animals and carry a homologue of the enteropathogenic *E. coli* (EPEC) *eaeA* (*E. coli* attach and efface) locus. In EPEC, the *eaeA* gene encodes intimin and is required for A/E lesion formation. To examine the importance of the *eaeA* homologue in EHEC O157:H7,

we modified an existing tissue culture assay to monitor adherence to epithelial cells. With this assay, we serendipitously discovered a novel adherence phenotype, which we designated "log jam", that was shared among intestinally-derived *E. coli*. The assay was also used to compare the in-frame *eaeA* deletion mutation strain 86-24*eaeΔ10* with the wild-type parent. The mutant strain, 86-24*eaeΔ10*, did not adhere to HEp-2 cells or colonize the spiral colon of infected piglets. By contrast, 86-24 formed microcolonies on HEp-2 cells, colonized the cecum and colon, and induced intestinal A/E lesions in gnotobiotic piglets. *In vitro* attachment and *in vivo* lesion formation by 86-24*eaeΔ10* was fully restored by a clone of EHEC 86-24 *eaeA*. To further characterize intimin, histidine::intimin fusions were constructed and purified over a nickel affinity column. Extracts of the fusions enhanced binding of wild-type 86-24 to HEp-2 cells and conferred HEp-2 cell adherence on 86-24*eaeΔ10* and an *eaeA*$^-$ O91:H21 EHEC strain. Polyclonal antisera against the His::intimin fusion proteins recognized a 97 kDa outer membrane protein in EHEC and EPEC. Furthermore, intimin-specific antibodies blocked adherence of EHEC to HEp-2 cells. These studies demonstrate that intimin acts as a primary adhesin in EHEC attachment to epithelial cells, is required for A/E lesion formation in the intestinal mucosa, and, as an isolated protein, confers adherence of *eaeA*-negative strains to epithelial cells.

ADHERENCE OF ENTEROHEMORRHAGIC *Escherichia coli* TO HUMAN EPITHELIAL CELLS: THE ROLE OF INTIMIN

by

Marian Little McKee

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To Daddy -

Preacher, Teacher, and Healer

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Table of contents

Materials and Methods

List of Tables

List of Figures

Introduction

I. The role of adherence in the pathogenesis of enteric infections.

To persist in a niche in the environment or the human body, a bacterium must first attach to the surface (Beachey, 1981). Indeed, an important early aspect of microbial pathogenesis is adherence to and subsequent colonization of body surfaces (Smith, 1977). To colonize a mammalian host, many microbial infections begin on the mucous membranes of the respiratory, urogenital, or gastrointestinal tract. On the mucosal surface, the bacterium constantly battles such host defense mechanisms as peristalsis (Formal *et al.*, 1983). Thus, most organisms caught in the mucus layer of the small intestine are cleared by the propulsive forces of this organ (Formal *et al.*, 1983). Additionally, an infecting bacterium must compete with a resident population of commensal microbes (Simon and Gorbach, 1986). After the bacterium contacts the mucosal surface by use of attachment factors (to be discussed), there are three documented outcomes of the resultant infection: i) colonization of the intestinal mucosa by the bacterium without penetration of or damage to the mucosa (e.g., enterotoxigenic *E. coli*, *Vibrio cholerae*, and *Bordetella pertussis*); ii) damage to the epithelial cell microvilli induced by the bacterium with subsequent deformation of the epithelial cell plasma membrane. This damage may occur with or without limited bacterial penetration of mucosal cells (e.g., enteropathogenic *E. coli* and *Citrobacter fruendii* 4280); or, iii) overt bacterial invasion of the epithelial layer of the mucosa. This

invasion may occur between or through the epithelial cells (e.g., *Salmonella typhi*, *Yersinia enterocolitica*, *Shigellae*, and enteroinvasive *E. coli*) (Smith, 1977; Formal *et al.*, 1983).

To accomplish the initial mucosal attachment step, the bacterium must express a factor or factors that mediate adherence. Specific bacterial adherence plays two important roles in bacterial infection. Many bacterial adhesins have been described, and a few representative ones from pathogenic *E. coli* are listed in Table 1 (Smith, 1977; Formal *et al.*, 1983; Krogfelt, 1991). Bacterial adhesins can be categorized as structured (fimbriae or pili) or amorphous (Krogfelt, 1991). Fimbriae, long, thread-like protein polymers found on the bacterial surface, are "adhesive appendages" by Beachey's definition (Beachey, 1981). Fimbriae make up the majority of the attachment factors described for *E. coli* (Klemm, 1985; Krogfelt, 1991). Of the adhesins listed in Table 1, only the afimbrial adhesin of uropathogenic *E. coli*, intimin of enteropathogenic and enterohemorrhagic *E. coli* (EPEC and EHEC, respectively), and AIDA-I of diffusely adherent *E. coli* are not fimbriae. The afimbrial adhesin intimin of EHEC is the subject of this study.

II. Overview of diarrheagenic *E. coli*

Strains of the species *Escherichia coli* are found in abundance as part of the normal flora of the gastrointestinal tract. However, pathogenic strains of

Footnotes for Table 1.

[a]UPEC, uropathogenic *E. coli*; ETEC, enterotoxigenic *E. coli*; EPEC, enteropathogenic *E. coli*; EAggEC, enteroaggregative *E. coli*; DAEC, diffusely adherent *E. coli*.

[b] C, chromosomally encoded; P, plasmid encoded.

Table 1. Representative adhesins of pathogenic *E. coli* (adapted from Muhldorfer and Hacker, 1994).

E. coli subtype[a]	Adhesin	Gene designation (location)[b]	Reference
All	Type I fimbriae	*fim* or *pil* (C)	(reviewed in Orndorff and Bloch, 1990)
All	curli	*csg* (C)	(Olsen et al., 1989)
UPEC	P-fimbriae	*pap* (C)	(Lindberg et al., 1984)
	S-fimbriae	*sfa* (C)	(Moch et al., 1987)
	Afimbrial adhesins	*afa* (C)	(Labigne-Roussel et al., 1984)
ETEC	K88	*fae* (P)	(Jones and Rutter, 1974)
	K99	*fan* (P)	(Burrows et al., 1976)
	CFA/I	*cfa* (P)	(Evans et al., 1975)
	CFA/II family	*coo* , CS2 and CS3 genes (C, P)	(Evans and Evans, Jr., 1978)
EPEC	Bundle-forming pilus	*bfp* (P)	(Giron et al., 1991a)
	Intimin	*eae* (C)	(Jerse et al., 1990)
EAggEC	Aggregative adherence fimbriae I	*aaf/I* (P)	(Nataro et al., 1992; Savarino et al., 1994)
DAEC	F1845 fimbriae	*daa* (C)	(Bilge et al., 1989)
	AIDA-I	AIDA/I (P)	(Benz and Schmidt, 1989; Benz and Schmidt, 1992)

E. coli that cause distinct syndromes of diarrheal disease have been recognized since the early twentieth century. In 1987, Levine divided these pathogenic *E. coli* into five groups based on the presence of specific virulence determinants, the nature of the interactions with the intestinal mucosa, the types of clinical syndromes caused by the group, epidemiological patterns, and O:H serotypes (Levine, 1987). The typing scheme of Levine divided the diarrheagenic *E. coli* into i) enterotoxigenic (ETEC), ii) enteroinvasive (EIEC), iii) enteropathogenic (EPEC), iv) enterohemorrhagic (EHEC), and v) enteroadherent (EAEC) *E. coli*. The fifth category was less well defined than the other groups, in part because little was known about the pathogenesis of EAEC-mediated disease. Since Levine's comprehensive review, more data have emerged for each of the five categories, and the number of diarreheagenic *E. coli* groups has now expanded into six (Table 2). The original EAEC group of Levine (1987) is now organized into two distinct units: enteroaggregative (EAggEC) and diffusely adherent (DAEC) *E. coli* based on the distinct patterns of HEp-2 cell adherence of isolates from each class (reviewed in Savarino, 1993). While each of the 6 current classes of diarrheagenic *E. coli* forms a discrete group, there are certain underlying pathogenic commonalities. For example, enterotoxins or cytotoxins are produced by many of these agents. Additionally, all of the groups have virulence-associated determinants encoded by a plasmid.

ETEC are a major cause of diarrhea in domestic animals as well as the primary agent of travelers' diarrhea (Gorbach *et al.*, 1971; Merson *et al.*, 1976).

Footnotes for Table 2.

[a] ST, heat-stable toxin; LT, heat-labile toxin; SLT, Shiga-like toxin; EAST-1, Enteroaggregative heat-stable toxin.

[b] CFA, colonization factor antigen;ND, not described; LA, localized adherence to HEp-2 cells; A/E, attaching and effacing lesion in vivo; DA, diffuse adherence to HEp-2 cells; AIDA, adhesin involved in diffuse adherence; AA, aggregative adherence on HEp-2 cells.

[c] EPEC and EHEC are members also of the AEEC (attaching and effacing *E. coli*) group.

[d] DAEC and EAggEC were formerly called EAEC (enteroadherent *E. coli*) by Levine (1987).

Table 2. Characteristics of diarrheagenic *E. coli*.

***E. coli* groups**	**Enterotoxin produced[a]**	**Adherence phenotype / adhesin[b]**	**Location of colonization**
ETEC	ST/ LT	CFA/I; CFA/II family	small intestine
EIEC	enterotoxin; cytotoxin	ND	colon
EPEC[c]	none	LA / A/E;intimin; BFP	small intestine
DAEC[d]	none	DA ; AIDA-1; F1845	small intestine
EAggEC[d]	EAST-1	AA; AAF/I	small intestine
EHEC[c]	SLT	LA / A/E; fimbriae; intimin	colon

ETEC are also a major cause of dehydrating diarrhea in infants in developing countries (Black *et al.*, 1981; Black *et al.*, 1982). Members of this class of diarrheagenic *E. coli* elaborate heat-stable (ST) and/or heat-labile (LT) toxins which are the primary virulence factors that provoke the watery diarrhea associated with an ETEC infection (Levine *et al.*, 1983). To colonize the small bowel, ETEC express a full complement of colonization factors. These adhesins are fimbrial structures that include CFA/I and the CFA/II family (CS 1-6) (Table 1). Specific colonization fimbriae are associated with particular O:H serotypes of ETEC (Evans and Evans, Jr., 1978; Knutton *et al.*, 1985). Recently, ETEC have also been reported to have the capacity to invade human epithelial cells *in vitro* (Elsinghorst and Kopecko, 1992), although the same strains are negative in the guinea pig conjunctivitis model of invasion. Two separate loci (*tia* and *tib*) have been cloned and confer the adherent and invasive phenotypes on a noninvasive K-12 *E. coli* strain (Elsinghorst and Weitz, 1994).

EIEC were confirmed as pathogens in volunteer studies. The volunteers presented with an invasive, dysentery-like form of diarrheal disease (Dupont *et al.*, 1971). The bacterial strains isolated from these patients resemble *Shigella* and can invade and multiply within epithelial cells (Dupont *et al.*, 1971). This invasive capacity of EIEC is encoded on a large plasmid (Black *et al.*, 1982) that is similar to the virulence-associated plasmid of *Shigella*. Recently, Fasano and colleagues (1990) demonstrated the presence of distinct enterotoxic and cytotoxic activities in culture filtrates and cell lysates of EIEC strains. Neither of

the toxic activities are neutralized by anti-SLT-I or anti-SLT-II antibodies. The function of these toxins in EIEC -mediated disease is unknown.

EPEC were incriminated as the cause of infant diarrhea in the 1940s and 1950s (Bray, 1945; Giles *et al.*, 1949; Taylor *et al.*, 1949). Until the mid-1970s, EPEC were identified only by serotype, because no specific virulence determinants were attributed to them. EPEC do not elaborate LT or ST (Gross *et al.*, 1976) or high levels of SLTs (O'Brien *et al.*, 1982). Subsequently, two independent observations led to an intensified research effort on EPEC pathogens. Polotsky *et al.,* (1977) noted a distinct lesion at the site of EPEC adherence to the human small intestinal enterocyte. This lesion, called the attaching and effacing (A/E) lesion, was experimentally reproduced by Moon (1983) and Tzipori *et al.* (1985) in the intestines of gnotobiotic piglets. The A/E lesion is characterized by intimate association of the bacterium with the epithelial cell, cupping of the epithelial cell membrane around the bacteria, and the accumulation of cytoskeletal elements at the site of adherence which results in the dissociation of the terminal web and effacement of the microvilli (Staley et al., 1969; Moon *et al.*, 1983). These intimately associated bacteria appear to be seated on a pedestal of actin. The second finding that propelled EPEC investigation forward was the observation by Cravioto *et al.* (1979) that a particular phenotype of adherence to HEp-2 cells is associated with 80% of EPEC isolates from the Colindale Laboratory Collection. This adherence assay developed by Cravioto, et al., (1979) was then used by Jerse *et al.* (1990) to identify a locus required for the capacity of EPEC to cause

localized adherence (LA) to HEp-2 cells and the A/E lesion on Caco-2 cells. The eaeA locus encodes a 94 kD adhesin called intimin. The A/E lesion, the HEp-2 cell adherence phenotype, and intimin of EPEC will be discussed in greater detail later in this introduction.

The large plasmid present in all classic EPEC strains, called the EAF plasmid [EPEC adherence factor (Baldini *et al.* 1983)], encodes fimbriae, called bundle forming pili (bfpA), that are involved in localized adherence to epithelial cells *in vitro* (Girón *et al.*, 1993b). The BFP mediate a bacterium-bacterium as well as a bacterium-host cell interaction (Girón *et al.*, 1991a; Giron *et al.*, 1993b). Internalization of EPEC bacteria by epithelial cells *in vitro* has been reported (Andrade *et al.*, 1989; Donnenberg *et al.*, 1989; Donnenberg *et al.*, 1990b). Although several loci have been identified that are required of the internalization of EPEC (Donnenberg *et al.*, 1990a), no pathogenic role has been defined for the invasion phenotype.

The DAEC group is characterized by the diffuse pattern of HEp-2 cell adherence exhibited by these bacteria (Benz and Schmidt, 1989; Bilge *et al.*, 1989). Several epidemiological studies (Mathewson *et al.*, 1987; Giron *et al.*, 1991b; Baqui *et al.*, 1992; Savarino, 1993) have implicated this group of *E. coli* as agents of diarrhea in the developing world, but no definitive data on causality have been reported. Two adhesins, F1845 (Bilge *et al.*, 1989) and AIDA-1 (Benz and Schmidt, 1989; Benz and Schmidt, 1992) are present on some DAEC strains, and the genes from these adhesins have been cloned (Table 1).

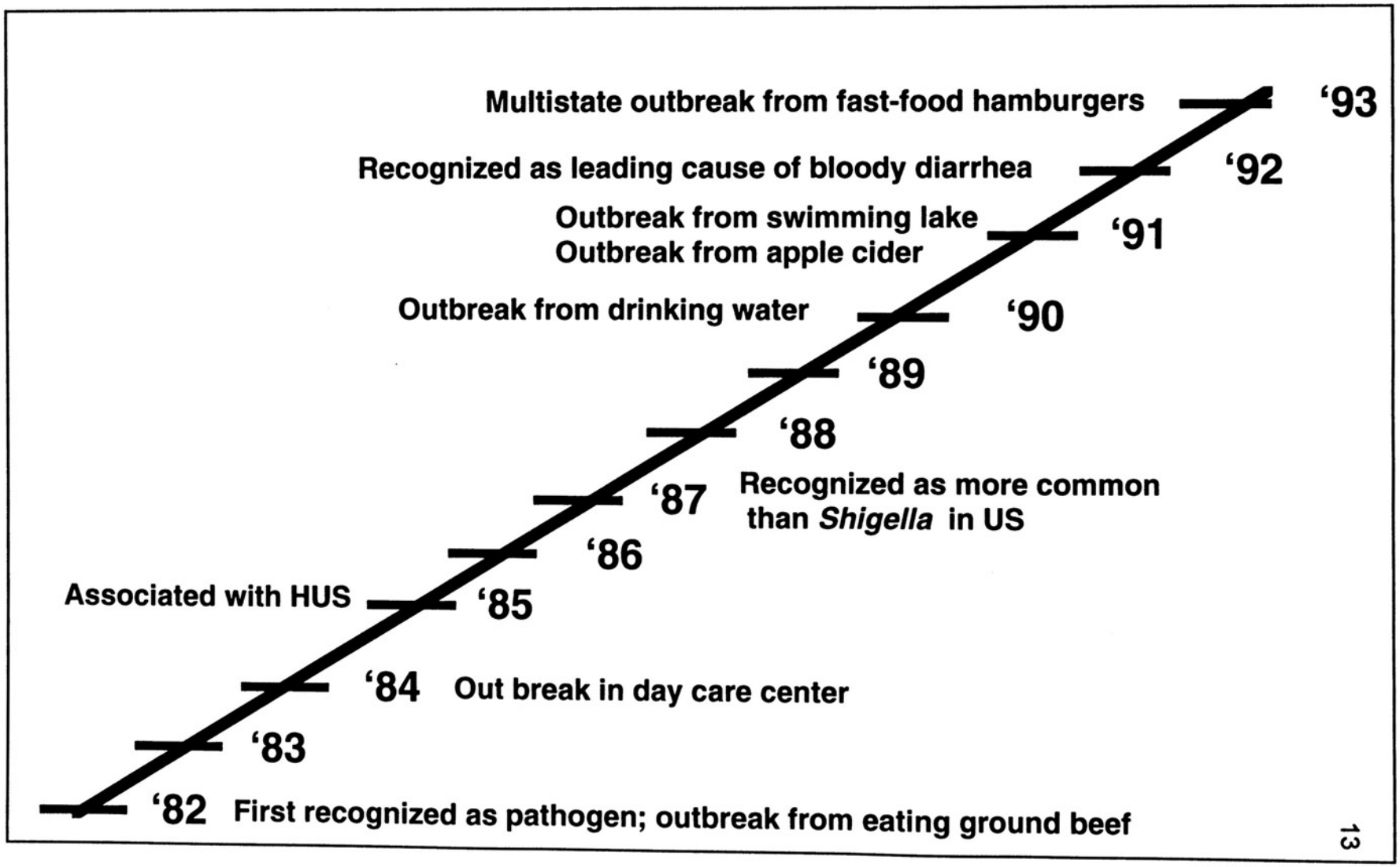
EAggEC are associated with persistent (≥14 days) diarrhea in children in developing countries (Savarino, 1993). EAggEC are easily distinguished by the pattern by which the organisms adhere to HEp-2 cells. Aggregative adherence (AA) refers to a phenotype in which bacteria adhere to each other to form a stacked brick-like lattice; this bacterial lattice can be observed on the epithelial cells as well as the slide on which the cells are grown (Savarino, 1993). AA is mediated by AAF/I, the aggregative adherence fimbriae (Nataro *et al.*, 1992; Savarino *et al.*, 1994). Members of this *E. coli* subtype elaborate EAST-1, a heat-stable enterotoxin that is encoded on a plasmid shared among EAggEC isolates (Savarino *et al.*, 1991; Savarino *et al.*, 1993).

The sixth group of diarrheagenic *E. coli*, EHEC, is the focus of this dissertation. EHEC are described in detail below.

III. Enterohemorrhagic *E. coli* (EHEC)

History, epidemiology, and diagnosis. *Escherichia coli* serotype O157:H7 first came to the attention of the clinical and scientific community in 1982 as the causative agent of an acute syndrome of bloody diarrhea known as hemorrhagic colitis (HC) that occurred in individuals who had consumed contaminated undercooked ground beef (Riley *et al.*, 1983). At the time it was first identified, O157:H7 was thought to be a rare *E. coli* isolate. However, over the last 15 years, a significant number of large outbreaks as well as sporadic cases of *E. coli*

Figure 1. Emergence of *Escherichia coli* O157:H7 as a pathogen in the United States (1982-1993) (Centers for Disease Control and Prevention, 1994).



Adapted from MMWR (1994) **43**:7

O157:H7-mediated disease have occurred across the United States (Fig. 1). These cases *of E. coli* O157:H7 infection have gained EHEC a place on the list of emerging infectious diseases targeted by Centers for Disease Control and Prevention (Centers for Disease Control and Prevention, 1994). In fact, *E. coli* O157:H7 is now recognized as the leading cause of bloody diarrhea in the US (Centers for Disease Control and Prevention, 1994).

Among EHEC, O157:H7 is the most commonly isolated serotype. EHEC are not only associated with hemorrhagic colitis but also the resultant sequelae, the hemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP). The EHEC group is comprised of organisms from a range of serogroups also including, but not limited to, O26, O91, and O111 (Levine, 1987; World Health Organization, 1992; Lindgren *et al.*, 1993).

The primary mode of EHEC transmission is the ingestion of contaminated beef products (most often as undercooked hamburger), but EHEC O157:H7 has also been isolated from lamb, pork, seafood, and poultry (Doyle and Schoeni, 1987; Samadpour *et al.*, 1994). Contamination of salad vegetables (Abdul-Raouf *et al.*, 1993), raw milk (MacDonald *et al.*, 1988), and unpasteurized apple cider (Besser *et al.*, 1993) has also been documented. One case of direct transmission from calves to a thirteen-month old boy has been reported (Renwick *et al.*, 1993). EHEC outbreaks have occurred in nursing homes and day care centers as a result of person-to-person spread (Samadpour *et al.*, 1993; Belongia *et al.*, 1993).

Diagnosis of EHEC infection is relatively straightforward if the clinical laboratory is made aware of a suspected case early enough in the infection. EHEC O157:H7 are unable to ferment sorbitol, a biochemical characteristic which distinguishes EHEC from most other fecal *E. coli* isolates (Wells *et al.*, 1983). Therefore, plating a stool specimen on sorbitol-MacConkey (SMAC) agar will identify sorbitol-negative colonies for further confirmation. Commercial agents are available to easily and rapidly serotype suspect isolates. However, until very recently, most clinical laboratories did not routinely plate diarrheic stools on SMAC agar; this omission has led to an under representation of the magnitude of EHEC O157:H7-related disease. The CDC now recommends that this test be routinely performed in clinical laboratories (Centers for Disease Control and Prevention, 1994).

Diseases caused by EHEC. Infection with EHEC O157:H7 is responsible for a spectrum of illnesses in humans which include: HC (~90% of diagnosed cases), nonbloody diarrhea (~10% of diagnosed cases), and associated intestinal and extraintestinal complications of enteric infection (≤5% of cases) (Tarr, 1995). HC is characterized by abdominal cramping and bloody diarrhea with radiologic or colonoscopic evidence of mucosal edema, erosion, or hemorrhage (Riley, 1987). Fever occurs in a minority of cases and fecal leukocytes are typically absent (Griffin *et al.*, 1988).

In approximately 10% of infected patients less than 10 years of age, HUS develops 1 week after the onset of diarrhea (Tarr, 1995). HUS is thought to result from damage to the vascular endothelium and kidney glomeruli following the absorption of SLT from the gut (Milford and Taylor, 1990; Pickering *et al.*, 1994). The sequela is defined by a triad of signs that include microangiopathic hemolytic anemia, thrombocytopenia, and acute renal dysfunction (Pickering *et al.*, 1994). TTP resembles HUS histopathologically but tends to affect adults (Tarr, 1995).

Virulence mechanisms associated with EHEC. The three primary characteristics of EHEC that have been linked to virulence include: i) production of the Shiga-like toxins, ii) presence of the 60 MDa plasmid, and iii) the capacity to produce an attaching and effacing intestinal lesion *in vivo*. Other products produced by EHEC include an exopolysaccharide capsule (Junkins and Doyle, 1992), cytolethal distending toxin (Johnson and Lior, 1988), and an α-hemolysin [formerly referred to as enterohemolysin (Beutin *et al.*, 1989; Beutin *et al.*, 1990; Beutin *et al.*, 1993; Schmidt *et al.*, 1994; Schmidt *et al.*, 1995)]. None of these products has been associated with EHEC pathogenicity and will not be discussed further here.

Shiga-like toxins (SLTs). SLTs, also known as Vero toxins (VTs), were originally identified on the basis of cytotoxicity for HeLa and Vero cells that

could be neutralized by anti-Shiga toxin antibodies (Konowalchuk *et al.*, 1977; O'Brien *et al.*, 1983). All EHEC strains produce SLTs (for reviews see O'Brien and Holmes, 1987; Tesh and O'Brien, 1992). In the prototypic EHEC isolates, the *slt* operons are located on toxin-converting lambda-like lysogenic phage (O'Brien *et al.*, 1984; Strockbine *et al.*, 1986; O'Brien *et al.*, 1989) or the chromosome (O'Brien *et al.*, 1989). The role of SLTs in disease has not been conclusively demonstrated, because there is no human model of infection. However, animal models and epidemiological data strongly implicate the SLTs as responsible for the neurologic and hemorrhagic components of HC and for the secondary sequelae of HUS (Karmali *et al.*, 1983; Pickering *et al.*, 1994).

60 MDa EHEC plasmid. The importance of the 60 MDa EHEC plasmid has been investigated both *in vitro* (Karch *et al.*, 1987; Junkins and Doyle, 1989; Toth *et al.*, 1990; Fratamico *et al.*, 1993) and in animal models of EHEC infection (Tzipori *et al.*, 1987; Wadolkowski *et al.*, 1990). The EHEC 60 MDa plasmid is similar to, but distinct from, the EAF plasmid of EPEC. Data in the literature are conflicting as to whether the EHEC large plasmid encodes fimbriae.

Karch *et al.* (1987) reported that a nonfimbriated K-12 strain expressed fimbriae when transformed with the large plasmid from 933, but Toth et al. (1990) did not observe fimbriation under similar conditions. Evidence concerning the role of the large plasmid in epithelial cell adherence is also contradictory. Junkins and Doyle (1989) reported that the plasmid-cured derivative

actually adhered better than the wild-type parent strain, whereas Fratamico (1993) observed equivalent epithelial cell adherence levels for the plasmid-cured and the plasmid-containing parent. Conversely, Karch *et al.*, (1987) and Toth *et al.* (1990) reported that O157 strains cured of the 60 MDa plasmid did not bind either HEp-2 or Henle 407 cells. Data on the significance of the plasmid in EHEC colonization *in vivo* are also at variance. Tzipori *et al.* (1987; 1989) found that both plasmid-containing and -cured derivatives of O157:H7 isolates colonized the large intestine, generated A/E lesions, and caused watery diarrhea. However, in the streptomycin-treated mouse model of EHEC infection, Wadlokowski, et al. (1990) reported that a plasmid-cured EHEC strain was unable to compete with wild-type parent for colonization of the bowel. When fed individually, the plasmid-cured derivative and the wild-type parent strain colonized equally well.

The attaching and effacing lesion. The third virulence characteristic of EHEC isolates is the capacity to cause the A/E lesion on the colonic epithelium of infected animals. Potter *et al.*, (1985) and Francis *et al.*, (1986) orally inoculated infant rabbits and gnotobiotic piglets, respectively, with clinical isolates of O157:H7. In both investigations, histopathologic lesions on the intestinal mucosa were observed. Francis *et al.* (1986) noted that bacteria colonized cecal and colonic surfaces diffusely and that, at bacterial attachment sites, the microvilli were effaced and the epithelial cells were irregularly shaped, rounded, or detached. These findings are characteristic of the A/E lesion described for EPEC

interaction with the small intestinal epithelial surface of pigs, rabbits (Staley *et al.*, 1969; Moon et al., 1983) and humans (Rothbaum *et al.*, 1982).

IV. Models of EHEC adherence and A/E lesion formation.

***In vitro* models of EHEC adherence.** Conclusions from early reports of the adherence of EHEC O157 strains *in vitro* are contradictory. In 1984, Scaletsky *et al.* reported that of three O157 strains tested none were able to bind to HeLa cells. In 1987, Karch *et al.*, (1987) stated that 14 O157:H7 strains were negative for HEp-2 cells adherence, that these isolates did adhere to Henle 407 cells, albeit in small numbers. In that same year, Sherman *et al.* (1987), showed that 5 different O157:H7 EHEC strains were adherent to either HEp-2 or Henle 407 cells, but not all strains tested in that study were equally adherent to both cell types. Ratnam *et al.*, (1988) reported similar findings; of 174 isolates of O157:H7, ~70% of the strains demonstrated variable degrees of either a localized or diffuse pattern of adherence to HEp-2 cells and Henle 407 cells Lastly, Knutton *et al.* (1989) observed that two O157:H7 isolates adhered in a localized manner to HEp-2 and HEL (human embryonic lung carcinoma) cells but not Caco-2 colonic epithelial cells and that this localized adherence caused actin polymerization at the site of bacterial attachment (a positive FAS test). A comparative evaluation of the conclusions of these reports is hampered by the fact that none of these groups of

investigators used the same method for the adherence assay or for measuring attachment of bacteria to the epithelial cells.

***In vivo* models of EHEC infection and A/E lesion formation.** A number of animals have been tested as models of EHEC infection. These models include infant rabbits, piglets, and mice. The A/E lesion has been reported in conventional and gnotobiotic piglets (Francis *et al.*, 1986; Tzipori *et al.*, 1989), rabbits (Pai *et al.*, 1986; Sherman *et al.*, 1988), and chickens (Beery *et al.*, 1985), as well as in naturally or experimentally infected calves (Mainil *et al.*, 1993).

Infant rabbits were among the first animals experimentally infected with O157:H7 clinical isolates (Farmer, III *et al.*, 1983; Potter *et al.*, 1985; Pai *et al.*, 1986). Rabbits infected intragastrically with large doses of EHEC developed watery diarrhea and histopathologic lesions characteristic of EHEC infection. The animals did not demonstrate bloody diarrhea or kidney involvement. The histopathology observed in the colon and cecum of infected rabbits was reproduced by intragastric administration of SLT alone (Pai *et al.*, 1986).

Francis *et al.* (1986) found that an O157:H7 strain colonized the cecum and colon of gnotobiotic piglets and caused diarrhea and the A/E lesion. More recently, the gnotobiotic piglet infection model has been employed to define the role of toxin in EHEC-mediated disease (Tzipori *et al.*, 1988; Gannon and Gyles, 1990; Dykstra *et al.*, 1993). SLT has been shown to cause neurological

abnormalities by damage to cerebral vessels as well as vascular damage and ischemic necrosis in the intestines (Tzipori *et al.*, 1988; Dykstra *et al.*, 1993).

Wadolkowski *et al.* (1990) developed a streptomycin-treated mouse model of intestinal colonization. As previously mentioned, an EHEC strain cured of the 60 MDa plasmid or the wild-type parent strain colonized the mouse intestine equally well. When the two strains were fed in competition, the plasmid-cured derivative was unable to stably colonize the mouse intestine (Wadolkowski *et al.*, 1990). No A/E lesions were observed in the infected mouse intestinal tissues. However, damage to the kidney which included renal cortical tubular necrosis was observed in mice that died as a result of EHEC infection (Wadolkowski *et al.*, 1990; Lindgren *et al.*, 1993). The streptomycin-treated mouse model appears to be a model of toxin-mediated damage to the kidney after oral infection, but it is not a model of HC or HUS. In sum, no single animal model suffices to mimic all clinical aspects of EHEC-mediated disease.

V. Discovery and characterization of the *eaeA* locus.

As noted above, EHEC strains cause the A/E lesion in the intestines of experimental animals, a virulence characteristic shared with EPEC. The A/E lesion is characterized by degeneration of epithelial cell microvilli and "pedestals" of densely clustered cytoskeletal proteins, that include F-actin (Moon *et al.*, 1983). Until Knutton *et al.* (1989) developed a novel method for visualizing this

cytoskeletal rearrangement, the A/E lesion could be visualized only by electron microscopic examination. Knutton's method, called the fluorescence actin staining (FAS) assay, utilizes fluorescein isothiocyanate-labeled phalloidin to detect polymerized actin at the site of bacterial attachment. Phalloidin is a mushroom phallotoxin from Amanita mushrooms (Wieland and Faulstich, 1978) that binds to actin filaments (F-actin) much more tightly than it binds to actin monomers (G-actin) (Estes *et al.*, 1981).

Armed with the FAS test, investigators began the search for EPEC loci involved in the pathologic mechanisms that lead to the A/E lesion. In 1990, Jerse *et al.* (1990) described a genetic locus required for A/E lesion formation by EPEC strains: eae, *E. coli* attaching and effacing. The chromosomal gene was identified by screening transposon mutants of EPEC strain JPN15 [a plasmid-cured derivative of strain E2348/69 (Levine *et al.*, 1985)] for a negative FAS test on HEp-2 cells and the absence of the A/E lesion on Caco-2 cells (Jerse *et al.*, 1990). The results with the Tn*phoA* mutagenized *eae* strain were confirmed by an *eae* deletion mutant, CVD206 (Donnenberg and Kaper, 1991). The *eae* gene, now called *eaeA* since other genes have been identified in the *eae* gene cluster (Donnenberg *et al.*, 1993c), is a 2817 bp open reading frame which encodes a 94 kDa outer membrane protein. A probe derived from the EPEC *eaeA* sequence hybridizes with DNA isolated from other attaching and effacing bacteria that include: EHEC, RDEC-1, an enteropathogenic strain of rabbits (Jerse *et al.*, 1990), *Citrobacter freundii* biotype 4280 a strain associated with colonic murine hyperplasia (Schauer

and Falkow, 1993), and *Hafnia alvei* which was isolated from a child with diarrhea (Albert *et al.*, 1992). Studies by Jerse *et al.* and Gómez-Duarte and Kaper have shown that at least two loci on the EAF plasmid enhance expression of the eaeA gene (Gómez-Duarte and Kaper, 1995). These results may explain the observation that both the EAF plasmid and the *eae* gene are required for efficient A/E lesion formation (Francis *et al.*, 1991).

The role of the *eaeA* gene in EPEC pathogenesis was demonstrated by Donnenberg *et al.* (1993a) who found that 4 of 11 volunteers fed the eaeA mutant (CVD206) developed diarrhea as compared to 11 of 11 individuals who received the wild-type parent. Those recipients of CVD206 who developed diarrhea had lower stool volumes than those who received the parent isolate. In a separate study, immune sera collected from volunteers 28 days after infection with EPEC recognized the *eaeA* gene product, intimin.

VI. Specific aims of this dissertation.

The hypothesis upon which this dissertation project is based is that intimin, the product of the chromosomal *eaeA* locus of EHEC, is required for intimate adherence of EHEC to colonic epithelial cells *in vitro* and is required to cause the attaching and effacing lesion *in vivo*. Three aims were devised to test these hypotheses. The first goal of this project was to establish a reliable *in vitro*

model of EHEC adherence and FAS activity. The parameters by which to assess EHEC adherence to human epithelial cells in culture were optimized with a panel of EHEC strains. The assay also permitted detection of a novel adherence phenotype by which intestinally-derived *E. coli* adhere to human ileocecal cells. Second, the *eaeA* locus in EHEC O157:H7 strain 86-24 was inactivated. The role of this locus in HEp-2 cell adherence and the correlation with the A/E lesion in gnotobiotic piglets was determined. The wild-type *eaeA* allele from 86-24 was cloned and tested for the capacity to complement the mutant phenotype both *in vitro* and *in vivo*. The third aim focussed on characterization of the intimin protein encoded by *eaeA*. Histidine::intimin fusion proteins were constructed and studied for EHEC adherence-blocking activity. Taken together, these aims demonstrate the essential role of the *eaeA* locus in the pathogenesis of EHEC.

Materials and Methods

Cells, bacterial strains and plasmids. HEp-2, human laryngeal epithelial cells (ATCC# CCL23) and HCT-8, human ileocecal epithelial cells (ATCC# CCL244), were obtained from the American Type Culture Collection, Rockville, Md. Eukaryotic cell cultures were maintained by serial passage in complete MEM [Eagles minimal essential medium (BioWhittaker, Md.), 10% fetal calf serum, 20 mM L-glutamine, 100 µg/ml gentamicin and 100 U penicillin G].

The bacterial strains and plasmids used in this study are described in Tables 3 and 4, respectively. EHEC strain 86-24 (O157:H7) was isolated in 1986 from a patient with hemorrhagic colitis and was provided by Dr. Phil Tarr, Seattle, Wash. (Griffin *et al.*, 1988). Strain 933 (O157:H7), isolated from a patient in the 1982 outbreak of HC in the pacific northwest, was obtained from Dr. Kay Wachsmuth, formerly of the CDC (Riley *et al.*, 1983). *Escherichia coli* DH5α [Bethesda Research Laboratories (BRL), Gaithersburg, Md.] was used as a negative control in the adherence assays. The *eaeA* gene from 86-24 was cloned into plasmid vector pBRKS$^-$ (Schmitt *et al.*, 1994). Briefly, this is a pBR328-based vector that includes the multicloning site from pBlusecriptKS$^-$ (Stratagene) as well as the promoter of *lacZ* (P_{lac}) in one orientation and the T7 phage promoter (P_{T7}) in the opposite orientation (Schmitt *et al.*, 1994). The cloning vectors used to construct the histidine:intimin fusion proteins were obtained from Qiagen, Inc

(Chatsworth, Calif.). The fusion constructs were expressed in an M15 or SG13009 background containing pREP4 which encodes the *lac* repressor (see Table 3).

Media, enzymes, biochemicals and radionuclides. Bacterial strains were routinely grown on LB or MacConkey (Difco Laboratories, Detroit, Mich.) agar or in L broth (5g NaCl per liter). When necessary, antibiotics (Sigma Chemical Co., St Louis,Mo.) were added to the medium at the following concentrations: ampicillin 100 µg/ml, chloramphenicol 20 µg/ml, kanamycin 50 µg/ml, and tetracycline 12.5 µg/ml. These concentrations were varied as indicated in a specific assay. To screen clones that contained DNA inserts which inactivated β-galactosidase, the gratuitous inducer of the *lac* operon, IPTG (isopropyl-β-D-thiogalactopyranoside), and the colorimetric substrate, X-gal (5-bromo-4-chloroindolyl-β-D-galactoside) were added at 100 mM and 20 µg/ml, respectively, to solidified LB agar. Restriction endonucleases and calf intestinal alkaline phosphatase were purchased from BRL, Boehringer Mannheim Biochemicals (Indianapolis, Ind.), or New England BioLabs (Beverly, Mass.). Enzymes and kits were used according to the particular manufacturers instructions unless otherwise stated. Radionuclides were purchased from New England Nuclear Research Products (Boston, Mass.).

Table 3. Bacterial strains used in this study.

Strain	Relevant characteristics	Reference
86-24	Wild-type EHEC O157:H7; *eaeA* positive	(Griffin *et al.*, 1988)
EDL933	Wild-type EHEC O157:H7; *eaeA* postive	(Riley *et al.*, 1983)
933D	Derivative of 933; lost *sltII*-encoding phage	(O'Brien *et al.*, 1989)
933cu	90 kb plasmid-cured derivative of 933	(Karch *et al.*, 1987)
933II$^-$	*sltII* knockout derivative of 933	This study
933curev	Spontaneous derivative of 933cu after mouse passage with 933	(Wadolkowski *et al.*, 1990)

Strain	Relevant characteristics	Reference
4494	Wild-type EHEC O157:H7; *eaeA* positive	(O'Brien *et al.*, 1993)
B2F1	Wild-type EHEC O91:H21; *eaeA* negative	(Ito *et al.*, 1990)
86-24*eaeΔ10*	86-24 *eaeA* deletion mutant	This study
E2348/69	Wild-type EPEC O127:H6	(Levine *et al.*, 1978)
HS	Nonpathogenic normal flora isolate	(Levine *et al. 1978)*
H414-36/89	*Wild-type EHEC O91:H21;* *eaeA* negative	(Lindgren *et al.*, 1993)
S1191	Wild-type EHEC edema disease strain; O139; *eaeA* negative	(Marques *et al.*, 1987)
DH5α	Cloning host	BRL
XL1-BlueF'	Cloning host; *lacI*^{q}	Stratagene

Strain	Relevant characteristics	Reference
M15 (pREP4)	Expression host	Qiagen
SG13009 (pREP4)	Expression host	Qiagen
GM119	*dam-3 dcm-6*	(Arraj and Marinus, 1983)

Table 4. Plasmid vectors and clones used in this thesis study.

Plasmid	Relevant characteristics	Reference
Vectors		
pBRKS$^-$	Cloning vector; P_{T7} , P_{lac}	(Schmitt *et al.*, 1994)
pGP1-2	T7 RNA polymerase	(Tabor and Richardson, 1985)
pAM450	Suicide vector, *amp*, *sacB/R*, *ts*	Melton-Celsa
pBluescriptSK$^+$	Cloning vector	Stratagene
pQE32	Cloning vector for histidine fusion	Qiagen
pQE16	6XHis::*dhfrs*; source of His::DHFR	Qiagen
pMAK705	Suicide vector; *ts*	(Hamilton *et al.*, 1989)
pUC4-KSAC	Source of *kan* cassette	Promega
Clones		

Plasmid	Relevant characteristics	Reference
pEB290	86-24 *eaeA* in pBluescriptSK$^+$	This study
pEB300	pEB290 with internal *Bcl*I fragment deleted	This study
pEB305	*eaeA* with internal deletion in pAM450	This study
pEB310	86-24 *eaeA* in pBRKS$^-$ from P_{T7}	This study
pEB311	86-24 *eaeA* in pBRKS$^-$ from P_{lac}	This study
pEB312	6XHis::'*eaeA*; source of RVHdHisEae	This study
pEB313	6XHis::'*eaeA*; source of RIHisEae	This study
pCKS107	*sltII* in pMAK705	(Schmitt *et al.*, 1991)
pMS200	*sltII*::*kan* in pMAK705	This study
pCVD419	Source of 3.4kb *Hin*dIII probe for EHEC large plasmid	(Levine *et al.*, 1987)
pCVD434	*eaeA* probe in pBR325	(Jerse *et al.*, 1990)

Plasmid	Relevant characteristics	Reference
pCVD436	Cosmid clone of *eae* from E2348/69	(Jerse *et al.*, 1990)
pCVD438	*eaeA* from E3248/69	(Jerse *et al.*, 1990)
pCVD444	*eaeA* from 933 in pUC19	(Yu and Kaper, 1992)

HeLa or Vero cell cytotoxicity assay. Samples of bacterial culture supernatants or sonically disrupted bacterial cell pellets were tested for Vero or HeLa cell cytotoxicity by the method of Gentry and Dalrymple (1980) as modified by Marques *et al.* (1986). The cytotoxic titer per milliliter culture is expressed as the reciprocal of the highest dilution required to kill 50% of the cells in a given well (CD_{50}/ml).

Adherence assay. (I) Qualitative. Adherence of E. coli to either HEp-2 or HCT-8 cells was assessed by a modification of the method of Cravioto *et al.* (1979). Specifically, semiconfluent monolayers of HEp-2 cells on glass coverslips in 24 well tissue culture dishes or in 8 well Permanox Chamber Slides (Nunc, Naperville, Ill.) were overlaid with adherence assay medium (EMEM supplemented with 0.4% sodium bicarbonate and 1% mannose) which contained 20 µl/ml (v/v) of an overnight culture of the bacteria to be tested in LB. Each inoculum contained $\geq 10^7$ bacteria which resulted in an approximate multiplicity of infection (MOI) of 100:1. The infected monolayers were incubated at 37°C in a 5% CO_2 atmosphere. After three hours, the medium with the nonadherent bacteria was aspirated and the monolayers washed once with sterile 10 mM phosphate buffered saline, pH 7.4 (PBS: sodium chloride, sodium phosphate dibasic, and potassium phosphate monobasic). Fresh adherence assay medium was added

to the cells with adherent bacteria, and the infected cells were then incubated for an additional 3 hours. The monolayers were then washed six times with PBS to remove nonadherent bacteria. Each wash was gently removed by aspiration in an attempt to avoid disturbing the monolayers. Each assay was done ≥2 times and duplicate slides were prepared to permit both Giemsa and FITC-phalloidin (FAS) staining. For Giemsa staining, the HEp-2 cells and adherent bacteria were fixed with 70% (v/v) methanol (glass coverslips) or graded acetone washes (chamber slides) and stained with 1:10 Giemsa (Sigma) for 20 minutes. To assess the FAS phenotype, the FITC-Phalloidin (Sigma) staining procedure of Knutton *et al.* (1989) was used. Phalloidin is a mushroom phallotoxin that specifically binds filamentous, not globular, actin. FITC-phalloidin-stained preparations were examined by both phase contrast and fluorescent microscopy using an on Olympus model BHS microscope with a model BH2-RFL reflected light fluorescence attachment (Olympus Optical Co., Ltd., Tokyo, Japan). Photomicrographs were taken with an Olympus PM-10AD photomicrographic system (Olympus) as a record of each assay.

Adherence assays with HCT-8 cells were done by the procedure described above for HEp-2 cells, but the bacteria were allowed to interact with the HCT-8 cells for 2.5 hours before the first wash and an additional 2.5 hours before

terminating the assay. All assays with HCT-8 cells were carried out in 8 well permanox Chamber Slides.

To assess the effect of anti-intimin antibodies on EHEC adherence, mouse or rabbit anti-intimin antisera (or normal sera as controls) were added to the bacteria suspended in adherence media, and the bacteria-antisera mixtures were incubated at 37°C for thirty minutes prior to infection of the HEp-2 cells. Antisera were maintained in the adherence media throughout the assay. To evaluate the effect of exogenously added intimin fusion proteins, the purified His-intimin fusion proteins were added to the epithelial cell monolayers before addition of bacteria as indicated in each experiment. Other specific manipulations of the adherence assay are discussed in the results section.

(ii) Quantitative. For quantitative analysis of bacterial adherence, the assay was done as described above with the following modifications. Epithelial cell monolayers were seeded in 24 well culture dishes and then infected with 20 µl of bacteria from an overnight culture. At the end of the infection period, cells and attached bacteria were removed from the dish with 0.25% trypsin in PBS (5 minutes at 37°C). The trypsinized mixture was removed from the plates to a plastic tube and vortexed vigorously to break any residual bacterial-cell or bacterial-bacterial interactions (as verified by microscopic evaluation). This solution was serially diluted in PBS then plated onto MacConkey agar (with or

without antibiotics as appropriate) to obtain bacterial counts. Dilutions that gave counts between 30 and 300 were used for calculation of the number of adherent bacteria per well. The formula used for this calculation was: adherent CFU/ml = (CFU on agar plate) x (dilution factor) x 10 (factor of 0.1 ml plated onto the agar) x 2 (factor for 0.5 ml trypsin solution used to remove cells and adherent bacteria from tissue culture well). To calculate an adherence index (AI), the CFU adherent per well was divided by the CFU in the inoculum and multiplied by 100%. In preliminary experiments, the viability of the HEp-2 cells before and after the adherence assay was not significantly affected as determined by trypan blue exclusion.

Invasion assays. Invasion of HCT-8 cells was assessed according to the method of Elsinghorst and Kopecko (1992). Stationary phase bacteria in adherence medium were allowed to interact with the HCT-8 monolayer for three hours at an MOI of 100:1, then the bacteria:HCT-8 mixture was washed with sterile 10 mM PBS, pH7.4. Half of the cell sample was then solubilized in 0.1% Triton X-100 (Sigma) and processed to determine the total number of bacteria associated with the cells before gentamicin treatment. The other half of the sample was overlaid for an additional hour with fresh medium containing 100 µg/ml gentamicin. The infected monolayer was washed extensively with sterile PBS and solubilized

with 0.1% Triton X-100. This solution was serially diluted, plated on MacConkey agar, and colonies were counted to determine the CFU/ml associated with the HCT-8 cells after gentamicin treatment. Adherence and invasion of each strain were assessed in triplicate in each of three assays. The methods of calculation are given in the legend of Table 10.

Oral infection assays. (i) Piglet infection. Since we do not have the facilities required to maintain infected large animals in isolation, both piglet infection assays reported in this dissertation were performed by others. The first experiment was initiated by Dr. Michael Donnenberg (CVD, Baltimore) and conducted by Dr. Saul Tzipori (Tufts University) (Donnenberg *et al.*, 1993b). The second set of experiments was initiated by us and conducted by Dr. David Francis (South Dakota State University) (McKee *et al.*, 1995). The conditions were similar for both studies. In our study (i.e., a comparison of 86-24 with its in-frame deletion derivative), pairs of colostrum-deprived, 24-hour-old piglets from the same litter were fed ~10^9 organisms of either 86-24, 86-24*eae*Δ*10*, 86-24*eae*Δ*10*(pEB310), B2F1, or EDL933 (as the positive control). The piglets were sacrificed 48 hours after challenge (72 hours old). Animals fed 86-24*eae*Δ*10*(pEB310) were treated with ampicillin at a dose of 250 mg per os per day to ensure maintenance of the recombinant plasmid. At the time of sacrifice, all piglets were evaluated clinically

for signs of dehydration, rectal staining, and overall condition. Tissue samples obtained on necropsy were examined visually and then processed for histological sections. Intestinal samples from the duodenum, jejunum, proximal ileum, terminal ileum, cecum, spiral colon (two sections), and rectum were fixed in 3% glutaraldehyde (in 0.1 M sodium cacodylate buffer, pH 7.4 with 5% sucrose) for electron microscopic (EM) observation. Fixed tissues were embedded in Epox 812 (Ernest Fullam, Latham, N.Y.) and ultrathin sections (70 nm) were mounted on copper grids then stained with uranyl acetate and lead citrate. Electron micrographs were obtained at USUHS with a Joel 100CX microscope set at 80kV. Bacterial contamination of the pigs was monitored by anaerobic and aerobic culture of the colonic and cecal contents of the animals (done by D. Francis). Maintenance of the recombinant plasmids in the EHEC strains tested in the piglets was confirmed in our laboratory by extraction and analysis of plasmid DNA from the recovered bacteria.

(ii) Mouse colonization. Streptomycin-treated male CD-1 mice were fed 10^{10} CFU of bacteria (i.e., 86-24 Str^r, 86-24*eae*Δ*10* Str^rNal^r, or both strains in combination) in 20% sucrose as previously described (Wadolkowski *et al.*, 1990) and monitored for colonization of the intestine, as follows. Briefly, one gram of fecal material was harvested at 1 or 2 day intervals, homogenized in sterile PBS,

diluted and spread onto appropriate media. Colonies were counted and the CFU per gram of feces calculated.

DNA manipulation and isolation. Cloning procedures and plasmid manipulations were carried out in XL1Blue or DH5α, essentially according to the procedures described by Maniatis *et al.*, (1975). Plasmid DNA was isolated by alkaline-lysis (Maniatis *et al.*, 1975) for small-scale preparations or by the Qiagen Plasmid Miniprep (Qiagen) for large scale restriction digests and automated sequencing reactions. Plasmids were moved into the host strains by $CaCl_2$ transformation (Mandel and Higa, 1970) or electroporation (Sizemore *et al.*, 1991) at 1.25kV, 25μF, 1000Ω with a Bio-Rad Pulse Controller (Bio-Rad).

The *eaeA* gene was amplified from the chromosome of EHEC strain 86-24 by the polymerase chain reaction (PCR) with primers designed from the published sequences of EHEC *eaeA* (Yu and Kaper, 1992; Beebakhee *et al.*, 1992). The 5' primer spanned bases 20-41 of the CL8 *eaeA* sequence, and the 3' primer spanned bases 3061-3082 of the sequence from 933 and was designed so as to generate a new *Xba*I restriction site after PCR amplification.

Total bacterial DNA was isolated according to the following method (Ausubel *et al.*, 1989). Briefly, 5 ml of a saturated overnight culture was centrifuged, and the pellet was resuspended in TE with SDS and Proteinase K.

Polysaccharides were removed from solution after the addition of CTAB (hexadecyltrimethyl ammonium bromide) at 65°C, and the precipitated protein was removed by successive extraction with chloroform and phenol:chloroform. Total cellular DNA was then precipitated by addition of 0.6 volumes of isopropanol.

DNA fragments were purified from agarose gels or from PCR reaction mixtures with GeneClean (Bio101, LaJolla, Calif.). The fragment of interest was excised from the agarose gel and weighed. Two and a half volumes of sodium iodide (NaI) solution was then added to the gel slice, and the mixture was incubated for 5 minutes at 50°C. A 10-15 µl volume of Glassmilk™ solution was added to this mixture and the tube containing the solution was inverted several times. The Glassmilk-NaI-DNA mixture was then incubated 30 minutes at room temperature, washed three times with ice-cold NewWash™, and the DNA eluted from the Glassmilk with 15 µl of sterile dH_2O for 30 minutes at room temperature. In some cases, a second elution was done at 4°C overnight.

Single stranded DNA templates for nucleotide sequencing reactions were isolated by rescue of the positive strand from clone pEB290 by the method of Messing and coworkers (Messing, 1983; Norander *et al.*, 1983). Host strain DH5αF' transformed with pEB290 was grown to log phase in the presence of the helper phage. Supernatant from the induced culture was treated with 20% PEG and 2.5M NaCl to precipitate the filamentous phage containing the rescued DNA.

After extraction with phenol to remove the proteinaceous material, the DNA was precipitated with ethanol and resuspended in TE.

DNA sequencing. Nucleotide sequence analysis was done by the dideoxy chain termination method (Sanger *et al.*, 1977). The ends of the *eaeA* gene fragment cloned into pEB290 were sequenced manually with the Sequenase kit (USB) following the procedure supplied by the manufacturer. The DNA insert in pEB310 was subcloned in pUC19 for sequencing with the Prism Ready Reaction Cycle Sequencing kit (Applied Biosystems, Inc.) according to the manufacturers protocol. Automated sequencing reaction products were separated and analyzed on an Applied Biosystems Model 373 Automated DNA sequencer (Applied Biosystems, Inc.)

DNA-DNA hybridizations. Restricted total DNA or plasmid DNA fragments were separated by agarose gel electrophoresis and probed by in situ gel hybridization (Kidd, 1983; Schmitt *et al.*, 1994) with either the 1kb *SalI-StuI eaeA* from pCVD434 or with the 3.4 kb *Hin*dIII fragment from pCVD419. The probes were labeled with [^{35}S]dCTP according to the manufacturer's directions provided with the DNA Nick Translation Labeling Kit (BRL). The DNA probes generated for other specific experiments are described in the Results section.

Potential recombinant clones of *eaeA* from 86-24 were screened for the presence of the insert DNA fragment by nonradioactive colony blot hybridization with the 1 kb *Sal*I-*Stu*I *eaeA* fragment that had been biotinylated by the incorporation of Biotin-14-dATP (BRL). Briefly, 5 µl of an overnight bacterial culture was spotted onto a nitrocellulose filter and allowed to air dry. The membrane-bound bacteria were lysed, and the DNA was then denatured by successive 5 minute treatments with 0.5M NaOH, 1M Tris, pH 7.5, 1.5 M NaCl/0.5M Tris, pH 7.5, and 0.3M NaCl. Air dried filters were incubated with the biotinylated *eaeA* fragment, and positive hybrids were identified according to the manufacturers specifications in the BRL BluGENE Kit protocol (BRL).

To screen intestinal *E. coli* isolates for the presence of *eaeA*, the DNA probe fragment was radiolabled with [^{35}S]dCTP by the BRL Nick Translation Kit. Positive hybridization reactions were visualized by autoradiography.

Protein expression. The *eaeA* gene was cloned into vector pBRKS$^-$ in the orientation of the T7 RNA polymerase-dependent promoter (P_{T7}) to allow expression of the protein products. Plasmids pEB310 and pEB311 (as a negative control) were independently transformed into DH5α which contained plasmid pGP1-2 (Tabor and Richardson, 1985). Proteins were selectively expressed under P_{T7} control and pulse-labeled with [^{35}S]methionine as described (Tabor and

Richardson, 1985). Plasmid pGP1-2 encodes a temperature inducible copy of the T7 RNA polymerase which is expressed at 42°C. Log phase cultures of DH5α(pGP1-2) carrying the plasmid with the gene(s) to be expressed were induced at 42°C and grown in the presence of 100μg/ml rifampin to inhibit host RNA polymerase activity. Radiolabeled products in the whole cell lysates or fractionated cell components were separated by discontinuous denaturing sodium dodecyl sulfate -polyacrylamide gel electrophoresis (SDS-PAGE; 4% stacking gel with 10% separating gel) (Laemmli, 1970) on the Mini Protean II slab cell apparatus according to the instruction manual provided by the manufacturer (BioRad). Proteins that had incorporated the [^{35}S]methionine were visualized by autoradiography.

To prove that the deletion mutation constructed in *eaeA* in strain 86-24 was in-frame, linear DNA templates were derived by PCR amplification of the *eaeA* sequences from the chromosomes of either the mutant or wild-type EHEC strains with the primers Sn20 and MM2 and subjected to an *in vitro* transcription/translation reaction with the *E. coli* S30 Extract Prokaryotic Translation System for Linear DNA (Promega). Resultant protein products were labeled with [^{35}S]methionine. One fifth of each reaction was separated by 10% SDS-PAGE as described above, and the gels were subjected to autoradiography for visualization of the proteins.

Expression and purification of fusion proteins. Fusion protein expression was induced in log-phase cultures by the addition of 0.1mM IPTG for 2 hours. Proteins were purified in buffers containing 8M urea according to the protocols supplied by Qiagen. Fusion protein preparations were purified over Ni-NTA resin spin columns and dialyzed extensively against 10mM PBS, pH 7.4 (PBS) to remove the urea and renature the protein. Concentrations of the fusion proteins were determined by the Bradford method (BCA Protein Quantification Kit, Pierce Chemical, Co., Rockford, Ill.).

Immunization of mice and rabbits. Four female BALB/cJ mice (Jackson Laboratory, Bar Harbor, Maine) were prebled then injected three times intraperitoneally with 25µg RIHisEae fusion protein in TiterMax adjuvant (Vaxcel, Inc., Norcross, Ga.) over 6 weeks. Blood samples were obtained by tail vein nicking. When the mouse serum from each animal achieved high titer, all animals were sacrificed by exsanguination.

Two male New Zealand white rabbits (HRP, Inc., Denver, Penn.) were injected at four sites with 100µg (total) RIHisEae intramuscularly in TiterMax then boosted twice with the same protein in PBS at three week intervals. Titers of the mouse or rabbit antisera were assessed by the enzyme-linked immunosorbant assay (ELISA) described below.

ELISA. Fifty nanograms of RIHisEae (or RVHdHisEae, where specified) were plated onto polystyrene 96-well microtiter plates as an antigen substrate to test the reactivity of rabbit or mouse sera against intimin. After an overnight incubation at 4°C, the coated plates were washed with PBS and unoccupied sites on the wells blocked with 3% BSA in PBS. Two hour incubations with primary and secondary [either horseradish peroxidase-conjugated goat anti-mouse or donkey anti-rabbit (Amersham Corp., Arlington Heights, Ill.) where appropriate] antibodies were carried out at room temperature with PBS washes between each step. TMB (3',3',5',5'-tetramethylbenzidine) Peroxidase (BRL) was added to each well as the substrate. After 10-30 minutes of incubation, the enzymatic reaction was terminated by the addition of 1N H_2SO_4 which caused the mixture to turn yellow. The intensity of the yellow color reaction was monitored on a Titertek Multiscan MC (Flow Laboratories, McLean, VA). The optical density values were plotted for each sample. The anti-intimin titer was defined as an A_{450} value 0.2 units above the negative control value which was usually the value obtained with normal sera.

Bacterial membrane fractionation. Bacterial membrane fractionation was done essentially according to the method of Achtman *et al.* (1983). Overnights of bacteria were subcultured into fresh L broth for 2 hours at 37°C.

Pelleted bacteria were resuspended in 10mM Tris with 100µg/ml PMSF (phenyl sulfonyl methyl fluoride) and sonically disrupted. The lysates were subjected to low speed centrifugation to remove intact cells, and the resultant supernatant was centrifuged at 100,000 x *g* for 1 hour to harvest the bacterial membranes. Outer membrane proteins were extracted with 1.67% sarkosyl in 11.1 mM Tris, pH 7.6 at room temperature then pelleted by centrifugation at 4°C. The sarkosyl insoluble material was resuspended in Tris/PMSF, quantitated by the Bradford method (BCA Quantification Kit), and subjected to SDS-PAGE followed by immunoblot analysis.

Immunoblot procedure. Proteins separated by SDS-PAGE were electroblotted onto nitrocellulose according to the method of Towbin (1979). The membranes were blocked with 5% nonfat dried milk (Carnation Company, Los Angeles, Calif.) in Tris-buffered saline, pH 7.2 with 0.1% Tween-20 (v/v) (TBS-T), washed, then incubated with either mouse or rabbit anti-intimin (as indicated in each experiment). The membranes were washed 3 times with TBS-T then overlaid with a 1:5000 dilution of either horseradish peroxidase-conjugated goat anti-mouse Ig (BMB), donkey anti-rabbit Ig (Amersham), or sheep anti-human Ig (Amersham). Antigen-antibody complexes were visualized by chemiluminescence with the ECL™ Western blotting detection kit (Amersham).

Agglutination tests. The capacity of antisera raised against the intimin fusion proteins to agglutinate wild type bacteria was assessed by slide agglutination tests with sera diluted 1:10 in PBS or neat protein preparations. The same standard technique was used to confirm the serotype of O157 strains used throughout this study.

Results

I. *In vitro* model of EHEC adherence.

The first objective of this project was to establish a reproducible *in vitro* model of EHEC adherence and FAS activity.

Cell types. Cravioto *et al.*, (1979) had successfully used HEp-2 cells to study and quantitate the adherence of EPEC strains, and these investigators found that EPEC attachment to HEp-2 cells correlated with the capacity of EPEC strains to cause diarrhea in infants. We began our adherence studies with HEp-2 cells based on Cravioto *et al.*'s observations and because these cells are of epithelial origin (human laryngeal epithelial cells). Moreover, Sherman (1987) and Knutton (1989) found that some EHEC strains adhere to HEp-2 cells.

Once the adherence assay was established, HCT-8 cells were substituted for HEp-2 cells, because they are of ileocecal origin (Tompkins *et al.*, 1974) and, thus, more physiologically relevant than HEp-2 cells. Also, in a non-polarized state, HCT-8 cells are thin enough to allow easy microscopic evaluation of bacterial-host cell interaction. Other human intestinal epithelial cell lines were tested, but no other lines were as useful as HEp-2 or HCT-8 cell. Specifically, Caco-2 [human small intestine; (Fogh and Trompe, 1975)] and T_{84} [human colonic; (Madara *et al.*, 1987)] cells were too thick to obtain a good plane of focus for observation by light microscopy of bacteria adherent to the tops of the monolayer.

HCT-116 [human colonic; (Brattain *et al.*, 1981)] epithelial cells did not stick to any of the culture substrates used in this study. The aforementioned cell lines were tested for sensitivity to SLTs before use in adherence assays (Table 5). Only HCT-116 cells were sensitive to SLTs.

EHEC strains. Table 6 summarizes the panel of EHEC isolates and control *E. coli* strains used to establish the adherence assay. These clinical isolates were chosen because they span the range of SLT-producing phenotypes and include a plasmid-cured derivative of the prototypic EHEC O157:H7 strain, EDL933 (Riley *et al.*, 1983). EPEC isolate E2348/69 (Levine *et al.*, 1978) was the positive control in all assays. E2348/69 binds to HEp-2 cells (Baldini *et al.*, 1983), produces the A/E lesion (Knutton *et al.*, 1987) and causes diarrheal disease in adult human volunteers (Levine *et al.*, 1978). A nonpathogenic K-12 strain, DH5α, was the negative control. In the presence of mannose, which blocks type1 fimbrial binding, K-12 strains are not adherent to epithelial cells *in vitro* (Ofek *et al.*, 1977). For some later adherence assays with HCT-8 cells, *E. coli* HS, a normal gut flora strain (Levine *et al.*, 1978), was included as a negative control.

Qualitative analysis of EHEC adherence to HEp-2 cells. Adherence of EHEC isolates was monitored in three ways: by i) fluorescein-conjugated phalloidin staining and fluorescent microscopy in the FAS test, ii) Giemsa staining and light microscopy, or iii) removal of adherent bacteria from the epithelial cells and

Table 5. Sensitivity of epithelial cell lines to the Shiga-like toxins.

Cell line	Anatomical origin	SLT-I CD_{50}[a]	SLT-II CD_{50}[b]
HeLa	Cervix	10^6	10^{4-5}
HEp-2	Larynx	<1:10	<1:2
HCT-8	Ileocecum	<1:10	<1:2
HCT-116	Colon	10^{1-2}	10^3
T_{84} (polarized or not)	Colon	<1:10	<1:10
Caco-2 (polarized)	Colon	$<10^2$	$<10^2$

[a] Sensitivity of each cell type to SLT-I was determined by incubation with dilutions of either whole cell sonic extracts from 933D or partially purified SLT-I from recombinant clone pNAS13 (Strockbine *et al.*, 1988).

[b] Sensitivity of each cell type to SLT-II was determined by exposure to dilutions of either whole cell sonic extracts from 86-24 or partially purified toxin from recombinant clone pJES120 (J. Samuel).

Footnotes for Table 6.

[a]All of the strains are O157:H7 EHEC except E2348/69 which is an O127:H6 EPEC isolate, HS an O9:H4 intestinal *E. coli* isolate, and DH5α which is a rough:K12 strain.

[b]Plasmid profiles were determined for each strain by plasmid DNA extraction and analysis as described in the Material and Methods. The presence of the 60 MDa plasmid was confirmed by hybridization with the 3.4 kb *Hin*dIII fragment from pCVD419 (Levine *et al.*, 1987). The 60 MDa plasmid carried by EPEC E2348/69 is distinct from the plasmid of the same size in EHEC strains (Levine *et al.*, 1987).

[c]The toxin phenotype was determined by neutralization of HeLa or Vero cell cytotoxicity with anti SLT-I or anti SLT-II antibodies. Strain 87-23 was isolated from a HC patient. Although the isolate is believed to have originally carried SLT-II-converting phage, 87-23 is nontoxigenic as determined by cytototoxicity analysis and DNA hybridization (Tarr *et al.*, 1989).

[d] HC, hemorrhagic colitis; ND, not determined.

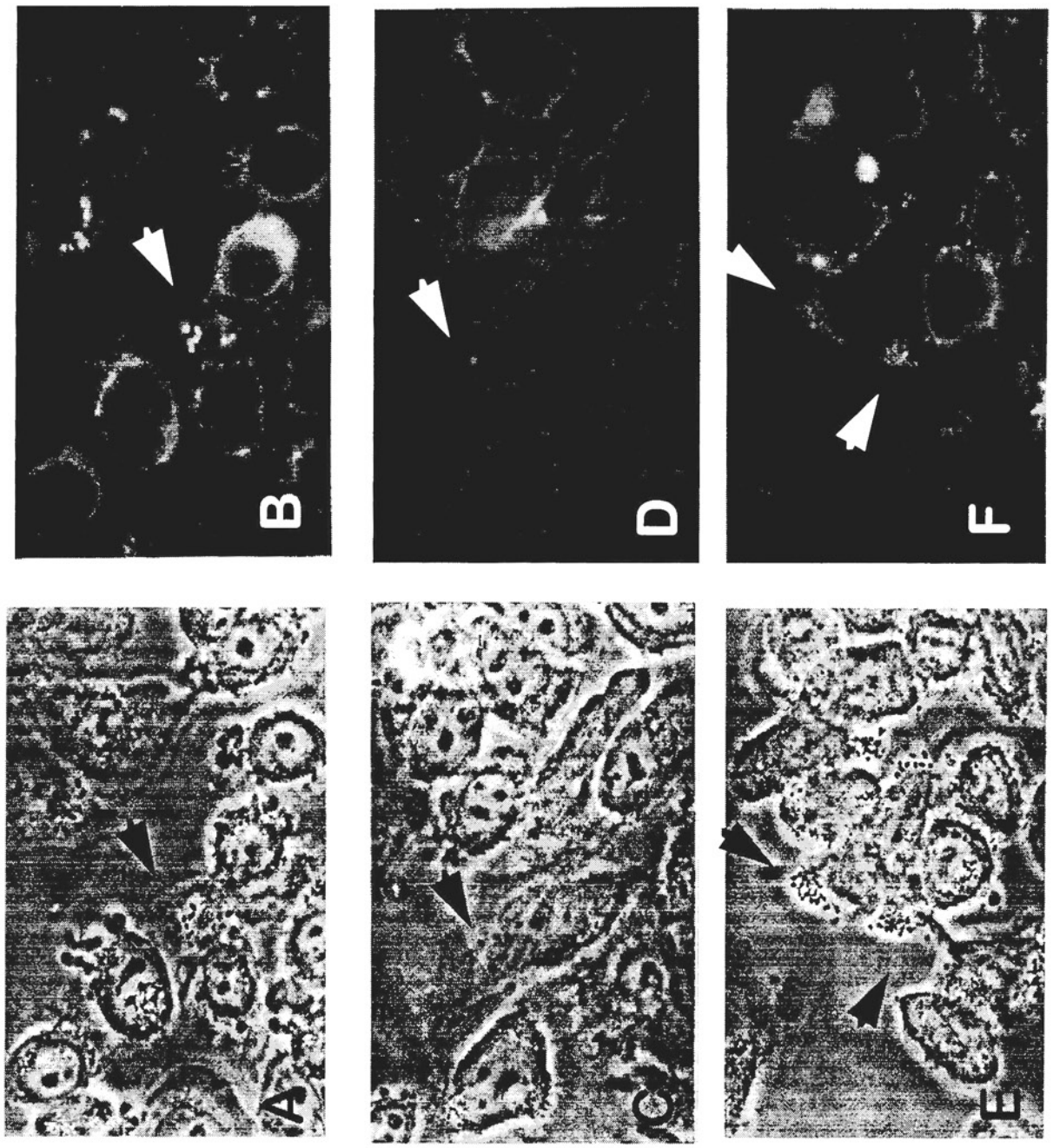
Table 6. Characteristics of bacterial strains used to establish the in vitro model of EHEC adherence.

Strain[a]	Plasmid profile[b] (MDa)	SLT type produced[c]	Clinical findings[d]
EDL933	60; 2.5	I;II	HC
933D	60; 2.5	I	ND
933cu	2.5	I;II	ND
86-24	60; 2.5	II	HC
87-23	60; 2.5	None	HC
E2348/69	60	None	Infantile diarrhea
DH5α	None	None	Nonpathogenic
HS	None	None	Nonpathogenic

quantitation of bacterial counts as described in Materials and Methods. As detailed earlier, the punctate fluoresence observed at the site of bacterial attachment observed with the FAS test is correlated with the capacity of these organisms to cause the A/E lesion *in vivo* (Knutton *et al.*, 1989). The Giemsa stain allows assessment of the adherence phenotype which may be localized, diffuse, or aggregative. The Giemsa stain procedure was typically done in parallel with the FAS test. When microcolonies were observed by Giemsa stain, the phalloidin stain usually revealed an FAS positive phenotype. Singly or diffusely adherent bacteria were occasionally FAS positive.

In the first series of adherence assays, HEp-2 cells were incubated for 3 hours with a panel of *E. coli* strains that included: 933, 933D, 933cu, 86-24, 87-23, DH5α (the negative control) and E2348/69 (the positive control). Only strain 87-23 and the EPEC control strain adhered in a localized manner (Fig. 2). Small microcolonies (5-8 bacteria/microcolony) of 87-23 were observed on a minority of the eukaryotic cells. Areas of punctate actin accumulation were present under the attached bacteria as revealed by FITC-phalloidin stain. In contrast, microcolonies formed by the EPEC control strain at three hours were large with at least 20 bacteria in each microcolony. As previousy reported (Knutton *et al.*, 1989), the EPEC strain was FAS positive. Isolate 933cu bound the HEp-2 cells in pairs, and this bacterial-HEp-2 cell interaction was FAS negative. Strain 933D lined up at the edges of the HEp-2 cells, was poorly adherent, and the FAS reaction was negative. EDL933 and 86-24 were unable to adhere as was the K-12 control.

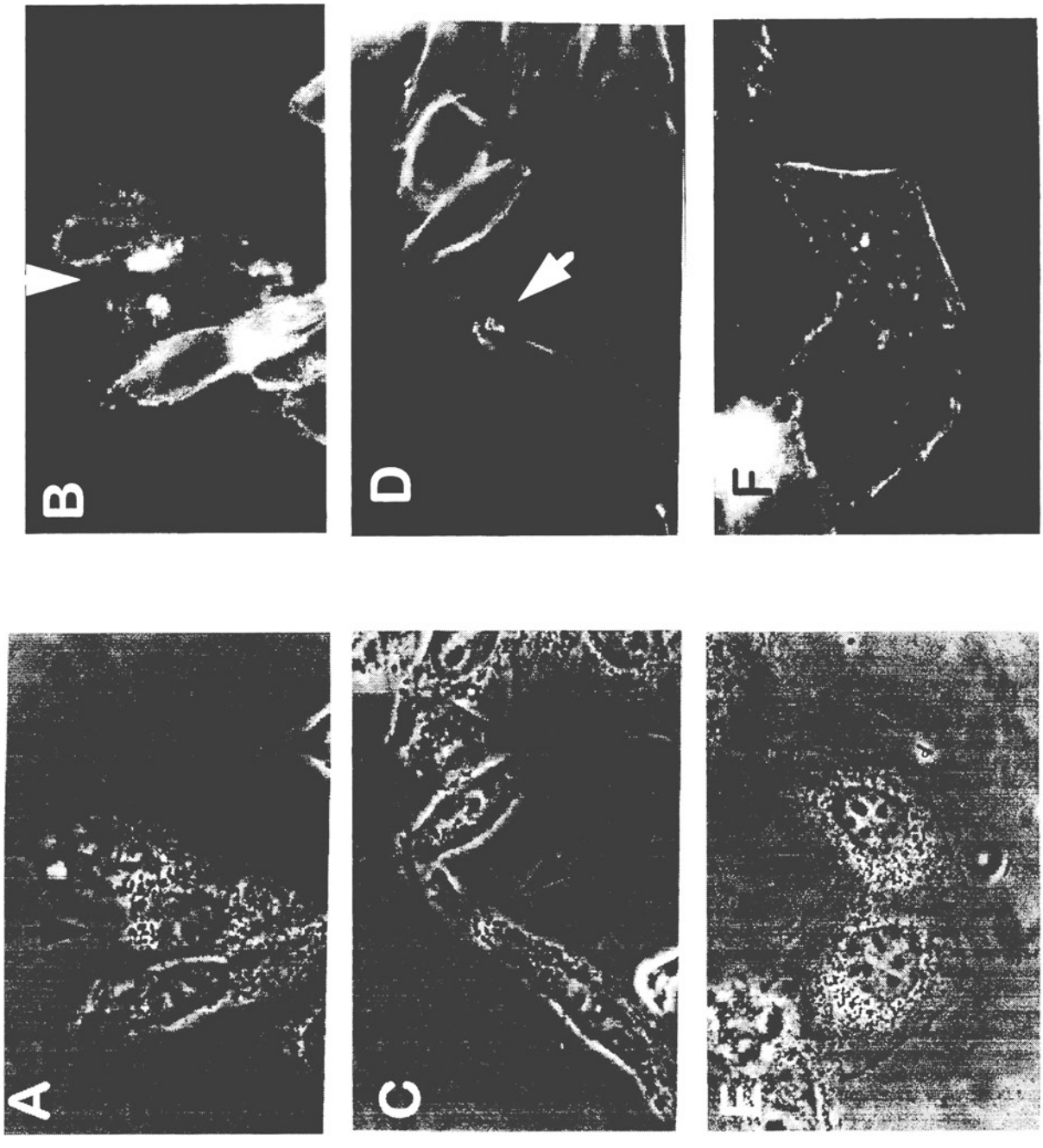
Figure 2. Phase contrast and fluorescent micrographs of HEp-2 cells infected for three hours with EHEC strain 87-23 (A and B), EHEC 933cu (C and D) or EPEC strain E2348/69 (E and F). Microcolonies of adherent bacteria (arrows) were observed on top of the cells in the phase contrast micrographs (A, C, and E). Bright fluorescence with the FITC-phalloidin stain (arrows) indicates condensation of F-actin under the microcolonies by fluorescent microscopy (B, D and F). The K-12 strain, DH5α, was unable to adhere to HEp-2 (i.e., the adherence phenotype was the same as 933cu).


A
B
C
D
E
F

In the second set of adherence experiments, the incubation time was extended to 6 hours, and the 87-23 microcolonies were larger than they were after the 3 h assay. Furthermore, strains 86-24, 933D, and 933cu became positive for microcolony formation (LA) and actin accumulation (FAS) (Fig. 3). The proportion of the infected HEp-2 cell population with a microcolony also increased at 6 hours. However, strain 933 remained negative for LA/FAS, but a few bacteria were aligned along the edges of some of the HEp-2 cells (data not shown). An occasional DH5α bacterium was seen associated with a cell. From these data, we concluded that an extended incubation time of 6 h is required to allow maximal association of the EHEC bacteria with the eukaryotic cells and to permit microcolony formation.

Quantitative analysis of EHEC adherence to HEp-2 cells. Once the microscopic correlates of the *in vitro* assay were defined, we sought to establish a quantitative means of evaluating attachment. Adherence to epithelial cells has been reported as a percent or index of the infecting inoculum (e.g., Sherman *et al.*, 1987). The adherence index is the proportion of bacteria attached to the epithelial cells divided by the inoculum. To compare our data with those of other groups, we developed the quantitative aspect of the adherence assay. As described in Materials and Methods and illustrated in Fig. 4, at the end of the six hour incubation period, HEp-2 cells with attached bacteria were removed from the

Figure 3. Phase contrast (A, C, and E) and fluorescent (B, D, and F) micrographs of HEp-2 cells after a six hour incubation with 87-23 (A and B), 933cu (C and D) or DH5α (E and F). The microcolonies of attached bacteria (arrows) were noticeably larger than those observed at the three hour time point for 87-23 (Fig. 2). At six hours, the adherence patterns of strains 86-24 and 933D were similar to that of 933cu. Isolate 933 was negative for HEp-2 cell adherence as was DH5α (E and F).


A
B
C
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F

culture dishes with trypsin. The bacterial-eukaryotic cell suspension was serially diluted and plated on MacConkey agar to obtain viability counts. Each condition tested was done in triplicate or quadruplicate. The geometric mean adherent CFU was calculated and used for all statistics. Differences between adherence of experimental strains that had been observed micoscopically were reflected as differences in the magnitude of the recovered CFU/ml in the quantitative assay (Fig. 5). Adherence levels of EPEC strain E2348/69 were greater than or equal to that of EHEC strains 87-23 and 86-24 (5.37×10^7 versus 1.71×10^7 and 1.2×10^7 adherent CFU/ml, respectively in a representative assay). The negative control, DH5α, gave values at or below 10^5 adherent CFU/ml. The minimum level of detection for the assay is 10^4adherent CFU /ml. EHEC strains 933cu and 933D also exhibited high levels of attachment (~1×10^7). Isolate 933 demonstrated levels consistently one log less than the other O157:H7 strains tested ($2.75\text{-}8.5 \times 10^6$). This difference was reproducible and observed across a panel of other SLT-I- and SLT-II- producing isolates that included O157:H7 strains CL40 and A8959-C7 (5.6×10^6 and 6.9×10^6, respectively). On microscopic evaluation, 933 and other SLT-I and SLT-II- producing EHEC were observed lined up at the edges of HEp-2 cells, and these interactions resulted in a FAS negative phenotype. When the quantitative adherence data were expressed as a proportion of the starting inoculum (given as an adherence index) (Table 7), the resultant values indicated that a net growth of bacteria had occurred over the course of the six hour assay.

Figure 4. Schematic of the in vitro adherence assay used to evaluate *E. coli* attachment throughout this dissertation. When HEp-2 cells were used as the substrate for bacterial attachment, the bacteria were incubated with the eukaryotic cells for a total of 6 hours. In assays with HCT-8 cells as the substrate the bacteria were allowed to associate for a total of 5 hours. The other parameters of the assay are discussed in detail in the text.

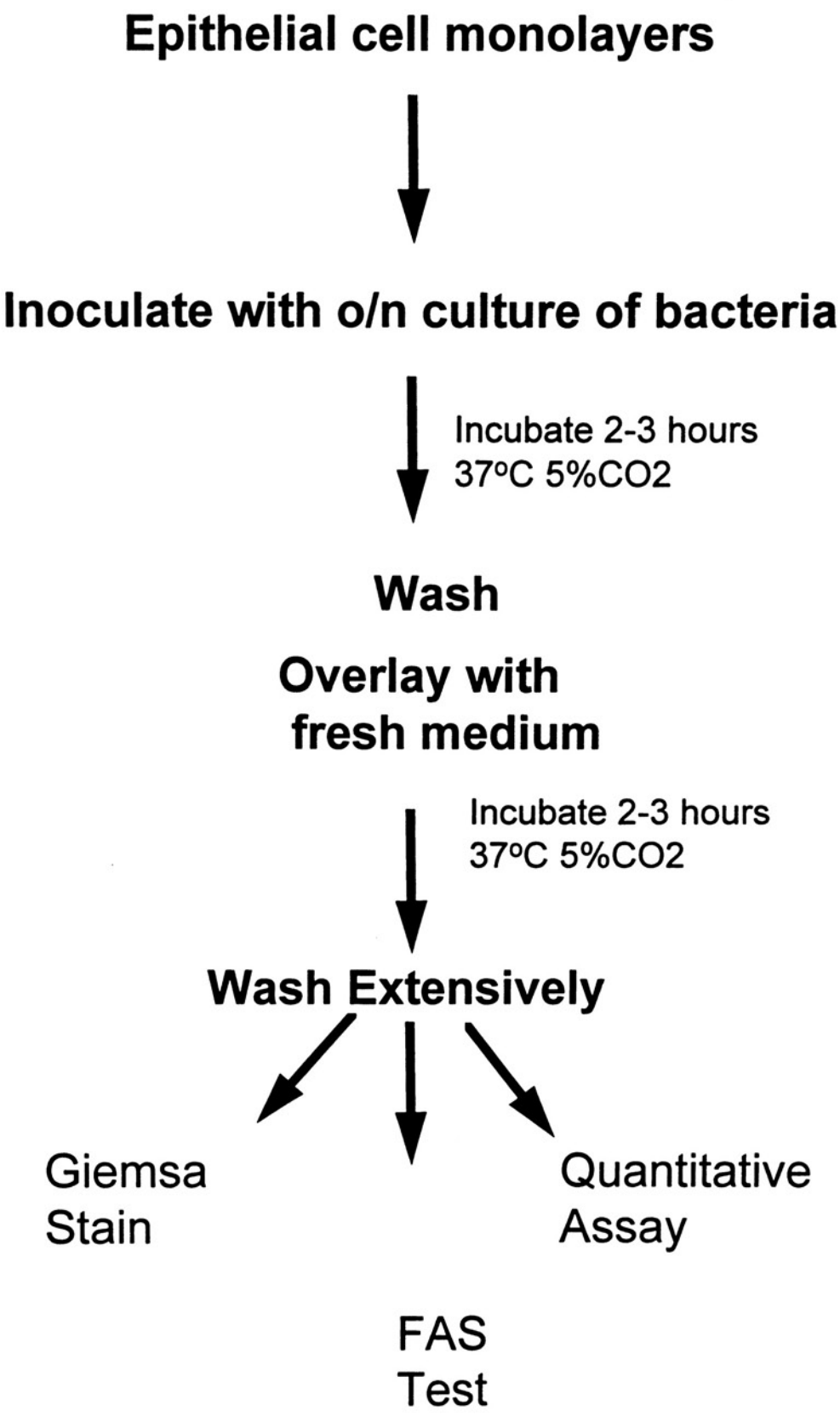
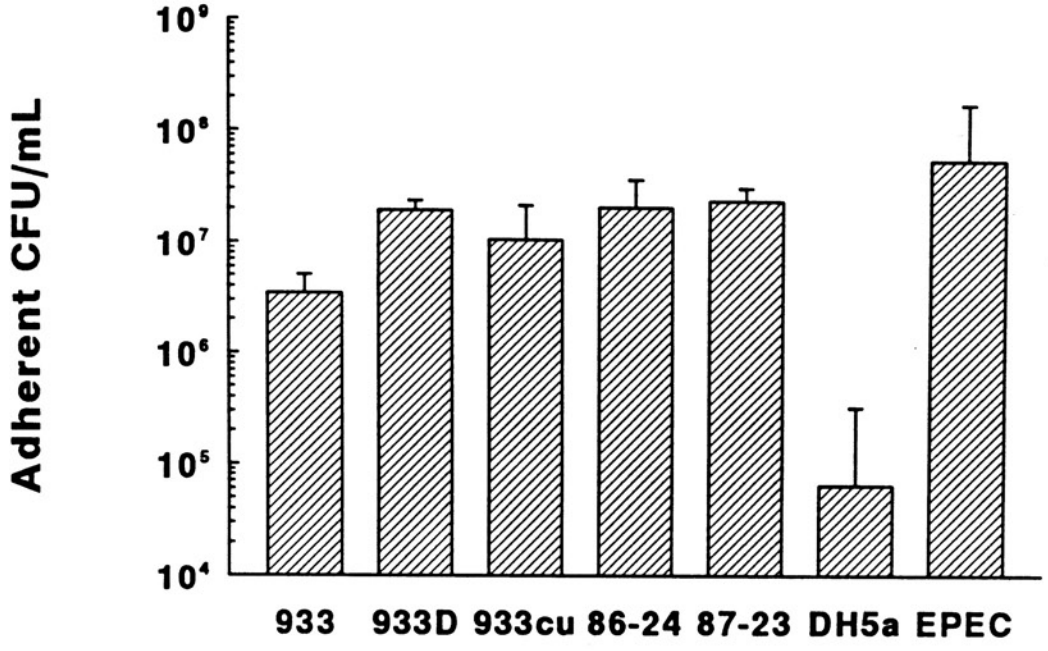

Epithelial cell monolayers
Inoculate with o/n culture of bacteria
Incubate 2-3 hours
37°C 5%CO2
Wash
Overlay with
fresh medium
Incubate 2-3 hours
37°C 5%CO2
Wash Extensively
Giemsa
Stain
Quantitative
Assay
FAS
Test

Figure 5. Comparison of the HEp-2 cell- adherence levels of a panel of EHEC strains with a K-12 strain, DH5α, and the positive control, EPEC E2348/69. Geometric mean values from quadruplicate samples are shown + 2 SD.


Adherent CFU/mL
10⁹
10⁸
10⁷
10⁶
10⁵
10⁴
933
933D
933cu
86-24
87-23
DH5a
EPEC

The trend in the quantitative data correlated with the qualitative data. However, we were concerned that the average values for the negative control were 10^5 adherent DH5α CFU/ml, yet no bacteria were observed microscopically on the HEp-2 cells. Several hypotheses were proposed to explain the quantitative binding of the K-12 strain. These theories included: i) the concentration of mannose in the adherence medium was not sufficient to block all type I pili-mediated attachment; ii) DH5α may not be an appropriate K-12 strain to use as a negative control; and/or, iii) the bacteria recovered in the quantitative assay bound to the plastic but not the HEp-2 cells. Each of these suppositions was tested independently as described below.

Effect of mannose concentration on binding of *E. coli* K-12 to HEp-2 cells. The standard assay medium consisted of EMEM with 1% sodium bicarbonate (for buffering) and 0.5% D-mannose. Mannose was included in the medium to block any type I pili-mediated adherence to the epithelial cells (Ofek *et al.*, 1977). The background of 10^5 adherent DH5α/ml (as determined by the quantitatitive assay not by microscopic evaluation) may indicate that 0.5% mannose is not sufficient to block all Type I adherence. To test this hypothesis, DH5α and EHEC strains were incubated with the HEp-2 cells in the presence of 0.5, 1, 2, or 5% mannose (Table 8). While an increase in mannose concentration from 0.5% to 1.0% did not have a significant effect on 87-23 or 86-24 adherence levels, this same change in concentration decreased the binding of DH5α

Footnotes for Table 7.

[a]Dilutions that resulted in counts between 30 and 300 were used for calculation of the number of adherent bacteria per milliliter (adherent CFU/ ml): CFU on agar plate x dilution factor x 10 (factor of 0.1 ml plated onto the agar) x 2 (factor for 0.5 ml trypsin solution used to remove cells and adherent bacteria from tissue culture well).

[b]To calculate an adherence index, the CFU adherent per well was divided by the CFU in the inoculum and multiplied by 100%. The number of bacteria in the inoculum for the calculations presented here was estimated based on the average from other assays.

[c]MLD = minimum level of detection for the assay; $\leq 10^4$ adherent CFU/ml.

Table 7. Representative adherence indices of EHEC strains with HEp-2 cells.

Strain	Adherent CFU/ml[a]	Adherence Index[b] (%)
933	3.47×10^6	34
933D	1.91×10^7	191
933cu	1.05×10^7	105
86-24	2.04×10^7	204
87-23	2.34×10^7	234
DH5α	≤MLD[c]	ND

approximately 7 fold (2.2% to 0.32%). As the concentration of mannose in the medium was increased, the viability of the HEp-2 cells began to be affected. The low adherence indices for all three strains reflect a decrease in the HEp-2 cell population at 2 and 5% mannose (Table 8). From this experiment, we concluded that a proportion of the background counts seen with DH5α was due to mannose-sensitive adherence. To block this effect, all subsequent adherence assays were conducted in the presence of 1% mannose. In a different assay, both 86-24 and 87-23 were allowed to interact with HEp-2 cells in the presence or absence of mannose (1%). In that study, both isolates gave values of 10^7 CFU adherent/ml regardless of the presence of the sugar. These data, taken together with the findings in Table 8, indicate that the proportion of EHEC adherence mediated by type I fimbriae is minimal compared to the proportion due to other mechanisms of attachment.

Comparison of the extent of adherence to HEp-2 cells by various K-12 strains. The increased mannose concentration in the adherence medium was not sufficient to abolish the attachment of DH5α to HEp-2 cells. Therefore, we tested the adherence of other K-12 strains to HEp-2 cells in a search for a less adherent negative control. Two other isolates of DH5α and HB101 were tested for the capacity to bind HEp-2 cells. All three of the K-12 strains adhered at equivalent levels in the presence of 1% mannose (~1 x 10^5 CFU adherent/ml) with an adherence index of less than 10% of the inoculum. In contrast, the EHEC

Table 8. Effect of increasing mannose concentration in the assay medium on the adherence indices of EHEC and K-12 strains.

	Mannose concentration (%)			
	0.5	**1.0**	**2.0**	**5.0**
***E. coli* Strain**				
EHEC 86-24	96%	77%	49%	23%
EHEC 87-23	140	131	80	39
K-12 DH5α	2.2	0.32	0.64	0.33

strains gave values of 1-3 x 10^7 adherent CFU/ml (AI = >100%). Based on these data, we concluded that there were no significant differences in the adherence of a variety of K-12 *E. coli* strains tested. Therefore, we elected to retain DH5α as the negative control for all subsequent assays.

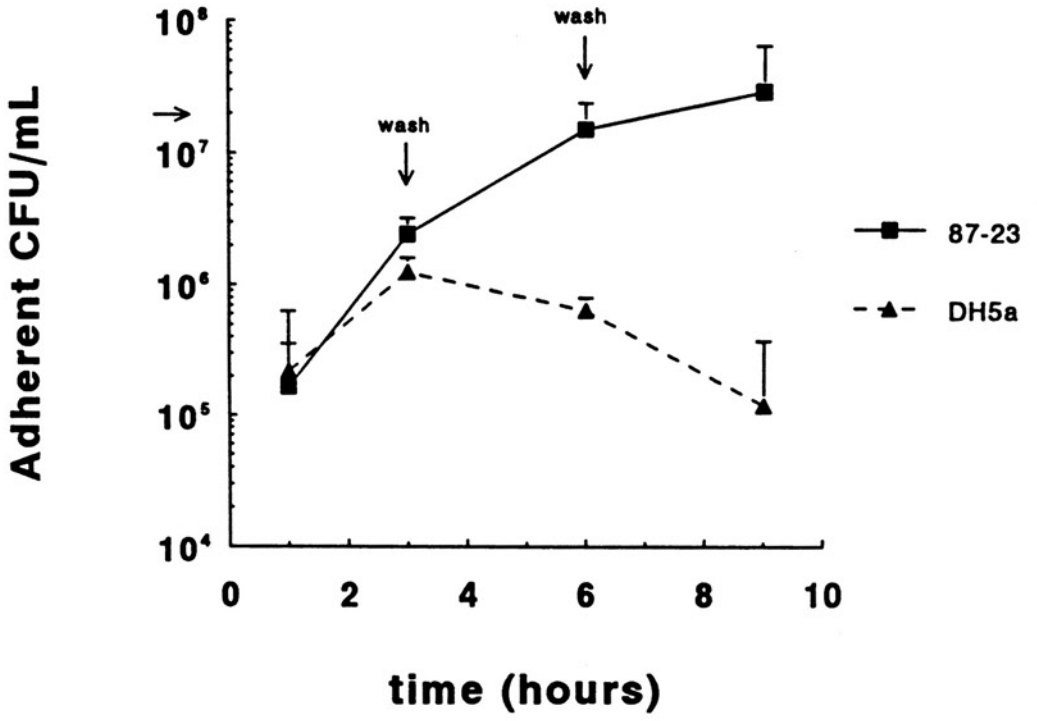
Adherence of K-12 strains to plastic. Another possible explanation for the residual adherence counts of DH5α is that the bacteria are binding to the plastic surface of the tissue culture dish in which the assay is conducted. One might envision a nonspecific, hydrophobic interaction between the bacterium and the negatively charged tissue culture dish surface. To test this hypothesis, 10^7 DH5α in adherence medium containing 0.5% mannose were inoculated into duplicate wells; one of the wells was seeded with HEp-2 cells and the other was preincubated with media alone. At the end of 6 hours, the wells were washed 6 times with PBS, as per the standard protocol, and the bacteria were removed by trypsinization. Those wells that had been inoculated with HEp-2 cells had 10^5 CFU adherent, whereas those wells that did not contain eukaryotic cells had no detectable counts. From these data, we surmised that DH5α do not bind to plastic and that this nonspecific means of attachment does not explain the adherence counts obtained with this control strain.

The actual location of the ~10^5 organisms in an infected monlayer of HEp-2 cells remains unclear. As seen in Fig. 3, DH5α bacteria were not routinely observed on microscopic examination of HEp-2 cells infected for 6 hours with this

K-12 strain. This discordance between the microscopic and quantitative data probably reflects the fact that there were 1 x 10^5 HEp-2 cells per well or ~1 K-12 bacterium per eukaryotic cell. Indeed, whenever any K-12 bacteria were seen, they appeared as single bacterium and never as clusters. We elected to use microscopic observations, particularly by the FAS technique, as the primary criteria by which to classify a bacterial strain as adherent or nonadherent, because we felt that exclusive reliance on the quantitative assay might lead to false positive results.

Kinetics of attachment of EHEC strains to HEp-2 cells. One of the primary differences between the adherence assay described in this dissertation (Fig. 4) and those protocols previously reported is the increase in the incubation time of EHEC with the epithelial cells from 3 to 6 hours. As mentioned above, the difference between the two incubation times had significant effects on the results of adherence in that no attachment was observed at 3 hours for some strains (e.g., 86-24), but clear microcolony formation was evident at 6 hours with several of the strains, including 86-24. The apparent lag in binding of certain EHEC strains to the HEp-2 cells was evaluated by a kinetic assay. Fig. 6 shows the attachment of 87-23 to HEp-2 cells at 1, 3, 6 and 9 hours after inoculation. At the two early timepoints, there was not a significant difference between the adherence of 87-23 and DH5α. However, at 6 and 9 hours, the difference between the EHEC strain and the K-12 was marked. Overall, the level of DH5α attachment hovered around

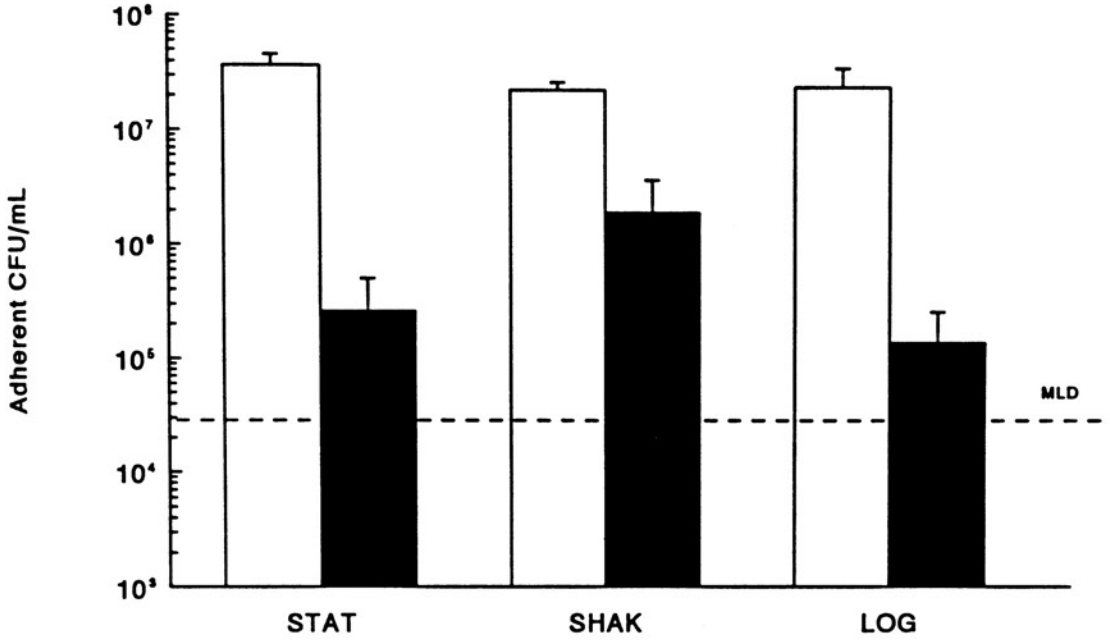
Figure 6. Comparison of the kinetics of EHEC strain 87-23 and *E. coli* K-12, DH5α, adherence toHEp-2 cells. Geometric mean values are shown + 2 SD for each time point. The initial inoculum of 2 x 10^7 bacteria in 20 µl is denoted by the arrow on the ordinate. The assay medium with nonadherent bacteria was removed at 3 and 6 hours (indicated by the arrows). The monolayers were then washed once with sterile 10 mM PBS and overlaid with fresh medium for the duration of the incubation period.


Adherent CFU/mL
10^8
10^7
10^6
10^5
10^4
wash
wash
0
2
4
6
8
10
time (hours)
87-23
DH5a

10^{5-6} CFU adherent/ml while the values for 87-23 continued to rise throughout the assay from 1.66×10^5 (95% CI = 4.35×10^4; 6.34×10^5) at 1 hour to 2.95×10^7 (95% CI = 1.34×10^7; 6.49×10^7) at 9 hours. These data show a lag in EHEC attachment that was reflected in the microscopic analysis (data not shown). The lag was not due to a varying of the MOI with time, because counts of the bacteria free in the medium at each timepoint revealed an equivalent number of organisms (e.g., 2.19×10^8 & 5.72×10^8 CFU for 87-23 at 1 and 9 hours, respectively). The lag was also not a result of a growth defect of the organism in the assay medium. The growth curves for 87-23 in adherence media and LB were indistinguishable as were the growth curves for DH5α in the same media (data not shown). The bacteria entered the log phase on the growth curve after the first hour in the medium and were into stationary phase by the end of the assay (9 hours). These results indicate that the adherence capacity of the bacteria is greater when the bacteria are in log phase or actively growing (e.g., at 3-6 *hours after inoculation)* rather than in the early stages of outgrowth after saturation (e.g., 1 hour).

Effect of growth conditions of the bacteria on HEp-2 cell adherence. Since the kinetics data described above indicated a lag in HEp-2 cell adherence of 87-23 similar to the lag in the bacterial growth of the EHEC strain in the assay medium, we assessed the adherence capacity of strain 86-24 at different phases of growth. Bacteria were grown in LB broth with or without shaking overnight to saturation or were subcultured from an overnight culture for 2 hours such that the organisms

Figure 7. Evaluation of effect of bacterial growth conditions on attachment of EHEC 86-24 or *E. coli* K-12 DH5α to HEp-2 cells. The open bars represent EHEC 86-24 adherence and the dark bars represent attachement of DH5α. STAT, overnight stationary culture grown to saturation; SHAK, overnight shaking culture grown to saturation; LOG, subculture of bacteria grown to log phase with shaking; MLD, minimum level of detection for the quantitative adherence assay.


Adherent CFU/mL
10⁸
10⁷
10⁶
10⁵
10⁴
10³
MLD
STAT
SHAK
LOG

were in log phase. Samples of these bacterial cultures were then compared forthe capacity to bind HEp-2 cells by a quantitative assay. The results in Fig. 7 demonstrate that strain 86-24 adhered to HEp-2 cells equally well regardless of the growth condition or growth phase tested. However, the level of adherent DH5α was higher in the shaking overnight culture. Based on these experimental findings, the standard assay was carried out with bacteria from a static overnight culture of bacteria.

Effect of chloramphenicol or heat treatment on HEp-2 cell adherence. Chloramphenicol was added to EHEC bacteria throughout the course of an adherence assay to evaluate the role of bacterial protein synthesis in adherence. When twice the minimum inhibitory concentration (MIC) of chloramphenicol was added to the bacterial-HEp-2 cell milieu either i) during the first three hours of incubation, ii) the second three hours, or iii) throughout the six hour incubation period, microcolony formation by 86-24 was ablated. However, a single bacterium was observed attached to HEp-2 cells. By contrast, microcolonies were present on HEp-2 cells in wells infected with 86-24 in the absence of the drug. From these data, we concluded that *de novo* protein synthesis is required by the bacterium for microcolony formation, but the role of protein synthesis in the initial attachment event is unclear since bacteria were observed singly adherent to some of the HEp-2 cells. These conclusions were also supported by the finding that heat-treated EHEC 86-24 (80°C for 10 minutes) were nonadherent to HEp-2 cells (data not

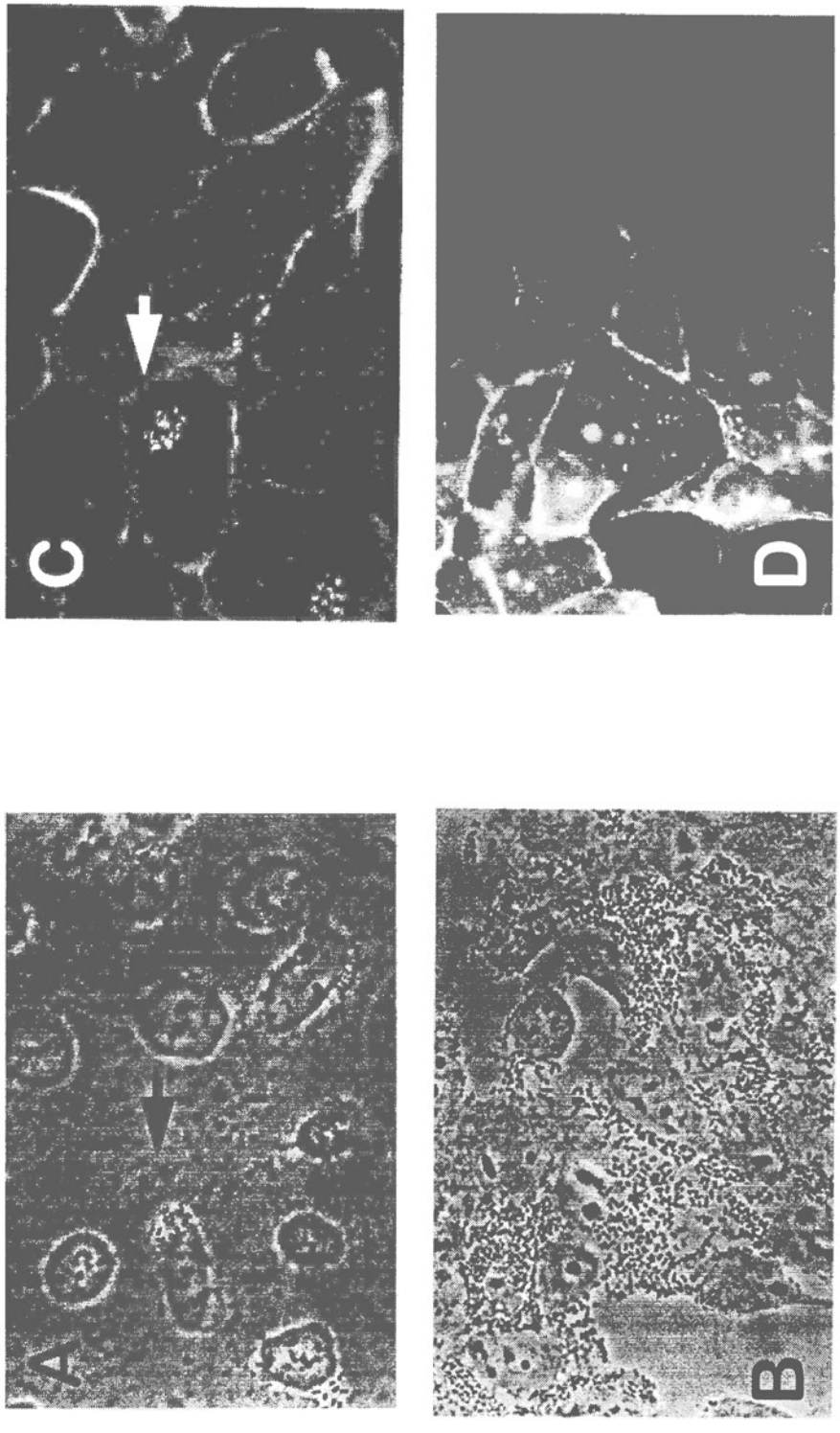
shown). Effect of Shiga-like toxin on HEp-2 cell adherence. As mentioned previously, the attachment phenotype (i.e., the time to attachment and microcolony formation) varied among the EHEC strains tested. At the 3 hour timepoint, we noted that only the strain that did not produce SLT, 87-23, formed microcolonies on the HEp-2 cells (Fig. 2- 87-23). Although HEp-2 cells were not sensitive to either SLT-I or SLT-II by standard cytotoxicity analysis (Table 5), we hypothesized that the SLT elaborated when a bacterium is in close association with a eukaryotic cell may have an effect on the cell that results in an inability of the bacterium to bind. Several approaches were used to test this possibility. First, strain 87-23 was incubated with HEp-2 cells in the presence of exogenous toxin preparations. Dilutions of 1:10 or 1:50 of lysates from 933 (SLT-I and SLT-II; CD_{50} =10^6/ml), 933D (SLT-I; 10^8/ml), or 86-24 (SLT-II; 10^6/ml) were added simultaneously with the bacteria (87-23) to the HEp-2 cells. By Giemsa stain analysis, the addition of toxin did not appear to affect the adherence of 87-23 at 3 or 6 hours. The control strains, E2348/69 and DH5α, were also unaffected by the addition of exogenous toxin. Second, excess toxin was incubated with the HEp-2 cells for one hour before infection with 87-23. Again, no effect of toxin on the adherence of 87-23 to HEp-2 cells after a 6 hour assay was noted. To test our supposition that EHEC produced SLT during the adherence assay, samples of media overlaying the infected cells were taken after a 3 hour assay and were tested on Vero cells for cytotoxicity. We found that toxin was elaborated by both 933 and 86-24 during the adherence assay (CD_{50} = 10^4 for both). Third, the overnight bacterial cultures in

one assay were washed twice with PBS to remove exogenous toxin before addition of the inocula to the HEp-2 cells. Prior washing did not have any observable effect on the adherence of 87-23 to HEp-2 cells after a 3 hour assay. Taken together, these results indicate that toxin does not have a direct effect on the adherence capacity of the EHEC strains.

The next two sections of this portion of the dissertation are taken nearly verbatim from McKee and O'Brien, 1995.

Adherence of EHEC and other *E. coli* strains to HCT-8 cells. We hypothesized that EHEC strains might adhere better or differently to HCT-8 cells, which are derived from the human ileocecum, than the less physiologically relevant, but more commonly used, laryngeal epithelial cell-derived HEp-2 cell line. We found that EHEC 86-24 adhered to and formed microcolonies on HCT-8 cells (Fig. 8), and that actin rearrangement occurred at the site of microcolony formation in a pattern similar to that seen with HEp-2 cells. However, EHEC strains that were poorly adherent to HEp-2 cells gave a clear LA/FAS phenotype on HCT-8 cells (data not shown). Moreover, an additional pattern of adherence of EHEC to HCT-8 cells, but not Hep-2 cells, was noted (Fig. 8). Bacteria appeared to be adherent to and lined up at the junctions between the HCT-8 cells, but no organisms were seen sticking to the plastic slide. We called this phenotype "log jam" adherence because the bacteria resembled a mass of logs crowded together floating down

Figure 8. Adherence of EHEC to HCT-8 human ileocecal epithelial cells. EHEC strain 86-24 exhibits an LA/FAS phenotype (arrows) on HCT-8 cells (A, phase contrast; B, fluorescein-conjugated phalloidin stain by fluorescence microscopy). In a different plane of focus, 86-24 also shows the log jam pattern of adherence to HCT-8 cells. The bacteria are between or at the sides of the cells (C). This pattern of adherence is FAS negative (D). Some areas of positive FAS are present on the tops of the cells in panel D which illustrates that both adherence phenotypes occur simultaneously.


C
D
A
B

a river. This attachment phenotype was FAS negative (Fig. 8) and limited to the HCT-8 cells.

We sought to determine whether the log jam pattern of adherence to HCT-8 cells was specific for EHEC or whether other *E. coli* strains exhibited this phenotype. Therefore, we examined the interaction of both pathogenic and nonpathogenic *E. coli* isolates with HCT-8 cells. The log jam adherence pattern was observed among intestinally-derived pathogenic and nonpathogenic *E. coli* strains (Table 9). Even normal flora *E. coli* isolates formed log jams on the HCT-8 cells, but neither of the two laboratory K-12 strains tested, DH5α and HB101, did so. All of the EHEC O157:H7 strains and the one nonmotile O157 strain (E32511) tested were both LA/FAS and log jam positive. The wild type EPEC strains formed large FAS positive microcolonies only. A derivative of EPEC E2348/69 mutated at the bundle-forming pilus (*bfp*) locus [mutant designation 10-1-1(1), (Donnenberg *et al.*, 1990a)] was able to form log jams. From this observation, we concluded that the tight interbacterial association among the EPEC organisms masked the log jam pattern. Enteroaggregative *E. coli* adhered to the top of the HCT-8 cells in such large numbers that the junctions of the HCT-8 cells, where log jams typically were observed, could not be seen. Thus, the aggregative or stacked brick pattern of EAggEC (Savarino, 1993) may have obscured any low level or background adherence such as the log jam. ETEC strains either adhered diffusely or formed microcolonies on the HCT-8 cells in addition to the log jam pattern. The EIEC strain tested was diffusely adherent and log jam positive. Only the *E. coli*

Footnotes for Table 9.

[a] EHEC serotypes O157:H7 and O157:H$^-$; EPEC serotypes O127:H6, O111:NM, and O114:H2; ETEC serotypes O78:H11, O7:NM, and O25:NM; EIEC serotype O143:ND; EAggEC serotypes O44:H18 and O3:H2; and K-12, DH5α and HB101.

[b] MC = microcolony; FAS = fluorescence actin staining by FITC-phalloidin; DA = diffuse adherence; log jam = bacteria adherent at the junctions between HCT-8 cells.

[c] The *eae* genotype was determined by DNA dot blot hybridization with an internal *eae* probe derived from a subclone of 86-24 *eaeA*.

[d] Strain 933cu (Karch *et al.*, 1987).

[e] This EHEC *eae* mutant is an in-frame deletion mutant in the *eaeA* locus of strain 86-24 (86-24*eaeΔ10*, described in this thesis).

[f] The EPEC*bfp* (bundle forming pilus) mutant is a Tn*phoA* insertional mutant of strain E2348/69 that no longer produces bundle forming pili (Donnenberg *et al.*, 1990a).

[g] The O8$^+$ and O8$^-$ strains represent an isogenic set of *rfb* mutants, strains 2443 and AB1133, respectively (Meier-Dieter *et al.*, 1989; Meier-Dieter *et al.*, 1992).

Table 9. Adherence of pathogenic and nonpathogenic *E. coli* to HCT-8 cells.

***E. coli* type[a] (no. strains tested)**	**Adherence phenotype[b]**	***eae* genotype[c]**
EHEC (5)	MC/FAS$^+$; log jam	+
EHEC cured[d]	MC/FAS$^+$; log jam	+
EHEC*eae*Δ*10* [e]	log jam	in-frame deletion
EPEC (3)	large MC/FAS$^+$	+
EPEC*bfp* [f]	log jam; FAS$^{+/-}$	+
ETEC (3)	MC/FAS$^-$ or DA/FAS$^-$; log jam	-
EIEC (1)	DA/FAS$^-$; log jam	-
EAggEC (2)	Aggregative/ stacked brick; FAS$^-$	-
Normal Flora (1)	log jam	-
O8$^+$ [g]	DA/FAS$^-$; log jam	-
O8$^-$ [g]	DA/FAS$^-$; log jam	-
K-12	non-adherent	-

carrying the *eaeA* locus (i.e., EHEC and EPEC) were FAS positive. An in-frame deletion mutant in *eaeA* of 86-24 (described below and McKee *et al.*, submitted) was LA/FAS negative but remained log jam positive. Strain 933cu (Karch *et al.*, 1987) adhered to the HCT-8 cells by both the LA/FAS and log jam mechanisms; therefore, the capacity of EHEC to form log jams is not mediated by the 60 MDa plasmid present in EHEC strains.

Because we observed the phenotype across a range of LPS serogroups, the log jam pattern does not appear to be LPS type specific. Further, an LPS rough *E. coli* strain and its isogenic smooth derivative (serotype O8) (Meier-Dieter *et al.*, 1989; Meier-Dieter *et al.*, 1992) were able to adhere to HCT-8 cells in the log jam manner. Since all adherence assays were carried out in the presence of 1% mannose, we concluded that the log jam phenotype was mannose resistant.

Invasive potential of EHEC strains with epithelial cells. Recently, there has been one report that EHEC strains can invade HCT-8 cells in a microfilament-dependent manner (Oelschlaeger *et al.*, 1994). To test the possibility that the log jam phenotype represented intracellular bacteria, we examined O157:H7 strains for the capacity to enter the HCT-8 cells. EHEC strains 933 and 86-24 were assessed for the capacity to invade the HCT-8 cells as determined by survival after gentamicin treatment (Table 10). Both 86-24 and 933 adhered better than

the normal flora *E. coli* isolate, HS, (17.9% and 36.8%, respectively, versus 13.0% for HS). The K-12 strain, DH5α, exhibited minimal adherence (column 1, Table 10) but was not visible by light microscopy. It should be noted that the adherence indices presented here reflect both the bacteria adhering in a LA/FAS manner as well as those bacteria in the log jams. One cannot compare these values directly to the adherence indices with HEp-2 cells because of the additional log jam adherence mechansim with HCT-8 cells. When compared to the normal flora values, invasion percentages for the two EHEC strains tested were indistinguishable (86-24, 0.16%; 933, 0.17%; and HS, 0.22% by Method I). Further, the percent of EHEC bacteria that survived gentamicin treatment (column 2, Table 10) was substantially below that of EPEC or *Shigella flexneri* 2457T, 2.6% and 1.21%, respectively. The difference between the invasive capacity of *S. flexneri* and the EHEC strains was even more marked when the percent invasive bacteria was calculated based on the total bacteria associated with the monolayer at the time of gentamicin treatment (Method II). Both EHEC strains gave invasion values within the range of the normal flora isolate regardless of the method used to calculate invasion. Thus, we concluded from our data that EHEC do not invade HCT-8 cells to any significant extent.

Light microscopy was used to examine infected HCT-8 cell monolayers both before and after gentamicin treatment (Fig. 9). For all strains tested except DH5α, bacteria were associated with the cells after the samples were treated with gentamicin. For EHEC and HS, the remaining bacteria were not exclusively those

Footnotes for Table 10.

[a] Percent adherence = [(CFU associated with the HCT-8 cells at 3 hours) minus (CFU surviving 100µg/ml gentamicin treatment for 1 hour)] / CFU inoculated x 100%.

[b] Method I: Percent invasion = (CFU surviving gentamicin treatment) / (CFU inoculated) x 100%.

[c] Method II: Percent invasive/total bacteria associated with the HCT-8 cells = (CFU surviving gentamicin treatment) / (CFU associated with the HCT-8 cells after 3 hours) x 100%.

Table 10. Invasive potential of EHEC O157:H7 strains.

Bacterial type/ Strain	%Adherence[a] (range)	%Invasion Method I[b] (range)	%Invasion Method II[c] (range)
EHEC O157:H7 86-24	17.9 (10.1-32.2)	0.16 (0.15-0.19)	1.1 (0.5-1.85)
EHEC O157:H7 933	36.8 (14.3-47.8)	0.17 (0.12-0.20)	0.49 (0.25-1.38)
Normal flora HS	13.0 (11.0-14.4)	0.22 (0.22-0.22)	1.7 (1.5-1.98)
K-12 DH5a	2.2 (1.4-3.1)	0.02 (0.002-0.025)	0.7 (0.08-1.7)
EPEC E2348/69	16.8 (14.6-19.1)	2.6 (2.3-2.9)	13.5 (10.9-16.5)
S lla fl r 2457T	4.4 (2.3-7.8)	1.21 (0.9-1.5)	21.4 (10.7-34.8)

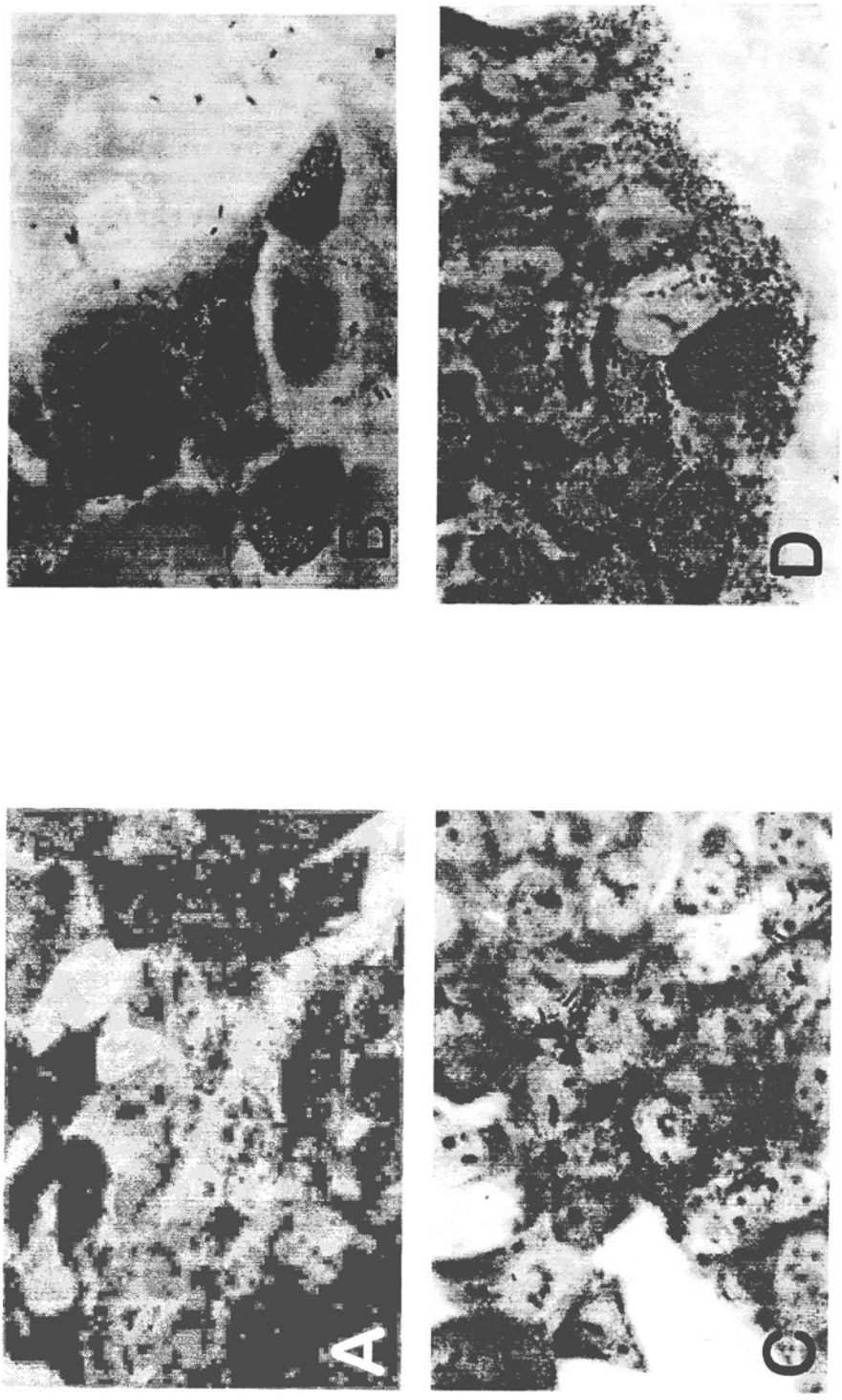
bacteria in the log jams. By light microscopy, we were unable to determine whether those bacteria which survived gentamicin treatment were intracellular or extracellular. The EPEC strain E2348/69 was still contained within formed microcolonies, but the clusters were smaller than those microcolonies observed without gentamicin treatment (Fig. 9). The EPEC survivors may have been protected from gentamicin by the close interbacterial association or the intimate association of the bacterium with the HCT-8 cell membrane.

In a separate assay with HEp-2 cells, only 0.2% and 0.1% of EHEC strains 933D and 87-23, respectively, survived gentamicin treatment. The invasive potential of EHEC strains for HEp-2 cells was calculated by Method I (Table 10). None of the K-12 strain DH5α bacteria survived gentamicin treatment. From these data, we concluded that EHEC do not invade HEp-2 cells.

II. Genetic approach to EHEC adherence -- *eaeA*.

Presence of *eaeA* homologue in EHEC strains. When Jerse *et al.* (1990) discovered the eaeA locus (then *eae*) in EPEC strains, these investigators also reported the presence of homologues (as determined by Southern hybridization) of the *eaeA* gene in 29/30 EHEC isolates of serogroups O157:H7 and O26:H11 as well as in RDEC-1, an EPEC of weanling rabbits. Based on this information and with the availability of the newly established *in vitro* assay for EHEC

Figure 9. Infected HCT-8 cell monolayers giemsa stained after treatment with gentamicin. HCT-8 cell monolayers infected with wild-type 86-24 and the normal flora *E. coli* HS were similar in appearance (A and B). EPEC bacteria associated with HCT-8 cells appeared to remain in formed microcolonies (D), *albeit* smaller than those observed in the absence of gentamicin. *Shigella flexneri* 2457T is shown as a positive control for epithelial cell invasion (C). No K-12 *E. coli* remained after treatment of infected monolayers with gentamicin (data not shown).

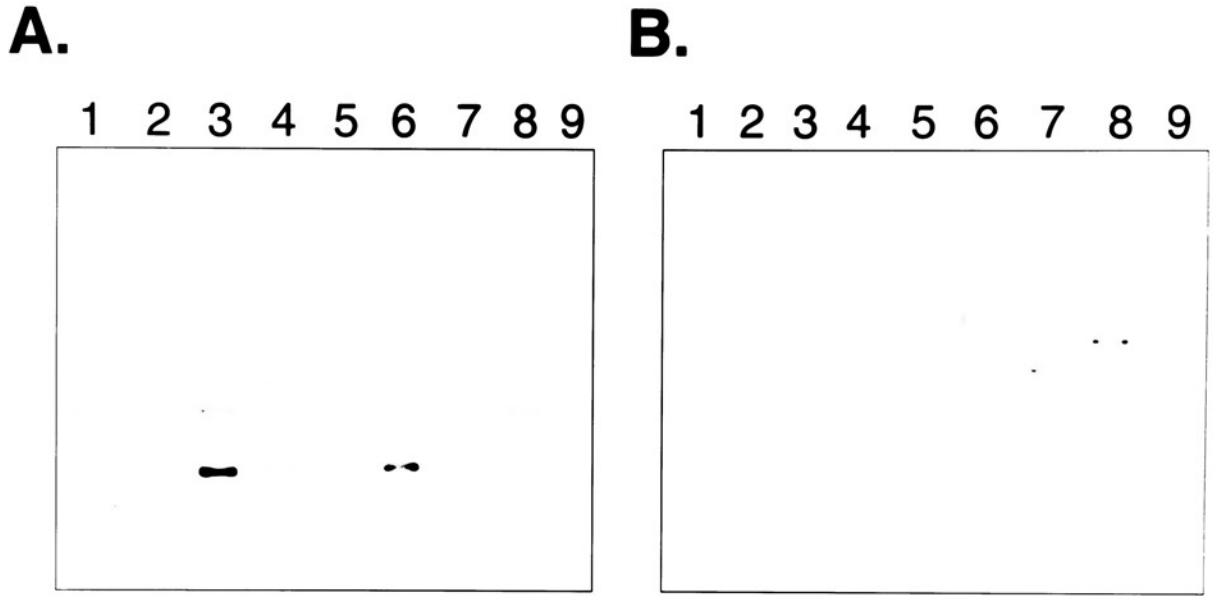

A
B
C
D

adherence and FAS, we began an in-depth study ofthe *eaeA* homologue in EHEC. First, selected EHEC strains were screened for the presence of *eaeA* by DNA hybridization. The 1kb *Sal*I-*Stu*I fragment from pCVD434 recognizedDNA fragments in strains 86-24, 87-23, E32511, 933, 933cu, and 933curev, but not in B2F1, H414-36/89 or S1191 (Fig. 10). The isolates that were *eaeA* probe positive are all of the O157 serogroup, whereas the negative strains are O91:H21 (B2F1 and H414-36/89) or 0139:K82B [S1191, an isolate from a pig with edema disease (Marques *et al.*, 1987)]. Even when hybridizations were conducted at lower stringency, B2F1 *and* H414-36/89 *remained eaeA negative.*

Because EHEC strain 86-24 was of the commonly isolated O157:H7 serotype and consistently LA/FAS positive in the *in vitro* assay, we chose this clinical isolate as the prototype for all further analysis of the role of *eaeA* in EHEC adherence. We elected an approach that would fulfill the Molecular Koch's Postulate (Falkow, 1988) to define the role of the *eaeA* locus in EHEC adherence to epithelial cells *in vitro* and the capacity to cause the A/E lesion *in vivo*.

Cloning the *eaeA* gene from EHEC 86-24. The second step in fulfilling the Molecular Koch's postulates is to isolate the implicated gene for the phenotype in question (Falkow, 1988). Therefore, we sought to clone the *eaeA* gene from strain 86-24. The first strategy was to clone the gene directly from the chromosome of 86-24. To determine the smallest continuous segment of the chromosome that encodes the *eaeA* gene in 86-24, a series of chromosomal

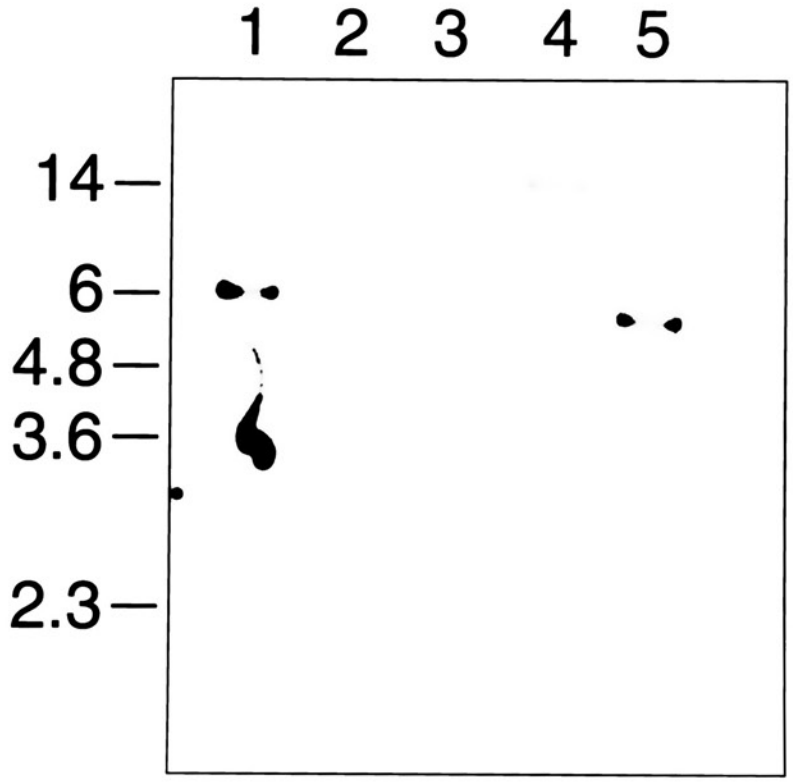
Figure 10. Hybridization of *eaeA* sequences with total DNA isolated from EHEC strains. Panel A, chromosome preparations were digested with *Hin*dIII (odd numbered lanes) or *Eco*RI (even numbered lanes) from 933 (lanes 1 and 2), 933cu (lanes 3 and 4), and 933curev (lanes 5 and 6). Chromosomal DNA from 86-24 was digested with *Nru*I (lane 7), from EPEC E2348/69 (*Hin*dIII, lane 8) or DH5α (*Eco*RI, lane 9). In panel B, chromosomes isolated from *eaeA* negative EHEC strains were digested with *Eco*RI (odd numbered lanes) or *Nru*I (even numbered lanes) then reacted with the 1 kb *eaeA* probe (Jerse *et al.*, 1990): B2F1, (lanes 1 and 2), H414-36/89 (5 and 6) and S1191 (*Hin*dIII digest, lane 9). The *eaeA* positive control, strain 86-24, digested with *Eco*RI (lane 7) or *Nru*I (lane 8) reacted with probe. K-12 *E. coli* DH5α (3 and 4) is not recognized by the pCVD434 probe.


A.
1 2 3 4 5 6 7 8 9
B.
1 2 3 4 5 6 7 8 9

digests were hybridized with the pCVD434 probe. Single bands were obtained with *Eco*RI, *Sal*I, *Stu*I, and *Hin*dIII (Fig. 11). *Hin*dIII digestion resulted in the smallest fragment (about 6 kb) that encompassed the entire open reading frame. While these preliminary studies were under way, our collaborators at the Center for Vaccine Development in Baltimore were sequencing the *eaeA* locus from EDL933. The 933 *eaeA* sequence revealed that *Eco*RI, *Sal*I and *Stu*I all cut within, the coding sequence of the gene, but *Hin*dIII did not. Therefore, we constructed a bank of 6-8 kb *Hin*dIII chromosomal fragments in pBluescriptSK$^+$, and screened the recombinants on LB-amp agar containing IPTG and X-gal. Ten white colonies were obtained, and the plasmid DNA from them was further analyzed by restriction digestion with *Hin*dIII and hybridization with the *eaeA* probe. The restriction digests revealed that these subclones were different from one another, but none of the subclones contained the *eaeA* probe.

With the availabilty of the then unpublished sequence for the *eaeA* gene of 933, we began an alternate approach to clone the *eaeA* locus from 86-24. Primers based on the known sequence were derived for polymerase chain reaction (PCR) amplification of the gene directly from the chromosome (primers MM1 and MM2, Fig. 12). The resultant amplimer was restricted with *Sca*I and *Xba*I and cloned into the *Sma*I/*Xba*I sites of pBluescriptSK$^+$. The recombinant plasmid was transformed into host strain. Fourteen true white colonies were obtained out of 100 potential colonies, and these were passed through a secondary screening on IPTG-X-gal-containing medium. Restriction analysis of plasmid DNA isolated from

Figure 11. Southern hybridization of restricted total DNA from EHEC strain 86-24 probed with the 1kb *Sal*I-*Stu*I fragment from pCVD434 {45}. Extracted DNA was digested with *Eco*RI (lane 1), *Pst*I (lane 2), *Sal*I (lane 3), *Stu*I (lane 4), or *Hin*dIII (lane 5) to determine the smallest fragment of chromosomal DNA that carried the entire coding sequence.


1 2 3 4 5
14
6
4.8
3.6
2.3

2 of these 14 colonies appeared to have the correct *eaeA* insert. Potentially positive recombinants were screened further by colony blot hybridization with the pCVD434 probe. Six of the fourteen white colonies yielded probe positive clones. Of these 6, only 1 gave fragments close to the predicted size when digested with either *Hin*dIII or *Eco*RV. DNA sequencing of the ends of this clone, designated pEB290, revealed that the *eaeA* had been cloned, but that the 3' 250 bases had been lost by comparison with the published sequences of EHEC *eaeA*. Therefore, pEB290 is an incomplete clone that contains the *eaeA* locus starting at the nucleotide sequence of the second codon and ending 250 base pairs upstream of the transcription termination sequence.

The complete *eaeA* locus from 86-24 was finally cloned by PCR amplification of the gene fragment directly from the wild-type chromosome. At that time, another group had published the sequence of the *eaeA* gene from EHEC O157:H7 strain CL8 (Beebakhee *et al.*, 1992). The CL8 published sequence starts 205 bases upstream of the ATG codon for *eaeA*. We speculated that an *eaeA* clone with more of the flanking sequences might stabilize the recombinants. New primers were derived based on the composite of the two known EHEC *eaeA* sequences. The 5' primer, Sn20 - CGTTGTTAAGTCAATGGAAAC, spanned bases 20-41 of the CL8 sequence. The 3' primer, MM2 - TCTAGAGAGAAAACGTGAATGTTGTCTCT, spanned bases 3061-3082 of the sequence from 933 with an added *Xba*I site at the 3' end. This amplification resulted in a 3144 bp fragment that encoded the entire *eaeA* ORF and included

Figure 12. Strategy for plasmid constructs of the wild-type 86-24 *eaeA* gene (large dark bar) were derived from the Sn20MM2 PCR product as described in the text. The primers used for amplification reactions are indicated by the small arrowheads. The direction of transcription is indicated by the large arrow. Plasmid pEB310 includes the entire 3144bp amplicon cloned into the *Eco*RV site of pBRKS$^-$ in the orientation of the T7 RNA polymerase promoter (P_{T7}). Plasmid pEB290 is a clone of the fragment from a reaction with primers MM1 and MM2 in vector pBluescriptSK$^+$. Plasmid pEB300 is pEB290 with the internal *Bcl*I fragment deleted. The *Xba*I/*Hin*dIII fragment of pEB300 which includes the deleted *eaeA* gene was ligated into the suicide vector pAM450 to create construct pEB305. Primers: MM1 - ATAACATGAGTACTCATGGTTG; Sn20 - CGTTGTTAAGTCAATGGAAAC; and MM2 - TCTAGAGAGAAAACGTGAATGTTGTCTCT. RI=*Eco*RI; B=*Bcl*I; RV=*Eco*RV.

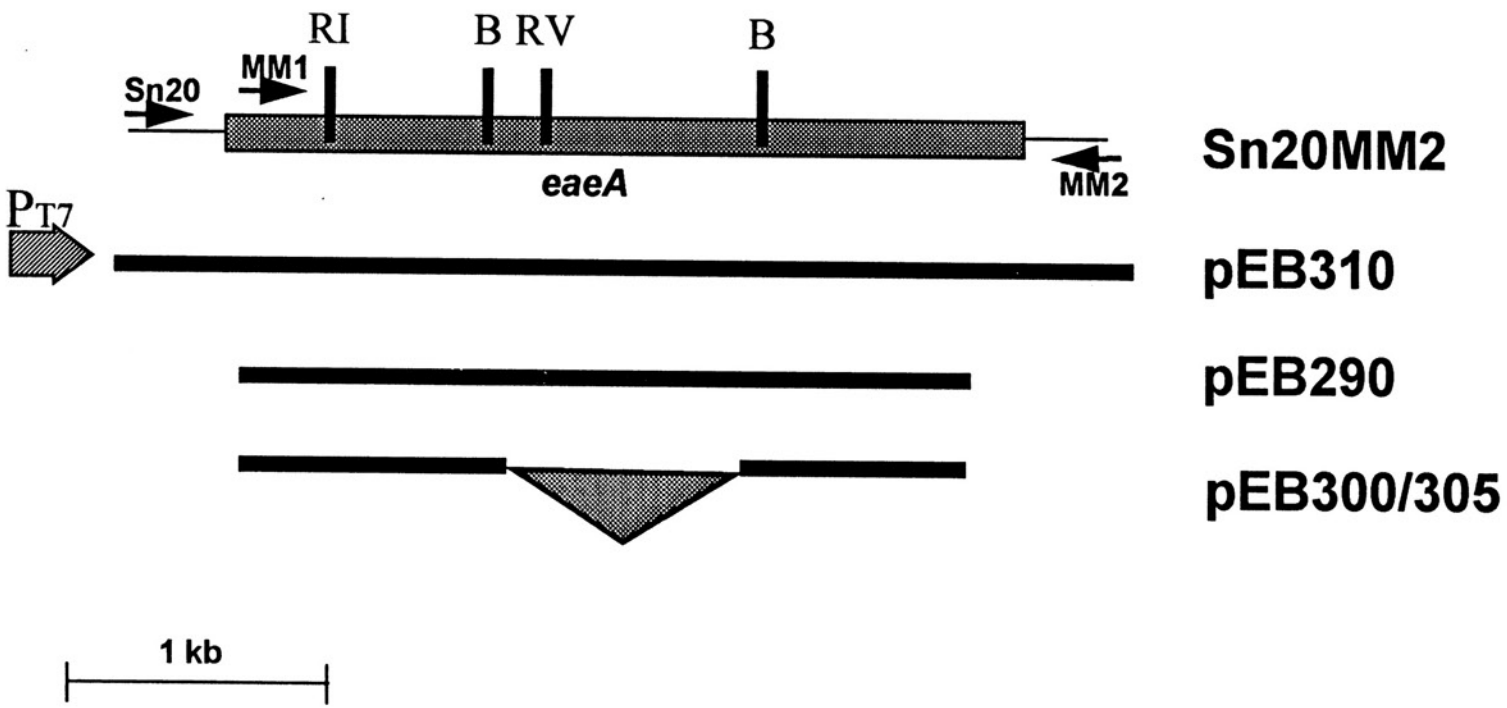
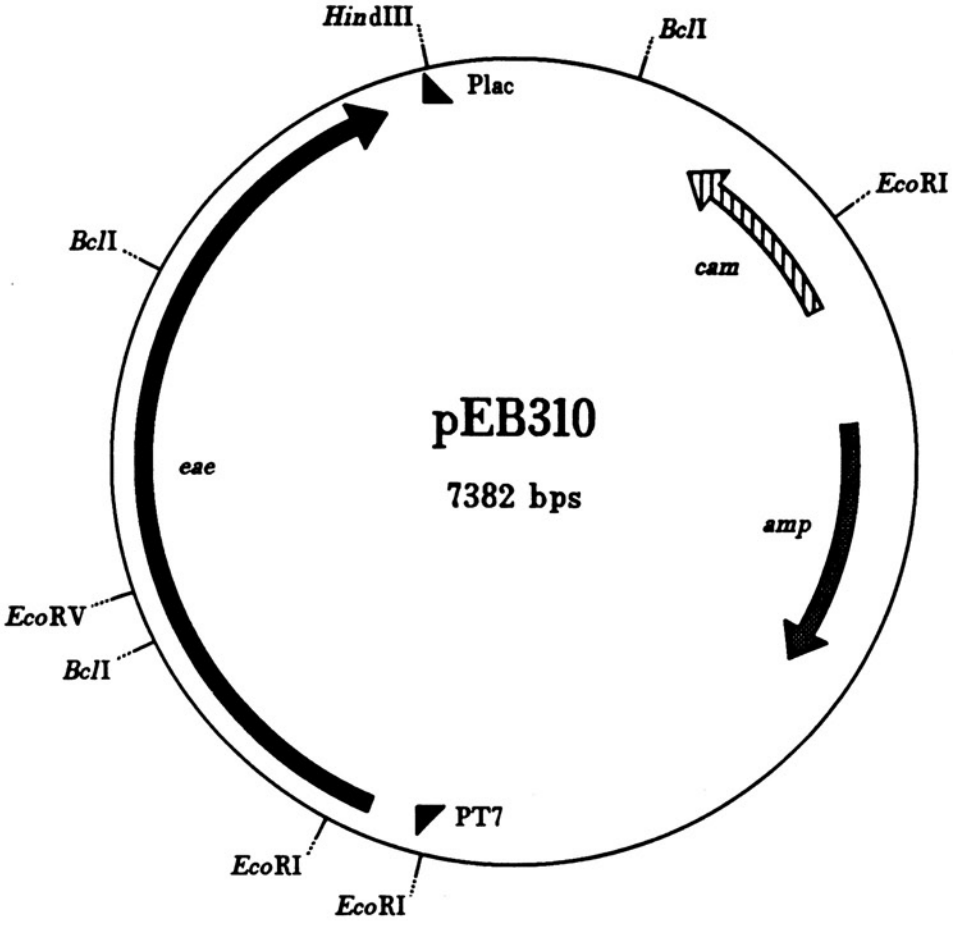

RI
B RV
B
MM1
Sn20
eaeA
MM2
Sn20MM2
PT7
pEB310
pEB290
pEB300/305
1 kb

Figure 13. Map of plasmid pEB310. This construct encodes the entire *eaeA* open reading frame from EHEC 86-24 in vector pBRKS^{-}. The construction of this recombinant plasmid is described in the text.


HindIII
BclI
Plac
EcoRI
BclI
cam
pEB310
7382 bps
eae
amp
EcoRV
BclI
PT7
EcoRI
EcoRI

186 bp upstream (Fig. 12). The PCR product was processed to create blunt ends and was ligated into the *Eco*RV site of the vector pBRKS$^-$ (Schmitt *et al.*, 1994). The gene was cloned in both orientations to allow transcription both from either P_{lac} and from P_{T7} under the appropriate conditions. The constructs were called pEB311 and pEB310, respectively (pEB310 is shown in Figs. 12 and 13). The recombinants were maintained under the constitutive control of the lac repressor in host strain XL1BlueF', because some of the previous failed attempts to clone *eaeA* suggested that expression of *eaeA* might be lethal to the host *E. coli* strain. The lower copy number of the pBRKS$^-$ vector along with the control of the lac repressor obviated these problems.

The next three sections of this portion of the dissertation are taken nearly verbatim from McKee, et al., 1995 (submitted for publication).

Construction of an EHEC *eaeA* mutant. To create the in-frame deletion in the chromosomal copy of 86-24 *eaeA*, the wild-type copy of the gene was replaced by double homologous recombination with an internally-deleted copy (Fig.14). Plasmid pEB290 was transformed into GM119 [*dam-6*, *dcm-3* (Arraj and Marinus, 1983)] to obtain unmethylated DNA which was sensitive to the restriction endonuclease *Bcl*I. Plasmid DNA was isolated and restricted with *Bcl*I to remove an internal 1125 bp fragment from the gene. The resulting sticky ends were ligated to create pEB300. The deleted *eaeA* gene was excised by digesting

pEB300 with *Xba*I and *Hin*dIII, and the fragment containing the *eaeA* sequence was ligated into the *Bam*HI site of the suicide vector, pAM450. Plasmid pAM450 is a derivative of pMAK705 (Hamilton *et al.*, 1989) that has a temperature sensitive (*ts*) origin of replication, carries the *sacB/R* locus from *Bacillus subtilis* which renders the host strain sensitive to sucrose (Gay *et al.*, 1985; Lepesant *et al.*, 1972), and encodes ampicillin resistance. These features allow homologous recombination and positive selection for a second recombination event resulting in resolution and loss of vector sequences. The suicide:*eaeA* construct, pEB305, was transformed into wild type 86-24 by electroporation. Double recombinants that had been cured of the vector sequences were selected by growth on medium containing sucrose and then screened for ampicillin sensitivity (Blomfield *et al.*, 1991). Resolution products that had been cured of the suicide vector sequences were sucrose resistant, ampicillin sensitive, and able to grow equally well at 30° and 42°C. The chromosomal deletion was confirmed by: i) the reduced size of the *eaeA* fragment after PCR amplification with primers MM1 and MM2 (data not shown), ii) Southern blot analysis of the mutated chromosomal DNA (Fig. 15), iii) loss of restriction sites within the *eaeA* gene, and iv) the inability of an internal probe to recognize the mutated chromosome (Fig. 15). The resulting strain was designated 86-24*eaeΔ10*. The mutation was confirmed to be in frame by *in vitro* transcription and translation analysis of the PCR-derived product from 86-24*eaeΔ10*. A truncated protein product of the predicted size, about 68,000 Da, was identified by [^{35}S] methionine labeling of the translation product (Fig. 16). The

Figure 14. Strategy for construction of the in-frame mutation in EHEC 86-24 *eaeA*. The mutated gene from clone pEB290 was subcloned into the suicide vector pAM450 to create pEB305. The salient features of the pAM450 vector are discussed in the text. Plasmid pEB305 was electroporated into the wild-type 86-24. Resolution products after allelic replacement were selected and screened as described in the text. The "external probe" is denoted by the dashed line between the *Eco*RV sites; the "internal probe" is denoted by the dotted line between the *Eco*RV and *Sal* I sites.

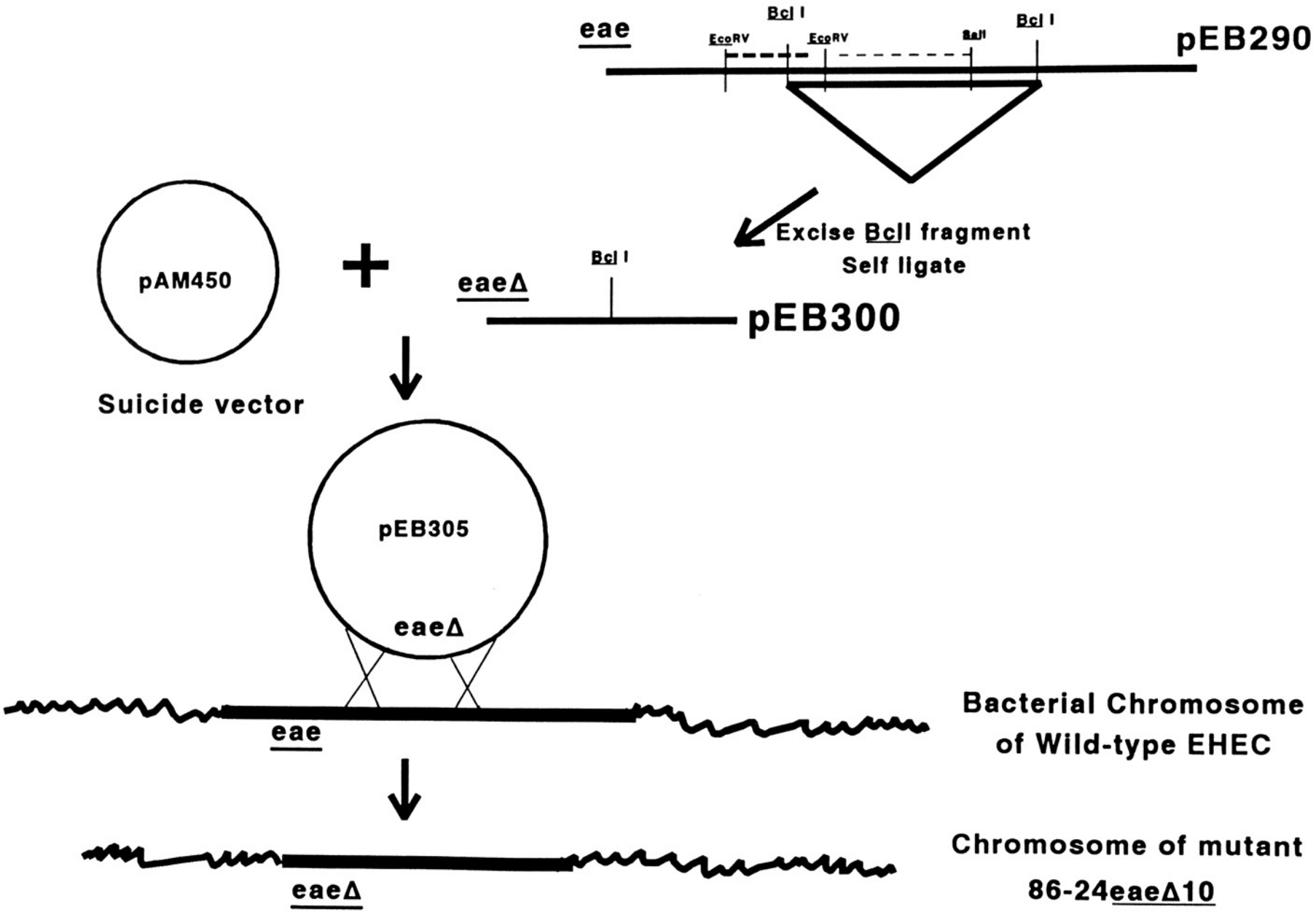
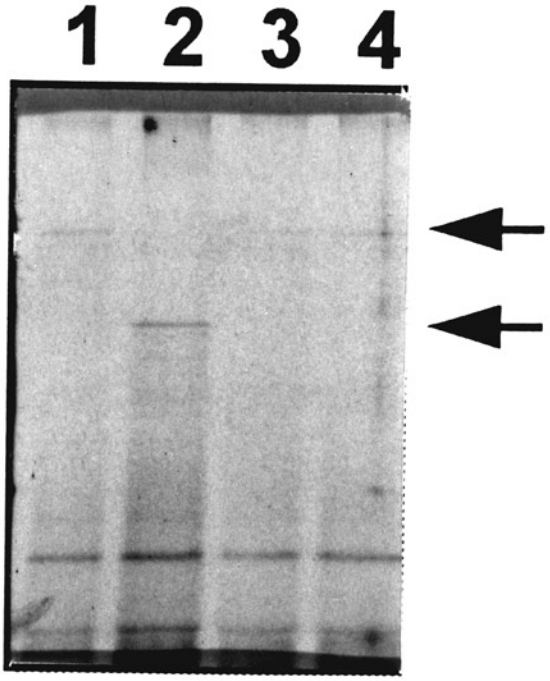

eae
BclI
EcoRV
EcoRV
SalI
BclI
pEB290
Excise BclI fragment
Self ligate
pAM450
+
eaeΔ
BclI
pEB300
Suicide vector
pEB305
eaeΔ
eae
Bacterial Chromosome
of Wild-type EHEC
eaeΔ
Chromosome of mutant
86-24eaeΔ10

Figure 15. Hybridization of wild-type 86-24 and the in-frame deletion mutant of *eaeA* DNA probes internal (A) or external (B) to the deleted region. The restriction endonucleases with which extracted total DNA was digested are indicated. W, wild type 86-24 chromosome; M, 86-24*eaeΔ10* chromosome. DNA size markers (kb) are indicated.

A.
EcoRV
M W
BglII
M W
EcoRI
M W
23
9.4
5.6
4.8
B.
EcoRV
M W
BglII
M W
EcoRI
M W
14
8.4
7.2
5.6
4.3

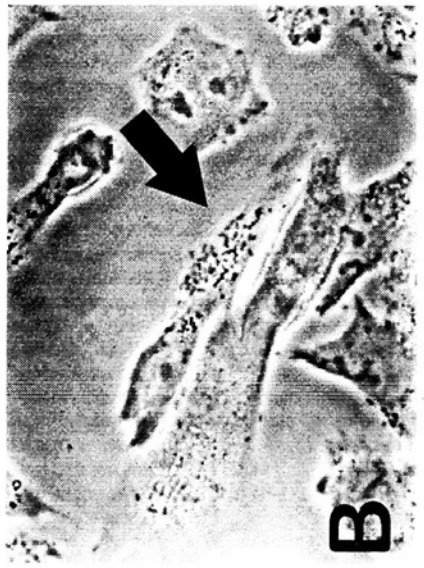
Figure 16. *In vitro* transcription/translation products from templates derived from wild type EHEC isolates 933 (lane 1), 86-24 (lane 3), and 4494 (lane 4). Full-length intimin at 97,000 Da is indicated by the upper arrow. Lane 2 demonstrates that the deletion in the *eaeA* locus in 86-24*eaeΔ10* is in frame. A truncated product of the predicted size (~60 kDa) is observed (lower arrow).

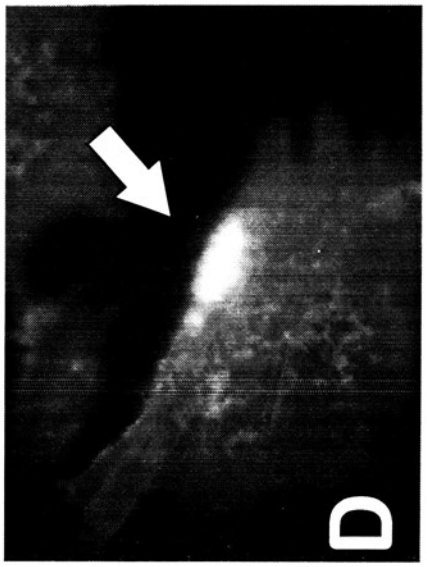

1 2 3 4

eaeA mutant strain was identical to wild type 86-24 in all characteristics tested including: growth in LB, agglutination with O157 and H7 antisera, inability to ferment sorbitol, and growth on MacConkey agar at 37°C.

The role of *eaeA* in EHEC adherence *in vitro*. We then tested our isogenic strains, 86-24, 86-24*eaeΔ10* and 86-24*eaeΔ10* carrying pEB310, for adherence to HEp-2 and HCT-8 cells. Wild type 86-24 formed microcolonies when the bacteria interacted with HEp-2 or HCT-8 cells. This localized adherence was FAS positive which indicates the polymerization of F-actin at the site of bacterial attachment (Fig. 17). The mutant 86-24*eaeΔ10* was unable to adhere to HEp-2 cells (Fig. 17). When *eaeA* was introduced into 86-24*eaeΔ10* on either pEB310 or pEB311, the LA/FAS phenotype was fully restored (Fig. 17), an observation which demonstrated that intimin alone complements the mutation. Since both of the clones complemented the *eaeA* mutant, the native promoter for *eaeA* is probably present in the PCR amplified sequences. B2F1, a naturally *eaeA*-negative O91:H21 EHEC isolated from a patient with hemolytic uremic syndrome (Ito *et al.*, 1990), was also tested in the *in vitro* adherence assay. The few B2F1 bacteria that bound to HEp-2 cells did so in a diffuse pattern. In contrast, B2F1 carrying pEB310 exhibited an LA pattern with a weakly positive FAS phenotype (data not shown). From these data, we conclude that *eaeA* is sufficient to render B2F1 LA positive. We also tested 86-24*eaeΔ10* carrying pCVD444 or pCVD436 in the adherence assay. Plasmid pCVD444 contains the *eaeA* locus from EHEC strain

Figure 17. Phase contrast and fluorescent micrographs of the EHEC *eaeA* mutant adherence phenotype to HEp-2 and HCT-8 cells. The *eaeA* mutant, 86-24*eaeΔ10*, was unable to adhere to the HEp-2 cells (A). Adherence of the mutant is restored by transformation with plasmid pEB310 carrying the wild-type *eaeA* locus from 86-24 (B - HEp-2 cells, C and D - HCT-8 cells). Complementation of 86-24*eaeΔ10* by plasmid pEB310 restored FAS activity on both epithelial cell types (D for HCT-8 cells).


B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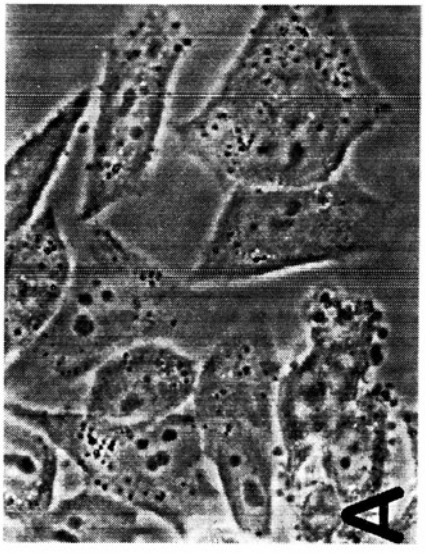
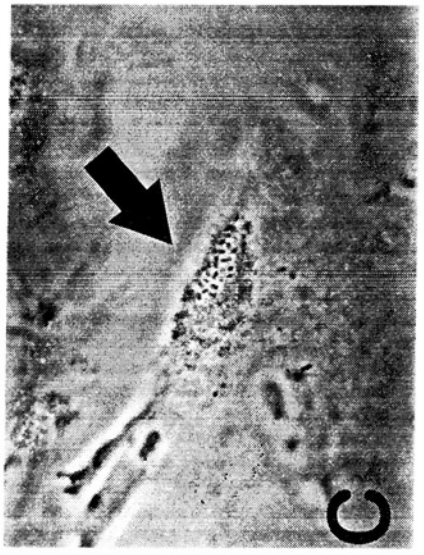
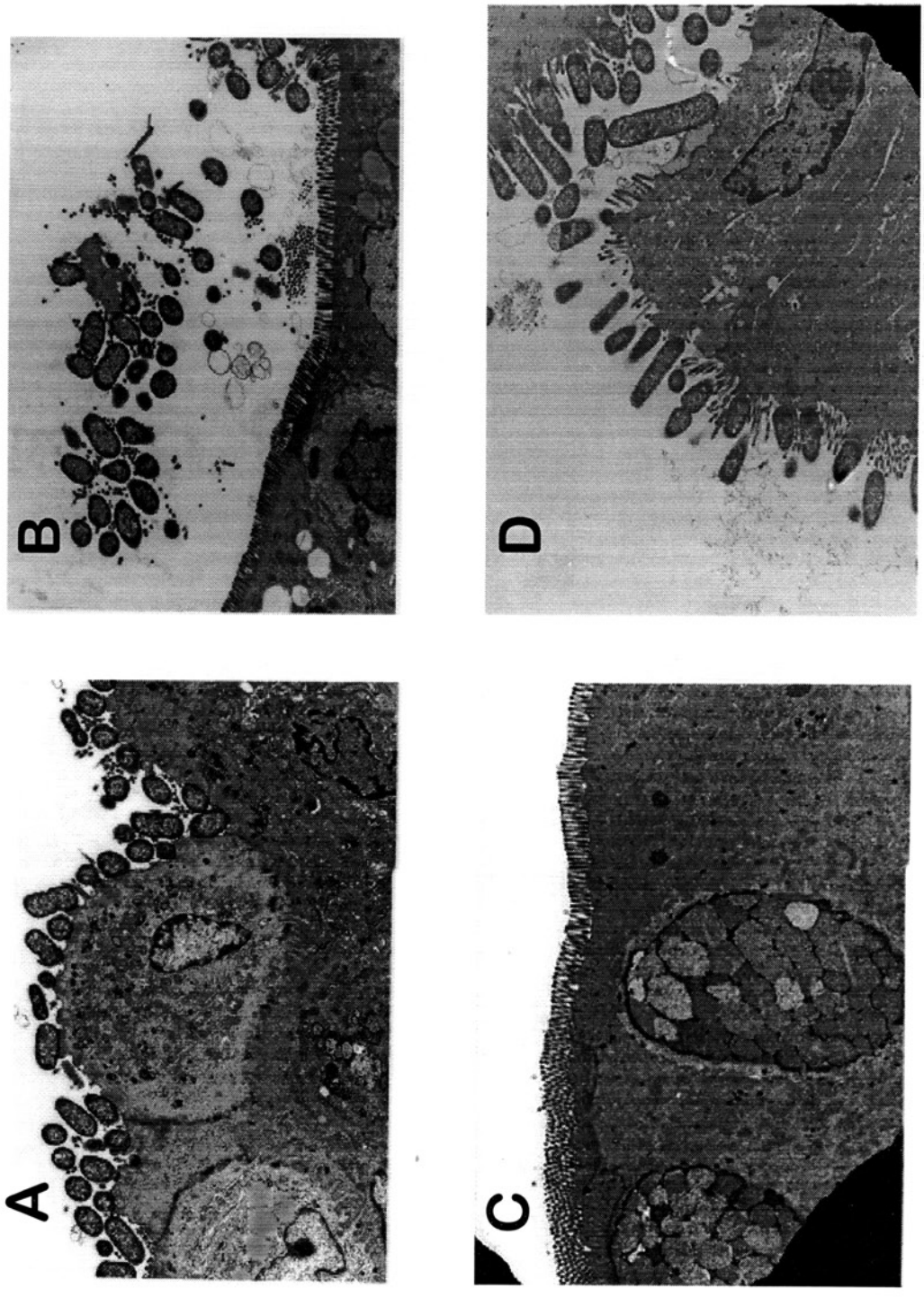

A


C

EDL933 (Yu and Kaper, 1992) and was previously shown not to complement UMD619 *in vitro* (Donnenberg *et al.*, 1993b). Cosmid pCVD436 contains the entire *eae* gene cluster from EPEC E2348/69 (Jerse *et al.*, 1990) and also did not complement UMD619. However, both of these constructs complemented the in-frame *eaeA* mutation in 86-24. Conversely, pEB310 was unable to render UMD619 adherent to the HEp-2 cells (data not shown). We concludethat pEB310, pEB311, pCVD436, and pCVD444 produce a functional intimin product, but only 86-24*eaeΔ10* is able to express the protein in such a way that intimin was functional. Alternatively, the other *eaeA* negative strain may have other functional defects related to adherence, possibly due to polar effects.

The role of *eaeA in vivo*. (i) Gnotobiotic piglet infection model. Next, the role of intimin in intestinal colonization, A/E lesion formation, and EHEC-mediated colitis and diarrhea in the gnotobiotic piglet was evaluated by the method of Francis *et al.* (1986). The in vivo challenge results are summarized in Table 11. Both pairs of piglets inoculated with the wild-type parent strain, 86-24 and the *eae*-positive control strain, EDL933 developed diarrhea and had edema in the mesentery of the spiral colon at necropsy. Histologically, strains 86-24 and EDL933 primarily colonized the cecum and spiral colon. Minimal multifocal bacterial adherence was also seen in the terminal ileum of one of two piglets inoculated with EDL933. Histologically and by culture, no evidence of bacterial dissemination to the liver, kidney, lung, or brain was detected with either strain.

Figure 18. Electron micrographs of enterocytes in the spiral colon from piglets infected with wild-type 86-24 (A and B), the in-frame deletion mutant 86-24*eaeΔ10* (C), or 86-24*eaeΔ10* that carried pEB310 (D). Bacteria were observed intimately associated with the epithelial cells on which microvilli had been effaced (magnification = X5,000). In some instances, microvilli had sloughed into the gut lumen with bacteria still attached (B). All regions along the spiral colon appeared normal in piglets infected with the mutant strain (C), but when the same strain carried pEB310, wild-type attaching and effacing activity was restored (D). Intestinal sections from piglets infected with B2F1 resembled those infected with 86-24*eaeΔ10*.


B
D
A
C

Footnotes for Table 11.

[a]The presence or absence of *eaeA* was determined by in situ hybridization of total DNA extracts probed with the 1 kb *Sal*I-*Kpn*I fragment from pCVD434 (Jerse *et al.*, 1990).

[b]In vitro adherence to HEp-2 cells was assessed in a six hour assay and observed microscopically (McKee and O'Brien, 1995). LA, localized adherence; FAS, fluorescence actin staining; and NA, nonadherent.

[c]The capacity of each organism to cause the attaching and effacing lesion was determined with the gnotobiotic piglet oral infection model (n=2 per strain) and electron microscopic evaluation as described in the text. +, A/E lesion positive as defined by Staley *et al.* (Staley *et al.*, 1969); -, nonadherent to intestinal mucosa and therefore A/E negative.

[d]Typhlocolitis, inflammation of the cecum and spiral colon, characterized by scattered neutrophils in the lamina propria and accumulation of serous fluid and perivascular lymphocytes and macrophages in the submucosa. +, colitis detected; -, colitis not detected.

[e]EDL933 bacteria line up at the edges of the HEp-2 cell with rare, small microcolonies which are weakly FAS positive observed (O'Brien *et al.*, 1993)

Table 11. Comparison of *in vitro* and *in vivo* activities of *eaeA*-positive and *eaeA*-negative strains.

Strain	*eaeA* genotype[a]	HEp-2 adherence[b]	A/E lesion[c]	Typhlocolitis[d]
86-24		LA/FAS	+	
86-24*eae*Δ*10*		NA	-	
86-24*eae*Δ*10* (pEB310)		LA/FAS		
B2F1	-	NA/weak	-	-
EDL933		DA/weak FAS[e]		

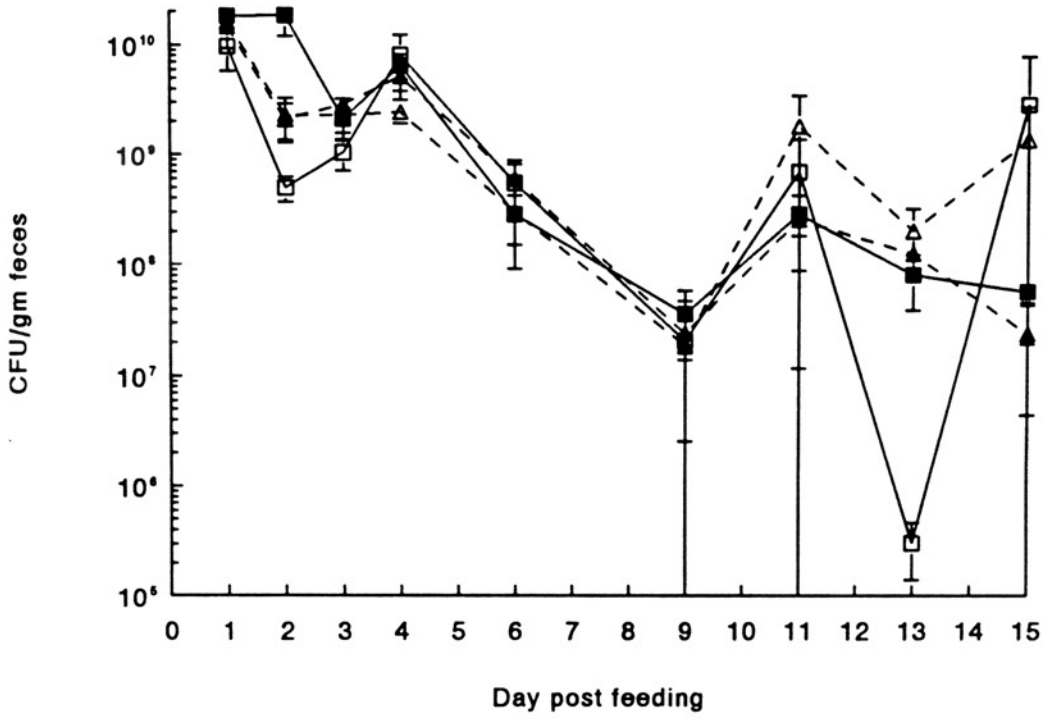
Intimate bacterial adherence and A/E lesions, as described by Staley (Staley *et al.*, 1969) and Moon (1983) for EPEC, were evident by both light and EM examination of cecum and colon of piglets infected with either EDL933 or 86-24 (Fig. 18A). A/E lesions included the accumulation of electron-dense material at the site of attachment. In some areas, sloughed enterocyte fragments and microvilli with attached bacteria were noted in the gut lumen (Fig. 18B). In histologic sections of the cecum and spiral colon of piglets infected with 86-24 or EDL933, an inflammatory infiltrate was seen. Inflammation was characterized by scattered neutrophils in the lamina propria and mild diffuse accumulation of serous fluid and perivascular lymphocytes and macrophages in the submucosa.

Both piglets inoculated with the mutant strain, 86-24*eaeΔ10* had formed feces at necropsy. Histologically and by EM examination, there was no evidence that strain 86-24*eaeΔ10* was able to colonize piglet intestine and cause the A/E lesion (Fig. 18C). The few bacteria seen by light and EM examination were in the mucus overlying the mucosal epithelium of the cecum and spiral colon. One of two piglets inoculated with 86-24*eaeΔ10* had slight mesocolonic edema, but no other gross or microscopic lesions were seen in either piglet. Piglets inoculated with 86-24*eaeΔ10*(pEB310) had pasty feces and mesocolonic edema at necropsy. Strain 86-24*eaeΔ10*(pEB310) intimately adhered to mucosal enterocytes and caused A/E lesions in the cecum and spiral colon (Fig. 18D). Histologically, perivascular lymphohistiocytic typhlocolitis, similar to that caused by wild type 86-24 and EDL933 was also seen. One of two piglets inoculated with

strain B2F1 had pasty feces and mesocolonic edema at necropsy; the other piglet had formed stool and no gross lesions. Neither piglet had microscopic colitis and in both piglets, strain B2F1 rods in the intestines were rare in number, non-adherent to mucosal enterocytes, and mainly seen in the gut lumen by histologic and EM examination (data not shown). B2F1 transformed with pEB310 was not tested in the gnotobiotic piglet model.

(ii) Mouse colonization model. EHEC strains colonize the intestinal tract of streptomycin treated mice (Wadolkowski *et al.*, 1990). The presence of the 60-megadalton (MDa) plasmid is not required for intestinal colonization of the mice, but two-thirds of the mice co-fed 933 and its cured derivative, 933cu, selectively clear 933cu. We sought to determine the role, if any, of the *eaeA* gene in EHEC colonization of the mouse gut. Mice were fed 10^{10} CFU of either the *eaeA* mutant, the wild type parent, or both strains in competition. The infected animals were monitored for colonization by each organism and for survival over the next 15 days. All infected mice were colonized within 24 hours, as determined by fecal excretion of the infecting organism (Fig. 19). Levels of bacterial colonization remained high over the course of the experiment (10^{7-9} CFU/g of feces). The parent and 86-24*eaeΔ10* competed equally well for attachment sites in the mouse intestine. The standard errors were large by day 13 of the trial, because some of the mice in each group began to clear the infecting organisms. One mouse from each of the three groups died in the course of the experiment (mean time to death

Figure 19. Colonization of the streptomycin-treated mouse (n=4) intestine by EHEC strain 86-24 (■ and □) or 86-24*eae*Δ*10* (▲ and Δ) fed alone (open symbols) or in competition with each other (filled symbols). The mean CFU/gram feces is given ± 2SD at each time point. At days 8 and 9, one mouse in each group died which resulted in an n of 3 for the remainder of the study. On days 11, 13, and 15, one mouse per group had cleared the infecting organism.


CFU/gm feces
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Day post feeding

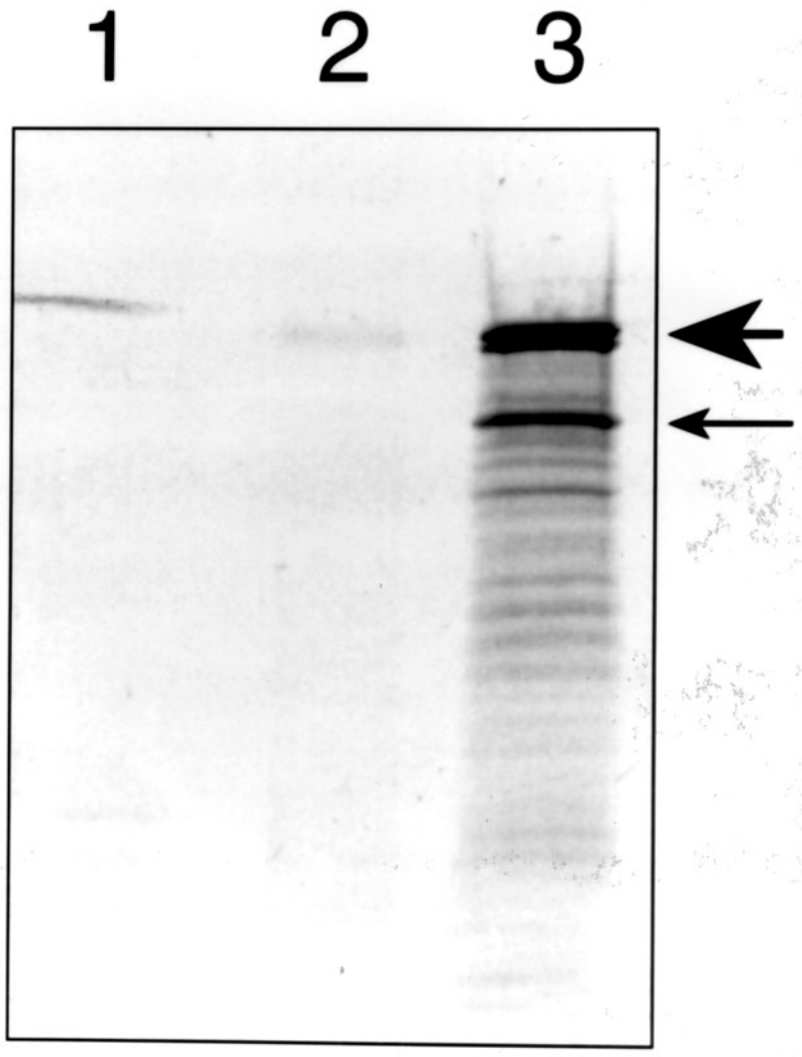
= 8.67days). From these data, we concluded that the *eaeA* locus does not play a role in the colonization of streptomycin treated mice.

III. Analysis of the *eaeA* gene product - Intimin

Expression of *eaeA* product under control of the T7 promoter. Because the data of Donnenberg *et al.* (1993b) and our results indicated a critical role for the eaeA product of EHEC O157:H7 adherence to epithelial cells *in vitro* and *in vivo* in the piglet intestine, we sought to characterize intimin at the protein level. Plasmid pEB310 contains the wild type *eaeA* locus cloned in an orientation such that the T7 RNA polymerase-dependent promoter (P_{T7}) can drive transcription of the gene. The intimin protein encoded by pEB310 was over-expressed in a background containing a temperature induced copy of the T7 RNA polymerase on plasmid pGP1-2. Three major products of the cloned insert were observed (Fig. 20). The major bands were a doublet at 97 kDa and a singlet at approximately 80,000 Da. The majority of the intimin expressed by this method remained associated with the bacterial membrane fraction even after sonic disruption of the host bacterium and addition of mild detergent to the extraction buffer.

Construction and purification of intimin fusion proteins. The insolubility of the intimin protein, combined with the abundance of other native *E. coli* protein species

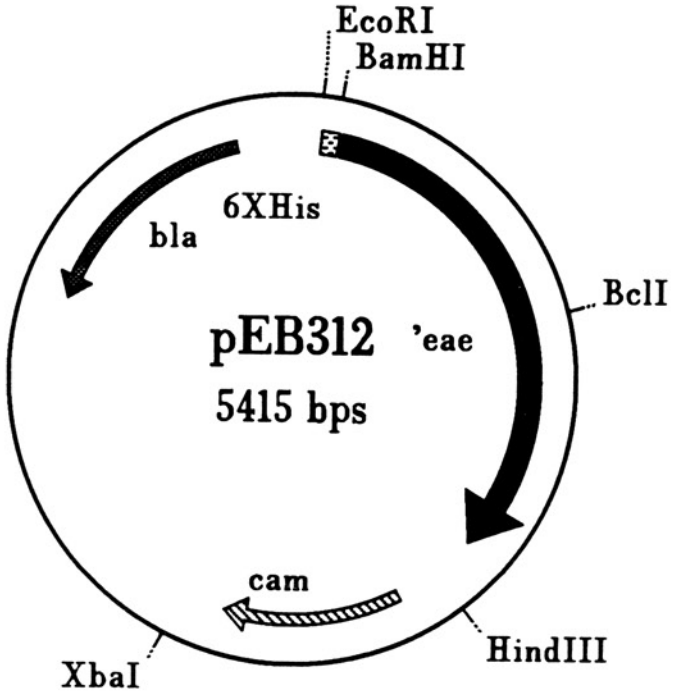
Figure 20. Expression of the product of the EHEC *eaeA* locus under control of the T7 RNA polymerase dependent promoter. Lane 1, DH5α(pGP1-2, pBRKS$^-$); lane 2, [^{14}C]-labeled carbonic anhydrase marker (M_r = 97,400), lane 3, DH5α(pGP1-2, pEB310). The large arrow is pointing to the doublet thought to be intimin; the smaller arrow indicates the minor product at M_r = 80,000.


1
2
3

in the 97 kDa range, made purification of the native protein difficult. For these reasons, we sought to create fusion proteins for rapid and easy purification of intimin. In a first attempt, we sought to create a *malE*::*eaeA* fusion construct that would result in an amino terminal fusion of intimin starting at the second amino acid with the maltose binding protein (MBP). The maltose binding protein binds readily to maltose and maltose analogues, a feature that can be used to purify the MBP fusion protein. Several attempts to create the MBP::intimin fusion were unsuccessful. Recombinant plasmids recovered after ligation of a PCR-derived DNA fragment encoding *eaeA* with the *malE* vector plasmids resulted in deletions and rearrangements of the predicted product (data not shown).

Next, we attempted fo create fusions with the 6X histidine (6xHis) fusion protein system of Qiagen, Inc. The histidine fusion method was particularly attractive for three reasons: i) a small peptide is added to the amino terminal end of the protein rather than a bulky protein like MBP, ii) the 6XHis tag binds tightly to a nickel affinity matrix which facilitates purification of large quantities of material for further studies, and iii) the expression system maintains tight control of the 6XHis fusion proteins to prevent any possible lethal effects of the recombinant protein on the host *E. coli* strain. Two fusions were constructed as outlined in Fig. 21. The longer fusion, RIHisEae, was ~101 kDa and encoded 900 out of 935 predicted amino acids. This fusion was constructed such that the amino terminal 35 amino acids were deleted to remove any potential signal sequence that might target the fusion protein to the membrane or lead to cleavage of the Histidine tag

Figure 21. Plasmid maps of pEB312 (RVHdHisEae) and pEB313 (RIHisEae), the constructs that result in the His::intimin fusions. Inserts were cloned into vector pQE32 to create fusions in-frame with the 6XHis sequences (dotted boxes). 'eae, truncated *eaeA* gene; bla, β-lactamase; cam, chloramphenicol acetyl transferase.


EcoRI
BamHI
6XHis
bla
BclI
pEB312
'eae
5415 bps
cam
XbaI
HindIII

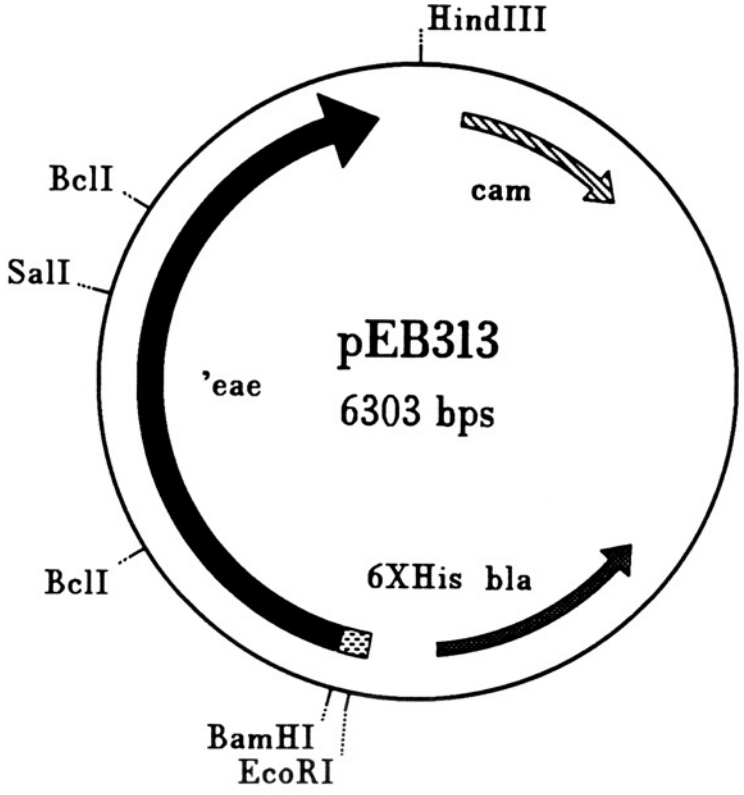
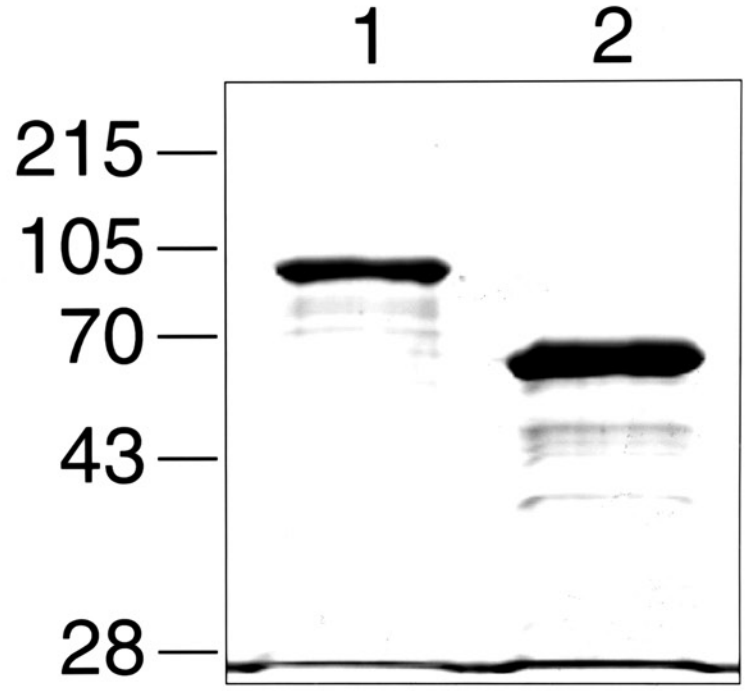

HindIII
BclI
cam
SalI
pEB313
'eae
6303 bps
BclI
6XHis bla
BamHI
EcoRI

Figure 22. Coomassie stained SDS-PAGE gel of His::intimin fusion proteins that have been purified over the Ni-NTA resin. RIHisEae is 101 kDa (lane 1) and RVHdHisEae is 65,000 kDa (lane 2). The M_r of the markers is indicated to the left.


1
2
215
105
70
43
28

from the intimin. The shorter fusion, RVHdHisEae, was ~65 kDa and encoded 604 amino acids. This construct contains the carboxyl two thirds of the wild-type intimin protein. As with the P_{T7} -expressed intimin, the fusions remained primarily in the insoluble pellet after sonic disruption of the host *E. coli*. Therefore, we included urea which allowed extraction of the fusion proteins from the insoluble pellet. Both His::intimin fusion constructs were purified to homogeneity over nickel affinity columns (Fig. 22).

Antisera to His:intimin fusion proteins. Intimin specific antibodies were produced in both mice and rabbits that had been injected with RIHisEae fusion protein. All of the antisera achieved high titer (>10,000 for the rabbit sera and 1300-5000 for the mouse antibodies) in the ELISA assay. The antisera raised in mice was monospecific and recognized both of the fusion proteins (data not shown) and the native intimin expressed under the control of the T7 promotor in pEB310 (Fig. 23). The antisera obtained from the rabbits recognized additional *E. coli* proteins when reacted with extracts in an immunoblot (data not shown).

Blocking of HEp-2 cell adherence by anti-intimin antibodies. The anti-intimin antibodies raised in mice blocked adherence of strain 86-24 to HEp-2 cells when preincubated with the bacteria for 30 minutes prior to the infection of the monolayer (Fig. 24). At a 1:1000 dilution of of the anti-intimin sera, partial blocking of bacterial adherence was observed. As the concentration of anti-intimin

Figure 23. Reactivity of mouse anti-intimin antibodies against intimin expressed from plasmid pEB310. Lane 1, DH5α(pGP1-2, pBRKS$^-$) membrane associated fraction; lane 2, DH5α(pGP1-2, pEB3 DH5α(pGP1-2, pEB310) supernatant fraction disrupted cells; lane 3, DH5α(pGP1-2, pEB310) membrane associated fraction. The arrows indicate the 97 kDa protein, intimin.

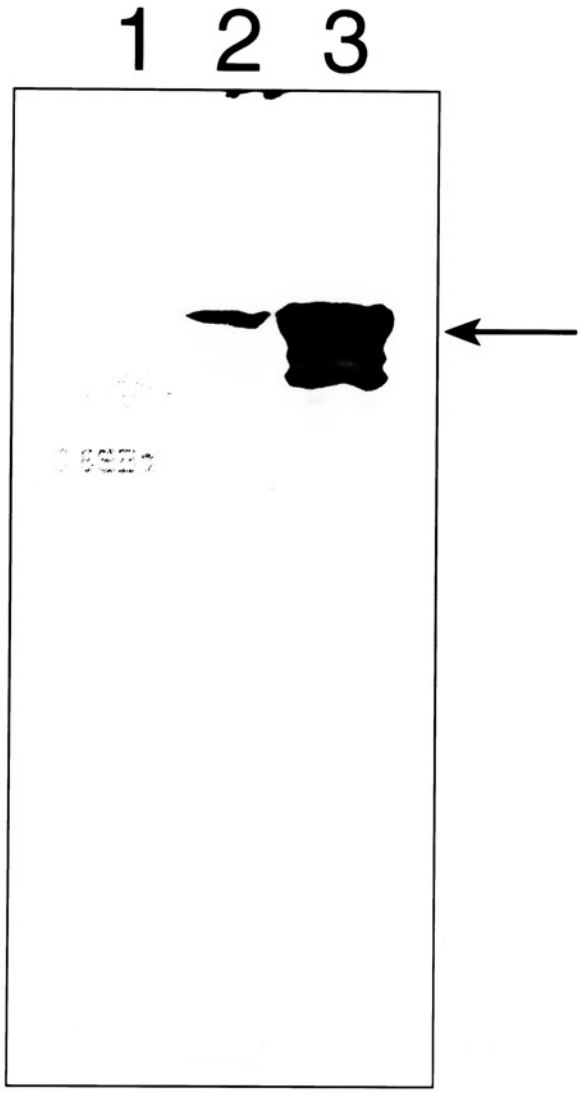
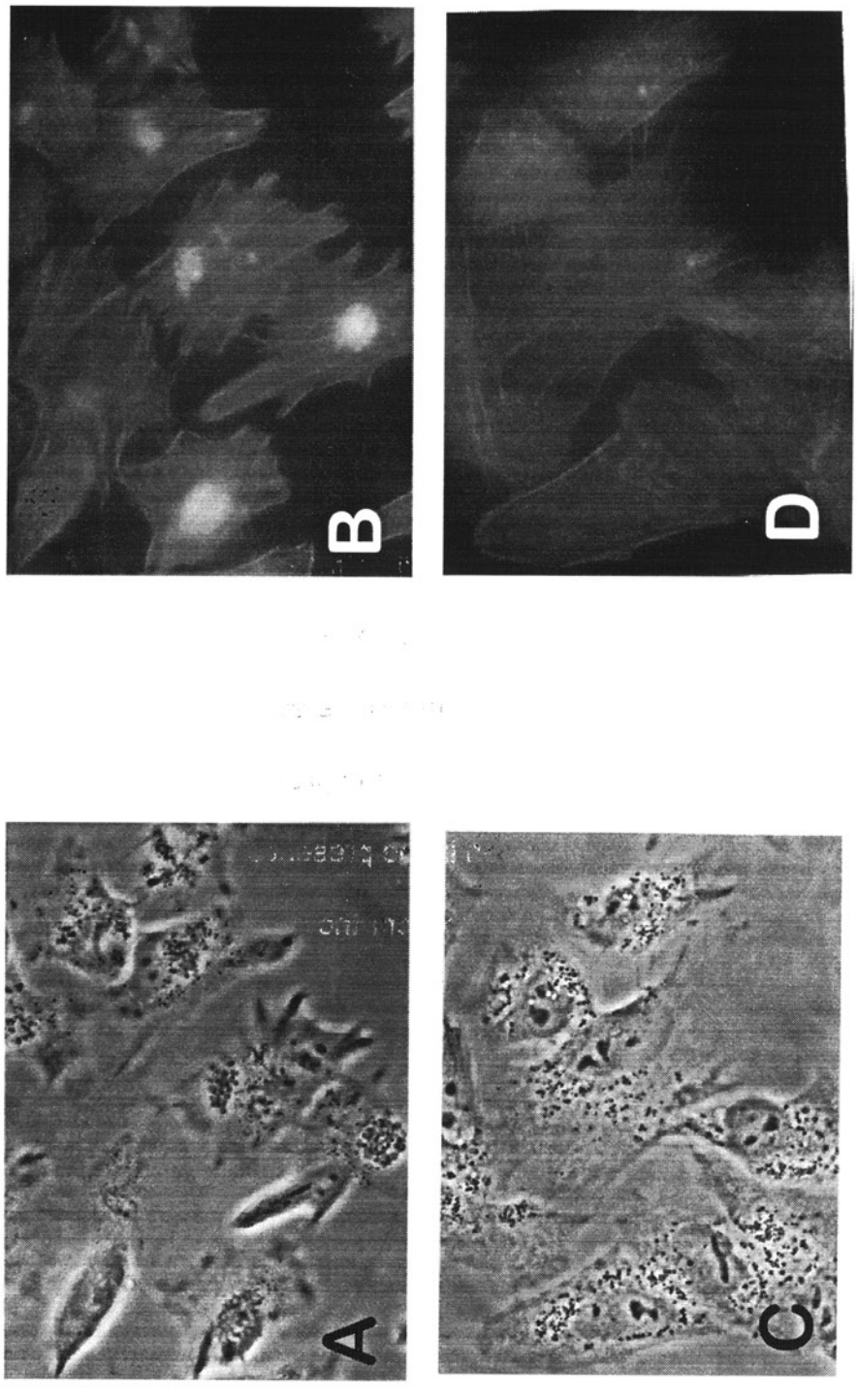

1 2 3

Figure 24. Adherence of EHEC strain 86-24 to HEp-2 cells is blocked by anti-intimin antibodies. The standard HEp-2 cell attachment assay with wild-type 86-24 was conducted in the presence of a 1:100 dilution of mouse antisera specific for intimin. Microcolonies formed in the absence of antisera (A - phase contrast and B - FAS) were not observed in the presence of anti-intimin antibodies (C - phase and D - FAS). Incubation in the presence of preimmune mouse sera gave results that were indistinguishable from those obtained in the absence of antisera.


B
D
A
C

antibodies increased to a 1:10 dilution, total blocking of EHEC 86-24 adherence to HEp-2 cells was achieved. With preimmune sera, some interference was seen at dilutions ≤ 1:100.

Recognition of 97 kDa bacterial outer membrane by anti-intimin antisera. Antibodies to the intimin fusion protein recognized a 97 kDa protein in the sarkosyl insoluble fraction of extracts from 86-24, 933, and 933cu, as well as from EPEC (representative data are shown in Fig. 25, panels A and B). The mouse anti-intimin sera did not recognize any proteins in outer membrane extracts from the K-12 *E. coli* DH5α or the *eaeA*⁻ O91:H21 isolate, B2F1. The anti-intimin antisera also recognized a 50 kDa protein in the fraction of bacteria that included both the cytoplasmic and periplasmic contents. This 50 kDa species was only present in preparations that had been stored at 4° C or those preparations that had gone through at least one freeze (-20°C)-thaw cycle. From these data, we conclude that EHEC intimin is a 97 kDa outer membrane protein expressed by bacterial strains that carried the *eaeA* locus and that the initimins expressed by EHEC and EPEC are immunologically related.

Recognition of EHEC proteins by HC patient sera. Convalescent immune sera tested from hemorrhagic colitis patients (kindly provided by T. Barrett at the CDC, Atlanta, GA) reacted with P_{T7}-expressed intimin preparations in a Western immunoblot (Fig. 26, A and B). To decrease reactivity of the hemorrhagic colitis

Figure 25. Immunoblots of mouse anti-intimin antisera against bacterial fractions from wild-type 86-24, the *eaeA* mutant with and without plasmid pEB310, or the K-12 strain XL-1 Blue. 1, low speed supernatant; 2, high speed supernatant; 3, high speed pellet fraction; 4, sarkosyl precipitate from the high speed pellet. Molecular weight markers are to the left; the arrow indicates intimin.

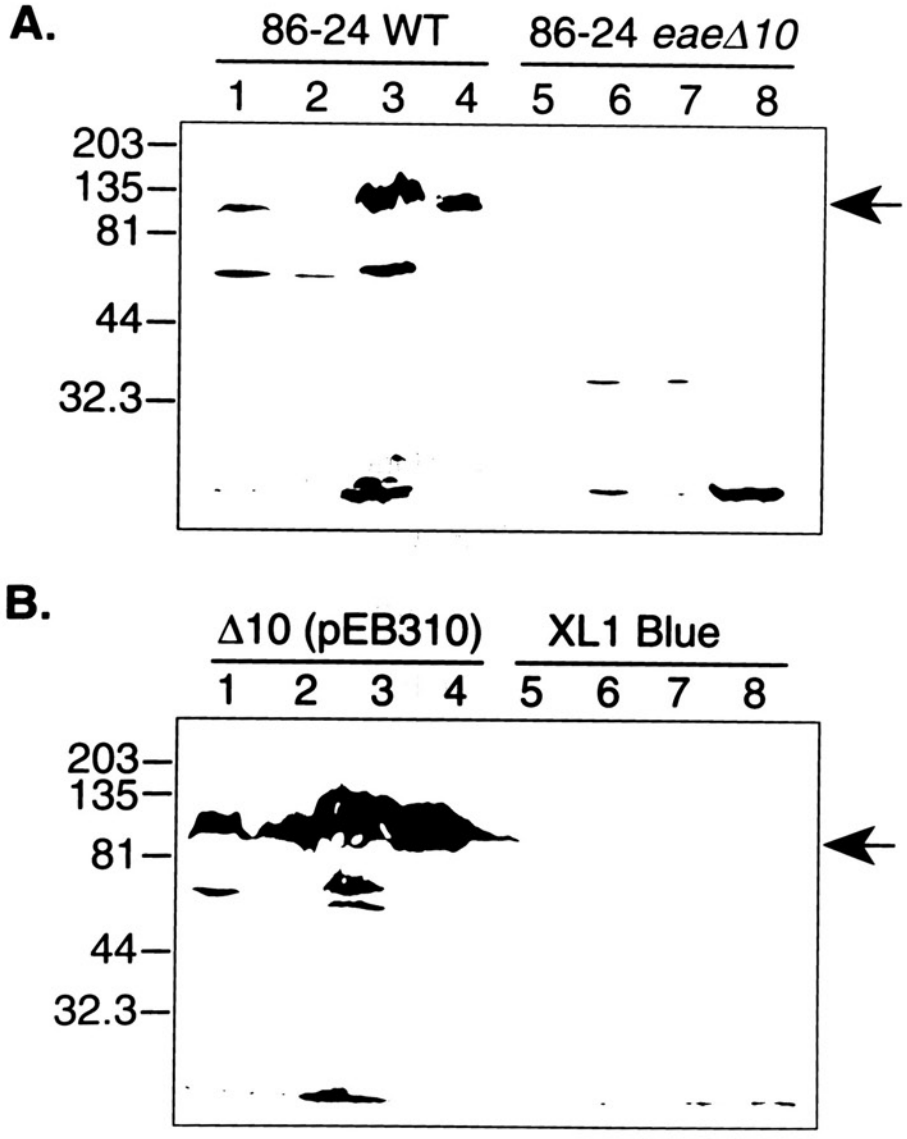
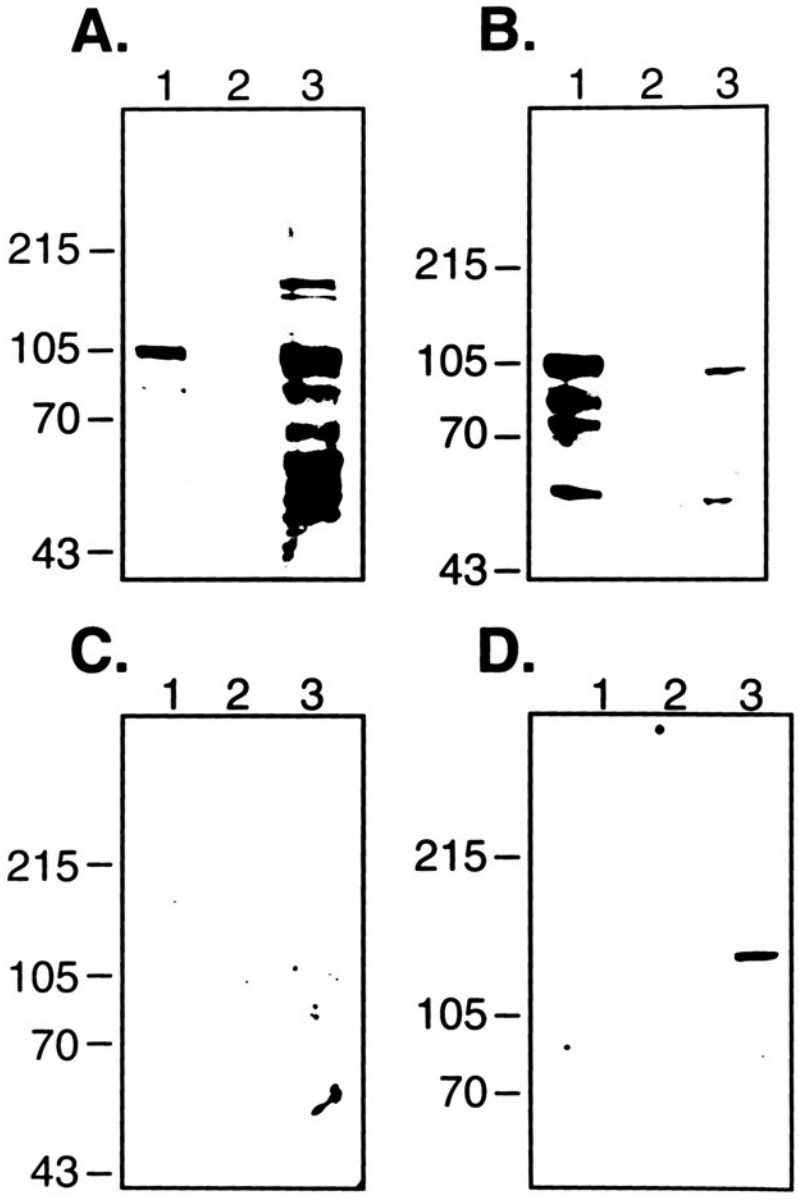

A.
86-24 WT
86-24 eaeΔ10
1 2 3 4 5 6 7 8
203
135
81
44
32.3
B.
Δ10 (pEB310)
XL1 Blue
1 2 3 4 5 6 7 8
203
135
81
44
32.3

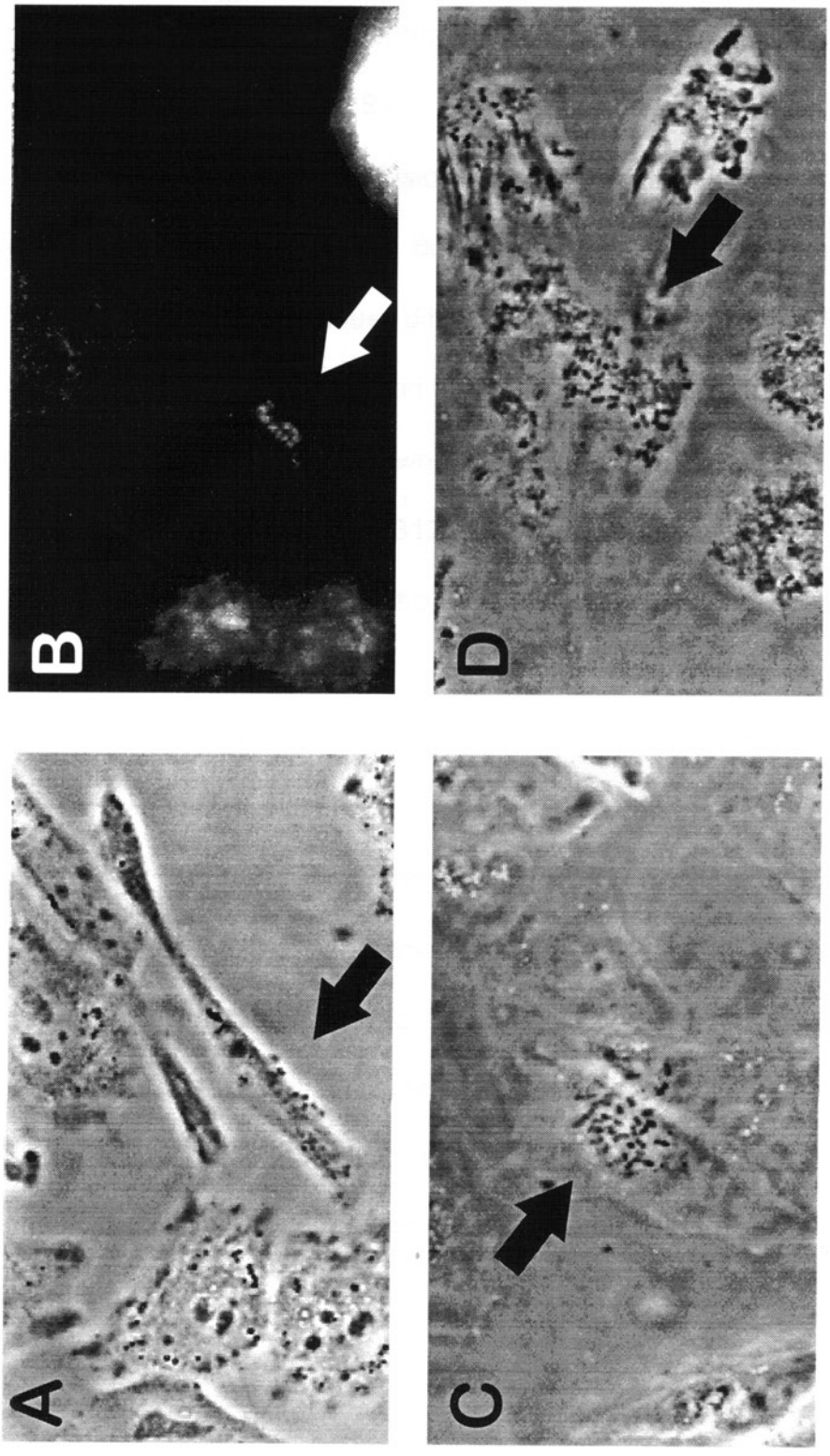
Figure 26. Immunoreactivity of HC patient sera against EHEC intimin. Proteins encoded by plasmid pEB310 were expressed under control of the T7 RNA-dependent polymerase and extracted by sonic disruption of the bacteria as described in the text. Patient sera absorbed against *E. coli* containing pGP1-2 and the expression vector were reacted at 1:1000 against the proteins which were separated by denaturing PAGE and transferred to nitrocellulose (Panel A and B). Panels C and D demonstrate reactivity of two normal human sera control samples. Lane 1, ammonium sulfate precipitate fraction of pEB310-encoded proteins; lane 2, vector control proteins; lane 3, whole cell sonic extracts from induced cultures of DH5α(pGP1-2, pEB310). The molecular weight markers (kDa) are indicated. The intense protein band at ~97 kDa is thought to be intimin.


A.
1 2 3
215
105
70
43
B.
1 2 3
215
105
70
43
C.
1 2 3
215
105
70
43
D.
1 2 3
215
105
70

patients' sera with *E. coli* proteins in the expression system, sera samples were adsorbed with whole cell extracts of DH5α transformed with pGP1-2 and pBRKS⁻ (the expression vector). After adsorption, some residual activity with the normal sera controls versus the protiens in the pEB310 expression system reamained, but was weak (Fig. 26, C and D). After adsorption, the HC patient sera still recognized many *E. coli* proteins, but the reaction with intimin remained strong.

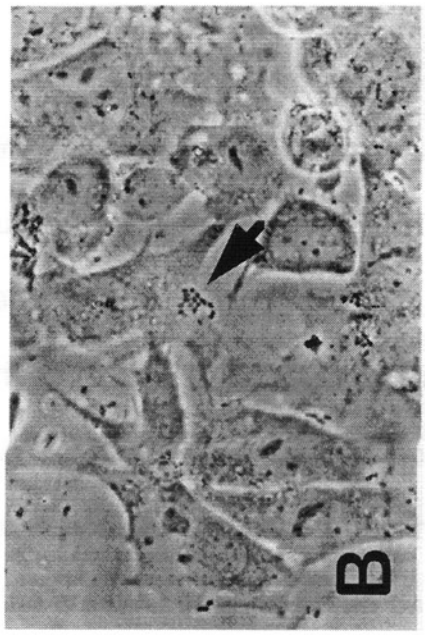
Effect of addition of His:intimin fusion proteins to the adherence assay. Both of the fusion proteins were tested for the capacity to block binding of 86-24 to HEp-2 or HCT-8 cells *in vitro*. We hypothesized that preincubation of the eukaryotic cells with excess His:intimin would occupy most or all of the receptor binding sites available on the cell surface for intimin and block any further adherence by the wild-type bacteria that expressed intimin on its outer surface. To test this theory, we incubated HEp-2 cells with 20ng - 20μg of RIHisEae for 30 minutes prior to the addition of 86-24 to the monolayer. The infected monolayers were then washed extensively, stained with FITC-phalloidin, and observed microscopically. The results are presented in Fig. 27. Contrary to the expectation that the fusion proteins would block adherence, the fusions enhanced binding of 86-24 to HEp-2 cells. The size of the 86-24 microcolony as well as the total number of HEp-2 cells with adherent microcolonies increased as the concentration of RIHisEae increased. At high doses (20 μg), the fusion protein caused the HEp-2 cells to show aberrant appendages and processes (data not shown). For this

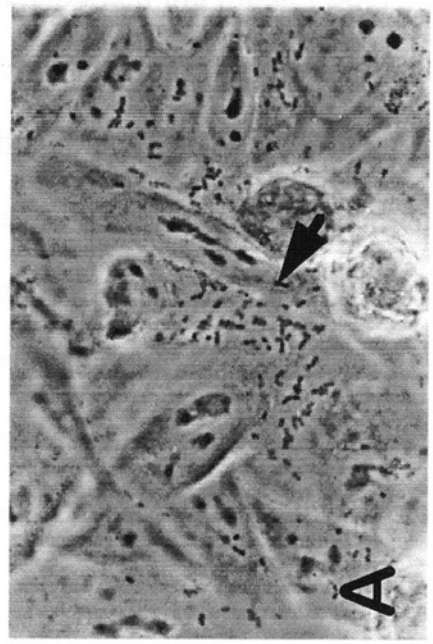
Figure 27. Enhancement of microcolony formation of strain 86-24 by addition of the intimin fusion protein, RIHisEae. Panels A and B show LA (indicated by the arrows) exhibited by wild-type 86-24 on HEp-2 cells in the presence of 20 ng of the histidine fusion protein. In the presence of 2 μg or 20μg RIHisEae, microcolonies (indicated by the arrows) were larger and a greater proportion of the HEp-2 cell surface was covered by bacteria (C and D, respectively).


B
D
A
C

reason, we picked 1-2 μg as the optimal dose for further studies. When added exogenously to HEp-2 cells, RIHisEae complemented the HEp-2-cell binding defect of 86-24*eaeΔ10* (Fig. 28 A) and rendered B2F1 capable of adherence to HEp-2 cells (data not shown). The shorter fusion protein, RVHdHisEae, also complemented foradherence (Fig. 28 B with 86-24*eaeΔ10*). A similar amino terminal fusion of 6 histidine residues to mouse dihydrofolate reductase (HisDHFR) did not enhance the adherence of 86-24 nor did it confer binding on nonadherent strains (B2F1 or K-12 *E. coli*) (data not shown). Moreover, the plasmids that encoded the intimin fusion proteins, pEB312 and pEB313, were able to complement 86-24*eaeΔ10* for attachment *in vitro*. The fusion proteins localized to the insoluble pellet fraction after sonic disruption of the host strains which suggested that these proteins are localized to the membrane. Plasmid pQE16 which encodes the His::DHFR fusion did not complement 86-24*eaeΔ10* (data not shown). That the irrelevant protein fusion with the 6 histidine residues did not confer HEp-2 cell adherence on the *eaeA* mutant indicates that the histidine residues added to intimin are not responsible for the activity observed for the exogenously added His::intimin fusions. The enhancement or complementation of EHEC binding to HEp-2 cells observed with exogenous RIHisEae and RVHdHisEae suggests that intimin is capable of interacting with both the bacteria and the epithelial cell.

Figure 28. The histidine-intimin fusion proteins render mutant 86-24*eaeΔ10* adherent to HEp-2 cells. Adherence assays with the in-frame deletion mutant strain conducted in the presence of 1 µg RIHisEae (A) or 2 µg RVHdHisEae (B) resulted in microcolony formation by the bacteria (indicated by the arrows).


B


A

Discussion

The major findings of this dissertation study which paralleled the specific aims listed in the introduction were as follows. First, a tissue culture model of EHEC adherence was established and used to identify a new adherence phenotype among intestinally-derived *E. coli*, designated log jam, as well as to assess the requirement for *eaeA* in EHEC O157:H7 LA/FAS positive adherence. Second, the role of the *eaeA* gene in EHEC adherence to epithelial cells *in vitro* and *in vivo* was demonstrated by the creation of an in-frame deletion mutation in the *eaeA* locus of EHEC O157:H7 strain 86-24. The mutant, 86-24*eaeΔ10*, did not adhere to HEp-2 cells or to cause the A/E lesion in gnotobiotic piglets. A complete clone of the *eaeA* gene fully complemented the mutant both *in vitro* and *in vivo*. Third, the capacity of a histidine-intimin fusion protein to enhance EHEC adherence to HEp-2 cells rather than block the adhesin-receptor interaction was described. These findings were extended by the restoration of HEp-2 cell adherence of the *eaeA* mutant and O91:H21 EHEC strain B2F1 by the His::intimin fusion proteins. Conversely, antibodies to intimin blocked adherence of EHEC strains to HEp-2 cells. Finally, the EHEC *eaeA* gene encodes a 97 kDa outer membrane protein immunologically similar to the intimin protein expressed by EPEC strains.

I. Adherence of EHEC strains to epithelial cells in culture. By extending the incubation period of the bacteria with the HEp-2 cells in the prototypic adherence assay from 3 h to 6 h, EHEC were found to be LA/FAS positive. Although EHEC strains had been reported to adhere to certain types of epithelial cells in culture (Karch *et al.*, 1987; Sherman and Soni, 1988; Knutton *et al.*, 1989; Toth *et al.*, 1990), only Knutton *et al.* (1989) had reported the localized adherence phenotype for EHEC. Reports with Henle 407 cells by Karch *et al.* (1987) for strain EDL933 and by Toth et al. (1990) for EHEC strain 7785 showed bacteria adherent singly or in pairs or lined up at the edges of the epithelial cells rather than in microcolonies on top of the cells. In the present study, some variability in the adherence phenotype of EHEC strains was also noted. The prototypic EHEC O157:H7 isolate EDL933 was poorly adherent to HEp-2 cells; only isolated bacteria were observed adherent to the epithelial cells. By contrast, a majority of the other EHEC isolates tested were LA positive and formed distinct clusters on the epithelial cell membrane. The reasons for the poor adherence of EHEC 933 were not determined. However, other SLT-I- and SLT-II-producing EHEC O157:H7 strains were also less adherent than the SLT-II- only producing strain 86-24.

The kinetics of attachment of strain 87-23 (O157:H7, SLT$^-$) revealed a lag phase before the adherence levels became statistically different from those of the K-12 E. coli DH5α or the LA phenotype was observed. The events that occur during this 2-3 hour period were not defined. Finlay *et al.* (1989) reported that interaction of Salmonella spp. with epithelial cells in culture or that growth of

these bacteria in the culture medium led to the induction of at least six new proteins and the down-regulation of a minimum of seven proteins when compared with bacteria in the assay supernatant. The newly synthesized proteins are essential for *Salmonella* adherence to and invasion of epithelial cells *in vitro* (Finlay *et al.*, 1989). Lee and Falkow (1990) concluded that the capacity of *Salmonella* to enter host cells is influenced by the growth state of the organisms and by the oxygen limitation the bacteria encounter when in close association with or internalized by host cells. In this study, *de novo* protein synthesis or up-regulation of EHEC bacterial proteins in the presence of the HEp-2 cells may explain the delay in the bacterial adherence as measured by LA morphology. The results of the chloramphenicol and heat treatment assays, taken together with the microscopic correlate of the kinetic assay, support this possibility.

The role of the 60 MDa EHEC plasmid in the adherence of EHEC isolates to epithelial cells has not been conclusively demonstrated by others or by this investigation (Karch *et al.*, 1987; Tzipori *et al.*, 1987; Toth *et al.*, 1990). Indeed, we found that EDL933 was poorly adherent to HEp-2 cells, but its plasmid-cured derivative, 933cu, was LA/FAS positive (Figs. 2 and 3). Our results with this nonfimbriated (Karch *et al.*, 1987) plasmid-cured derivative differ from those of Karch (1987) and Toth (1990) who reported that EHEC strains cured of the large plasmid no longer bound epithelial cells in culture. It is possible that had Karch *et al.* (1987) conducted the adherence assays for 6 h instead of the then standard

3 h, 933cu would have been adherent. Our findings indicate that the presence of the EHEC plasmid may negatively affect the LA/FAS phenotype.

EPEC strains cured of the EAF plasmid were originally found not to adhere to HEp-2 cells (Baldini *et al.*, 1983). However, more recent results indicate that EAF$^-$ EPEC require a longer incubation time (6 h) to give an LA/FAS phenotype of attachment (Jerse *et al.*, 1990). We now know that EAF plasmid-encoded factors positively modulate expression of chromosomally encoded EPEC adhesins. Indeed, EAF$^+$ EPEC strains express more intimin (Jerse *et al.*, 1991; Gómez-Duarte and Kaper, 1995). Furthermore, the EAF plasmid itself encodes additional factors (BFP - Girón *et al.*, 1993a; Girón *et al.*, 1993b; and EAF - Nataro *et al.*, 1985; Donnenberg and Kaper, 1992) which contribute to EPEC adherence. Further analysis is required to define the role of the EHEC 60 MDa plasmid in adherence and to establish the presence of regulatory factors encoded by the plasmid. Strain 86-24 may be a better candidate than EDL933 for dissecting this issue since this EHEC strain consistently binds HEp-2 cells in an LA/FAS manner.

Some disparity between the quantitative and qualitative analyses was evident. For example, 10^5 adherent CFU/ml *E. coli* K-12 DH5α were recovered by quantitation of bacterial counts, but bacteria were rarely observed by microscopic observation. Conversely, on occasion the quantitative data were similar for two different strains, but the microscopic findings revealed significant differences between the adherence phenotype of the two strains. As mentioned earlier, we

elected to use the microscopic findings as the primary criteria for assessing the adherence capacity of wild-type bacterial strains as well as mutants.

Another variable in the adherence procedure was that the number of bacteria present in a microcolony on a HEp-2 cell differed depending on the staining procedure followed. Bacterial-epithelial cell preparations that had been fixed with formalin for the FAS test had larger microcolonies than those slides that were fixed with methanol or acetone for Giemsa staining (data not shown). These disparate findings may be indicative of the strength of the interbacterial bond in a microcolony or of the bacterial adhesin-receptor interaction. The level of manipulation and washing required for each of the techniques could have led to the discrepancies between the two methods.

A novel pattern of EHEC attachment visible only on HCT-8 cells was described for the first time in this dissertation. This log jam pattern of adherence was characterized by bacteria attached to and lined up at the junctions between the HCT-8 cells. The interaction was mannose-resistant, *eaeA* independent, LPS type nonspecific, and not due to invasion of the HCT-8 cells. Because the log jam pattern was shared among a variety of intestinally-derived *E. coli* of both pathogenic and nonpathogenic types, this phenotype does not appear to be associated with virulence. The correlation between the log jam pattern of adherence and the intestinally-derived epithelial cells suggests that this phenotype may represent a basal adherence mechanism that allows a variety of *E. coli* to bind to and colonize the human intestine whether or not the organism expresses

additional specific adhesive factors. Further, our data indicate that EHEC isolates do not invade HCT-8 cells, but rather that this ileocecal cell line may nonspecifcally take up a small proportion of the EHEC or the normal flora control strain to which the cells are exposed. It should be noted that the invasion percentages we calculated for EHEC are similar to those reported by Oelschlaeger *et al.* (1994). However, no normal flora control was included in that report. Additionally, electron microscopic evaluation of the intestine of gnotobiotic pigs infected with EHEC 86-24 revealed that the organisms attached to but did not invade the mucosal surface (Donnenberg *et al.*, 1993b). To our knowledge, there have been no reports to date of EHEC found in tissue samples from biopsies of hemorrhagic colitis patients.

II. The role of *eaeA* in the adherence and A/E lesion formation of EHEC O157:H7.

In this investigation the pivotal role of the *eaeA* locus both *in vitro* (by LA/FAS on HEp-2 and HCT-8 cells) and *in vivo* (by A/E lesion formation in gnotobiotic piglet) was established through the use of a mutant with an in-frame deletion in EHEC O157:H7 *eaeA*. The *in vivo* data confirm the previous finding of Donnenberg *et al.* (1993b), that the *eaeA* gene product, intimin, is required for A/E lesion formation *in vivo*. By contrast our *in vitro* results differ from those of Donnenberg *et al.* (1993b), who found that an insertion-deletion mutation in eaeA (UMD619) could not be complemented for adherence to HEp-2 cells by plasmids

encoding intimin; the same plasmids were able to complement 86-24*eaeΔ10*. Our *in vitro* data are also different from those of Louie *et al.* (1993), who reported that an insertional inactivation of the eaeA locus in EHEC strain CL8 abolished FAS activity but not cytoadherence by the bacterium. An explanation for these discrepant results is that the *eaeA* mutations in both UMD619 and CL8-KO1 are polar on downstream genes (Donnenberg *et al.*, 1993c; McDaniel *et al.*, 1995) necessary for intimate attachment *in vitro*.

UMD619 was constructed by deletion of an internal *Bcl*I fragment from the cloned *eaeA* gene of 933 and the insertion of a tetracycline resistance determinant to interrupt the coding sequence of the gene (Donnenberg *et al.*, 1993b). The entire construct was recombined into the chromosome of EHEC 86-24 to replace the wild-type copy of the *eaeA* locus. The insertion of additional sequences into UMD619 may have introduced termination signals that interrupt expression of genes downstream of *eaeA*. CL8-KO1 was constructed by recombination of a plasmid carrying a mutated copy of the *eaeA* locus from EHEC strain CL-8 into the chromosomal copy of *eaeA* (Louie *et al.*, 1993). The entire recombinant plasmid was integrated into the bacterial chromosome to interrupt the *eaeA* gene. Additionally, the CL8 strain has been found to bind to HEp-2 cells in a pattern similar to that of enteroaggregative *E. coli* (S. Savarino, personal communication) an observation that could confound interpretation of the adherence phenotype of the CL8-KO1 mutant.

The in-frame mutation in 86-24*eaeΔ10* described here appeared to have no such polar effects, a hypothesis supported by the fact that the mutation was complemented by intimin alone. The apparently polar nature of the previous *eaeA* mutants suggests that multiple gene products are involved in EHEC adherence to epithelial cells. More precise mutations are required to discern all of the loci involved in the steps of attachment and lesion formation.

In support of the polarity argument, the plasmids that were unable to complement UMD619 for HEp-2 cell adherence were found to complement the 86-24*eaeΔ10 in vitro*. We conclude that pEB310, pEB311, pCVD436, and pCDV444 produce a functional intimin product, but only 86-24*eaeΔ10* expresses the additional factor(s) required for full LA/FAS on HEp-2 cells. The hypothesized additional factors may not be directly involved in the adherence of EHEC *in vitro*, but rather may aid in the localization or presentation of intimin by the bacterium. These additional factors may not be required *in vivo* but may be necessary for the LA/FAS phenotype *in vitro*. Neither plasmid pEB310 nor pEB311 was able to confer HEp-2 cell adherence on a K12 host strain (DH5α or XL1-Blue). These data are consistent with the findings that neither EPEC *eaeA* (Jerse *et al.*, 1990) nor EHEC *eaeA* is sufficient to confer adherence on K-12 strains.

To date, three EHEC factors have been implicated in intimate adherence to epithelial cells and the capacity to cause the A/E lesion *in vivo*. These factors include: i) the 60 MDa plasmid [termed pO157 by Toth *et al.* (1990) and Karch (1987)], ii) a 94 kDa outer membrane protein required for EHEC

adherence to Henle 407 cells (Sherman and Soni, 1988; Sherman *et al.*, 1991), and iii) intimin. Dytoc *et al.* (1993) demonstrated that the factor(s) encoded by pO157 were distinct from the *eaeA* product and the 94 kDa OMP. Nor is intimin the 94 kDa OMP identified by Sherman. Additionally, two independent Tn*phoA* mutants of EHEC strain CL-8 (O157:H7) were isolated and found deficient in bacterial factors necessary for A/E lesion formation. Neither the cloned *eaeA* locus nor pO157 transformed separately into an *E. coli* K-12 background mediate A/E lesion formation *in vitro* (HEp-2 cells) or *in vivo* (ligated rabbit ileal loops) (Dytoc *et al.*, 1993). The gene encoding the 94 kDa protein described by Sherman *et al.* (1991; Sherman and Soni, 1988), has not been cloned, but antibodies that recognize the 94 kDa OMP block adherence of EHEC strains to epithelial cells in culture (Sherman et al., 1991). These same antibodies do not recognize intimin (Dytoc *et al.*, 1993). These results from Dytoc and colleagues support the supposition that multiple determinants are required by EHEC strains to cause the A/E lesion formation.

More recent data demonstrate the genetic complexity of EHEC adherence. McDaniel *et al.* (1995) described a 35 kb region of the EPEC chromosome that is shared by attaching and effacing enteric bacteria: EHEC, RDEC-1, C. freundii, and *H. alvei* strains. The 35 kb region, called LEE (locus of enterocyte effacement) encodes at least 3 identified genes (including *eaeA*) as well as two loci thought to be involved in secretion of EPEC virulence factors (McDaniel *et al.*, 1995). The LEE locus appears to be analogous to the pathogenicity islands

of UPEC (Blum *et al.*, 1994) and, in fact, is inserted into the *E. coli* chromosome at the identical site (McDaniel *et al.*, 1995). Further studies will be required to determine if the mutations in EHEC A/E lesion-associated loci identified by Dytoc and colleagues (1993) map within the LEE region of EHEC.

Intimate adherence in the gnotobiotic piglet model of EHEC infection appears to be necessary for the development of pathological findings other than the A/E lesion, such as enterocyte sloughing and inflammation. Both piglets infected with wild-type 86-24 showed signs of mesocolonic edema. By contrast, one of the piglets fed the *eaeA* mutant developed mild edema in the absence of bacterial adherence as did one of the animals fed B2F1 (O91:H21, *eaeA* negative). The edema in the spiral colon has been associated with the production of SLTs by the infecting organisms (MacLeod *et al.*, 1991; Dykstra *et al.*, 1993). The edema in the absence of bacterial adherence may be evidence of the effects of SLT absorbed directly from the gut lumen, since SLT-IIe can bind to pig intestinal epithelium (Waddell *et al.*, 1994), and SLT-I (Dykstra *et al.*, 1993) and SLT-II (MacLeod *et al.*, 1991) injected parenterally induce this lesion in pigs. That the mesocolonic edema was more severe in the piglets infected with the wild-type *eaeA*$^{+}$ strain may indicate a role for intimate adherence in toxin delivery to the epithelial cells and ultimately to the submucosa. Studies with other *E. coli* types have indicated that close adherence to cells results in enhanced toxicity (Zafriri *et al.*, 1987). To address the hypothesis that intimate adherence is required for toxin delivery by EHEC, an isogenic set of intimin and toxin mutants should be tested

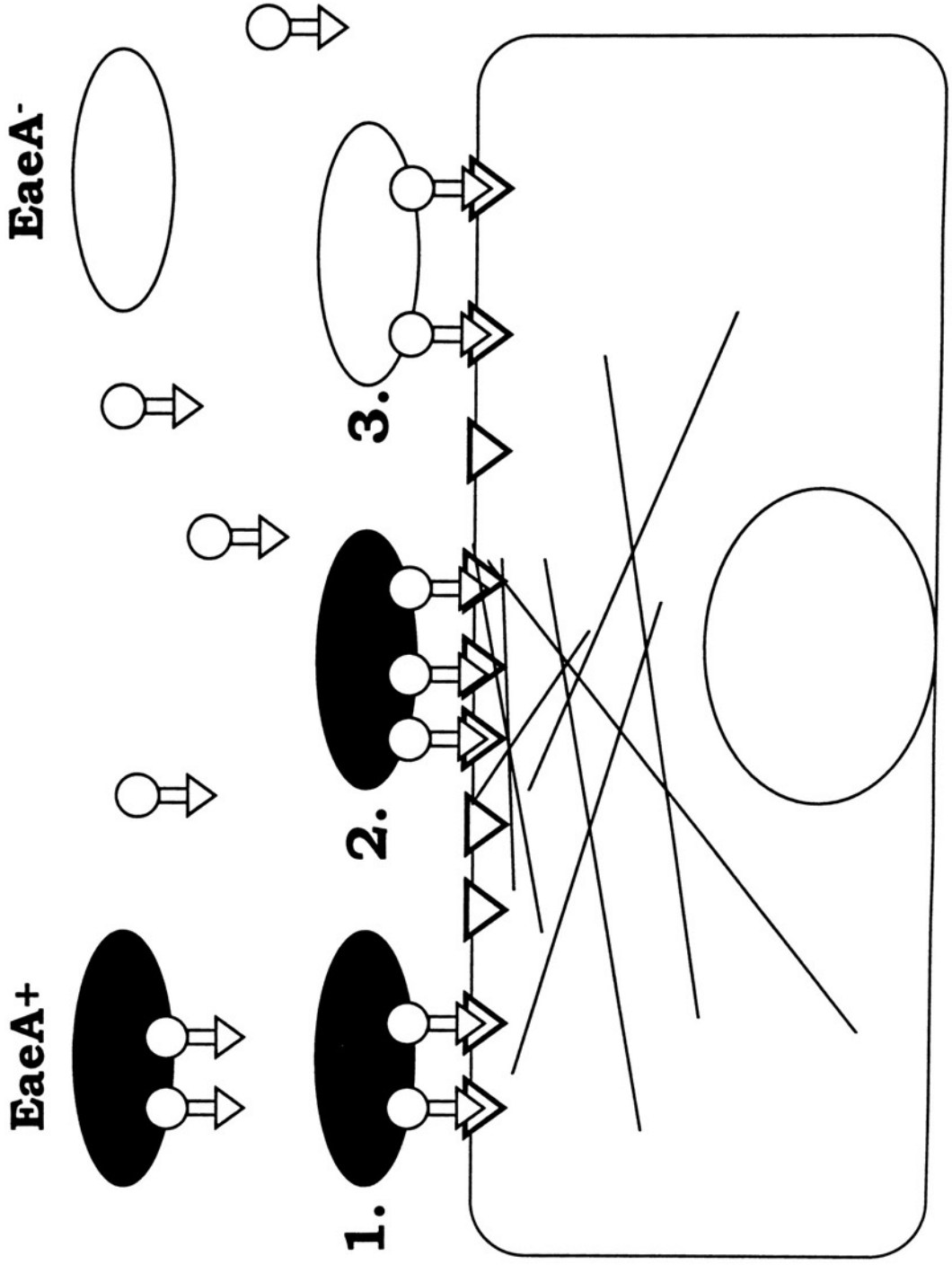
in piglets over an extended experimental period. In such a study, the capacity to cause fulminant disease in the pig, which includes diarrhea, edema, and pathologic lesions in the brain and neural tissue (Dykstra *et al.*, 1993), should be monitored.

Both the *eaeA* mutant and the wild-type parent strain colonized the mouse intestine equally well when fed individually or in competition. The lack of a requirement for the *eaeA* locus in mouse colonization by a single strain is not surprising when one considers that K-12 *E. coli* strains are capable of colonizing the intestines of streptomycin-treated CD-1 mice (Cohen *et al.*, 1983; McCormick *et al.*, 1989; Wadolkowski *et al.*, 1990). However, Wadolkowski *et al.* (1990) demonstrated that the 60 MDa EHEC plasmid is required for colonization when a plasmid-cured strain is fed to mice in competition with the plasmid-carrying parent. We found no analogous requirement for the eaeA locus in a competition assay.

III. Functionality of the EHEC intimin protein.

The enhancement of EHEC adherence to HEp-2 cells by addition of the 6XHis::intimin fusion proteins was surprising. The expected result was an inhibition or blocking of the adherence of strain 86-24 to epithelial cells due to occupation of the intimin receptors by the fusion protein. One model to explain the enhanced adherence is shown in Fig. 29. In this model, the intimin protein has two distinct ends: one that interacts with the "receptor" on the eukaryotic cell surface and the other end that interacts with the outer membrane of the bacterium acting as a bridge between the epithelial cell and the bacterium. According to

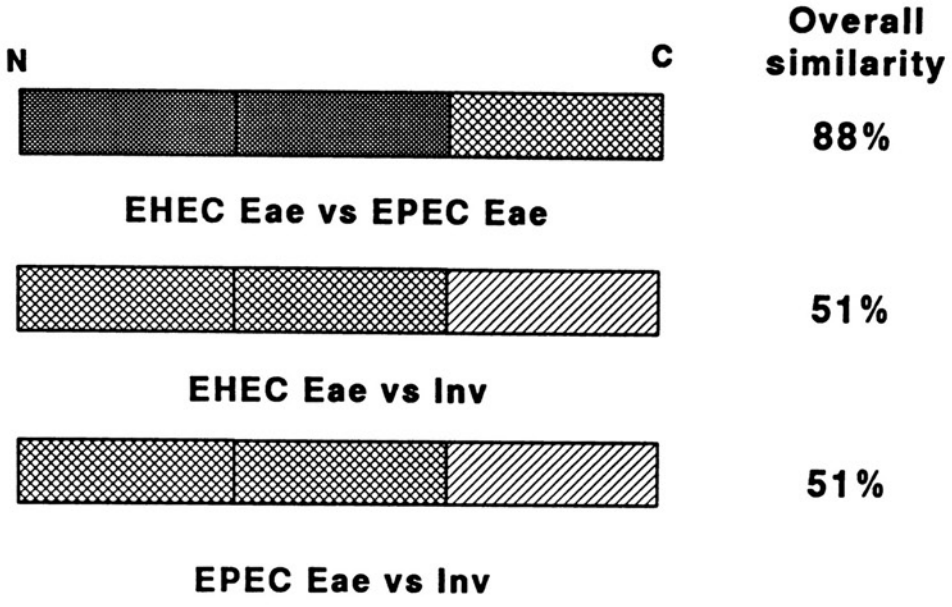
Figure 29. Model of intimin interaction with the bacterium and the epithelial cell. Intimin is depicted as a molecule with distinct ends; one end interacts with the bacterium and the other end interacts with the eukaryotic cell. 1) Wild-type bacteria express intimin as an outer membrane protein although no canonical signal sequence is present. This protein interacts with a receptor on the eukaryotic cell and results in the condensation and rearrangement of cytoskeletal components (lines within epithelial cell). 2) When exogenous intimin (in this study as a his::intimin fusion protein) is added to wild-type EHEC (dark rods), the LA/FAS phenotype is enhanced. 3) When the his::intimin fusions are added to EHEC bacteria that do not express intimin (i.e., 86-24*eae*Δ*10* or B2F1, shown here as open rods), the protein interacts with both the bacterium and the eukaryotic cell to form a "bridge" which confers the LA phenotype.


EaeA+
EaeA-
1.
2.
3.

the definition put forth by Beachey (1981), receptors are "the complementary adhesive structures on the surfaces of host cells" (Beachey, 1981) and include both known and putative entities to which an adhesin binds (Krogfelt, 1991). In this model, when RIHisEae was added to the 86-24-HEp-2 cell milieu, the excess intimin molecules formed additional bridges which led to larger microcolonies on the HEp-2 cells. When the fusion protein was added to 86-24*eaeΔ10* which does not express an intact intimin, the fusion proteins acted as a bridge to render the nonadherent mutant adherent to the HEp-2 cells. This effect should also be concentration-dependent. Consistent with this model, the intimin fusion proteins rendered B2F1, a naturally *eaeA* negative EHEC isolate that does not bind HEp-2 cells in an LA/FAS manner, capable of forming microcolonies on HEp-2 cells. The weak FAS activity reported with both the deletion mutant and the B2F1 strain may be due to i) an imprecise association of the intimin protein with the bacterium or the epithelial cell when added exogenously, ii) the fact that the entire intimin molecule is required for the full LA/FAS phenotype (neither of the fusion proteins described contained the full predicted amino acid sequence), or iii) an effect of the 6 histidines on the amino terminus of the intimin protein. The shorter fusion, RVHdHisEae, also complemented 86-24*eaeΔ10*, which implies that the regions of intimin that associate the bacterium with the epithelial cell are not in the amino terminal one-third of the molecule. Further studies with a nested set of deletions at both termini would test the validity of this model.

The reported homology between EPEC intimin and the invasin protein of *Yersinia pseudotuberculosis* extends to EHEC intimin and invasin (Jerse *et al.*, 1990; Yu and Kaper, 1992) (Fig. 30). Invasin is the major protein involved in the penetration of intestinal epithelial cells by *Y. pseudotuberculosis* (Isberg and Falkow, 1985; Isberg *et al.*, 1987; Isberg and Leong, 1988) and has a pivotal role in the initiation of infection of *Y. enterocolitica* (Pepe and Miller, 1993). Isberg and Leong (1990) identified the β_1 family of integrins as the cellular receptor for invasin. Overall, the EHEC intimin and invasin predicted amino acid sequences are 31% identical and 51% similar (Yu and Kaper, 1992). However, when the sequences are compared by thirds the similarities are more striking (Fig. 30). The carboxyl termini of the three proteins are the most divergent. The carboxyl terminal 192 amino acids of invasin comprise the receptor binding domain (Leong *et al.*, 1990; Leong *et al.*, 1993; Leong *et al.*, 1995). In fact, the aspartate residue at position 911 is critical for integrin binding by invasin (Leong *et al.*, 1995). Frankel *et al.* (1994) demonstrated by use of maltose binding protein (MBP) fusions that the C terminal 280 amino acids of intimin from EPEC, EHEC, H. alvei, or *C. freundii* 4280 were capable of binding and mediating the attachment of staphylococci to HEp-2 cells. The purified MBP-invasin fusion proteins themselves were also able to bind HEp-2 cells. However, the pattern of attachment of the isolated MBP-intimins was different from the pattern demonstrated for the MBP-invasin fusion protein (Frankel *et al.*, 1994). These investigators did not explore the potential nucleation of F-actin as a result of the binding of the intimin

Figure 30. Comparison of the predicted amino acid sequences of intimin from EPEC and EHEC [derived from the CL-8 (Beebakhee *et al.*, 1992) and EDL933 (Yu and Kaper, 1992) nucleotide sequences] to each other and to the invasin protein of *Yersinia pseudotuberculosis* (Isberg and Falkow, 1985). The darker the shading of each region of the protein, the greater the homology (i.e., dark cross-hatched bars = > 90% similarity, cross-hatched bars = 50-90% similarity, and the single hatched bars = < 50% similarity between the predicted amino acid sequences).


N
C
Overall similarity
88%
EHEC Eae vs EPEC Eae
51%
EHEC Eae vs Inv
51%
EPEC Eae vs Inv

carboxyl domain to the HEp-2 cells. Two cysteine residues essential for binding of invasin to its integrin receptor (Leong *et al.*, 1993) are shared by the intimins from all four of the A/E bacteria (Frankel *et al.*, 1994).

IV. Further studies and implications.

A search for the cellular receptor for intimin was beyond the scope of this dissertation project but is one of the next logical steps to further characterize the bacterial-epithelial cell interaction. Structure-function studies of the intimin protein are required to gain a better understanding of the mechanisms of adherence and lesion formation by attaching and effacing bacteria. Based on the observations of Frankel *et al.* (1994), information about EHEC intimin may be generalizable to the intimins produced by EPEC, *C. freundii* 4280, and *H. alvei*. Although not discussed here, there is evidence that the interaction between EPEC intimin and its cellular receptor initiates a protein phosphorylation event that begins a cascade of cellular messages and responses (Rosenshine *et al.*, 1992; Manjarrez-Hernandez *et al.*, 1992; Foubister *et al.*, 1994). An investigation of the signals initiated by EHEC intimin, if any, as a result of intimate adherence would elucidate the mechanism of A/E lesion formation. Such studies are underway elsewhere (Sherman *et al.* personal communication)

Intimin is a likely candidate as a component in an ideal EHEC vaccine for three reasons. First, an immune response against an adhesin (here intimin) would block the initial step in infection. We have shown that sera from HC patients recognize EHEC intimin, as well as other E. coli proteins. Whether this response is protective against EHEC can not be determined directly in the absence of a human model of EHEC infection. However, applicable information can be gained from EPEC intimin studies in volunteers. For example, a volunteer that had a pre-existing titer against EPEC intimin did not develop fulminant diarrheal disease when challenged with wild-type EPEC (Jerse and Kaper, 1991; Levine *et al.*, 1985). In another study, an EPEC strain mutated at the *eaeA* locus caused diarrhea in 4 of 11 volunteers, and those individuals that developed diarrhea had a reduced stool volume (Donnenberg *et al.*, 1993a). A second reason that intimin is a relevant EHEC vaccine candidate is the finding that in the absence of EHEC *eaeA*, EHEC are not capable of causing the A/E lesion in gnotobiotic piglets. A vaccine that blocks the intimin-receptor interaction either by antibodies or receptor analogues might lead to less severe disease. There is no direct evidence that EHEC cause the A/E lesion in the human bowel, but the hemorrhagic ulcerations observed macroscopically are indicative that such an event might occur (Riley, 1987; Griffin *et al.*, 1988; Griffin *et al.*, 1990). Prevention of the ulceration may lessen the severity or abolish disease. Third, if intimate association of EHEC with the intestinal mucosa is required for efficient toxin delivery by EHEC to the gut mucosa and ultimately to the blood system, then any

vaccine that blocks this intimate association may protect against the secondary sequelae, such as HUS in children and TTP in the elderly (Milford and Taylor, 1990; Pickering *et al.*, 1994; Tarr, 1995).

The ideal EHEC vaccine must include inactivated SLT or an immunogen that will elicit a protective response against SLT. Since clinical isolates of EHEC have been shown to elaborate SLT-I, SLT-II, as well as SLT-IIc, the ideal immunogen should elicit a response to all types of SLTs. The role of the other putative adhesins described for EHEC must be firmly established before these factors can be considered as additional vaccine candidates, but one can imagine that the best vaccine will be a cocktail of multiple EHEC components.

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