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Virulence of Enteropathogenic *Escherichia coli*, a Global Pathogen

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INTRODUCTION

Diarrheal illness is a major public health problem worldwide, with over 2 million deaths occurring each year, particularly among infants younger than 5 years (www.who.int). One of the most common causes of infantile diarrhea is enteropathogenic *Escherichia coli* (EPEC). Despite intensive research on this organism over the last two decades, however, much still remains to be learnt. Although other excellent reviews have been published in recent years (23, 75, 84, 94, 95, 178, 181), the field is fast moving, and here we provide an updated overview of the virulence mechanisms associated with EPEC and some of the more recent developments resulting from modern molecular and cell biological research.

Historically, EPEC strains were defined in terms of their negative characteristics, particularly their inability to produce enterotoxins or to demonstrate *Shigella*-like invasiveness. In recent years, however, the virulence mechanisms of EPEC have become better understood, due primarily to the advent of molecular and cell biological approaches. While EPEC invades tissue culture cells in vitro, this is not thought to occur in vivo (39, 56), and it is now clear that the virulence of EPEC depends primarily on the induction of a characteristic ultrastructural lesion in which the bacteria make intimate contact with the apical plasma membrane, causing localized destruction of the intestinal brush border and distortion of the apical enterocyte membrane. This contact results in gross cytoskeletal rearrangements, particularly the formation of an actin-rich cup-like pedestal at the site of bacterial contact, the so-called attaching and effacing (AE) lesion (125, 128). The formation of AE lesions is a dynamic process, and it has been shown that they can bend and undulate when viewed by video microscopy (150) and that individual attached bacteria can move on the host cell surface. It is proposed that the formation of AE lesions results in a reduction in the absorptive capacity of the intestinal mucosa, which inevitably leads to disruption of the electrolyte balance and subsequently to diarrhea. AE lesions have been demonstrated both in vivo (in biopsy specimens taken from infants with diarrhea) (24, 148, 173) and in vitro with a range of cell lines and organ explants (56, 106, 134). AE lesion formation is dependent on a number of physiological and environmental conditions (146) and is optimal in early to mid-logarithmic growth at 37°C (AE lesions are not induced at 28°C). Infection with enterohemorrhagic *E. coli* (EHEC) results in the formation of similar lesions at the point of bacterial contact; however, these lesions are different in composition (38, 64) and are localized to the terminal ileum or colon (82). The mouse pathogen *Citrobacter freundii* is also able to stimulate the production of AE lesions in vitro (5, 154).

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THE FOUR-STAGE MODEL OF EPEC LESION FORMATION

The pathogenesis of EPEC infection has been proposed to occur in four distinct stages (42, 108) (Fig. 1), although this model remains controversial and probably artificial. In the first stage and under the correct environmental conditions, EPEC cells express bundle-forming pili (Bfp), the intimate adhesin intimin, and short, surface-associated filaments (EspA filaments); the expression of these determinants is dependent on both plasmid and chromosomal genes. In the second stage, EPEC cells adhere to the epithelial cell via Bfp and EspA filaments, and a type III secretion system injects the translocated intimin receptor (Tir) and an as yet undetermined number of effector molecules directly into the host cell. Effector molecules activate cell-signaling pathways, causing alterations in the host cell cytoskeleton and resulting in the depolymerization of actin and the loss of microvilli. Tir is modified by the action of both protein kinase A and tyrosine protein kinase and inserts into the host membrane. In the third stage, the EspA

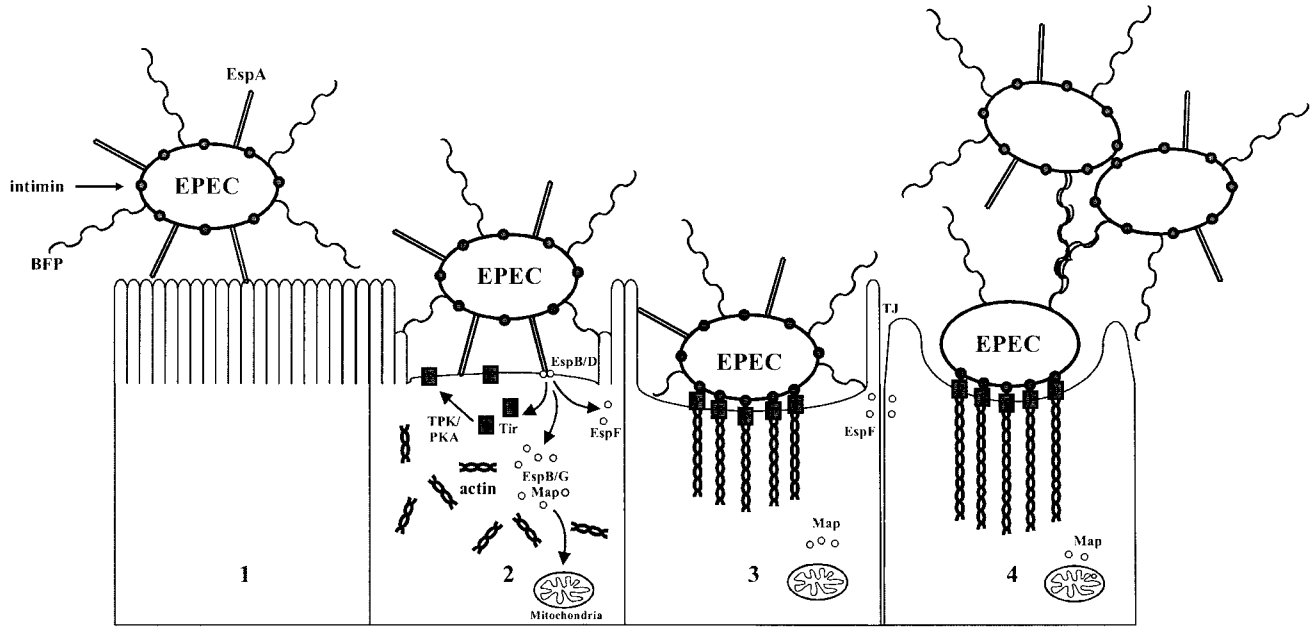

FIG. 1. Four-stage model of EPEC pathogenesis.

filaments are lost from the bacterial cell surface; the bacterial adhesin intimin binds to the modified Tir, resulting in intimate attachment; and accumulation of actin and other cytoskeletal elements occurs beneath the site of bacterial adherence. During the fourth stage, massive accumulation of cytoskeletal elements at the site of bacterial attachment results in the formation of the characteristic EPEC pedestal structure. The translocated effector molecules disrupt host cell processes, resulting in loss of tight-junction integrity and mitochondrial function, leading to both electrolyte loss and eventual cell death.

LOCALIZED ADHERENCE OF EPEC

EPEC bacteria adhere to epithelial cells in vitro in a so-called localized-adherence (LA) pattern. LA is an inducible phenotype, which occurs more rapidly in vitro if EPEC cells are preincubated with cultured cells (183). Thus, when EPEC bacteria that were nonadherent after 60 min of incubation with cultured HEp-2 cells were subsequently transferred to uninfected HEp-2 cells, LA occurred within 15 min compared with 30 to 60 min for noninduced bacteria. Interestingly, EPEC adherence experiments using the enterocyte-like HT-29 cell line suggested that LA of the bacteria occurred only when the HT-29 cells were differentiated, suggesting that LA requires an unknown host cell receptor that is expressed only after differentiation (64).

LA depends on both chromosomal genes and the *bfp* gene cluster carried on a ~92-kb (60-MDa) IncFII plasmid (11, 68), subsequently termed the EAF (for "EPEC adherence factor") plasmid (114). EAF plasmids are negative for alpha-hemolysin, colicin, and aerobactin synthesis, and they do not possess any recognized biochemical or antibiotic resistance markers (127). EAF-cured EPEC strains adhere poorly to HEp-2 cells, confirming that the plasmid is required for expression of the LA phenotype (11). Moreover, EAF-positive EPEC cells form tight, spherical, bacterial autoaggregates when cultured in defined media (but not in complex media) while EAF-cured EPEC do not (183); this autoaggregation is not inhibited by D-mannose, indicating that it is not due to the expression of type 1 pili. EAF plasmids from various EPEC strains show only 50 to 90% homology (127). Homology between the adherence plasmid of the rabbit AE-producing *E. coli* strain RDEC-1 (which, incidentally, is EAF probe negative) (127) and EPEC EAF plasmids is only 50%, suggesting that there may be uncharacterized genes on either of these plasmids involved in host specificity or virulence. Note that some EPEC strains do not carry the EAF plasmid; for example, an EPEC isolate of serotype O18ab was reported that possessed the gene for intimin but did not contain the EAF plasmid and did not therefore express Bfp (152). As expected, this strain did not show LA on cultured cells. EPEC have been classified according to whether they do (class I) or do not (class II) possess an EAF plasmid (128), although they are more commonly termed typical and atypical EPEC, respectively (91, 178). EPEC cannot, therefore, be considered to be a single group of enteropathogens; as such, these strains are ripe for analysis by modern molecular microbiological approaches. Typical and atypical EPEC strains often occur within the same serotype (178).

BUNDLE-FORMING PILI

Strains of *E. coli* produce various types of adherence pili, some of which may be involved in pathogenicity (78). The Bfp of EPEC, first described by Girón and colleagues (67), exist as bundles which are 50 to 500 nm wide and 14 to 20 μm long and that intertwine with Bfp of other bacterial cells to create three-dimensional networks. Bfp may be partially or wholly responsible for the LA phenotype by the recruitment of bacteria in the environment of the host cell (67). A receptor has not been

identified for Bfp; however, recent work (105) has identified Bfp binding to the lipid phosphatidylethanolamine, and it has been proposed that this could be responsible for Bfp interactions with both the host and other bacterial cells. The amino-terminal sequence of the major structural protein of Bfp indicates a type IV pilus (68) similar to those expressed by other pathogenic bacteria, such as *Pseudomonas aeruginosa*, *Neisseria gonorrhoeae*, *Moraxella bovis*, *Dichelobacter nodosus*, and *Vibrio cholerae* (168). The type IV pili, which were initially classified by their polar location and their association with twitching motility, all possess similar structural features, share a characteristic short leader peptide sequence, and have a highly conserved N terminus (66). Nevertheless, a PCR method has been developed for the detection of the *bfp* genes of EPEC (77) that showed no amplification of DNA from any other bacterial enteropathogens and was 100% specific for EPEC strains which exhibited the characteristic LA phenotype. Monoclonal antibodies have also been raised against Bfp (69), and these may prove useful both for diagnosis and for studying the interaction of Bfp with host cells.

Two independent laboratories have reported that the *bfp* gene cluster contains 14 genes (162, 168). The proteins encoded by these genes include some that have homologues that are involved in the biogenesis of type IV pili in other bacteria (168); however, several of the genes are unique of EPEC. The first gene to be cloned was *bfpA* (40), which encodes the major structural subunit of Bfp, "bundlin"; expression of BfpA is regulated at the transcriptional level (137), and optimal expression occurs during exponential growth, at temperatures of 35 to 37°C, and in the presence of calcium. Ammonium ions significantly reduce *bfpA* expression and the LA phenotype. Immediately downstream of *bfpA* is a region of dyad symmetry that is predicted to reduce the expression of downstream genes (168), an arrangement similar to that observed in the *tcp* operon of *V. cholerae.* The gene *bfpP* encodes a peptidase (193) that is homologous to prepilin peptidases of several other bacterial species; mutation of *bfpP* blocks signal sequence cleavage of prebundlin, Bfp biogenesis, and localized adherence. Mutations in any of *bfpG*, *bfpB*, *bfpC*, *bfpU*, *bfpD*, *bfpE*, *bfpI*, *bfpJ*, *bfpK*, or *bfpL* do not affect prebundlin expression or processing but do block Bfp biogenesis and localized adherence (6, 139, 140). The precise functions of the products of these genes in the assembly complex is still unproven; however, based on protein sequence and biogenic interactions, Ramer et al. (140) have proposed that BfpG and BfpB form an outer membrane secretin-like complex (155); that BfpC, BfpI, BfpJ, BfpK, and BfpL form an inner membrane component with the major pilin subunit bundlin; and that the soluble BfpU (156) forms a periplasmic component between the two. BfpE, which is required for the stability of all of the other components, has been proposed to serve two functions: it forms an inner membrane scaffold for the assembly complex, and it is responsible for transporting the other protein components across the inner membrane (15, 140). The function of BfpH is unknown; a mutation in *bfpH* has no effect on prebundlin expression and processing or on Bfp biogenesis and LA (6, 140). Mutations in *bfpF* result in bacteria that produce increased levels of Bfp and are more adherent in cell culture assays than their parents (7, 14). Furthermore, these mutants exhibit an altered autoaggregation phenotype; i.e., the bacterial agglutinates they form are irregular compared to the smooth autoaggregates formed by wild-type EPEC, and the agglutinates do not disperse back to single cells when transferred to noninducing conditions (14).

Genes external to the *bfp* gene cluster are also required for full expression of Bfp. For example, three open reading frames designated *perA*, *perB*, and *perC* (for "plasmid-encoded regulator"), also known as *bfpT*, *bfpV*, and *bfpW*, respectively, have been described which are located 6.7 kb downstream of the distal gene of the *bfp* gene cluster and are required for transcriptional activation of *bfpA* (72, 162, 177). In addition, mutational inactivation of the chromosomal locus *dsbA*, which encodes disulfide isomerase, leads to the loss of LA (192), suggesting that disulfide bond formation is required for biogenesis of Bfp. It has also been proposed that another chromosomal gene, *lspA*, which codes for lipoprotein signal peptidase, may be necessary for Bfp biogenesis (162).

Although it is clear that Bfp are responsible for the LA phenotype and may be responsible for species-specific adhesion (176), it remains unclear whether Bfp are involved in the initial stages of bacterial adhesion. Tobe and Sasakawa (175) reported that Bfp-expressing cells preferentially bound to cultured cells rather than to bacterial microcolonies already on the surface, indicating a role in initial attachment. However, EPEC mutants that expressed intimin normally but were Bfp negative have been shown to adhere and cause AE lesions on pediatric intestinal tissue, while an *eae* mutant expressing Bfp did not (85). This suggests that Bfp are not essential for full EPEC adherence to intestinal cells and therefore that a second adhesin may be responsible for initial interactions with the host cell. A similar conclusion was reached previously with the characterization of the AF/R1 adhesin of the rabbit EPEC strain RDEC-1 (190), and the concept is supported by recent evidence from a study of EHEC (44), which showed that serotype O157:H7 possesses an *eae* homologue but serotype O113:H21 does not, suggesting that the latter uses a different adherence mechanism. A second adhesin has been found in AE strains of *E. coli* that are pathogenic for rabbits, particularly strains of serotype O103:K$^-$:H2 (135). The major subunit of this adhesin, which enables the bacteria to adhere to HeLa cells with a diffuse pattern, can be purified from surface extracts of the bacteria and is a protein of 32 kDa. The presence of second adhesin would explain the events which occur during the initial interaction between bacteria and enterocytes. Although Bfp may not be solely responsible for the LA phenotype, recent data have confirmed that they are required for full virulence of EPEC (14). The response of human volunteers to wild-type EPEC, *bfpA*-negative EPEC, and *bfpT*-negative EPEC was studied in a randomized double-blind study; only administration of wild-type EPEC resulted in full diarrheal disease. In a parallel study, an EPEC strain with a modified *bfpF* had to be administered as a 200-fold greater bacterial inoculum to produce the same diarrheal response as the parent. This unexpected result led Bieber et al. (14) to speculate that while BfpF was not required for Bfp formation, its role in the dispersal of bacteria from autoaggregates might be essential for the colonization of additional epithelial sites within the host.

In addition to Bfp, two other EPEC surface structures, rod-like fimbriae and fibrillae, have been characterized and have been suggested to be involved in the interaction of EPEC with

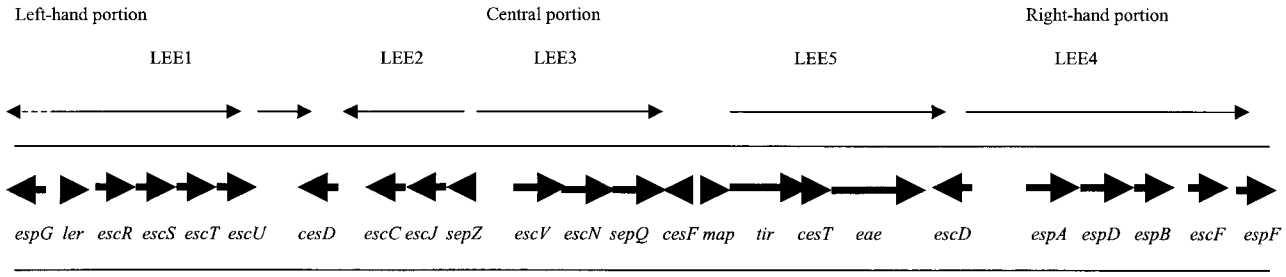

FIG. 2. Schematic representation of the EPEC LEE. It contains 41 predicted open reading frames arranged in at least five polycistronic operons, namely, LEE1, LEE2, LEE3, LEE4, and LEE5. The diagram shows the genes known to be required for EPEC virulence.

host cells (67) in a multifactoral process that also involves host cell factors. Other authors suggest that additional factors encoded by the EAF plasmid or chromosomal genes may also be required for adherence (168). LA may be due in part to surface hydrophobicity conferred by bacterial lipopolysaccharides and outer membrane proteins, although these probably play only a small role in overall adherence since other *E. coli* strains, including nonpathogenic strains, also exhibit similar surface hydrophobicity.

Recent work has suggested that flagella may also be involved in EPEC adherence to epithelial cells (70). EPEC mutants with mutations in the flagellar gene *fliC* were markedly impaired in their ability to adhere and form microcolonies. Futhermore, purified EPEC flagella and anti-flagellum antibodies were both effective in blocking the adherence of several EPEC serotypes. Additionally, it was observed that medium preconditioned by growth of cultured epithelial cells was stimulatory to EPEC for the production of flagella, leading to the proposal that epithelial cells may produce a signal that the bacteria can recognize (70, 163).

LOCUS OF ENTEROCYTE EFFACEMENT

The ability of EPEC to induce AE lesions is associated with a large chromosomal pathogenicity island called the locus of enterocyte effacement (LEE). Furthermore it has been demonstrated, by transferring the entire region cloned on a plasmid into *E. coli* K-12, that the LEE contains all of the genes required for production of AE lesions by EPEC (120). Other enteropathogens that also produce AE lesions (119), including EHEC, *C. freundii*, and the rabbit pathogen RDEC-1, also carry an LEE. Although *Helicobacter pylori* induces cytoskeletal rearrangements and causes tyrosine phosphorylation of host cell proteins, a similar chromosomal locus associated with these phenotypes has not yet been found (157), and it is probable that *H. pylori* adhesion is mediated by mechanisms distinct from those of EPEC adhesion (45). The chromosomal location of the LEE varies among EPEC and EHEC strains according to their evolutionary lineage (187), and it has therefore been suggested that it may have been acquired at several times during the evolution of these groups.

The fluorescent actin staining (FAS) test detects the accretion of actin at sites of intimate adherence by the use of fluorescein-labeled phalloidin, an actin-specific fungal toxin (106). The FAS test can be used to visualize all AE lesion-producing bacteria, including EPEC, EHEC, and *C. freundii.* A recent report suggests, however, that some bacteria may be negative in the FAS test even though they possess the LEE (187); it is likely that this is due to lack of adherence to certain cell lines used in the test rather than to the lack of genes required for actin accretion.

The LEE regions of EPEC, EHEC, and RDEC-1 have been sequenced and shown to contain 41, 54, and 41 predicted open reading frames, respectively (49, 132, 172, 195). The additional 13 genes found in the EHEC LEE fall within a putative P4 family prophage designated 933L; the prophage is not present in the prototype EPEC O127:H6 strain E2348/69 but is found in a closely related EPEC O55:H7 serovar and other O157:H7 isolates (132). In EPEC, the LEE open reading frames are arranged in five large, polycistronic operons, LEE1 to LEE5, and as a number of smaller transcriptional units (Fig. 2); these contain many genes known to be involved in AE lesion formation, including *eae* (*E. coli* AE), *esp* (*E. coli* secreted proteins), and *esc* (*E. coli* secretion [formerly known as *sep*, for secretion of EPEC proteins]) (49).

Recently, LEE pathogenicity islands (PAIs) have been characterized from two additional rabbit EPEC strains and compared with homologous loci from RDEC-1 and the human EPEC and EHEC strains E2348/69 and EDL933, respectively (172). Although the five PAIs differ in their overall size and content, they all contain a core 34-kb region and include an integrase gene, Int-phe, that can mediate site-specific integration of foreign DNA. Moreover, a LEE::*sacB* derivative of the rabbit EPEC strain 84/110-1 was shown to be capable of spontaneous PAI deletion (172). Taken together, these findings indicate a possible mechanism for the mobilization and integration of the LEE PAI among the different strains of EPEC.

The first gene identified within the LEE was *eaeA* (90), now known simply as *eae* following recent nomenclature changes (49). The protein product of *eae*, known as intimin, is present in all AE *E. coli* strains and is required to activate the signal transduction pathways that lead to remodeling of the eukaryotic cell surface at the site of bacterial attachment and pedestal formation (134). EPEC intimin mutants are unable to induce the production and elongation of microvillus-like processes that occur on bacterial attachment during infection. Intimin shows significant homology to the invasin protein from *Yersinia pseudotuberculosis*, which binds with high affinity to the β_1 family of integrins and allows efficient invasion of epithelial cells (41). There is significant sequence variation between the *eae* genes of animal and human AE *E. coli* strains of various serotypes (195), leading to the identification of at least 10

intimin types, which have been designated alpha, beta, gamma 1, gamma 2, delta, epsilon, zeta, eta, iota and kappa (3, 4, 130, 194). In EPEC O111 clones, the divergence of intimin types is so great that it suggests that in these EPEC lineages the genes of the LEE have been acquired at multiple times (171). Intimin type contributes to tissue specificity; i.e., when an EPEC *eae* mutant was complemented with either EPEC eae_{α} or EHEC eae_{γ} (133), the former adhered to small intestinal mucosa in the manner of the EPEC parent while the latter adhered to Peyers' patches, as do EHEC cells. Mutational analysis of intimin has demonstrated that key amino acid residues modulate its activity (141, 142) and, in particular, the molecular basis of intimin-mediated tissue specificity. The receptor-binding activity of intimin resides in the carboxy-terminal 280 amino acids (known as Int280) of the protein; while this region shows considerable sequence variation between intimin types, some residues, such as the four tryptophan molecules W117, W136, W222, and W240 (at positions 776, 795, 881, and 899 in the native protein) are conserved in many EPEC strains (3). Recently the crystal structure of the intimin-receptor complex has been deduced (115), and a structure has been proposed for the cell adhesion fragment of intimin (92). It is suggested that Int280 comprises three domains, two immunoglobulin-like domains and a C-type lectin-like module, with the latter defining a new family of bacterial adhesion molecules (92).

THE EPEC TYPE III SECRETION SYSTEM

Four secretion pathways (types I to IV) have been described for gram-negative bacteria (52, 86); there is also a possible type V system, but to date this has not been well characterized (86). Type III secretion systems (TTSS) are designated as *sec* independent; however, assembly of the actual secretion machinery system may require the *sec* pathway for some components. TTSSs are almost exclusively involved with virulence and have been shown to be involved with protein secretion in a number of bacterial species which are pathogens of both animals or plants (86). The EPEC LEE includes 10 genes that have been proposed to be involved with type III secretion (Table 1) (49, 89); originally designated *sep* (for "secretion of EPEC proteins"), these genes were recently renamed *esc* (for "EPEC secretion") (49), in line with the nomenclature used for TTSSs in other bacterial pathogens such as *Pseudomonas* (86), *Salmonella* (25), *Shigella* (9), and *Yersinia* (28, 29).

Many protein components of TTSSs show sequence similarities to those of flagella basal bodies (86), and the supermolecular structures of the TTSSs identified in both *Shigella* (16, 170) and *Salmonella* (110) indicate a similar structure. Kubori et al. (110) identified a "needle complex" (NC) which in *Shigella* has been characterized and found to be composed of a central needle, approximately 8 nm wide and 45 nm long, surrounded by a basal body consisting of two doublet rings 15 and 26 nm in diameter (170). The height of the basal body was 32 nm and therefore is sufficient to span both the peptidoglycan layer and the membranes. In *Shigella* the protein components of both the basal body and the needle have been identified to be MxiD, MxiG, MxiJ and MxiH, MxiI respectively (16, 170).

Sekiya et al. (158) investigated the EPEC TTSS by electron microscopy and were able to identify NCs which were smaller than (doublet ring diameters of approximately 17 and 18 nm) but reminiscent of *Shigella* basal bodies but which possessed unusually long needles with lengths up to 600 nm. These needle structures were also unusual in that they possessed a short thin "neck" region (approx 8 nm in diameter) close to the membrane followed by a wider "sheath" region (approximately 12 nm in diameter) extending outwards (158). Examination of NCs from EPEC mutants found that in *espB* mutants they were wild type, but that *espA* mutants and *escF* mutants failed to produce NCs at all. Since the product of the *escF* gene shows significant homology to the main *Shigella* needle component MxiH (170), it would be predicted to form part of the EPEC NC; indeed, an *escF* mutant is deficient in translocation of effector molecules and AE lesion formation (158, 188). Interestingly a complemented *espA* mutant produced sheath-like structures that were much longer than in the parent (158). EspA is one of three proteins, the others being EspB (previously EaeB) and EspD, which are all secreted in significant quantities by EPEC in a TTSS-dependent manner (43, 54, 103, 113) and which are required for effector protein secretion and AE lesion formation (1, 103, 113). Electron microscopy using gold-labeled EspA antibodies demonstrated that EspA forms novel filamentous surface appendages (EspA filaments) that span the space between bacteria and host in the early stages of EPEC infection (108). It has previously been proposed that these filaments are associated with the TTSS and form an extended needle structure through which effector molecules are translocated into the host (57, 108, 159). Wilson et al. (188) have shown that an *escF* mutant does not form EspA filaments, and it has been shown by two groups that EscF and EspA interact directly (32, 158). With the recent demonstration that EspA filaments have a diameter of 12 nm (32), it has been proposed that the EspA filament is the sheath-like structure that is directly linked to the TTSS via the EscF needle (32, 158).

The TTSS and the EspA filament form a physical link, akin to a "molecular syringe," between bacteria and host, along which bacterial effectors can travel (53). The EspB and EspD proteins are translocated into the host membrane in an EspA-dependent manner (108, 159), but they are also themselves required for the translocation of other effector molecules, such as Tir (57). Due to their homology to the *Yersinia* YopB/D proteins, which cause contact-dependent hemolysis of red blood cells (RBCs) (80), it is predicted that EspB and EspD form a pore complex in the host cell membrane which is the tip of the molecular syringe (53). Recent studies have shown that EPEC can also induce hemolysis of RBCs and that both the EspA filament and EspD are required for this process (159, 185). Although it is predicted that EspD is the major pore component, the failure of an *espD* mutant to form EspA filaments (108) suggests that it may also have other activities prior to host cell contact (188). The role of EspB is also ambiguous; it is required for effector protein translocation and for directly interaction with EspA but is not required for EspA filament formation (83). Wilson et al. (188) therefore proposed that the EspA-EspB interaction is a late event in infection and may modulate EspA filament activity, initiating the transition from an adhesive to a translocation function.

Ide et al. (88) have used the RBC model to demonstrate that culture supernatants (containing EspB and EspD) from dif-

TABLE 1. EPEC virulence genes

Gene	Alternative or previous name	Function	Reference(s)
bfpA		BFP biogenesis, major pilus subunit—bundlin	40
bfpB		BFP biogenesis, outer membrane	140, 155
bfpC		BFP biogenesis, inner membrane	140
bfpD		BFP biogenesis	162, 168
bfpE		BFP biogenesis, inner membrane scaffold and transporter	15, 140
bfpF		Required for dissociation of BFP bundles	7, 14
bfpG		BFP biogenesis, outer membrane	140
bfpH		Function unknown	162, 168
bfpI		BFP biogenesis, inner membrane	140
bfpJ		BFP biogenesis, inner membrane	140
bfpK		BFP biogenesis, inner membrane	140
bfpL		BFP biogenesis, inner membrane	140
bfpU		BFP biogenesis, periplasmic	140, 156
bfpP		BFP biogenesis, prepilin peptidase	193
bfpT	*perA*	Regulator of *bfp* and *ler*	72, 162, 177
bftV	*perB*	Accessory factor for PerA	72, 177
bfpW	*perC*	Accessory factor for PerA	72, 177
escC	*sepC*	TTSS biogenesis	49
escD	*sepE*	TTSS biogenesis	49
escF	*orf28*	TTSS biogenesis, needle component	158, 188
escJ	*sepD*	TTSS biogenesis	49
escN	*sepB*	TTSS biogenesis	49
escR	*sepI*	TTSS biogenesis	49
escS	*sepH*	TTSS biogenesis	49
escT	*sepG*	TTSS biogenesis	49
escU	*sepF*	TTSS biogenesis	49
escV	*sepA*	TTSS biogenesis	49
sepQ		TTSS biogenesis	49
sepZ		TTSS biogenesis	49
eae	*eaeA*	Adhesin binds Tir—intimin	90
tir		Translocated intimin receptor; initiates actin pedestal formation	103
espA		Forms filaments between bacteria and host; delivery of effector molecules	57, 107
espB	*eaeB*	Translocation pore; host cytosolic effector(?)	57, 88, 174
espD		Translocation pore	57, 88
espF	*orf30*	Disrupts tight-junction integrity; induces cell death	31, 121
espG	*rorf2*	Effector molecule (function unknown)	46
map	*orf19*	Damages mitochondria membrane integrity; induces filopodia formation	94, 99, 102
ler	*orf1*	Regulator for LEE genes and *espC*	48
cesD	*sepE*	Chaperone for EspB and EspD	184
cesT	*orf21*	Chaperone for Tir	36
cesF	*rorf10*	Chaperone for EspF	47

fusely adhering EPEC can induce hemolysis without the need for bacterial cell contact. Although this phenomenon does not occur with typical EPEC supernatants (88), cell damage has been reported after treatment of cultured cells with outer membrane protein extracts of EPEC that include maltoporin and EspB (111, 112). The lysed RBC membranes were found to contain both EspB and EspD and, when examined under both electron microscopy and atomic force microscopy, were found to contain pores comprising six to eight subunits and with an apparent outer diameter of 55 to 65 nm and an inner diameter of 8 nm but narrowing deeper into the pore (88). Additional osmoprotection experiments (88) confirmed that the diameter of the pore was 3 to 5 nm, which is similar to the size of the TTSS pores of *S. flexneri* (16) and *Yersinia* (80).

In addition to the EspA filaments seen in EPEC and EHEC, it is apparent that there are similar filaments associated with TTSSs in other bacteria, such as the HrpA pilus of *Pseudomonas syringyae* (144) and the HrpY pilus of *Ralstonia solanacearum* (182). Since these TTSSs are structurally distinct from the archetypal systems observed in *Shigella* and *Yersinia*, it has been proposed that they be redesignated as a filamentous type III secretion system (FTTSS) (32).

EPEC SECRETED PROTEINS

There are at least eight EPEC secreted proteins, of which six are encoded by genes within the LEE (Fig. 2); the two proteins not encoded within the LEE are EspC and the glycolytic pathway enzyme glyceraldehyde-3-phosphate dehydrogenase. The *espC* gene, which is located within a second PAI at 60 min on the *E. coli* genome (124), encodes a 110-kDa immunoglobulin A protease-like protein (167); it is not necessary for mediating signal transduction in epithelial cells and does not play a role in EPEC adherence or invasion of tissue culture cells in vitro. Furthermore, EspC is secreted independently of the LEE type

III secretion apparatus and has been recently found to be an enterotoxin (124).

The first three LEE-encoded secreted proteins to be characterized were EspA, EspB and EspD, which are 25, 37, and 39 kDa, respectively (79, 103) and are encoded by the LEE4 operon (Fig. 2). The role of these proteins in the EPEC FTTSS has already been covered, but EspB also appears to have other functions in EPEC virulence. After contact, the EspB protein is translocated into the host cell membrane (146), but it is also targeted to the host cell cytoplasm (173), where it has been proposed to act as a cytoskeletal toxin causing actin redistribution (174). A recent study of EHEC by using the two-hybrid system (109) has demonstrated that the N-terminal region of EspB interacts directly with the host cytoskeletal protein α-catenin. Additional immunofluorescence studies indicate that α-catenin is then recruited to the site of actin accumulation in an EspB-dependent manner. Furthermore, expression of either the N terminus or whole EspB protein from a transfected mammalian expression vector actually inhibited actin accumulation at the site of bacterial infection in HeLa cells. Interactions between EspB and α-catenin have not been demonstrated in EPEC; however α-catenin is recruited to the pedestal (109). It has recently been found that CesD (for "chaperone for *E. coli* secreted protein") is required for EspB and EspD secretion; this protein shows sequence homology to other chaperone proteins of type III secretion pathways (184).

A fourth secreted protein encoded by the LEE, known as Tir (for "translocated intimin receptor"), with a predicted mass of 78 kDa, acts as the intimin receptor in host cells (98). The protein homologue in EHEC has been termed EspE (34), but Tir is still the widely accepted name. After translocation into the host cell, which is dependent on EspA, EspB, EspD, and the TTSS (98), Tir is observed in the apical membrane with an apparent molecular mass of 90 kDa; indeed, Tir was originally thought to be a mammalian cell protein and was previously known as Hp90 (for "Host protein 90") (55, 145, 147). Efficient transfer of Tir to the host cell is also dependent on the presence of a LEE-encoded chaperone protein, CesT (36). The difference in the predicted and observed molecular masses of Tir is due to modification by phosphorylation on both serine and tyrosine residues by the host-encoded enzymes protein kinase A and tyrosine protein kinase, respectively (74, 96, 186). The processes involved in Tir translocation and modification are, however, even more complex; Kenny and Warawa (104) demonstrated that the artificial introduction of Tir into the host cell in the absence of EPEC did not result in full modification, indicating a requirement for additional EPEC-encoded factors. Tir translocation can occur separately from host cell-mediated modifications (160), indicating that the EPEC type III protein translocation apparatus does not require eukaryotic cell functions for the successful delivery and insertion of Tir into host cell membranes. However, in EPEC, actin accumulation and pedestal formation absolutely require phosphorylation of Tir at the tyrosine residue Y474 (19, 76). Although conserved, the Y474 residue is not phosphorylated in EHEC Tir (38), indicating that differences exist in the signaling pathways for formation of the pedestal structure between the two pathogens. Moreover, the Tir molecule of EHEC cannot be interchanged with that of EPEC due to the need for tyrosine phosphorylation of the Y474 residue (93).

After translocation and modification, Tir inserts into the host membrane with its central, intimin-binding region extracellular and both N and C termini in the cytoplasm to interact with host proteins. A large number of host cytoskeletal proteins are present in the AE lesion (73), and a number of these are known to interact directly with Tir. For example, NcK, a mammalian adaptor protein implicated in the initiation of actin signaling, binds to the 12-amino-acid sequence surrounding the tyrosine-phosphorylated Y474 residue of Tir (19, 76) and is essential for lesion formation. Additionally, vinculin (58), cortactin (20, 21), talin (21, 58), and α-actinin (58, 75) all interact directly with Tir, and the last three are required for the organization of the actin pedestal. Since Tir spans the membrane and since vinculin, talin, α-actinin, and many of the other cytoskeletal proteins found in the pedestal are all also components of focal adhesion plaques, it has been postulated that Tir might be acting in a manner similar to that of an β_1-integrin (73). This view is further supported by the observation that the intimin-binding domain of Tir is homologous to the extracellular matrix-binding domain of integrins (96).

The *espF* gene, which is at the extreme right hand end of the LEE (121), encodes an approximately 21-kDa proline-rich protein that requires the type III secretion apparatus for translocation into host cells but which is not required for AE lesion formation (121). However, an EPEC *espF* mutant, despite normal adherence and AE lesion formation, did not cause a loss of transepithelial electrical resistance in a polarized intestinal cell monolayer, indicating that EspF is required for EPEC-induced increases in monolayer permeability at tight junctions (122). Wild-type EPEC strains induce cell death in a manner similar to apoptosis (30); however, an EPEC *espF* mutant did not induce cell death (31). Furthermore, this effect was shown to be directly attributable to EspF activity since induction of *espF* from a transfected mammalian expression vector, in either HeLa or COS cells, was sufficient to induce cell death (31). It has also been recently reported that EspF requires a chaperone, *cesF* (previously *rorf10*), to be translocated to host cells (47).

Kenny and Jepson (102) have reported the translocation of a further LEE-encoded protein, the 203-amino-acid product of *orf19*. This protein did not associate with the actin pedestal but was found to be localized within the cell around the mitochondria and has therefore been renamed Map (for "mitochondrion-associated protein") (102). The first 44 amino acids are predicted to encode a mitochondrial targeting and cleavage signal, and some Map molecules are cleaved, indicating that they may indeed enter the mitochondria. Experiments using mitochondrion-specific fluorescent dyes (102) suggest that Map disrupts the mitochondrial membrane potential, an effect observed for many pathogens that induce apoptosis (17). However, the cell death observed in experimental EPEC infection has aspects of both apoptosis and necrosis, and so it is possible that Map may play an antiapoptotic role (94). Recent work (99) has shown that Map is multifunctional and that it is also involved in cytoskeletal rearrangements leading to the formation of filopodia early in EPEC infection. Formation of the previously undetected filopodia was distinct from pedestal formation and was dependent on the cytoskeleton-associated host GTPase Cdc42, which is not required for Tir-intimin-triggered events (99). Filopodia production is transient, and, unexpect-

edly, their down-regulation was found to be dependent on the interaction of Tir and intimin. Furthermore, down-regulation of filopodia, but not pedestal formation, was found to require a region in the Tir C terminus that has similarity to arginine finger regions (99); these motifs are also found in GTPase-activating-proteins, which can stimulate the activity of GTPases, such as Cdc42, resulting in their conversion to a GDP-bound inactive form (81). It was initially unclear why Tir-intimin should down-regulate Map-induced filopodia; however, the observation that overexpression of Map inhibits pedestal formation (99) indicates that this is a necessary step in AE lesion formation. Although Tir and Map are delivered to the host simultaneously, filopodia formation continues for up to 15 min before down-regulation occurs (99, 102); it is proposed that this timescale is dictated by the requirement for modification and membrane insertion of Tir prior to intimin binding (94). While the roles of filopodia formation and Cdc42 activation in EPEC pathogenesis remain unknown, maintenance of the pedestal-inhibitory Map activity suggests that they are important for EPEC virulence.

Finally, a 44-kDa secreted protein, encoded by the *espG* gene (previously called *rorf2*), has recently been described (46). Although EspG is translocated into host cells by the TTSS, an EPEC *espG* mutant was wild type for AE lesion formation. However, an *espG* mutant of a rabbit-diarrheagenic *E. coli* strain did show diminished intestinal colonization, indicating a role in virulence (46). EspG shows significant homology to the VirA protein of *S. flexneri* and enteroinvasive *E. coli*, which plays an accessory role in host cell invasion in these bacteria (37, 179). Furthermore, expression of EspG from a plasmid is sufficient to complement the reduced intracellular persistence phenotype of a *S. flexneri virA* mutant (46). Intriguingly, EPEC also encodes a second *virA* homologue, *orf3*, which is present in the *espC* PAI (124) and which can also complement the *S. flexneri virA* mutation (46). It was proposed that the lack of phenotype of an EPEC *espG* mutant might be due to *trans*-complementation by *orf3*; however, an *espG orf3* double mutant was phenotypically similar to parent and so the function of EspG remains unknown (46).

REGULATION OF EPEC VIRULENCE DETERMINANTS

Tight negative regulation of virulence genes and their subsequent expression in response to changes in environmental conditions are key elements in the pathogenic systems of many bacteria. The problem of inappropriate expression of bacterial virulence proteins is twofold: first, it is metabolically expensive for the infecting bacterium, and second, it may alert the host defense systems to the presence of the bacteria prior to successful colonization. Additionally, many bacterial species have distinct stages in infection, such as attachment, invasion, or cell-to-cell spread, which are mediated by separate suites of genes, which in turn are differentially expressed in response to the current bacterial environment.

The expression of EPEC virulence determinants is virtually undetectable when the bacteria are grown in rich laboratory media (97); however, transfer to a defined minimal medium, such as Dulbecco minimal essential medium, results in rapid expression of Bfp and the secretion of proteins to the supernatant (57, 79, 101). Optimal expression is observed during exponential growth, at temperatures of 35 to 37°C, at pH 7, and at physiological osmolarity, in the presence of calcium and sodium bicarbonate (2, 97, 137, 146). It is unclear why EPEC virulence determinant expression is maximal in the early growth phase, in contrast to many other bacterial virulence systems, which are initiated only in stationary phase (143); however, thermoregulation is a common feature in both gram-negative and gram-positive bacterial virulence systems (87). Ammonium ions significantly reduce *bfpA* expression and the LA phenotype in vitro; it is proposed that in vivo this effect would occur as EPEC passed from the low-ammonium environment of the ileum into the high-ammonium environment of the colon, where EPEC cells do not adhere and where down-regulation of virulence factors would therefore be required (137).

The first EPEC regulatory element to be identified was the previously described *per* locus of the EAF plasmid, which comprises *perA*, *perB*, and *perC* (also known as *bfpT*, *bfpV*, and *bfpW*, respectively) (72, 129, 177). The *bfpT* gene encodes a protein, belonging to the AraC family of transcriptional activators, which regulates transcription from the *bfp* operon (162, 177) and also of genes within the LEE (72); *bfpV* and *bfpW* encode proteins that enhance the activity of BfpT by an unknown mechanism (72, 177). Regulation of *per* is dependent on autoactivation by BfpT (118, 126) and on repression by GadX (161). GadX is a putative AraC-like transcriptional regulator that is also required for the activation of the *gadA* gene, which encodes glutamate decarboxylase, a protein involved in acid resistance in *E. coli.* It has been proposed that GadX may be involved in the response to the environmental shift encountered by EPEC cells as they move from the stomach, where they must resist acid conditions, to the ileum, where they are required to express virulence genes in order to colonize and cause AE lesions (161).

The BfpT-dependent regulation of LEE genes occurs via the activation of the *LEE1* and specifically requires the first gene of the operon, *ler* (for "Locus of enterocyte effacement regulator", previously known as *orf1*) (123). The *ler* gene encodes a distant homologue of H-NS (for "histone-like nucleoid-structuring protein"), a nucleoid-associated protein that is frequently involved in the response of enterobacteria to environmental stimuli (8). It has been demonstrated that Ler is required for expression of the LEE operons *LEE2*, *LEE3*, *LEE4*, and *LEE5* and the LEE genes *espF*, *espG*, and *map* (48, 63, 123, 149, 165); additionally, it is required for expression of the non-LEE located gene *espC* (48). In addition to its requirement for BfpT, the expression of *ler* (and the *LEE1* operon) is dependent on the function of the integration host factor (63), and the early growth phase regulator Fis (for "factor for inversion stimulation") (71). Recent work on the thermoregulation of the LEE operons (180) has shed light on the mechanism by which Ler activates gene expression. It had previously been observed that the protein H-NS acts as a negative regulator of *LEE2*, *LEE3*, and *map* (18, 149), and therefore it had been proposed that the observed activation of LEE operons by Ler was due to negation of the H-NS repression. Using an EPEC *hns* mutant, Umanski et al. (180) have shown that H-NS binding to promoter sequences represses the expression of *LEE2*, *LEE3*, *LEE4*, *LEE5*, and *espG* at both 27 and 37°C; however, it represses the expression of *LEE1* (including *ler*)

only at 27°C. Thus, when a culture of wild-type bacteria is shifted from 27 to 37°C, H-NS repression of *ler* expression is alleviated, and it was proposed that Ler antagonization of H-NS then leads to expression of *LEE2*, *LEE3*, *LEE4*, *LEE5*, and *espG* (180). Use of transcriptional reporters in EPEC *ler* and *hns* single mutants and *ler hns* double mutants has demonstrated that this hypothesis is mainly correct; however, in an *hns* mutant, which should in theory be derepressed for *LEE5* expression at all temperatures, there was still a requirement for Ler, indicating that it must also activate this operon by another, H-NS-independent mechanism (180).

Quorum sensing is a mechanism by which bacteria regulate their gene expression in response to cell density. The bacteria produce small hormone-like compounds, called autoinducers (AI), that accumulate in the medium until they reach a threshold concentration, at which they interact with regulatory molecules and induce gene expression. EPEC strains do not produce the classical AI of gram-negative bacteria, the acyl homoserine lactone family (131), but they do contain the gene *luxS* (169), which encodes an enzyme that produces the furanone-like molecule AI-2 (22, 153, 189), which has been proposed as a new quorum-sensing molecule (169, 189). It has been demonstrated that LEE gene expression and lesion formation are down-regulated in a *luxS* mutant (166), and it was proposed that this was due to the action of an AI-2-dependent regulator (164). Sperandio et al. (164) reported the characterization of the QseA protein (for "quorum-sensing *E. coli* regulator A"), of the LysR family of regulators, which is activated by AI-2 and which induces Ler expression leading to LEE gene expression in EPEC and EHEC. QseA does not itself bind AI-2; however, quorum-sensing systems frequently involve complex regulatory cascades (35), and other regulators remain to be identified.

While host cell protein phosphorylation has long been recognized as a key element in AE lesion formation, it has only recently been realized that prokaryotic protein tyrosine kinase activity may also be an important modulator of EPEC virulence. TypA (for "tyrosine-phosphorylated protein A") is a tyrosine-phosphorylated GTPase encoded by the o591 sequence, which has significant homology to ribosome-binding elongation factor G (136). TypA is notable as the first *E. coli* protein unequivocally shown to be phosphorylated on tyrosine in vitro and in vivo (51, 59–62). Tyrosine phosphorylation of TypA is observed in EPEC strains but, with the exception of an *E. coli* K-12 L-form (60), has not so far been demonstrated in laboratory strains (51, 59, 62), suggesting that phosphorylation of TypA may play a role in EPEC virulence. For instance, the TypA homologue in *Salmonella enterica*, BipA, is considered to be a global regulator involved in bacterial survival during invasion of the host (138). In EPEC, TypA has been implicated in the formation of actin-rich pedestals and in resistance to the cationic host defense peptide BPI (51, 138). Biochemical studies have shown that TypA is an autophosphorylating protein that affects the phosphorylation in vivo of UspA (for "universal stress protein A"), a regulatory protein that is induced by a variety of stresses and that determines bacterial survival during growth arrest (61, 62). Interestingly, cellular levels of TypA are low, typically less than 0.1% of total cellular protein (our unpublished data), compared with its homologue EF-G, which has levels equimolar with those of ribosomal proteins. This makes it even more intriguing that in an *E. coli typA* mutant the synthesis and/or posttranslational modification of several dozen proteins is markedly altered; these proteins include regulators such as CspA (for "carbon starvation protein A") and H-NS (61, 62). A role for TypA as a new class of EPEC global regulator must therefore be considered.

EPEC-INDUCED PROTEIN PHOSPHORYLATION AND SIGNAL TRANSDUCTION

The protein phosphorylation and signaling events that occur during EPEC infection are complex and involve proteins in both the infecting bacteria and the host cell. Host cell protein tyrosine phosphorylation has been reported as a potential virulence mechanism in a number of pathogenic bacteria (33, 54, 55). In addition to Tir phosphorylation, EPEC has been observed to induce the tyrosine phosphorylation of a 150-kDa host protein (Hp150), as well as the tyrosine dephosphorylation of a number of proteins, including a 240-kDa host protein (Hp240) (100). The Hp150 band is heterogeneous in composition but includes phospholipase C-γ, which, on tyrosine phosphorylation, is known to lead to calcium and inositol phosphate (IP) fluxes (100). EPEC infection has also been observed to result in an increase in the levels of IPs within HeLa cells (39). Since IPs regulate, among other things, the release of calcium from calcium-sequestering compartments within the cell, this result appears to be consistent with early studies that demonstrated increased levels of intracellular free calcium in EPEC-infected cells. A plausible model for microvillus effacement and AE lesion formation invoked increased intracellular calcium levels as the cause of the breakdown of cytoskeletal actin (13). However, more recent data obtained using a sensitive ratiometric technique (in which the risk of calcium compartmentalisation is eliminated) indicated that increased calcium levels could not be detected at the site of the AE lesion in EPEC- or EHEC-infected cells, thus implying that they may not be necessary for AE lesion formation (10). Moreover, the EPEC-induced increases in IP levels observed took approximately 2 h (55), compared with normal hormone responses of seconds.

Nontyrosine protein phosphorylation also occurs on attachment of EPEC cells to the host (12, 116). The major phosphoprotein observed in EPEC-infected small intestinal mucosal biopsy specimens and Caco-2 cells (116) is myosin light chain, a 20-kDa cytoskeletal component which, when phosphorylated, regulates cell actin organization in nonmuscle cells (117). It has been proposed (117) that extensive serine/threonine phosphorylation of myosin light chain in host cells leads to irreversible destruction of microvillous function and accretion of actin at the site of bacterial attachment. Increased phosphorylation of myosin light chain due to EPEC infection also results in perturbation of the function of the intestinal epithelial barrier (191). This is probably an additional contributory factor to EPEC diarrhea. It has also been shown in vitro that there is a transient increase in the short-circuit current after AE lesion formation by EPEC on Caco-2 cells (27), which is dependent on EspA, EspB, and EspD (26). This is possibly linked to an influx of sodium and amino acids across the apical membrane. A second serine/threonine phosphoprotein of 29 kDa has been

identified in EPEC-infected HEp-2 cells, but its role, if any, in pathogenesis is currently unknown (12).

OTHER EPEC PLASMIDS

EPEC strains possess a number of plasmids, with some possessing as many as seven or more (151), but the relevance of many of these plasmids is unknown. EPEC strain E2348/69 possesses two plasmids, the large EAF plasmid designated pMAR2 (which, as described above, codes for Bfp) and a small 5.5-kb cryptic plasmid. The function of the latter is not known, but it may play a role in adherence since strains of E2348/69 that had been cured of the EAF plasmid were still able to adhere to cultured epithelial cells. Loss of both plasmids resulted in lack of adherence (our unpublished data). Two regions of the cryptic plasmid have 96 and 88% sequence similarity, respectively, to the mobilization and partitioning regions of plasmid pWQ799 of *S. enterica* serovar Borreze (our unpublished data). The role of a 6.6-kb plasmid in EPEC strain 0041 (serotype O111:H$^-$) has also been described (151); plasmid genes encode epithelial cell invasion and kanamycin resistance, the former being dependent on the synthesis of a 32-kDa protein. As mentioned above, some EPEC strains, referred to as atypical EPEC, do not carry an EAF plasmid and so do not express Bfp (152), yet they possess the *eae* gene and may still cause diarrhea. In such cases, is it possible that various small plasmids may have important functions in adherence.

CONCLUDING REMARKS

The elucidation of the molecular details of some of the virulence mechanisms of EPEC has led to a greater understanding not only of the pathogenesis of EPEC but also of that of other pathogenic bacteria in which genes homologous to virulence genes in EPEC have been identified. Nevertheless, EPEC remains a worldwide problem, causing high levels of morbidity and mortality among infants and young children in developing countries, and so identification of EPEC remains an urgent need if care and treatment of sick children are to be given early enough to be effective. Diagnosis may be achieved by the routine biochemical and serotyping methods that led to the original description of the "classical" serotypes of EPEC (50), but these methods are not 100% sensitive or specific. The FAS test, which uses fluorescein-conjugated phalloidin to bind filamentous actin in the AE lesion, is highly sensitive and specific for AE lesion-forming EPEC, EHEC, *H. alvei*, and *C. freundii.* Moreover, DNA probes for the major virulence factors of EPEC are now available, but they seem only to complicate the issue. In one particular study, for example, 80% of classical and 25% of nonclassical EPEC serotypes possessed the *eae* gene, although 60% or fewer of classical EPEC serotypes possessed the genes required for LA (*bfp*) or for production of the AE lesion at sites of adherence as determined by the FAS test (65). In nonclassical EPEC serotypes, the incidence of such probe-positive serotypes was 10% or lower (65). FAS test-positive, EAF-negative strains of EPEC have been isolated (107), but their role in infantile diarrhea remains to be assessed. Similarly, EAF-cured EPEC strains are positive in the FAS test (106). Therefore, although our understanding of the complexities of EPEC virulence mechanisms has increased substantially over the past two decades, due largely to the availability of molecular techniques, it is clear that much more needs to be done to achieve the effective diagnostic and therapeutic advances that will help to overcome the worldwide scourge of EPEC diarrhea.

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