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MINIREVIEW

Adherence of Diarrheagenic *Escherichia coli* Strains to Epithelial Cells

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An important early step in the colonization of the human gastrointestinal tract by bacteria is the adhesion of the organism to the host surface. Although adhesion is essential to maintain members of the normal microflora in the intestine, it is also the critical early phase in all diarrheal infections caused by pathogenic *Escherichia coli* strains. It is important, therefore, to fully understand the mechanisms underlying *E. coli* adhesion and in that way to be able to develop methods of maintaining the intestinal normal microflora and to prevent pathogenic *E. coli* from initiating an infectious process.

Great progress has been made in recent years in the identification of the adherence factors of different diarrheagenic *E. coli* strains (Table 1). These protein structures are associated with the bacterial surface and can be subdivided into fimbrial and nonfimbrial adhesins (Fig. 1). In this minireview, we will discuss recent advances in the identification and characterization of previously known and novel adhesion factors from the six major categories of diarrheagenic *E. coli* strains: enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), and diffusely adhering *E. coli* (DAEC).

EPEC AND EHEC STRAINS

EPEC and EHEC strains are implicated in diarrhea and/or dysentery in humans. EPEC is a major etiological agent of infant diarrhea in developing countries. EHEC, a food-borne pathogen of worldwide importance, can cause nonbloody diarrhea, but the most serious manifestation of disease is bloody diarrhea that can progress to a fatal illness due to acute kidney failure (hemolytic uremic syndrome), particularly in children (reviewed in reference 88). EPEC is an inhabitant of the small intestine, while EHEC colonizes primarily the large human intestine. These two *E. coli* pathotypes are distinct from other pathogenic *E. coli* strains because they produce a distinct histopathological lesion on intestinal epithelial cells known as the attaching and effacing (A/E) lesion. A/E lesions are marked by localized degeneration of the intestinal brush border surface, loss of epithelial microvilli, and assembly of highly organized pedestal-like actin structures in the epithelial cells at the sites of bacterial attachment (reviewed in references 42 and 88). All the proteins associated with the formation of A/E lesions are encoded on a chromosomal pathogenicity island known as the locus of enterocyte effacement (LEE) (for a recent overview of the LEE genes, see reference 133). The LEE contains the *eae* (stands for *E. coli* attaching and effacing) gene, encoding the outer membrane protein intimin (60). This protein mediates intimate adherence to target eukaryotic cells upon interaction with its translocated receptor Tir (stands for translocated intimin receptor), a protein encoded upstream of the *eae* gene on the LEE. In the following sections, we will discuss current data that help us to understand the detailed interactions between intimin and Tir and intimin and its eukaryotic receptor and the function of intimin as a mediator of tissue tropism. In addition, we will present recent progress towards the identification of additional adhesive molecules in EHEC and EPEC strains. A complete overview of the adhesion properties of EPEC strains to host cells has recently been published (93).

(i) An intimate situation: intimin, Tir, and the eukaryotic receptors. Our concept of how EPEC and EHEC strains causing A/E lesions adhere to mammalian cells has been enhanced by the description of the crystal structure of intimin coupled to the Tir receptor (78). That study resulted in a model that indicates that, once translocated, the Tir protein spans the host cell membrane, adopting a hairpin loop structure featuring both its N and C termini in the host cytoplasm and a central extracellular domain that binds intimin (4, 75, 78). In addition to serving as a receptor for intimin, the Tir protein is capable of interacting with host cytoskeletal and signaling components using its N- and C-terminal domains located in the host cell cytoplasm. The numerous host proteins that accumulate in the A/E lesion around Tir and the mechanisms by which Tir exploits the host cell signaling networks resulting in actin cytoskeletal rearrangements have been reviewed elsewhere (see reference 22).

Biochemical and biophysical studies have indicated that intimin can be subdivided into a flexible N-terminal region (including a periplasmic domain [residues 40 to 188]), a central membrane-integral β-barrel (residues 189 to 549), and a surface-exposed C-terminal region (includes four extracellular domains named D0 to D3 [residues 550 to 939]), where the receptor-binding activity resides (52, 78, 135). Originally, the receptor-binding region was mapped to a region comprising the 280 C-terminal amino acids of intimin (65). Further crystallographic studies indicated that the last 190 amino acids within this region mediate this binding. The 190 C-terminal

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TABLE 1. Proposed major adhesion factors of the principal pathotypes of diarrheagenic *E. coli*

Pathotype	Fimbrial adhesin(s)	Adhesin(s) and/or invasin(s)
EPEC	Bfp	Intimin, flagella, LifA/Efal
EHEC	Lpf, Sfp	Intimin, Iha, OmpA, Saa, Efal, ToxB
ETEC	CFs	Tia, TibA
EAEC	AAFs	AfaE-VIII, dispersin
EIEC	Unknown	Invasion-plasmid antigens (Ipas), IcsA, HAF
DAEC	F1845 and related fimbriae	AIDA-I

amino acids of intimin comprise an N-terminal immunoglobulin domain that is coupled to a novel C-type lectin domain, which in turn forms a flexible rod that extends from the bacterial surface and serves as a tip that binds the target cell (4, 74, 78). Furthermore, a direct link between intimin binding and Tir clustering has recently been demonstrated (135). The association of intimin with Tir triggers a host cell response leading to pedestal formation. Although the formation of actin pedestals by EHEC and EPEC strains is a phenotype best characterized in vitro for epithelial cell lines, pedestal formation correlates with the ability of the A/E organisms to colonize the intestine and cause disease in human and other animal hosts (88).

In addition to binding Tir (its primary receptor), there is evidence that intimin promotes initial adherence by binding to endogenous host cell receptors independently of its interaction with Tir (114). An initial study reported that intimin from EPEC can bind β1-chain integrins in vitro (40), but a subsequent study challenged these results and indicated that these host cell proteins are not essential for intimin-mediated cell attachment or EPEC-mediated actin polymerization (74). Although the location of β1-integrins in the basolateral membrane of intestinal epithelial cells seems to preclude interaction of EPEC with the apical membrane, a recent study showed that EPEC induced alterations in tight junctions, resulting in the redistribution of β1-integrin and other basolateral membrane proteins to the apical cell surface, thereby providing an opportunity to interact with EPEC intimin (85). Despite these discrepant results, additional indirect evidence favors the concept that intimin interacts with an additional eukaryotic cell receptor. Such evidence includes intimin-mediated tissue tropism (see below), nucleotide sequence divergence in the intimin domain mediating receptor binding, and the results of mutagenesis analysis of this region indicating that intimin binding to Tir is separated from the intimin domain mediating adherence to host cells (41). Moreover, the fact that purified intimin displays biological activity provides additional evidence indicating that intimin binds directly to host cells, inducing colonic hyperplasia in mice (55) and activating T cells (50). Based on these premises, Sinclair and O'Brien (114) identified a eukaryotic receptor for EHEC intimin on HEp-2 cells. Those investigators isolated a protein from HEp-2 cell extracts that bound to EHEC intimin, and amino acid sequence analysis identified the protein as nucleolin. This protein colocalized to areas in the HEp-2 cell surface where intimin was present, and antibodies directed against nucleolin reduced binding of EHEC to HEp-2 cells when they were added prior to or at the time of bacterial infection (114). Recently, the same investigators have shown that intimins α and β (see nomenclature below) bound nucleolin expressed on the surfaces of HEp-2 cells with an affinity similar to that of EHEC intimin (intimin γ) (115). The cumulative data indicate that nucleolin can serve as a host receptor for EPEC and EHEC intimin and that the nucleolin-intimin interaction occurs early in the infectious process.

(ii) Different types of intimin molecules and tissue tropism. As shown by experimental infections, intimin is required for the colonization and pathogenesis of EPEC in humans and of EHEC in calves and pigs (28–30). Intimin proteins of EHEC and EPEC strains show high conservation in the N-terminal region and variability in the last 280 C-terminal amino acids of the protein. The antigenic polymorphism at the C-terminal region, where binding to enterocytes and Tir occurs, has led to the classification of intimins into at least 10 different types (Table 2) (1, 61, 96).

The importance of the different intimin types in colonization of the intestine has been emphasized, with recent data suggesting that differences in the amino acid sequences of the intimin proteins influence the pattern of colonization and tissue tropism in the host (37, 101).

The initial studies using genetic and immunological approaches provided evidence for the existence of at least four distinct intimin types known as intimin α, β, γ, and δ (1, 2). A subsequent study revealed the presence of a fifth type, intimin ε, in *E. coli* strains of serogroup O103 (96). It has also been proposed that intimins α, β, and γ can be further subdivided based on restriction analysis into α1, α2, β1, β2, γ1, and γ2, with β2 being identical to δ (96). Recently, three independent studies have proposed that additional intimin types based only on subtle differences at the nucleotide level can be found in other EPEC or EHEC strains. These new groups have been classified as intimins ζ, η, θ, ι, and κ (61, 125, 149).

The correlation between the expression of some of these intimin types and the tissue tropism of different *E. coli* strains has been demonstrated experimentally with in vitro human intestinal organ cultures. While intimin α is expressed by EPEC clone 1 and confers specificity for the human proximal and distal small intestines as well as follicle-associated epithelia of Peyer's patches (102), intimin β is associated with human and animal EPEC 2 and EHEC 2 strains and is expressed in rabbit Peyer's patch lymphoid follicles (24). As for intimin γ, its expression correlates with colonization of the human ileal follicle-associated epithelia of Peyer's patches by EHEC O157:H7, EPEC O55:H7, and EPEC O55:H$^-$ (37, 102). Although intimin ε is expressed by human and animal EHEC strains other than those of serogroup O157, the tissue distribution of these strains is similar to that observed with EHEC O157:H7 strains expressing intimin γ (38). The host tissue distribution of EPEC and EHEC strains is probably multifactorial, but characterization of the different intimin types may yield important information regarding tissue tropism.

(iii) EPEC Bfp and flagella. Initial contact of EPEC with the host cells involves the generalized nonintimate interaction of bacterial microcolonies in a pattern known as localized adherence (LA) (109, 140). The LA phenotype is mediated by the plasmid-borne type IV fimbriae known as bundle-forming pili (Bfp) (46). Initial in vitro experiments employing cultured ep-

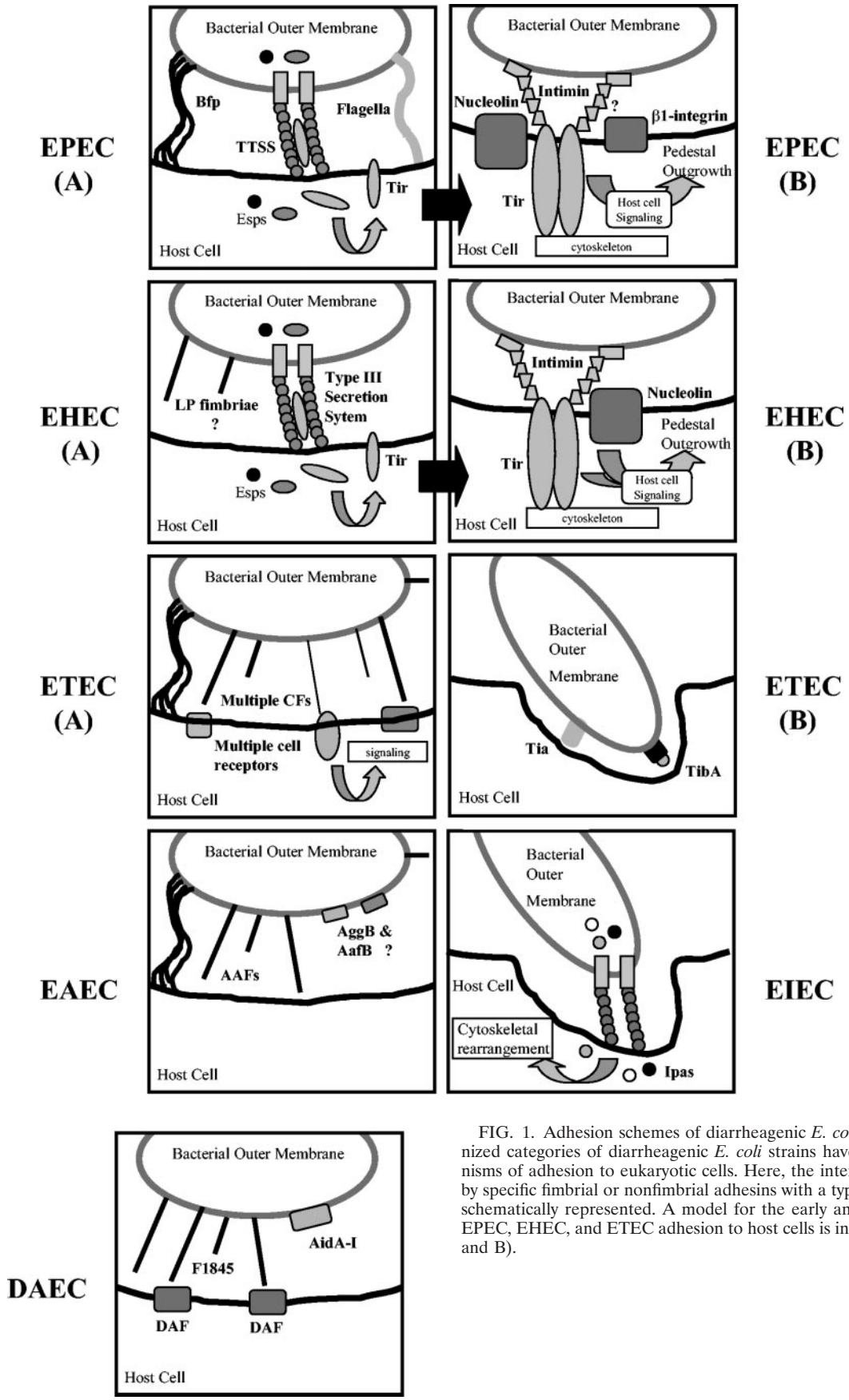

FIG. 1. Adhesion schemes of diarrheagenic *E. coli*. The six recognized categories of diarrheagenic *E. coli* strains have unique mechanisms of adhesion to eukaryotic cells. Here, the interaction mediated by specific fimbrial or nonfimbrial adhesins with a typical target cell is schematically represented. A model for the early and later events in EPEC, EHEC, and ETEC adhesion to host cells is included (panels A and B).

TABLE 2. Different intimin alleles, their origins, and tissue tropism

Intimin type	Category(ies) of *E. coli*,[a] serotypes, or species carrying the alleles	Host and/or tissue specificity[d]
α (alpha)[b]	EPEC 1	Human proximal and distal small intestines as well as FAE
β (beta)[b]	EPEC 2, EHEC 2, and *Citrobacter rodentium*, RDEC-1[e]	Animal and human (Peyer's patch lymphoid follicles in rabbits)
γ (gamma)[b]	EHEC O157:H7, EPEC O55:H7, and O55:H$^-$	Human ileal FAE
δ (delta)[b]	EPEC O86:H34	Human
ε (epsilon)	EHEC O8, O11, O45, O103, O121, and O165	Bovine and human (O103 strains adhere to large intestine FAE)
ζ (zeta)[c]	O84:NM	Bovine
η (eta)[c]	O125: H$^-$, O84:H2	Human
θ (theta)[c]	O111:H8	Human
ι (iota)[c]	O145:H4, O98:H2	Human
κ (kappa)	O118:H5	Human
Untypeable	EPEC O127:H40	Human

[a] Categories are based on the clonal relationships described by Whittam et al. (143).
[b] By restriction analysis, these intimins have been subgrouped as α1, α2, β1, β2, γ1, and γ2 (β2 is also known as δ).
[c] These are rare intimin alleles not frequently found in human isolates.
[d] FAE, follicle-associated epithelium of Peyer's patches.
[e] RDEC-1, rabbit diarrheagenic *E. coli* strain.

ithelial cells of nonintestinal origin have shown that Bfp mediated the initial binding of EPEC strains, the formation of microcolonies, and interbacterial interactions that allowed the formation of three-dimensional bacterial aggregates at a later stage of infection (46, 119, 121). A subsequent study employing freshly harvested human intestinal mucosa in the in vitro human intestinal organ culture system indicated that Bfp is involved in the development of the three-dimensional structure of the microcolonies after intimate attachment rather than in initial adherence (54). The apparent discrepancy in these results has recently been evaluated using an intestinal cell line, Caco-2, as the in vitro system (129, 130). Those studies suggested that EPEC binding occurs through direct interaction with the host cell rather than an already-formed EPEC microcolony and that Bfp plays an important function in the cell type-dependent adherence of EPEC and in the progression to the later steps in EPEC adherence (129, 130). A recent study by Cleary et al. (25) also presented evidence showing that Bfp can mediate rapid adherence of EPEC to Caco-2 cells. The importance of Bfp as a virulence factor in human EPEC pathogenesis has been clearly demonstrated by a volunteer trial in which a *bfp* mutant was ca. 200-fold less virulent than the wild-type Bfp$^+$ parent strain (15).

In addition to Bfp, EPEC strains possess other surface-exposed structures, such as the flagellum organelle. Epidemiological studies have shown that EPEC strains isolated throughout the world express a restricted number of flagellar antigen types, raising the possibility that this surface structure may perform an additional function besides its role in motility. This idea was investigated by Girón et al. (47), who reported that flagella of EPEC are directly involved in the adherence of these bacteria to cultured HeLa cells. It was also demonstrated that EPEC flagellar expression is induced by a molecule secreted by eukaryotic cells and that molecular cross talk exists between flagella and other virulence-associated systems in EPEC (47). The eukaryotic factor inducing flagellar expression remains unknown, and a recent report found no evidence for such a factor or for the production of flagella by EPEC strains adherent to HeLa cells (148). Another recent paper reported the production of flagella by adherent EPEC cells but could not confirm a role for flagella in adherence (25). In addition to having a potential role as an adhesin, the flagellar filament and its flagellin monomer have recently been demonstrated to induce the activation of interleukin 8 in intestinal epithelial cells, raising the possibility than EPEC flagellin plays an important role in the intestinal inflammatory response during infection (150).

(iv) Potential EPEC adhesins. It has been proposed that a number of other adhesins mediate EPEC-host cell interactions. Recent completion of the genomic sequence of EPEC strain E2348/69 (http://www.sanger.ac.uk/cgi-bin/BLAST/submitblast/escherichia_shigella) has revealed the presence of at least 9 regions with homology to potential fimbrial adhesin gene clusters and 10 regions encoding putative nonfimbrial adhesins in addition to the LEE-encoded intimin. One of these regions encodes type I fimbriae, which have been investigated as a possible EPEC adhesin. Although volunteers challenged with wild-type EPEC developed an immune response against type I fimbriae (64), mutagenesis of the type I fimbriae in EPEC strain E2348/69 failed to show any effect on levels or patterns of adherence of EPEC in vitro (34). The role of the EPEC type I fimbriae in human enteric infections is still unclear.

Another putative adhesin is encoded by the *lifA* gene. LifA was originally characterized for EPEC strains as the toxin lymphostatin, which inhibits the transcription of multiple lymphokines and inhibits lymphocyte proliferation (66, 67). Two nearly identical genes are found in strains of EHEC O111 (*efa-1*) and O157:H7 (*toxB*), and they have been implicated in adhesion to epithelial cells (see below). An initial study of a *lifA* mutant of strain E2348/69 found no difference in adherence to cultured epithelial cells (67), but a subsequent study of a *lifA* mutant of a E2348/69 derivative cured of the EAF plasmid encoding Bfp found a reduced adherence, thereby suggesting a potential role for LifA/Efa1 in adherence in the absence of Bfp (3).

Finally, EPEC was recently shown to exhibit a type III secretion-dependent, contact-mediated hemolytic activity requiring the EspA, EspB, and EspD secreted proteins (141). EspA is the major component of a large filamentous structure that is proposed to provide a conduit between the type III translocon and a host cell membrane pore (68). Experimental evidence

indicates that EspA filaments mediate the binding of EPEC to red blood cells and that close contact is not a requirement for EPEC-induced hemolysis (110). More importantly, Cleary et al. (25) recently showed that EspA participates in the initial attachment to the brush borders of Caco-2 cells. Even though the predominant role for Bfp adherence was also demonstrated for bacteria expressing EspA and Bfp, EspA filaments have been proposed as adhesins mediating initial attachment in EPEC strains that lack Bfp.

(v) EHEC fimbrial adhesins. Although an extensive search has been conducted to discover other adherence factors in EHEC strains besides intimin, very little is known about the identities of fimbrial proteins that mediate the binding of these organisms to the human intestine. The completion and analysis of the genomic sequence from two prototypic EHEC strains of serotype O157:H7 have indicated that at least 12 regions encode putative adhesins (i.e., regions encoding homologues to fimbrial and nonfimbrial proteins) (53, 100). One of these regions contains genes closely related to the long polar (LP) fimbrial (*lpf*) operon of *Salmonella enterica* serovar Typhimurium (100). The introduction of the EHEC *lpf* operon into a nonfimbriated *E. coli* K-12 strain resulted in increased fimbria expression and increased adherence to tissue culture cells (131). Isogenic *lpf* mutants of wild-type EHEC showed slightly reduced adherence to epithelial cells and formed fewer microcolonies than the wild-type strain (131). Sequences homologous to the proposed major LP fimbrial subunit gene, *lpfA*, have been detected in human and bovine EHEC isolates from serogroups O157 and O145 (123). These two serogroups belong to the same LEE genotype, based on the typing of genes necessary for the production of the A/E lesion, and this result suggests that LP fimbriae may be associated not just with particular serotypes but with a specific genotype.

A second operon in the chromosome of EHEC O157:H7 also has homology to LP fimbriae. This region contains five open reading frames predicted to encode fimbrial proteins that share an overall 31% identity (49% similarity) to proteins encoded by the other *lpf* operon in EHEC O157:H7. A similar region in a Shiga toxin-producing *E. coli* (STEC) strain of serotype O113:H21 has previously been characterized. This operon is found in the same chromosomal location in both strains. The mutation in the O113:H21 LP fimbriae resulted in decreased adherence of this strain to epithelial cells, suggesting that these fimbriae function as an adhesin, at least in this particular isolate (31). The *lpfA* gene from STEC O113 has been found in diverse *E. coli* strains isolated from humans and animals. This kind of fimbria is found in LEE-negative *E. coli* isolates and thus may represent an important adherence factor in this group of pathogens (95). In fact, the role of these fimbriae in adherence in rabbit-specific EPEC, a closely related pathogen, has been studied. With the rabbit model, it has been shown that LP fimbriae play a role early in adherence and that the expression of LP is important for the development of severe diarrhea (91). Despite the data produced in vitro, a role in EHEC pathogenesis for either of the two LP fimbrial operons has not yet been defined.

(vi) EHEC nonfimbrial adhesins. The most convincing data showing a role for a nonfimbrial factor encoded outside of the LEE in the adherence of a particular EHEC strain were published by two independent research groups (92, 120). Nicholls et al. (92) initially characterized a chromosomal genetic locus that they named *efa-1* (stands for EHEC factor for adherence) in an clinical EHEC O111:H$^-$ isolate and observed that this locus mediates adherence to cultured Chinese hamster ovary (CHO) cells. Stevens et al. (120) mutated the *efa-1* gene in O5 and O111 strains of EHEC and showed that this mutation dramatically reduced the number of bacteria associated with the bovine intestinal epithelium even though the mutants still retain their ability to form both microcolonies and A/E lesions (120). While EHEC O157:H7 lacks the full-length *efa-1* gene, a truncated version of *efa-1* exists in the chromosomes of the two fully sequenced EHEC strains EDL933 and O157Sakai (53, 100). This gene has been proposed to influence bacterial adhesion and intestinal colonization, although no experimental data supporting this proposal have been published (128). Furthermore, a large gene with significant homology to *efa-1* also exists in EHEC O157:H7 strains and is known as *toxB*. This gene is located in the EHEC pO157 virulence plasmid, and strains lacking *toxB* exhibit reduced adherence to cultured epithelial cells (127). The role of this gene in adherence was inferred from studies where mutants deficient in adherence were obtained (128). The authors concluded that although *toxB* is required for adherence to epithelial cells, this gene only indirectly influences adherence by modulating the production and secretion of LEE-encoded effector proteins that are required for the formation of A/E lesions (127). Therefore, the function of *toxB* and *efa-1* genes in EHEC O157:H7 adherence remains to be established.

Other reports have implicated EHEC proteins, in addition to Efa1, as mediators of adherence but with no obvious role in pathogenesis. They include Iha (*Vibrio cholerae* IrgA homologue adhesin), Cah (calcium-binding antigen 43 homologue), and OmpA (outer membrane protein A). Iha is associated with adherence to HeLa cells only when it is expressed in a nonfimbriated *E. coli* stain (126). Cah is a surface-expressed and heat-extractable protein that causes autoaggregation and changes in bacterial shape when it is expressed as a recombinant protein and also participates in the formation of EHEC biofilms (134). Finally, there is OmpA, which was identified during the screening of an EHEC O157:H7 transposon insertion mutant library for hyperadherent mutants. The isogenic *ompA* mutant strain showed a reduction in adherence to HeLa cells compared with the adherence of the parent strain, and antibodies directed against OmpA inhibited their adhesion (132). Additional experiments are required to elucidate whether the adhesive properties of OmpA in vitro also play a role during colonization.

(vii) STEC fimbrial and nonfimbrial adhesins. The term STEC is broadly used to describe any *E. coli* strain that expresses Stx, whereas the term EHEC is used to describe a subset of STEC strains that also contain the LEE. LEE-negative STEC strains are frequently isolated from animals and occasionally from humans (88, 98). The absence of intimin in these strains indicates that other adhesins are involved in adherence to host cells.

The best-characterized adhesin in LEE-negative STEC strains is a novel autoagglutinating adhesin, designated Saa (stands for STEC autoagglutinating adhesin), that has been described to occur in an O113:H21 STEC strain (97). This adhesin is encoded in a large plasmid of this strain and is

associated with both an increase in adherence to Hep-2 cells when it is expressed as a recombinant protein in *E. coli* and with a reduction in adherence when *saa* is mutagenized in the wild-type strain (97). Recent data have indicated that *saa*-positive strains are more frequently found in bovine STEC strains than in human strains, suggesting that Saa may have a more important role in attachment to the bovine gut than the human intestine (59).

In addition, a fimbrial operon has been characterized for a nonmotile strain of EHEC O157 that is able to ferment sorbitol (SF EHEC O157:H$^-$). Sfp (stands for sorbitol-fermenting EHEC O157 fimbriae, plasmid encoded) fimbriae are encoded by a cluster of genes located on pSFO157, a plasmid which is present only in SF EHEC O157:H$^-$ strains. The expression of the *sfp* cluster produces fimbrial structures on the surface of a laboratory strain of *E. coli* and also mediates mannose-resistant hemagglutination (21). The importance of this gene cluster in pathogenesis, whether for the small subset of SF O157:H7 strains or for other EHEC or STEC strains, remains to be established.

ETEC

ETEC is a major cause of traveler's diarrhea in adults from industrialized countries and children in developing countries worldwide (19). ETEC colonizes the proximal small intestine, and this category of *E. coli* was the first for which virulence factors were described. The mechanisms used by ETEC strains to cause disease and the characterization of the major virulence factors have been extensively described elsewhere (for reviews see reverences 88 and 144). In this section, we will briefly describe the major fimbrial colonization factors employed by ETEC to colonize the proximal small intestine.

(i) Fimbrial adhesins. Colonization of the human small intestine by ETEC depends mainly on the expression of several different proteinaceous surface structures generally known as colonization factors (CFs), which usually form pili (or fimbriae). These CFs are classified based on antigenic specificity and are called colonization factor antigens (CFAs), coli surface antigens (CS), and putative colonization factors (PCFs) (reviewed in references 43 and 144). The CFs of the major human ETEC strains are varied and include CFA/I, CFA/II (composed of CS1, CS2 and CS3, which are present in different combinations), CFA/III, CFA/IV (a complex of CS4, CS5 and CS6), CS7, CS17, CS19, CS20, CS22, and longus, and the PCFs include PCFO9, PCFO20, PCFO148, PCFO159, PCFO166, antigen 2230, and antigen 8786 (Table 3). The CFs can also be subdivided based on their morphology in fimbrial, fibrillar, helical, bundle-forming (longus), and nonfimbrial adhesins. ETEC strains expressing CFA/I or CFA/II seem to be the most prevalent, although a wide variety of CFs are found in different parts of the world (reviewed in references 43 and 88). At least 21 different CFs have been described for human ETEC strains (Table 3).

The terminology of CFs was rather confusing with the different terms for CFAs, CS, longus, and PCFs being used to describe these adhesins, and a uniform terminology that employs numbers derived from the order in which the CFs were described has been proposed (Table 3) (43).

Each CF has a unique subunit molecular mass, and most of them are usually encoded by genes on high-molecular-weight plasmids, which also encode other virulence factors, such as the heat-stable toxin (ST) and/or heat-labile toxin (LT). Moreover, surveys of ETEC strains have shown that most CFs are associated with a limited number of serotypes and enterotoxin types (144). The ability of several of these CFs to promote colonization and induce an immune response has been extensively studied in experimental infections of animals as well as human volunteers, and the importance of CFs in the pathogenesis of ETEC has made them a key component for the development of a vaccine against human ETEC (105).

TABLE 3. ETEC CFs[a]

CF	*E. coli* surface antigen	Morphology	Fimbrial diam[c] (nm)
CFA/I	CFA/I	Fimbrial	7
CS1	CS1	Fimbrial	7
CS2	CS2	Fimbrial	7
CS3	CS3	Fibrillal	3
CS4	CS4	Fimbrial	6
CS5	CS5	Helical	5
CS6	CS6	Nonfimbrial	NA
CS7	CS7	Helical	3–6
CFA/III	CS8	Fimbrial	7
2230	CS10	Nonfimbrial	NA
PCFO148	CS11	Fibrillal	3
PCFO159	CS12	Fimbrial	7
PCFO9	CS13	Fibrillal	ND
PCFO166	CS14	Fimbrial	7
8786	CS15	Nonfimbrial	NA
CS17	CS17	Fimbrial	7
PCFO20	CS18	Fimbrial	7
CS19	CS19	Fimbrial	7
CS20	CS20	Fimbrial	7
Longus	CS21	Fimbrial (bundle)	7
CS22[b]	CS22	Fibrillal	ND

[a] This table is adapted from reference 43
[b] CS22 is a novel adhesin related to CS15 (103).
[c] NA, not applicable; ND, not determined.

Although the nature of the intestinal cell receptors which interact with CFs is not completely known, many are likely to include sialoglycolipids (such as GM2), sialic acid-containing glycoconjugates, asialogangliosides, and several other glycoconjugates (glycolipids and glycoproteins) (43, 144). The interaction between ETEC CFs and their receptors seems to be host specific and depends on the oligosaccharides expressed on the mammalian cell surface. Thus, the CFs produced by ETEC infecting humans are different from those of animal strains.

(ii) *tia-pai* and *tib* loci. It has been observed that ETEC, in addition to CFAs that mediate intestinal colonization, has the ability to invade human intestinal cell lines (35). Invasion has been studied only in the prototype ETEC strain H10407 and has been linked to the fact that a significant number of ETEC strains causing human infection do not express identifiable colonization factor. This study led to the identification and characterization of two chromosomally borne invasion loci, designated *tia* and *tib* (toxigenic invasion loci A and B) (35, 36). It has been proposed that these two loci encode proteins that are sufficient to mediate the adherence and invasion of *E. coli* strains containing either the recombinant Tia or the recombinant TibA protein to intestinal epithelial cells (36, 39, 73). Furthermore, ETEC *tia* or *tib* locus deletion mutants are im-

paired in their ability to adhere to and invade epithelial cells (36, 39, 73). Regardless of the data, the presence of these invasion-associated loci in other ETEC strains needs to be further confirmed and their importance during ETEC infection needs to be fully established.

EAEC

EAEC is a category of *E. coli* strains that do not secrete the LT or ST enterotoxins but do adhere to epithelial cells in a characteristic stacked-brick pattern known as the aggregative adherence (AA) pattern (reviewed in reference 88). EAEC were originally recognized as predominant etiologic agents of persistent diarrhea in developing countries (14) and still remain an important cause of acute as well as protracted diarrhea in several parts of the world, including industrialized countries (reviewed in references 89 and 94). The pathogenesis of EAEC infection is not well understood. However, it is known that EAEC adheres to small and large bowel mucosal surfaces and stimulates mucus production, which leads to a thick mucus-containing biofilm encrusted with EAEC. Finally, EAEC strains secrete toxins, such as heat-stable enterotoxin 1 (EAST1), Pet, and Pic, which are associated with damage of the mucosa (88).

(i) Fimbrial adhesins. EAEC strains produce at least three fimbrial adhesins: AA fimbriae I, II, and III (AAF/I, AAF/II, and AAF/III, respectively). All three fimbriae are encoded on 60- to 65-MDa virulence plasmids and are required for expression of the AA pattern. AAF/I are flexible bundle-forming fimbriae that are 2 to 3 nm in diameter. They mediate adherence to HEp-2 cells and hemagglutination of human erythrocytes in EAEC strain 17–2 (86). The genes encoding AAF/I are separated into two regions (designated regions 1 and 2) by a 9-kb segment of DNA containing the gene encoding the enterotoxin EAST1 (86, 90, 108). Region 1 contains a cluster of four genes, *aggDCBA*, encoding the fimbrial structural subunit (AggA), a chaperone (AggD), and an usher protein (AggC). AAF/I fimbriae belong to the family of Dr adhesins that are characterized for their ability to mediate adherence to the Dr blood group antigen and whose members feature a conserved chaperone-usher-invasin-pilin genetic rearrangement (108). Region 2 encodes an AraC-like regulator named AggR that is necessary for AAF/I fimbrial expression (90).

Since only a minority of EAEC strains express AAF/I and since AAF/I-lacking strains still display the AA phenotype, a second factor, AAF/II, was soon discovered originally in strain O42 (27). AAF/II is 5 nm in diameter and is distinct from AAF/I genetically, phenotypically, and morphologically (27). AafA, the fimbrial structural subunit of AAF/II, is 25% identical to AggA of AAF/I. An isogenic *aafA* mutant strain was not able to adhere in vitro to human intestinal tissue, suggesting a role for AAF/II in intestinal colonization (27). Similar to those encoding AAF/I, the genes encoding AAF/II are separated into two regions, but the organization of the AAF/II genes is unique. Genes encoding the chaperone (*aafD*), the major fimbrial subunit (*aafA*), and the transcriptional activator (*aggR*) are present in region 1, while the genes encoding the silent chaperone (*aafD′*), usher (*aafC*), and invasin (*aafB*) are located in region 2 (33). Separation of the fimbrial subunit gene from the cluster containing genes associated with fimbrial biogenesis is a feature of the gene organization found in members of the Dr fimbria family. The third type of fimbriae, AAF/III, were identified in EAEC strain 55989 (11). Although the AAF/III fimbrial structural protein Agg-3A displays no significant similarity to any protein in the database, the sequences of Agg-3A, AggA, and AafA contain conserved residues that are typical of the family of adhesins encoded by *afa*-related operons (27). Furthermore, Agg-3A was demonstrated to function as an adhesin to HeLa cells, and unlike AAF/I or AAF/II, which formed bundles, AAF/III are commonly observed as individual filaments (11). The gene organization of the AAF/III cluster is very similar to that of AAF/I, and Agg-3BCD has 63 to 67% identity with the corresponding proteins of AAF/I and AAF/II (11).

(ii) Afimbrial adhesin AfaD-III. The existence of afimbrial adhesins in EAEC has recently been suggested (44). AggB and AafB, encoded by the *agg* and *aaf* adhesin operons of EAEC strains 17–2 and O42, respectively, have identity to AfaD-III, an afimbrial adhesin found in uropathogenic and diarrhea-associated *E. coli* strains (50.7 and 58.3% of these strains, respectively) (44, 45). AfaD and AfaE, encoded by the *afaD* and *afaE* genes, respectively, are located in the afimbrial adhesin (*afa-3*) gene cluster, and these two proteins have different roles in bacterium-HeLa cell interactions (44). Although at least eight different members of the *afa* family of gene clusters have been identified (72), the structure and function of AfaD, the protein required for the internalization of adherent bacteria, is conserved, while AfaE allows binding to HeLa cells (63). Purified His_6-AggB protein not only directs the entry of beads into HeLa cells, but also complements the invasion deficiency of an *afa-3*-expressing strain carrying a mutated *afaD* gene (45), suggesting that AggB belongs to this family of afimbrial invasins.

(iii) Dispersin and biofilm formation. One important characteristic of EAEC strains is that they adhere to small and large bowel mucosal surfaces in a thick aggregating biofilm (87, 137, 139). The presence of an EAEC mucosal biofilm may be important for the pathogenesis of the organism. For example, the biofilm formation may promote persistent colonization by presenting a barrier to the penetration of antibiotics and host antibacterial factors (94). The genetic regulation of biofilm formation in EAEC has been elucidated, and it was found that the fimbrial adhesin AAF/II is required for formation of stable biofilms (112). It was also found that *fis*, encoding an 11.4-kDa nucleoid-cell-associated protein, and *yafK* activate biofilm formation by regulating the transcription of the AAF/II biogenesis genes and of the activator *aggR* (112). Furthermore, a *yafK* mutant poorly adheres to human intestine mucosa, which suggests a role for the *yafK* gene in colonization (112).

A possible novel virulence factor, dispersin, was recently described to occur in EAEC. Dispersin, encoded by *aap*, is secreted into the extracellular milieu, where it remains noncovalently attached to the bacterial surface (111). Instead of causing biofilm formation during infection, the expression of the 10-kDa protein, which is controlled by AggR, probably participates in counteracting aggregation mediated by AAFs (111). Dispersin is expressed in vivo and is highly immunogenic in humans (111). Although it is hypothesized that dispersin is probably important in promoting bacterial spread across the

mucosa, its role in intestinal colonization needs to be investigated further.

EIEC

EIEC strains cause a watery diarrhea and dysentery in humans. Since EIEC strains are biochemically, genetically, and pathogenetically closely related to *Shigella* spp., our knowledge of the pathogenesis of EIEC has mostly been obtained from studies of *Shigella* (88, 104). EIEC and *Shigella* are inefficient at invading epithelial cells apically (84), and they invade colonic epithelium via M cells and macrophages to have access to the basolateral surfaces of enterocytes. The bacteria lyse the endocytic vacuole and multiply intracellularly and then spread from cell to cell within the epithelial cell layer (reviewed in reference 106). *Shigella* also induces apoptosis in macrophages via a caspase-1-dependent pathway leading to strong inflammatory responses (56, 107).

(i) Invasion-plasmid antigens. All genes required for invasiveness are carried on a 140-MDa plasmid in EIEC (118). The *ipa* (invasin plasmid antigen) locus encodes IpaB, IpaC, IpaD, IpaA, and IpgC (which is the chaperone of IpaB and IpaC) (82). The key effector proteins are secreted by the type III Mxi-Spa system, which is also encoded on the 140-MDa plasmid. IpaB, IpaC, and IpaD play an essential role in bacterial entry, whereas IpaA is only partially involved in invasion (83, 136). The Ipa complex can bind α5β1 integrin (142) and promote invasion to epithelial cells (81). IpaB binds to CD44, the hyaluronate receptor (116, 117), and caspase-1 (56). The IpaB-IpaC complex is inserted into epithelial cell membranes, forming a pore that allows the translocation of effector proteins, such as IpaA. The process triggers a series of signal transduction events in epithelial cells (106).

(ii) IcsA and cell-to-cell spread. After invasion, EIEC and *Shigella* rapidly lyse the surrounding vacuole and are released into the cytosol, where they quickly become coated with filamentous actin and ultimately form an actin tail at one pole of the bacterium (9). The ability of EIEC and *Shigella* to move within the host cell cytosol and into adjacent cells by polymerizing host actin requires the expression of the plasmid-encoded IcsA/VirG protein (80). IcsA is a 120-kDa autotransporter protein whose carboxy terminus forms a β-barrel in the outer membrane, through which the amino-terminal (α domain) portion of IcsA (contains the determinant for actin assembly) is transported and exposed on the bacterial surface (20, 48). IcsA recruits host cytosolic components to induce actin nucleation, and its asymmetric location ensures that actin assembly occurs in a directional manner (32, 48). The amount of IcsA α domain exposed on the bacterial surface correlates with the efficiency of actin tail formation (79).

(iii) HAF. A cell-associated mannose-resistant hemagglutinating factor (HAF) has been identified in an EIEC strain of serotype O124:H$^-$ (113). The HAF is probably a nonfimbrial putative adhesive factor that mediates the in vivo adherence of this EIEC strain to human epithelial cells, but additional information about its function or the role played, if any, in the adhesion of other EIEC strains has not yet been reported.

DAEC

DAEC strains are characterized by their diffuse adherence pattern on cultured epithelial cells (109), but the association of DAEC strains with diarrheal disease is not as strong as with the other categories of *E. coli* strains (57, 58, 124), and little is known about the mechanism of pathogenesis. So far, two adhesins capable of mediating the diffuse-adherence phenotype have been characterized for DAEC strains.

(i) Fimbrial adhesin F1845. F1845 is a fimbrial adhesin that mediates diffuse adherence of the DAEC strain C1845 to epithelial cells (18). F1845 fimbriae are a member of the Dr family of adhesins associated with the decay-accelerating factor (DAF; CD55) via binding to the Dr blood group antigen (10). The major F1845 fimbrial structural subunit DaaE can directly bind to DAF, suggesting that this protein confers specificity for binding (138). Adhesion of F1845-expressing strains to epithelial cells induces a variety of cellular responses, including elongation, nucleation of microvilli (10), formation of membrane projections (26, 145), F-actin rearrangements (99), and recruitment of CD55 and CD66e brush border-associated glycosylphophatidylinositol-anchored proteins (51). The F1845-expressing strains also stimulate the secretion of interleukin 8, the induction of mitogen-activated protein kinases, and the expression of DAF and promote PMN transepithelial migration in polarized T84 epithelial cells, suggesting that inflammation is a characteristic present in DAEC infections (12, 13). Despite the fact that DAEC is not considered an invasive organism, F1845-coated beads can mediate the invasion of epithelial cells, which suggests a potential role of the protein as an invasin (49).

F1845 fimbriae are encoded by five genes, designated *daaABCDE*, transcribed as a single transcriptional unit under the control of the *daaA* promoter (16). Expression of the *daa* locus is dependent upon leucine-responsive regulatory protein (Lrp) and integration host factor and subject to catabolite repression (16). The regulation of F1845 fimbrial-gene expression has been extensively investigated. *daaE* mRNA processing is independent of RNase III and RNase E (17) but dependent upon a tripeptide Gly-Pro-Pro sequence encoded by the *daaP* codon (76, 77). The significance of F1845 in virulence remains unclear because DAEC strains of fecal origin rarely express F1845 adhesin (23).

(ii) AIDA-I. The adhesin involved in diffuse adherence (AIDA-I) is a plasmid-encoded protein of the clinical DAEC strain 2787. AIDA-I precursor protein is encoded by the *aidA* gene, and its mature form mediates diffuse adherence to HeLa cells (5, 6, 8). The AIDA-I protein belongs to the family of outer membrane autotransporters (62) in which the translocation mechanism and adhesive properties are located towards the C-terminal portion of the precursor molecule (122). The N terminus of AIDA-I contains a signal sequence of 49 amino acids that is cleaved during transport through the inner membrane, generating a C-terminal AIDA^c or translocator protein and the passenger domain AIDA-I (69, 70). The translocator AIDA^c is integrated as a putative β-barrel structure in the outer membrane, whereas AIDA-I remains noncovalently associated with the bacterial surface (70, 122). AIDA-I adhesion is glycosylated by heptoses with the help of the autotransporter adhesion heptosyltransferase (Aah). The Aah protein is a specific (mono)heptosyltransferase that generates the functional

mature AIDA-I adhesin (7). AIDA-I binds to HeLa and HEp-2 cells in a saturable fashion, indicating the recognition of a finite number of distinct receptor proteins (5, 6). Recently, it has been demonstrated that the purified AIDA-I adhesin binds specifically to a high-affinity receptor on HeLa cells identified as an integral N-glycosylated surface membrane glycoprotein of about 119 kDa (gp119) (71).

In addition to F1845 fimbriae and AIDA-I, a 57-kDa protein that mediates diffuse adherence and hemagglutination has been identified in two *E. coli* strains of serotypes O73:H33 and O89:H$^-$, but its identity is still unknown (146). Furthermore, two surface proteins of 16 and 29 kDa (57) and an 18-kDa mannose-resistant HAF, also described to occur in an EIEC strain (147), have been implicated with DAEC adherence, but their specific functions have not been completely elucidated.

FINAL REMARKS

Significant progress has been made in understanding the initial adherence of diarrheagenic *E. coli* to epithelial cells in the past decade. Although the concept of fimbrial structures mediating adherence to host cell receptors is a long-standing paradigm for diarrheagenic *E. coli* and other bacterial pathogens, the elucidation of the interaction between Tir and intimin in EPEC and EHEC set a new paradigm of pathogenic bacteria and host cell interactions in cellular microbiology. Sequence differences and subtyping of intimin provide a possible explanation of the host tropism that indicates a continuous adaptation and evolutionary fitness of the organisms to their environment. Furthermore, new fimbrial as well as nonfimbrial adhesins have been found to be associated with the adhesion of the different diarrheagenic *E. coli* strains and have become novel targets for vaccine development. However, many of the adhesion studies have been carried out with in vitro culture cells not derived from intestinal cells. Even though such experiments provide information about the role played by these factors in adhesion in vitro, the relevance of these findings for in vivo infection are limited and still need to be validated in in vitro intestinal cell studies and in vivo with an appropriate animal model. Further characterization of these adherence factors will provide not only a better understanding of the pathogenesis of the different diarrheagenic *E. coli* strains, but also attractive targets for novel therapeutics and vaccine development.

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