The Locus of Enterocyte Effacement and Associated Virulence Factors of Enterohemorrhagic Escherichia coli

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ABSTRACT A subset of Shiga toxin-producing Escherichia coli strains, termed enterohemorrhagic E. coli (EHEC), is defined in part by the ability to produce attaching and effacing (A/E) lesions on intestinal epithelia. Such lesions are characterized by intimate bacterial attachment to the apical surface of enterocytes, cytoskeletal rearrangements beneath adherent bacteria, and destruction of proximal microvilli. A/E lesion formation requires the locus of enterocyte effacement (LEE), which encodes a Type III secretion system that injects bacterial proteins into host cells. The translocated proteins, termed effectors, subvert a plethora of cellular pathways to the benefit of the pathogen, for example, by recruiting cytoskeletal proteins, disrupting epithelial barrier integrity, and interfering with the induction of inflammation, phagocytosis, and apoptosis. The LEE and selected effectors play pivotal roles in intestinal persistence and virulence of EHEC, and it is becoming clear that effectors may act in redundant, synergistic, and antagonistic ways during infection. Vaccines that target the function of the Type III secretion system limit colonization of reservoir hosts by EHEC and may thus aid control of zoonotic infections. Here we review the features and functions of the LEE-encoded Type III secretion system and associated effectors of E. coli O157:H7 and other Shiga toxin-producing E. coli strains.

INTRODUCTION

Enterohemorrhagic Escherichia coli (EHEC) was first recognized as a cause of human disease in 1983 and is associated with diarrhea and hemorrhagic colitis, which may be complicated by life-threatening renal and neurological sequelae (reviewed in reference 242). EHEC strains are defined by their ability to produce one or more Shiga toxins (Stx), which mediate the systemic complications of EHEC infections (reviewed in reference 243), and to induce attaching and effacing (A/E) lesions on intestinal epithelia. The ability of EHEC to induce such lesions is shared by enteropathogenic E. coli (EPEC), Escherichia albertii (formerly classified as eae-positive Hafnia alvei), and the murine pathogen Citrobacter rodentium. The A/E histopathology was first described in gnotobiotic piglets challenged with a strain of EHEC serotype O157:H7 (1) but has subsequently been observed in ruminant reservoirs and diverse animal models (reviewed in reference 244).
A/E lesions are characterized by intimate bacterial adherence to the apical surface of enterocytes, sometimes on raised pedestals (pseudopodia), and destruction of nearby microvilli (Fig. 1A and B). Though reports of such lesions during human infection are lacking, Knutton et al. described that EPEC strains of many serogroups were able to form A/E lesions on human duodenal biopsies cultured ex vivo (2). Reasoning that the electron-dense pedestals may contain polymerized F-actin, the authors subsequently reported the use of fluorescein-labeled phallotoxin to stain F-actin at sites of adherence, forming the basis of a fluorescent-actin staining (FAS) test for A/E lesion formation by fluorescence microscopy (3) (Fig. 1C). Many other host cell proteins are recruited to A/E lesions, sometimes in a host-, pathotype-, serotype-, and time-dependent manner, and the molecular mechanisms underlying lesion formation have been the subject of intense study. This article focuses on the factors required for A/E lesion formation, their mode of action, and role in persistence, pathogenesis, and protection during EHEC infection.

THE LOCUS OF ENTEROCYTE EFFACEMENT

The locus of enterocyte effacement (LEE) is required for A/E lesion formation and is the most intensively studied of all virulence factors of attaching and effacing E. coli. The role of LEE-encoded genes in adherence to epithelial cells and nucleation of F-actin was first described after screening random TnphoA mutants of EPEC O127:H6 for mutants deficient in these processes. This identified the E. coli attaching and effacing (gae) gene encoding intimin (4) and other genes required for adherence and actin nucleation in a single ca. 35-kb locus that is conserved among A/E pathogens, including strains of EHEC serotypes O157:H7 and O26:H11 (5). Sequencing of a limited region of the LEE of the prototype EPEC O127:H6 strain E2348/69 identified four genes predicted to encode components of a Type III secretion system (T3SS), based on homology to Yersinia Lcr/Ysc and Shigella Mxi/Spa proteins (6). These genes were initially designated sepA–D (secretion of EPEC proteins) (6), but many were renamed esc to conform to the nomenclature of homologous Yersinia T3SS genes upon complete sequencing of the LEE of E2348/69 (7). Type III secretion is one of multiple pathways for export of proteins in bacteria and, consistent with a role in this process, the product of the sepB (escN) gene, which encodes a predicted inner membrane ATPase, was found to be required for secretion of several EPEC O127:H6 proteins required for A/E lesion formation, designated Esps (E. coli secreted proteins) (6). Secretion of Esps was also detected in EHEC O157:H7 and EHEC O26:H11 (8, 9) and found to be escN-dependent (9), supporting the existence of a conserved locus for secretion of proteins involved in A/E lesion formation.

The LEE of EPEC O127:H6 strain E2348/69 contains 41 open reading frames that are mostly organized in five polycistronic operons (7). Soon after the sequencing of the EPEC O127:H6 strain E2348/69 LEE, the sequence of the homologous region of the genome of the prototype EHEC O157:H7 strain EDL933 was reported (10), followed by the LEE of C. rodentium (11) and rabbit EPEC strains (12, 13). Many other LEE sequences have since emerged with the advent of high-throughput genome sequencing. These typically exhibit significantly lower %GC content than the flanking DNA (ca. 38% compared to the ca. 50% genomic mean), are inserted at tRNA loci, and contain remnants of mobile genetic elements, indicating that they are likely to have been acquired by horizontal transfer. The cloned LEE of EPEC O127:H6 strain E2348/69 is necessary and sufficient to confer the ability to form pedestals upon a laboratory-adapted E. coli K-12 strain (14); however, the cloned EHEC O157:H7 LEE cannot (15), consistent with the requirement for non-LEE-encoded proteins in pedestal formation by EHEC O157:H7, as discussed below.

The genetic organization of the EHEC O157:H7 strain EDL933 LEE is depicted in Fig. 2. The LEE region in this strain is inserted at the equivalent of 82 min on the E. coli K-12 chromosome, proximal to the selC selenocysteine tRNA locus, and shares the 41 genes found in EPEC O127:H6 strain E2348/69 in the same order, but is larger owing to the presence of a cryptic P4 family prophage at one end (10). Predicted structural components of the T3SS exhibit remarkable conservation among A/E pathogens; however, greater levels of amino acid sequence divergence exist among components that are predicted interact with host cells. Indeed, comparative analyses have suggested that the LEE comprises units under distinct selection pressures (16). Analysis of the insertion sites and flanking regions of the LEE in serogroup O26, O103, and O111 EHEC strains and atypical EPEC has indicated that while insertions tend to be restricted to the selC, pheU, and pheV tRNA loci, there is marked variation in the flanking regions (17, 18; reviewed in reference 19). For example, substrates for the LEE-encoded T3SS may be encoded in the flanking regions, such as Ibe5′ of the LEE in atypical EPEC (20), and a conserved cassette encoding NleE, NleB, and EspL 3′ of the LEE4 operon in EHEC O26,
FIGURE 1 (A) Transmission electron micrograph (TEM) showing A/E lesions induced by EHEC O111:H– strain E45035N in the spiral colon of a neonatal calf (note raised electron-dense pedestals and microvillus effacement relative to proximal uninfected enterocyte). From reference 233; scale bar = 1 μm. (B) TEM of A/E lesions induced by EHEC O157:H7 strain 85–170 12 h after inoculation of a bovine ligated ileal loop (note intimate adherence but relative absence of elongated pedestals). From reference 84; scale bar = 5 μm. (C) Fluorescence micrograph showing nucleation of F-actin under EHEC O103:H3 strain PMK5 adhering to a HeLa cell (green, F-actin detected with Oregon green514-phalloidin; red, bacteria stained with rabbit anti-O103 typing serum detected with anti-rabbit immunoglobulin-Alexa568). From reference 240; scale bar = 5 μm. doi:10.1128/Microbiolspec.EHEC-0007-2013.f1
O103, and O111 (18). In some strains the lifA/efa1 gene or a truncated variant thereof may be located 3′ of LEE4 as part of a mosaic island comprising the LEE and O-island 122 (18, 21). LifA/Efa1 and the truncated LifA/Efa1’ (Z4332) have recently been reported to be secreted by the LEE-encoded T3SS (22) and (together with other O-island 122-encoded genes) have been associated with isolates that cause epidemic and serious disease (23). In EHEC O103:H2 strain RW1374, the LEE, lifA/efa-1, and other virulence-associated genes form a pathogenicity-related island spanning ca. 111 kb at pheV (24), whereas in other cases effector genes may be present in the genome but not physically linked to the LEE, owing to activity of bacteriophages in the capture and transfer of genes encoding Type III secreted proteins (18, 25). As more genomes are sequenced, it is evident that the nature of lateral transfer of the LEE, and genes associated with the function of the T3SS, is highly variable, and further studies are required to define the consequences of such.

Regulation of the LEE is highly complex, with inputs from numerous global regulators, bacteriophage-encoded regulators, and LEE-encoded regulators. Control occurs at transcriptional, translational, posttranslational, and bacterial population levels (reviewed in reference 245). Of particular interest, LEE expression and the efficiency of pedestal formation are enhanced by passage in the mammalian host (26), and evidence exists that LEE expression is sensitive to host stress-related catecholamine hormones that are detected by bacterial adrenergic sensor kinases (reviewed in reference 27). Such studies reinforce the challenge of understanding LEE regulation and functions in cell-based systems devoid of cues from the host. Moreover, it is evident that the repertoire of regulators is strain-dependent and that studies on LEE regulation in single strains are therefore to be interpreted with caution. For example, lysogeny with Shiga toxin 2-encoding bacteriophages was recently reported to repress Type III secretion in phage-type (PT) 21/28 EHEC O157:H7 strains that are commonly isolated from humans in the United Kingdom and to account for a distinct level of T3SS activity relative to Stx2-minus PT32 strains (28).

The LEE-Encoded Type III Secretion System

T3SSs are key virulence factors of Gram-negative enteric pathogens and the four major genera of plant pathogenic bacteria and serve to inject bacterial proteins directly into host cells (reviewed in reference 29). A needle complex spans both the inner and outer bacterial membranes, and a subset of Type III secreted proteins forms a “translocon” that interacts with the eukaryotic cell membrane and mediates the delivery of secreted “effector” proteins into target cells. Effectors of A/E E. coli then modulate cellular processes to the benefit of the pathogen. Predicted functions have been assigned to LEE-encoded genes on the basis of homology with components of T3SSs in other bacteria (30); a schematic representation of the organization of the apparatus is shown in Fig. 3. Predicted protein-protein interactions within the T3SS apparatus have been confirmed experimentally in some cases, for example, by yeast-2-hybrid analysis (31) and analysis of purified needle complexes (32). Transmission electron microscopy of the purified EPEC O127:H6 Type III secretion needle complex has

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**FIGURE 2** Genetic organization of LEE of E. coli O157:H7. Open reading frames are represented by thick arrows, and putative polycistronic operons are designated by thin arrows. Clear arrows represent open reading frames of unknown function and are designated orf or rorf, depending on the direction of transcription relative to eae. doi:10.1128/microbiolspec.EHEC-0007-2013.f2
FIGURE 3 Schematic representation of the Type III secretion apparatus showing the predicted spatial organization of LEE-encoded proteins. Adapted from reference 30. doi:10.1128/microbiolspec.EHEC-0007-2013.f3

indicated that it comprises cylindrical inner and outer rings similar to the flagellar basal body (33), consistent with the shared evolution of such systems.

Systematic mutagenesis of the LEE in C. rodentium has been used to assess the role of all 41 genes in regulation of LEE expression, the level and hierarchy of Type III secretion, the ability to form pedestals via the FAS test, and virulence in mice (34). Nineteen genes essential for secretion of both translocator and effector proteins were identified (escR, escS, escT, escU, escC, escF, escV, escN, escD, escE, orf4, escL, rorf3, escl, orf12, escA, sepQ, and escG) with mutations in a further four genes (orf3, rorf6, orf16, and sepL) impairing secretion of translocators preferentially (34). Real-time imaging has subsequently indicated a distinct order in the efficiency of secretion of specific effectors in a manner dependent on intra-bacterial effector concentration, effector-chaperone interactions, and the efficiency of bacterial attachment to target cells (35). Although these studies indicated that EspZ is injected into cells early after infection, recent work has indicated that EspZ arrests translocation of effectors from within the host cell through an ill-defined “translocation stop” activity (36). Indeed, ectopic expression of EspZ inside eukaryotic cells renders them refractory to actin pedestal formation and prior infection of cultured cells with one A/E pathogen prevents superinfection by another (36). Mutants lacking espZ cause elevated cytotoxicity, probably owing to uncontrolled injection of effectors (36, 37). The importance of sepL, escD, sepQ, escV, rorf8, escC, escR, and orf4 in EspA secretion by EHEC O157:H7 was confirmed by analysis of transposon mutants that exhibit reduced adherence to Caco-2 cells in vitro (38).

Mutations affecting components of the needle complex and translocon were found to impair intestinal colonization of calves by signature-tagged transposon mutagenesis of EHEC O157:H7 (39) and EHEC O26:H– (40). A retrospective transposon-directed insertion-site sequencing analysis of the library of EHEC O157:H7 strain EDL933 mutants screened in calves by Dziva et al. identified 51 attenuating mutations in the LEE, many of which affected predicted needle complex components (41), extending earlier observations on the role of the LEE genes without further use of cattle. Screening of mutants in complex pools can exaggerate fitness costs; however, EHEC mutants with defects affecting the escN-encoded inner membrane ATPase were also found to be highly attenuated when tested in isolation in infant rabbits (42) and calves (39, 40), confirming a key role for the LEE-encoded T3SS in EHEC persistence and pathogenesis.

Inhibitors of Type III secretion in A/E pathogens have been identified (43–45). Many of these belong to a family of salicylidene acylhydrazides and appear to act in part by inhibition of transcription of LEE genes in EHEC O157:H7 (45), rather than by interacting with basal components of the T3SS apparatus and interfering with needle complex assembly as reported for Shigella sp. (46). Different salicylidene acylhydrazides produce distinct patterns of LEE repression (45), and the targets of such drugs have so far been mapped to genes outside the LEE (44, 47), providing insights into how LEE expression and T3SS function are controlled. The potential prophylactic and therapeutic applications of such inhibitors have received relatively little attention.

Translocon Components

A filamentous extension of the T3SS needle complex is transiently expressed on the surface of A/E pathogens in the early stages of lesion formation and comprises mostly the LEE4-encoded protein EspA (48, 49) (Fig. 4). EspA filaments in EPEC O127:H6 can vary in length up to 600 nm and are ca. 12 nm in diameter (33, 50). Such filaments form a physical bridge between the bacteria and host cells that is required for the translocation of EspB and Tir into host cells (48, 49, 51, 52) (Fig. 4A). This led to the hypothesis that EspA filaments comprise a hollow channel through which effector proteins are injected into host cells, a notion supported by resolution of the three-dimensional structure, which showed that
EPEC O127:H6 EspA subunits polymerized in a helical tube of 120 Å diameter with 5.6 subunits per turn enclosing a central channel of 25 Å diameter (53). Immunogold electron microscopy revealed that Tir is secreted from the tips of EspA filaments (54) (Fig. 4B and C), providing direct evidence that EspA filaments are hollow conduits through which effectors are secreted. Secretion and intracellular stability of EspA require the LEE1-encoded chaperone CesAB (55), and the filaments are elongated by addition of subunits at the growing tip (54). EspA binds directly to the needle complex protein EscF, which is required for EspA filament assembly and effector translocation (33, 56).

It remains unclear precisely how EspA filaments are connected to the host cell surface and the translocation pore is created. The LEE4-encoded Type III secreted EspB and EspD proteins are believed to mediate formation of the translocation pore on the basis of homology to the Yersinia YopB and YopD, their presence in the host cell plasma membrane, and ability to form pores in erythrocyte membranes (48, 57–61). EspD is required for assembly of EspA filaments (48, 58), interacts directly with EspA and EspB (62), and requires the chaperones CesD and CesD2 for secretion (63). EspB interacts with EspA and is required for the translocation of effectors, including Tir (64). However, EspA filaments can bind to host cell membranes in the absence of EspB, indicating that they may interact directly with cellular components (64). Indeed, studies using single, double, or triple mutants of EPEC O127:H6 lacking EspA filaments, intimin, and bundle-forming pili indicate that EspA filaments play a role in intimin-independent adherence to HEP-2 and Caco-2 cells, as an espA bfpA eae triple mutant was less adherent than a bfpA eae double mutant (65). EspA filaments appear shorter in EHEC O157:H7, and their expression is heterogeneous at the bacterial population level (66) but is coordinated with expression of LEE5 in single cells (67). EspA filaments are typically absent from bacteria at the time of intimate adherence to host cells on pedestals, and the basis of loss or disassembly of the translocon after initial attachment remains ill-defined.

It is clear that LEE4-encoded translocon components play pivotal roles in intestinal colonization by EHEC, as random and refined mutants are highly attenuated in cattle (39–41, 68) and in mice (69). Moreover, EspA is considered an important constituent of vaccines for control of EHEC in cattle (39–41, 68) and in mice (69). In this regard it is noteworthy that EspA filaments of EPEC and EHEC are antigenically distinct (72) and that variations in EspA primary sequences exist between serogroup O157 and non-O157 EHEC strains. Indeed, antibodies induced by immunization of cattle with Type III secreted proteins from EHEC O26:H11, O103:H2, or O111:NM failed

**FIGURE 4** (A) Scanning electron micrograph showing EspA filaments (arrow) of EHEC O26:H11 strain H19 attaching to the surface of an erythrocyte. From reference 241. (B) Transmission electron micrograph of an EspA filament of wild-type EPEC O127:H6 strain E2348/69 showing Tir issuing from the tip. EspA filaments were immunolabeled with anti-EspA conjugated to 5-nm diameter gold particles, and Tir was detected with anti-Tir conjugated to 10-nm diameter gold particles. (C) The specificity of Tir staining was confirmed using the same gold-labeled antibodies but an isogenic tir mutant. Panels B and C from reference 54. doi:10.1128/microbiolspec.EHEC-0007-2013.f4
to cross-react with EspA from EHEC O157:H7 (73), leading the authors to conclude that cross-serogroup protection due to EspA may be limited.

Although EspB is acknowledged to be a key part of the T3SS translocon, it can also be detected in the cytoplasm of infected cells (74), where it modulates cellular processes. For example, EspB binds to the host proteins α-catenin, α1-antitrypsin, and myosin that regulate the actin network leading to alterations in cell morphology (reviewed in reference 75). The myosin-binding domain of EspB inhibits the interaction of myosins with actin leading to microvillus effacement, and mutants lacking this domain lack the ability to efface enterocytes, suppress phagocytosis, or colonize the intestines of mice (76). EspB was also implicated in the ability of EHEC to suppress NF-κB activation and synthesis of proinflammatory cytokines (77); however, the extent to which this was dependent on EspB per se, or its role in delivery of other effectors, was not elucidated.

**Intimin**

Intimin is a 94- to 97-kDa outer membrane adhesin produced by all EHEC strains and related A/E pathogens and is encoded by the *eae* gene. Though first identified in EPEC O127:H6 (4), a homolog in EHEC O157:H7 that exhibits 83% amino acid sequence identity was subsequently reported to mediate bacterial adherence to cultured cells and intestinal colonization in gnotobiotic piglets (78, 79). Subsequent studies established that EHEC O157:H7 intimin plays a pivotal role in persistence and pathogenesis in mice, infant rabbits, neonatal calves and lambs, and adult cattle and sheep (80–84). It has also been reported to drive mucosal inflammatory responses; for example, it induces a T-helper cell type 1 (Th1) response characterized by mucosal thickening and infiltration of CD4+ T cells during infection of mice with *C. rodentium* (85), and can augment mitogen-stimulated proliferation of spleen CD4+ T lymphocytes and cells from organized lymphoid tissues (85, 86). Intimin is a component of chimeric and multivalent subunit vaccines that control EHEC in experimental models (reviewed in reference 246), and immunization with intimin alone has been reported to confer protection against intestinal colonization by EHEC O157:H7 when delivered by live-attenuated *Salmonella* sp. to cattle and mice (87, 88) and recombinant plants to mice (82). Moreover, neonatal piglets suckling dams vaccinated with EHEC O157:H7 intimin are passively protected (89), consistent with the ability of antibodies directed against the carboxyl-terminal domain to inhibit adherence of EHEC O157:H7 to HEP-2 cells (90).

Intimin shares significant homology with invasin proteins of *Yersinia pseudotuberculosis* and *Yersinia enterocolitica*, the carboxyl termini of which bind to β1-chain integrins to facilitate bacterial invasion of eukaryotic cells. Frankel et al. demonstrated that the carboxyl-terminal 280 amino acids of intimin (Int280) from EPEC O127:H6 and EHEC O157:H7 could directly bind to HEP-2 cells (91), and the same region also mediates binding of intimin from EHEC O26:H– to host cells (92). Furthermore, expression of the carboxyl-terminal two-thirds of intimin was sufficient to restore adherence of an EHEC O157:H7 Δeae mutant (93). Separate reports indicated that binding of purified intimin to eukaryotic cells could only be detected if the cells were pre-infected with EPEC or EHEC, indicating that a bacterial factor also influences intimin-mediated adherence (94, 95).

Studies to identify the host cell receptor for intimin initially focused on a 90-kDa membrane protein (Hp90) that became tyrosine phosphorylated during EPEC infection of epithelial cells and localized under adherent bacteria (96). EPEC intimin was later shown to bind to tyrosine-phosphorylated Hp90 but not to nonphosphorylated Hp90, and it was hypothesized that the bacteria signal to host cells to induce phosphorylation of a membrane protein that is subsequently bound by intimin (95). Kenny et al. later reported Hp90 to be the tyrosine-phosphorylated version of a 78-kDa EPEC O127:H6 protein that is translocated into the eukaryotic cell plasma membrane via the LEE-encoded T3SS where it acts as the receptor for intimin (51). Hp90 was thus renamed Tir (for translocated intimin receptor). Tir was independently discovered in EHEC O26:H– as EspE, an 80-kDa protein that is translocated into host cells where it appears as a 90-kDa tyrosine-phosphorylated protein associated with adherent bacteria (97). EHEC O157:H7 Tir is also delivered into host cells where it acts as a receptor for intimin; however, it is not tyrosine phosphorylated (97, 98).

The three-dimensional structures of the carboxyl-terminal domain of intimin and the Int280-Tir complex were first resolved for the EPEC O127:H6 protein by X-ray crystallography and nuclear magnetic resonance (99–101). These studies indicate that intimin contains four distinct domains within the carboxyl-terminal 380 residues that extend from the outer membrane. Domains D1, D2, and D3 belong to the immunoglobulin superfamily and are predicted to comprise an articulated rod connected to the membrane-anchored amino-terminal portion by a flexible linker comprising two glycine residues. The terminal D4 domain is predicted to be...
accessible to the target cell and shares similarity with C-type lectins, a family of proteins that recognize cell surface carbohydrates. The most distal immunoglobulin-like domain (D3) and D4 C-type lectin domain form a rigid superdomain that binds Tir (99), principally through contacts in a β-hairpin motif at the tip of the extracellular domain of Tir and an analogous region of the intimin D4 domain (101). This portion of Tir is critical for intimin binding (102–104). The crystal structure of the EPEC O127:H6 Int280-Tir complex reveals the intimin-binding domain of Tir to be a dimer, with the two parallel α-helices of the Tir extracellular domains aligning to form a four-helix bundle bound by two intimin molecules (101). Analysis of the crystal structure of the Tir-binding domain of EHEC O157:H7 intimin (Int188) at 2.8 Å resolution suggests that a similar conformation and contacts are adopted (105), extending earlier predictions from a yeast-2-hybrid analysis (106).

Although it is unclear if this structure is formed by the native proteins in vivo or by A/E pathogens with other intimin and Tir sequences, it predicts that the proteins bind in a plane roughly parallel to the surfaces of the bacteria and host cell, consistent with the estimated gap between the host cell plasma membrane and intimately attached bacteria of just 100 Å.

Early phylogenetic analysis of eae genes identified distinct subtypes, designated Int-α, -β, -γ, -δ, -ε, and -θ (107–109), though further intimin subtypes are now recognized. These differ in sequence in the carboxy-terminal cell-binding domain and are often associated with specific clonal lineages of EPEC and EHEC. For example, Int-α is associated with EPEC clone 1 serotypes O55:H6 and O127:H6, whereas Int-γ is found in EHEC clone 1 serotypes, including O157:H7 and its progenitor EPEC serotype O55:H7. As the carboxy-terminal domain of intimin mediates binding to Tir and host cell surfaces, it was anticipated that divergence of intimin subtypes may influence the avidity and specificity of adherence. Int-γ is required for colonization of the surface and glandular epithelium of the large intestine in gnotobiotic piglets (78). However, expression of Int-α from EPEC O127:H6 in an EHEC O157:H7 Δeae mutant was reported to cause a shift in intestinal tissue tropism, with colonization of the terminal ileum as well as the surface of the large intestine in a manner similar to wild-type EPEC (110). In subsequent studies, precise chromosomal replacement of EHEC O157:H7 eae with EPEC O127:H6 eae did not alter tissue tropism in piglets (111), and the basis of the disparity is unclear.

Support for the role of intimin in mediating intestinal tissue tropism derives from in vitro organ culture studies using human and porcine intestinal explants. Adherence of EHEC O157:H7 to human intestinal mucosa and the formation of A/E lesions are restricted to follicle-associated epithelium (FAE) overlying ileal Peyer’s patches (112), whereas Int-α-expressing EPEC O127:H7 can adhere to human small intestinal explants from a variety of sites (113). Expression of EPEC O127:H6 Int-α in an EHEC O157:H7 Δeae mutant without exchanging Tir results in an EHEC strain capable of adhering to explants from various intestinal sites from human (114) and pigs (115). A similar extension of tropism for human and porcine explants was observed when EHEC O157:H7 was engineered to express Int-β from C. rodentium (116). In reciprocal experiments EHEC O157:H7 Int-γ conferred upon an EPEC O127:H6 Δeae mutant a tropism for FAE overlying Peyer’s patches (113). FAE at the recto-anal junction has been reported to be a key site of persistence of EHEC O157:H7 in cattle (117); however, while Int-γ is known to be required for efficient colonization of this site by EHEC O157:H7 (118), it is unclear if different subtypes vary in their specificity for this region of the bovine gut. Indeed, non-O157 EHEC has been reported to colonize squamous epithelium in the bovine terminal rectum independently of intimin (119), and studies have indicated that factors other than those encoded by the LEE play a role in adherence of EHEC O157:H7 to rectal squamous epithelial cells in culture (120).

The ability of intimin to bind to eukaryotic cell surfaces in the absence of Tir and influence tissue tropism suggests the existence of a cellular coreceptor(s). Sinclair and O’Brien demonstrated that the carboxy-terminal domain of EHEC O157:H7 Int-γ binds in a specific and saturable manner to HEp-2 cells with a dissociation constant of 84 nM, consistent with the existence of a single host cell receptor (121). By affinity purification and sequencing of peptides derived from a 110-kDa intimin-binding HEp-2 cell protein, the receptor was identified as nucleolin, a protein involved in regulation of cell growth that can be expressed at the cell surface (121). Cell surface-localized nucleolin was observed to colocalize with bound purified Int-γ and with EHEC O157:H7 adhering to HEp-2 cells (121), and with EHEC O157:H7 adhering to porcine and bovine intestinal mucosa in vivo (122). Antinucleolin antibodies partially inhibit EHEC O157:H7 adherence to cultured cells (121). Interestingly, surface expression of nucleolin is enhanced by Stx2, and it is believed that this may partially explain the ability of Shiga toxin to promote EHEC O157:H7 adherence to Hep-2 cells and intestinal colonization in mice (122). Intimin subtypes α, β, and γ
bind to nucleolin with equal affinity, indicating that the distribution of nucleolin along the gastrointestinal tract is unlikely to determine tissue tropism of EHEC and EPEC (123). All three intimin subtypes bind to nucleolin with a lower avidity than to Tir. Furthermore, binding of intimin α, β, or γ to Tir in vitro blocks the interaction between intimin and nucleolin (123), suggesting that Tir and nucleolin compete for intimin binding.

Sites of adherence of EHEC O157:H7 to porcine and bovine tissue are also enriched in β1-chain integrins (122). Consistent with a role for such factors as coreceptors, EPEC O127:H6 intimin-α can bind to β1-chain integrins expressed on the surface of human lymphocytes or in enzyme-linked immunosorbent assays (124). However, β1-chain integrins were reported to be dispensable for intimin-mediated adherence as inactivation of the β1-chain integrin gene or the addition of antagonists of integrin function, such as EDTA or anti-β1 chain antibody, did not affect EPEC adherence or pedestal formation (94). Conversely, Muza-Moons et al. suggested that disruption of tight junctions by EPEC in an EspF-dependent manner leads to redistribution of β1-chain integrins from the basolateral to apical surface to promote bacterial adherence (125). Studies using isogenic single and double eae and tir mutants of EHEC O157:H7 in calves have indicated that intimin-Tir interactions are more significant than intimin-coreceptor interactions during colonization of the bovine intestines, as mutation of eae in a tir mutant did not exert further attenuation (84).

**Tir**

In addition to its role as the translocated receptor for intimin (above), Tir is required to activate actin assembly and recruit cytoskeletal proteins at the site of bacterial adherence. Intimin-Tir interactions are required to cluster Tir to initiate this process (126). The mechanism of insertion of Tir into the apical plasma membrane of enterocytes is ill-defined, but phosphorylated intermediates of EPEC O127:H6 Tir can be detected in the cytoplasm and a delay exists between Tir injection and intimin-Tir interaction (102, 127), implying that it may first be found in the cytoplasm prior to insertion. Phosphorylation of tyrosine residue 474 in a 12-amino-acid motif in the carboxyl-terminal domain of EPEC O127:H7 Tir is required to trigger local actin assembly through recruitment of the host cell adaptor protein Nck (102, 126, 128, 129). Such phosphorylation requires redundant cellular tyrosine kinases, including c-Fyn, Abl, Arg, and Erk, and was proposed to allow Nck to bind to a proline-rich domain of the neural-Wiskott Aldrich syndrome protein (N-WASP), which in turn stimulates the actin-nucleating activity of the cellular Arp2/3 complex (102, 129, 130). WASP-interacting protein (WIP), which binds Nck and a WASP-homology 1 (WH1) domain of N-WASP, may also act as linker between Nck and N-WASP, and such interactions may act in synergy with Nck interactions with the N-WASP proline-rich domain as interference in either interaction does not abolish pedestal formation (131). Recent studies have indicated that WIP is not essential for pedestal formation by typical EPEC (132).

N-WASP is also required for pedestal formation by EHEC O157:H7 (133); however, in contrast to EPEC O127:H6, the Tir of EHEC O157:H7 lacks the Y474 residue and is not tyrosine phosphorylated upon entry into host cells (98, 134). Instead, a conserved Asn-Pro tyrosine (NPY458) motif in a 12-amino-acid motif in the cytoplasmic domain of EHEC O157:H7 Tir is required for Nck-independent actin assembly (135–136) through recruitment of insulin receptor tyrosine kinase substrate (IRTKS) (137) and insulin receptor substrate p53 (IRSp53) (138). This motif is also found in EPEC O127:H7 Tir as NPY454 and can initiate actin assembly independently of Nck, albeit inefficiently (139). In EHEC O157:H7 the pathway of actin assembly mediated by recruitment of IRTKS/IRSp53 to Tir is amplified by another Type III secreted effector protein termed EspF1 or TccP (Tir cytoskeleton coupling protein), which contains 47-amino-acid proline-rich repeats that bind to IRTKS/IRSp53 (138) and the GTPase-binding domain of N-WASP (140, 141). Structural studies have indicated that the proline-rich repeats in EspF1/TccP compete with an auto-inhibitory domain of N-WASP for binding of its GTPase-binding domain, thereby relieving N-WASP auto-inhibition (142, 143). The number of such repeats varies in natural isolates and is associated with the efficiency of actin assembly (144, 145). Remarkably, the Arp2/3 complex is recruited to actin pedestals formed in a Tir- and EspF1/TccP-dependent process even in the absence of N-WASP, implying that redundant as yet unknown pathways are used to ensure actin assembly under adherent bacteria (146). Indeed, an EPEC O125:H6 strain unable to use the Nck or IRTKS/IRSp53 pathways was nevertheless able to form typical A/E lesions on human intestinal explants cultured ex vivo (147).

It is noteworthy that differences in the relative importance of Nck-dependent and Nck-independent pathways have been detected between immortalized epithelial lines in culture and human intestinal explants cultured ex vivo (148) as well as between infection of
cultured cells and animals (149). During C. rodentium infection in mice or EHEC infection of human intestinal biopsies, IRTKS, but not IRSp53, was recruited to sites of bacterial attachment sites, and N-WASP was recruited to pedestals even in the absence of tyrosine residues required for Nck or IRTKS binding (149). Thus, despite the key role of the Tir:Nck and Tir:IRTKS/IRSp53 pathways in actin polymerization in cultured epithelial cells, they appear not to be essential for N-WASP recruitment or A/E lesion formation on explants or in mice. C. rodentium mutants with Y451 or Y471 substitutions were outcompeted by the wild-type strain during mixed infection of mice (149), suggesting that such pathways do contribute to persistence. Further, while an EHEC O157:H7 espFU/tccP mutant exhibited no obvious defect in A/E lesion formation or early intestinal colonization of calves (84), such mutants appear impaired in the efficiency of formation and tissue distribution of A/E lesions in gnotobiotic pigs and infant rabbits (150).

The Tir:Nck and Tir:IRTKS/IRSp53 pathways appear conserved in isolates of EPEC belonging to lineage 1 and EHEC O157:H7, respectively (151). Typical isolates of EHEC O157:H7 carry espFU/tccP on prophage CP-933U/Sp14 but also harbor a related pseudogene (z1385/cecs2715) on prophage CP-933 M/Sp4 (tccP2/espFM). Sorbitol-fermenting EHEC O157:H7 strains contain an intact copy of tccP2/espFM, which can restore function to a espFU/tccP mutant and encodes a protein with near-identical proline-rich repeats but a distinct 80-amino acid amino terminus, which shares 42.7% identity with the amino terminus of EspFU/TccP (151). Interestingly, non-O157 EHEC isolates in general possess a Tir with a conserved Y474 residue for Nck recruitment and an intact copy of tccP2/espFM (151), suggesting that they are able to use both the Tir:Nck and Tir:IRTKS/IRSp53 pathways. The same applies to a high proportion of EPEC lineage 2 strains (152), indicating that varied and independent solutions to form pedestals have evolved. The repertoire of Tir, espFU/TccP, and EspFM/TccP2 pathways may explain the recruitment of distinct sets of cytoskeletal proteins to the pedestals formed by different A/E pathogens as well as distinct pedestal morphologies (Fig. 1A and B). Modulation of the cytoskeleton by A/E pathogens is summarized in Fig. 5.

Tir plays a key role in persistence and pathogenesis of EHEC, with tir mutants of EHEC O157:H7 being attenuated in infant rabbits (83), calves (84), and colonization of the terminal rectum of steers (118). Moreover, Tir is an important component of vaccines for control of EHEC O157:H7 in cattle (70, 71). Despite intensive research into the molecular mechanisms underlying actin assembly during A/E lesion formation, the advantages to the pathogen of forming pedestals remain open to question. It has been reported that N-WASP is required for efficient translocation of Type III secreted effectors (146), and a role in stabilizing the translocon and/or clustering Tir for interactions with intimin is plausible. In turn, stabilization of intimin-Tir interactions may help prevent bacterial detachment from cells under flow. Pedestals have also been reported to be mobile on the cell surface (153), and a role in spread between enterocytes and evasion of phagocytosis by remodeling of the cell surface cannot be excluded.

Other Type III Secreted Effectors

In addition to EspB and Tir, five other effector proteins are encoded within the LEE of the prototype EHEC O157:H7 strains EDL933 and RIMD 0509952 (EspF, EspG, EspH, EspZ, and Map) (Fig. 2). As discussed above, the boundaries of the LEE are less defined in some EHEC strains, with additional effector proteins encoded in the flanking regions. Upon sequencing of the complete genome sequences of the above strains (154, 155), more than 60 candidate Type III secreted proteins were identified on the basis of homology with effectors in other pathogens, of which 49 were judged to be potentially functional (25). The T3SS-dependent secretion of 31 candidate effectors of EHEC O157:H7 was confirmed by analysis of the secreted proteome of an EHEC O157:H7 ΔsepL strain with an isogenic ΔsepL ΔescR double mutant incapable of Type III secretion (25). SepL regulates the hierarchy of secretion in EHEC, and mutation of sepL causes the secretion of effectors in preference to translocon components (156). Subsequent studies have indicated that the ability of the carboxy-terminal portion of SepL to bind to Tir is sufficient to delay the export of effectors while the EspABD translocon components are secreted (157).

To confirm that candidate effectors identified by bioinformatics or proteomic analysis are translocated into eukaryotic cells in a T3SS-dependent manner, assays to detect the activity of reporter fusions have been used. Commonly, candidate non-LEE-encoded effectors have been fused to TEM-1 β-lactamase, enabling translocation to be detected by a shift in the wavelength of fluorescence emitted by target cells loaded with a fluorescent β-lactamase substrate (CCF2/AM) (158). Fusions to epitopes (e.g., FLAG) or Bordetella pertussis adenylate cyclase (CyaA) have also been employed for this purpose (25).
In total, 39 Type III secreted effector proteins were experimentally validated in EHEC O157:H7 (25). It is evident that lambdoid bacteriophages have played a key role in the lateral transfer of such effectors, both in EHEC O157:H7 (25) and non-O157 EHEC (18). Prophage-encoded effector genes are frequently carried downstream of tail fiber genes and exhibit a low %GC content relative to the prophage backbone (18, 25). Analysis of the content and insertion site of effector-encoding prophages in non-O157 EHEC suggests distinct evolutionary histories (18). Although many effector-encoding prophages identified by genome sequencing were initially considered defective, it is now evident that many are inducible and can release their DNA from EHEC O157:H7, possibly owing to recombination and other interprophage interactions (159). Many effectors encoded outside the LEE are designated Nle (non-LEE-encoded) effectors. Effector proteins of EHEC O157:H7 strain RIMD 0509952 and their activities, where known or inferred from studies with homologs in other A/E pathogens, are listed in Table 1. A notable absence from the genomes of the sequenced EHEC O157:H7 strains EDL933 and RIMD 0509952 is the non-LEE-encoded effector cycle-inhibiting factor (Cif), which was first described in an EHEC O103:H2 strain (160) and is found in many non-O157 EHEC. Cif arrests the cell cycle at G1/S and G2/M phases and inhibits other cellular processes by deamidation of ubiquitin or the ubiquitin-like protein NEDD8 that regulates cullin-RING-ubiquitin ligase complexes (reviewed in reference 161).

It is clear that effectors are often multifunctional. Indeed, EspF has been described as the “Swiss army knife” of A/E pathogens owing to its effect on epithelial barrier function, apoptosis, mitochondrial function, cytoskeletal components, microvillus effacement, and other processes (reviewed in reference 162). It is also evident that effectors may act in redundant, synergistic, and antagonistic ways. As a consequence, mutation of effector genes does not produce attenuation at the same level as defects affecting the translocon or needle complex, which result in loss of secretion all effectors. The LEE-encoded effectors EspF, EspG, EspH, and Map have been reported to be required for full colonization of the intestines of infant rabbits by EHEC O157:H7, albeit that the effect was modest and tissue-specific in some cases (42). Moreover, mutations affecting 29 or the 39 effectors of EHEC O157:H7 strain EDL933 were detected by screening pools of random transposon mutants in calves (39, 41); however, the extent of negative selection was often modest, and in the case of map and nleD mutants, could not be reproduced when the mutants were screened in isolation (39, 163). Functions
**TABLE 1** Type III secreted effector proteins of EHEC O157:H7 and their activities, where known or inferred from homologs in other A/E pathogens

<table>
<thead>
<tr>
<th>Effector</th>
<th>Number of alleles&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Subcellular localization</th>
<th>Interacting partners or substrates</th>
<th>Homologs</th>
<th>Function&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>EspB</td>
<td>1</td>
<td>Plasma membrane, cytosol</td>
<td>α1-antitrypsin, α-catenin, myosin-1c</td>
<td><em>Yersinia</em> YopD</td>
<td>Pore-forming translocon component; microvillus effacement; antiphagocytosis; disruption of adherens junctions</td>
</tr>
<tr>
<td>EspF</td>
<td>1</td>
<td>Plasma membrane, cytosol, mitochondria, nucleus</td>
<td>14-3-3zeta, ABCF2, actin, Arp2, CK18, N-WASP, profiling, SNX9, ZO-1/-2</td>
<td><em>EspF&lt;sub&gt;U&lt;/sub&gt; (TccP)</em></td>
<td>Disrupts mitochondrial function, nucleolus, tight junctions and intermediate filaments. Inactivates NHE3 and SGLT-1; activates SNX9 and N-WASP; inhibits PI3K-dependent phagocytosis and induces apoptosis</td>
</tr>
<tr>
<td>EspG</td>
<td>1</td>
<td>Cytosol, Golgi</td>
<td>Arf1/6, PAK1/2/3, tubulin, GM130, Rab1</td>
<td><em>Shigella</em> VirA</td>
<td>Disrupts microtubules, tight junctions and paracellular permeability; sequesters ADP-ribosylating factor (Arf) to modulate GTPase signaling; stimulates p21-activated kinases (PAKs) to inhibit endomembrane trafficking; binds GM130 and inactivates Rab1 to disrupt Golgi structure and protein secretion; induces calpain protease and necrosis in absence of Tir</td>
</tr>
<tr>
<td>EspH</td>
<td>1</td>
<td>Plasma membrane, pedestal</td>
<td>DH-PH Rho guanine nucleotide exchange factors (GEFs)</td>
<td>None known</td>
<td>Inhibits RhoGTPase signaling and FCγR-mediated phagocytosis; causes cell detachment via disassembly of focal adhesions and remodels brush border; promotes actin nucleation and pedestal elongation by recruiting N-WASP and Arp2/3 via WIP</td>
</tr>
<tr>
<td>EspZ</td>
<td>1</td>
<td>Plasma membrane, mitochondria</td>
<td>CD98, translocase of inner mitochondrial membrane 17b (TIM17b)</td>
<td>None known</td>
<td>Inhibits apoptosis and cytotoxicity; regulates Type III secretion via “translocation stop” activity</td>
</tr>
<tr>
<td>Map</td>
<td>1</td>
<td>Mitochondria</td>
<td>Na&lt;sup&gt;+&lt;/sup&gt;/H&lt;sup&gt;+&lt;/sup&gt; exchanger regulatory factor (NHERF)-1 and -2, Cdc42</td>
<td><em>Salmonella</em> SopE/SopE2, <em>Shigella</em> IpgB</td>
<td>GEF for Cdc42 that induces transient filopodia formation; causes mitochondrial dysfunction; inactivates sodium-D-glucose cotransporter (SGLT-1) in a manner that may result in net fluid secretion; disrupts tight junctions, causing loss of epithelial barrier integrity</td>
</tr>
<tr>
<td>Tir</td>
<td>1</td>
<td>Plasma membrane</td>
<td>Intimin, 14-3-3tau, α-actinin, cortactin, CK18, IQGAP1, IRTKS, IRSp53, Nck, PI3K, SHP-1, Talin, Vinculin</td>
<td>None known</td>
<td>Receptor for intimin, actin pedestal formation, regulates activities of Map and EspG</td>
</tr>
<tr>
<td>EspI/NleA</td>
<td>1</td>
<td>Plasma membrane, Golgi</td>
<td>Syntrophin, Sec23/24, MAL53, PDZK11, SNX27, TOCF1, NHERF-1 and -2, MAGI-3, SAP97 and -102, PSD-95</td>
<td>None known</td>
<td>Inhibits protein export from the endoplasmic reticulum by disrupting COPII function; disrupts tight junctions</td>
</tr>
<tr>
<td>EspJ</td>
<td>1</td>
<td>Cytosol, mitochondria</td>
<td>Unknown</td>
<td><em>Pseudomonas</em> HopF</td>
<td>Inhibits phagocytosis mediated by complement receptor 3- and Fcy-receptor</td>
</tr>
<tr>
<td>EspK</td>
<td>1</td>
<td>Cytosol</td>
<td>Unknown</td>
<td><em>Salmonella</em> GogB</td>
<td>Unknown, influences intestinal colonization of calves by EHEC O157:H7</td>
</tr>
<tr>
<td>EspL</td>
<td>4 (1)</td>
<td>Pedestal</td>
<td>Annexin 2</td>
<td><em>Shigella</em> OspD</td>
<td>Promotes F-actin bundling activity of annexin 2</td>
</tr>
<tr>
<td>EspM</td>
<td>2</td>
<td>Cytosol</td>
<td>RhoA</td>
<td><em>Salmonella</em> SopE/SopE2, <em>Shigella</em> IpgB</td>
<td>GEF for RhoA that inhibits actin stress fibers and modulates pedestal formation; disrupts tight junctions and monolayer integrity</td>
</tr>
</tbody>
</table>

<sup>a</sup> Data from the article by Stevens and Frankel.

<sup>c</sup> Functions are based on the effects described in the literature and are not exhaustive.
<table>
<thead>
<tr>
<th>Effector</th>
<th>Number of alleles</th>
<th>Subcellular localization</th>
<th>Interacting partners or substrates</th>
<th>Homologs</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>EspN</td>
<td>1</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Unknown</td>
</tr>
<tr>
<td>EspO</td>
<td>2</td>
<td>Unknown</td>
<td>Integrin-linked kinase (ILK)</td>
<td>Shigella OspE</td>
<td>Regulates EspM2-mediated RhoA activity and stabilizes focal adhesions to block cell detachment</td>
</tr>
<tr>
<td>EspR</td>
<td>4 (1)</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Shigella flexneri SF1757</td>
<td>Unknown</td>
</tr>
<tr>
<td>EspV</td>
<td>1 (1)</td>
<td>Cytosol</td>
<td>Unknown</td>
<td>Pseudomonas AvrA</td>
<td>Alters cell morphology</td>
</tr>
<tr>
<td>EspW</td>
<td>1</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Pseudomonas HopPmaA, HopW1</td>
<td>Unknown</td>
</tr>
<tr>
<td>EspX</td>
<td>7 (1)</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Shigella sonnei SSON_0027, Shigella dysenteriae Sd1012_0237</td>
<td>Unknown</td>
</tr>
<tr>
<td>EspY</td>
<td>5 (1)</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Salmonella SopD</td>
<td>Unknown</td>
</tr>
<tr>
<td>NleB</td>
<td>3 (1)</td>
<td>Cytosol</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)</td>
<td>Salmonella SseK</td>
<td>Inhibits TNFα-induced activation of NF-κB and proinflammatory responses by transferring N-acetyl-D-glucosamine to GAPDH, thereby disrupting TRAF2-GAPDH interaction to suppress TRAF2 polyubiquitination and NF-κB activation</td>
</tr>
<tr>
<td>NleC</td>
<td>1</td>
<td>Cytosol, nucleus</td>
<td>p65 (RelA), p50, c-Rel, IkB, acetyltransferase p3000</td>
<td>Photobacterium AIP56</td>
<td>Zinc metalloprotease that cleaves p65 (RelA), p50, c-Rel and IkB to inhibit NF-κB activation</td>
</tr>
<tr>
<td>NleD</td>
<td>1</td>
<td>Cytosol</td>
<td>c-Jun N-terminal kinase (JNK), p38 mitogen-activated protein kinase (MAPK)</td>
<td>Pseudomonas HopA1, HopH1</td>
<td>Zinc metalloprotease that cleaves JNK and MAPK to inhibit induction of apoptosis and proinflammatory responses</td>
</tr>
<tr>
<td>NleE</td>
<td>1</td>
<td>Cytosol</td>
<td>TAB2 and -3</td>
<td>Shigella OspZ</td>
<td>Blocks IkB degradation to inhibit activation of NF-κB, proinflammatory responses and neutrophil transepithelial migration. Uses S-adenosyl-L-methionine-dependent methyltransferase activity to modify Npi4 zinc finger domains in TAB2 and TAB3, which regulate NF-κB signaling</td>
</tr>
<tr>
<td>NleF</td>
<td>1</td>
<td>Unknown</td>
<td>Caspase-4, -8, and -9, Tmp21</td>
<td>None known</td>
<td>Inhibitor of caspase activation and apoptosis; binds the COPI-vesicle receptor Tmp21 involved in Golgi function and slows protein secretion</td>
</tr>
<tr>
<td>NleG/NleI</td>
<td>14 (5)</td>
<td>Cytosol</td>
<td>UBE2D2</td>
<td>Salmonella Typhi STY1076</td>
<td>E3 ubiquitin ligases analogous to eukaryotic RING finger and U-box enzymes</td>
</tr>
<tr>
<td>NleH</td>
<td>2</td>
<td>Plasma membrane, cytosol, endoplasmic reticulum</td>
<td>Bax-inhibitor 1, NHERF2, ribosomal protein S3 (RPS3)</td>
<td>Shigella OspG</td>
<td>Binds Bax-inhibitor 1 to block apoptosis; sequesters RPS3 to inhibit NF-κB signaling</td>
</tr>
<tr>
<td>TccP/EspFU</td>
<td>2 (1)</td>
<td>Pedestal</td>
<td>N-WASP, IRSp53, IRTK5, cortactin</td>
<td>EspF</td>
<td>Relieves auto-inhibition of N-WASP to stimulate the Arp2/3 complex to polymerize actin and form pedestals</td>
</tr>
</tbody>
</table>

1Adapted from reference 164.
2Allele number is shown for the Sakai outbreak strain of EHEC O157:H7 (RIMD 0509952). Values in parentheses denote the number of predicted pseudogenes.
3References in support of functions may be found in the text and reference 164.
of Type III secreted proteins of EHEC are considered below by functional categories and are reviewed in detail elsewhere (164).

MODULATION OF INNATE IMMUNITY

In recent years it has become clear that EHEC and other A/E pathogens inject multiple non-LEE-encoded effectors to suppress proinflammatory responses, converging on inhibition of the activation of host transcription factors including NF-κB and activator protein-1. NF-κB comprises a dimer of p50 and p65 subunits that are retained in the cytoplasm by a family of inhibitory proteins (IκB). Activation of NF-κB requires phosphorylation of IκB by the IκB kinase complex, which is subsequently ubiquitinated and degraded by the proteasome, allowing p50/p65 to translocate to the nucleus to bind consensus sequences upstream of genes associated with the inflammatory response. Remarkably, EHEC and other A/E pathogens disrupt these processes at multiple levels, as summarized in Fig. 6. For example, NleB suppresses tumor necrosis factor alpha-mediated NF-κB activation (165, 166) by transferring N-acetyl-d-glucosamine to the glycolysis enzyme glyceraldehyde-3-phosphate dehydrogenase, disrupting its ability to interact with the tumor necrosis factor receptor-associated factor 2 (TRAF2), which is required for NF-κB activation (167). In EPEC it appears that Tir also impairs TRAF function by interfering with the TRAF2 adaptor protein and inducing its proteasome-independent degradation (168). It has also been reported that EPEC Tir interferes with signaling via Toll-like receptors by binding to the host cell tyrosine phosphatase SHP-1 in a manner dependent on phosphorylated immunoreceptor tyrosine-based inhibition motifs in Tir (169). The association of Tir with SHP-1 facilitates the recruitment of SHP-1 to the adaptor TRAF6, thereby inhibiting the ubiquitination of TRAF6 and the subsequent induction of proinflammatory cytokines and intestinal immunity (169).

At another level, NleC inactivates the NF-κB subunits p50 and p65 by means of a zinc-dependent endopeptidase activity (170–173). Another zinc metalloprotease (NleD) degrades key kinases (c-Jun N-terminal kinase [JNK] and mitogen-activated protein kinase p38) involved in activation of activator protein-1, which like NF-κB controls expression of genes involved in the inflammatory response (170). A further effector, NleE, interferes with degradation of IκB to prevent translocation of NF-κB to the nucleus (165, 166, 174) and uses S-adenosyl-L-methionine-dependent methyltransferase activity to modify Npl4 zinc finger domains in the cellular proteins TAB2 and TAB3, which regulate NF-κB signaling (175). NleH

FIGURE 6 Diagram summarizing the activities of a subset of EHEC Type III secreted proteins on signaling pathways leading to inflammation and apoptosis. Effectors are represented by circles. Adapted from reference 164. doi:10.1128/microbiolspec.EHEC-0007-2013.f6
also acts in concert with these effectors, binding to a subunit of NF-κB transcriptional complexes (ribosomal protein S3), preventing its phosphorylation by the kinase IkB kinase complex-β and translocation to the nucleus \((176, 177)\). NleH1 and NleH2 also suppress IkB degradation by interfering with the ubiquitination of phosphorylated IkB, thereby aiding retention of NF-κB in the cytoplasm \((178)\). The functional significance of inhibition of proinflammatory responses through these pathways is evidenced by the fact that mutants lacking the effectors above typically elicit elevated proinflammatory cytokine responses and are attenuated in animal models, albeit that combination of mutations produces stronger phenotypes owing to functional redundancy. One should also caution that subtle differences may exist in the activities of homologs between EHEC and other A/E pathogens, as well as between members of the same family of effectors, as recently reported for NleH1 and NleH2 of EHEC \((179)\). NleH1 and NleH2 also suppress I protein S3, preventing its phosphorylation by the kinase \((180)\), whereas EspM acts \((190, 191)\).

NleH1 and NleH2 also suppress I protein S3, preventing its phosphorylation by the kinase \((180)\), whereas EspM acts \((190, 191)\). A

homologs between EHEC and other A/E pathogens, caution that subtle differences may exist in the activities of that combination of mutations produces stronger phenotypes owing to functional redundancy. One should also caution that subtle differences may exist in the activities of homologs between EHEC and other A/E pathogens, as well as between members of the same family of effectors, as recently reported for NleH1 and NleH2 of EHEC \((179)\). NleH1 and NleH2 also suppress I protein S3, preventing its phosphorylation by the kinase \((180)\), whereas EspM acts \((190, 191)\). A

The role of Tir and adaptor proteins in formation of actin pedestals is well established (above); however, it is becoming clear that other effectors are injected that act on the host cell cytoskeleton (summarized in Fig. 5). For example, mitochondrial-associated protein (Map) and EspM are WxxxE-family effectors homologous to the \(Shigella\) IpgB and \(Salmonella\) SopE proteins that act as guanine nucleotide exchange factors (GEFs) to activate GTPases that regulate the actin network. Map activates Cdc42 to trigger transient formation of filopodia at attachment sites \((127, 190, 191)\), whereas EspM activates RhoA to form actin stress fibers \((192, 193)\). Further WxxxE effector (EspT) has a putative GEF activity for Cdc42 and Rac1 to trigger membrane ruffling, and filopodia formation has been found in a minority of EPEC strains, but so far not in EHEC \((194)\). The relevance of the GEF activity of Map and EspM in vivo is not fully understood, but recent research with \(Salmonella\) sp. has indicated that activation of RhoGTPases by SopE triggers the NOD1 signaling pathway leading to inflammation, indicating that their activity may extend beyond changes to cell architecture \((195)\). Interestingly, A/E pathogens also inject EspH, which has an opposing activity to Map, EspM, and EspT. EspH inactivates cellular Dbl-homology and pleckstrin-homology (DH-PH) Rho family GEFs by competitively binding to tandem DH-PH domains, resulting in inhibition of activation of RhoA \((181)\). The bacterial GEFs Map, EspM, and EspT lack sequence homology with DH-PH domains, and recent studies have shown that EspH does not directly interfere with their activity \((196)\). Rather, EHEC and EPEC appear to use EspH to inactivate endogenous RhoGEFs while injecting their own GEFs to modulate Rho GTPase signaling to their advantage \((196)\). The interplay between these effectors is such that phenotypes detected by expression of effectors interactions between EspF and SNX9 have been reported to remodel the apical plasma membrane and facilitate EPEC invasion in epithelial cells \((189)\), indicating the potential for cell type-specific effects. Moreover, distinct \(espF\) alleles may confer distinct activities as comparative studies found EspF of EHEC O157:H7 to be far less effective in inhibiting macrophage phagocytosis than the variant in EPEC O127:H6, possibly owing to weaker interactions with SNX9 \((184)\). Conversely, EspF from EHEC O157:H7 was more effective than the EPEC O127:H6 EspF in preventing uptake by primary epithelial cells from the site of EHEC O157:H7 persistence in the bovine terminal rectum \((184)\).

**Modulation of the Cytoskeleton**

The role of Tir and adaptor proteins in formation of actin pedestals is well established (above); however, it is becoming clear that other effectors are injected that act on the host cell cytoskeleton (summarized in Fig. 5). For example, mitochondrial-associated protein (Map) and EspM are WxxxE-family effectors homologous to the \(Shigella\) IpgB and \(Salmonella\) SopE proteins that act as guanine nucleotide exchange factors (GEFs) to activate GTPases that regulate the actin network. Map activates Cdc42 to trigger transient formation of filopodia at attachment sites \((127, 190, 191)\), whereas EspM activates RhoA to form actin stress fibers \((192, 193)\). Further WxxxE effector (EspT) has a putative GEF activity for Cdc42 and Rac1 to trigger membrane ruffling, and filopodia formation has been found in a minority of EPEC strains, but so far not in EHEC \((194)\). The relevance of the GEF activity of Map and EspM in vivo is not fully understood, but recent research with \(Salmonella\) sp. has indicated that activation of RhoGTPases by SopE triggers the NOD1 signaling pathway leading to inflammation, indicating that their activity may extend beyond changes to cell architecture \((195)\). Interestingly, A/E pathogens also inject EspH, which has an opposing activity to Map, EspM, and EspT. EspH inactivates cellular Dbl-homology and pleckstrin-homology (DH-PH) Rho family GEFs by competitively binding to tandem DH-PH domains, resulting in inhibition of activation of RhoA \((181)\). The bacterial GEFs Map, EspM, and EspT lack sequence homology with DH-PH domains, and recent studies have shown that EspH does not directly interfere with their activity \((196)\). Rather, EHEC and EPEC appear to use EspH to inactivate endogenous RhoGEFs while injecting their own GEFs to modulate Rho GTPase signaling to their advantage \((196)\). The interplay between these effectors is such that phenotypes detected by expression of effectors interactions between EspF and SNX9 have been reported to remodel the apical plasma membrane and facilitate EPEC invasion in epithelial cells \((189)\), indicating the potential for cell type-specific effects. Moreover, distinct \(espF\) alleles may confer distinct activities as comparative studies found EspF of EHEC O157:H7 to be far less effective in inhibiting macrophage phagocytosis than the variant in EPEC O127:H6, possibly owing to weaker interactions with SNX9 \((184)\). Conversely, EspF from EHEC O157:H7 was more effective than the EPEC O127:H6 EspF in preventing uptake by primary epithelial cells from the site of EHEC O157:H7 persistence in the bovine terminal rectum \((184)\).
in isolation are to be interpreted with caution. For example, expression of EspH alone causes disassembly of focal adhesions, cell detachment, caspase-3 activation, and cytotoxicity, but its activity is normally offset by the opposing activity of EspM2 and, in EPEC, EspT (196). Overexpression of EspH has been reported to result in elongation of actin pedestals, and conversely, espH mutation causes them to shorten (197). It is now understood that EspH plays a role in recruitment of N-WASP and the Arp2/3 complex to assemble actin at sites of bacterial attachment (198). It is possible this involves recruitment of the WASP-interacting protein WIP, as the WIP-binding domain of N-WASP and carboxyl terminus of Tir are required for the effect (198). A further effector reported to subvert actin dynamics is EspV (199). Ectopic expression of EspV causes nuclear condensation, cell rounding, and formation of dendrite-like projections; however, it is relatively rarely found in EHEC and its mode of action is unknown.

Actin is not the only component of the cytoskeleton, and evidence is growing that effectors act on other structures involved in cell architecture. For example, EspG and EspG2 interact with tubulin, deplete microtubules at sites of attachment, and disrupt the integrity of tight junctions and epithelial paracellular permeability (200–204). In a further example of the multifunctional nature of effectors, recent work has also established that EspG also interferes with protein secretion, including release of interleukin-8, by disrupting endoplasmic reticulum to Golgi transport via interactions with ADP-ribosylation factors, p21-activated kinases, Rab1 GTPase, and Golgi matrix protein GM130 (205–208). Intermediate filaments are also targets for effectors of A/E pathogens, and evidence exists that Tir recruits the cytoskeletons CK8 and CK18 to sites of bacterial attachment (209) in a manner associated with direct binding of Tir to CK18 (209) and 14-3-3tau (210), which acts as an adaptor protein of CK18. Depletion of either CK18 or 14-3-3tau impaired pedestal formation (209, 210), implying a significant role. Spectrin, which polymerizes under the plasma membrane to form a scaffold important for cell shape and membrane integrity, is also recruited to EPEC O127:H6 and EHEC O157:H7 pedestals, and siRNA-mediated knockdown of spectrin and spectrin-associated proteins impaired pedestal formation (211).

Modulation of Apoptosis

Synergistic and antagonist interactions of Type III secreted effectors influence host cell survival during infection by A/E pathogens, as summarized in Fig. 6. For example, EspF, Map, and Cif induce apoptosis whereas NleD, NleF, NleH1, and NleH2 appear to counteract the process. EspF triggers apoptosis by damaging mitochondria and reducing the levels or function of Abcf2, which suppresses caspase 3 activation (212–216). Map also targets mitochondria, disrupting their membrane potential (127, 217), and may act in concert with EspF to trigger cell death via mitochondrial damage. In EPEC and some EHEC, Cif induces a delayed form of apoptosis characterized by activation of caspases, accumulation of cleaved caspase-3, and exposure of the phosphatidylserine on the cell surface (218). The basis of this effect is ill-defined but may be a consequence of Cif-mediated arrest of the cell cycle and subversion of the ubiquitin-dependent degradation pathway. The relevance of such events during infection is open to question. Indeed, one study reported a decrease in the number of apoptotic cells in the ileum and ileal Peyer’s patches of rabbits following infection with rabbit enteropathogenic E. coli (219).

NleH effectors block the induction of multiple apoptotic pathways, including those stimulated by staurosporine, brefeldin A, tunicamycin (220), and Clostridium difficile toxin B (221). This appears to be due to the ability of NleH1 and NleH2 to interact with the anti-apoptotic protein Bax inhibitor-1 (BI-1), as knockdown of BI-1 abolished their anti-apoptotic activity (220). NleH was also observed to inhibit cleavage of the procaspase-3 at sites of attachment of C. rodentium (222). NleH proteins have been reported to bind to Na(+)/H(+) exchanger regulatory factor 2 (NHERF2), and this may limit its ability to block apoptosis, as overexpression of NHERF2 appears to sequester NleH and impede its anti-apoptotic activity (222). In recent studies NleF has also been reported to prevent epithelial cell apoptosis in a manner associated with direct binding to caspase-4, -8, and -9 (223). NleD-mediated cleavage of JNK may also help reduce apoptosis as JNK stimulates this process.

Induction of Diarrhea

It is evident that the LEE-encoded T3SS and selected effectors play key roles in the induction of enteritis and fluid loss during infection of animals; however, the precise mechanisms involved in EHEC-induced diarrhea are not well understood. It is likely that a combination of factors lead to net fluid accumulation in the gut lumen, including a loss of absorptive capacity owing to microvillus effacement and enterocyte extrusion, disruption of tight junctions, inhibition of water adsorption channels (aquaporins), and dysregulation of ion and...
glucose transporters (reviewed in reference 224). EspB has been reported to contribute to loss of microvilli (76), and EspF, EspG, Map, Esp/NleA, and EspM disrupt tight junctions and monolayer integrity (201, 203, 225–227). EspF has been reported to downregulate the activity of Na+/H+ exchanger 3 (228), which is the main Na+-absorbing isomorph in the mammalian small intestine. It is not yet clear how binding of the Na (+)/H(+) exchanger regulatory factor 2 by Map, EspI, and NleH affects the activity of such ion exchangers. It is evident that EPEC O127:H6 EspF, Map, Tir, and intimin act cooperatively to inactivate the sodium-d-glucose cotransporter (SGLT-1), which plays a key role in the uptake of fluid from the lumen in the normal intestine (229). However, it is not clear if the same applies during EHEC infection, and a lack of tractable models of EHEC-induced diarrhea has hindered our understanding of the role of specific effectors in the process.

LifA/Efa1, Z4332 and ToxB

Quantitative analysis of the secreted proteome of EPEC O127:H7 by stable isotope labeling with amino acids in cell culture-based mass spectrometry recently revealed that LifA is a substrate for secretion by the LEE-encoded T3SS (22). LifA (lymphostatin) is a 366-kDa protein that inhibits mitogen-activated lymphocyte proliferation and proinflammatory cytokine synthesis (230) and is nearly identical to an adhesin of EHEC O111:H– termed EHEC factor for adherence 1 (Efa1) (231). LifA/Efa1 is highly conserved in non-O157 EHEC and plays a significant role in intestinal colonization of calves by EHEC strains of serotypes O5:H–, O26:H–, and O111:H– (232, 233). In each of these strains, mutations affecting lifA/Efa1 impair adherence, but in EHEC O5:H– and O111:H– this could not be separated from a posttranscriptional reduction in the expression and secretion of EspA and Tir (233). LifA/Efa1 of an EHEC O26:H– strain influences adherence independently of effects on EspA (232), and the basis of pleotropic effects of lifA/Efa1 mutation in non-O157 EHEC is unknown.

It is assumed that lymphostatin activity does not require Type III secretion as it is detectable in cell-free lysates and can be transferred to laboratory-adapted E. coli K-12 lacking a T3SS (230). Moreover, its ability to promote adherence independently of effects on Type III secretion and in a manner sensitive to antibody indicates that a portion of the protein may be surface localized (232, 234). It is evident, however, that some Type III secreted proteins can be secreted from E. coli by alternative pathways (e.g., the EPEC serine protease EspC) (235). Predicted glycosyltransferase and cysteine protease motifs of LifA/Efa1 were dispensable for intestinal colonization of calves by EHEC O26:H– (232), and an earlier report claiming roles for such motifs during C. rodentium infection of mice (236) can be explained by the fact that the motif deletions caused truncation.

Most EHEC O157:H7 strains lack lifA/Efa1 but contain a truncated variant (z4332) and a full-length pO157-encoded homolog (toxB). An extremely high frequency of carriage of lifA/efa1, z4332, and/or toxB exists in LEE-positive strains, with physical linkage of such genes to the LEE in many cases. Deng et al. recently confirmed that Z4332 and ToxB are also effectors of the T3SS (22). Mutation of either factor has been reported to impair Type III secretion at a posttranscriptional level (237, 238) but does not affect a lymphostatin-like activity of EHEC O157:H7 (239). Intestinal colonization of calves or lambs by a Stx-negative E. coli O157:H7 strain was not affected by single or double z4332 or toxB mutations (237). As with other Type III secreted effectors, it is likely LifA/Efa1 and ToxB are multifunctional and their location and activities inside host cells require study.

FUTURE PERSPECTIVES

Research on the LEE-encoded T3SS and the repertoire and functions of secreted effectors has expanded enormously. Today, it represents one of the most vibrant areas of study on E. coli O157:H7 and other EHEC strains, yet significant challenges remain. It is already clear that analysis of the activities of effectors in isolation cannot account for synergistic or antagonist activities with other effectors, or reproduce the timing and intracellular concentration of effectors delivered in vivo. Moreover, a relatively small number of prototype strains are used, and variation in the repertoire, sequence, and regulation of effectors between strains may exert distinct phenotypes. The sources of novel effectors and regulators of LEE function, and frequency and impact of acquisition of these on the virulence of EHEC, require further investigation. Moreover, research should recognize that EHEC can infect multiple hosts and that effectors that appear unimportant in surrogate rodent- or cell-based assays may play key roles in adaptation to humans or reservoir hosts. Unraveling the activities in effectors in reservoir hosts is presently hindered by the paucity of reagents to study signaling pathways and detect proteins in ruminant cells relative to murine and human systems.
While there is much merit in the intellectual pursuit of the molecular mechanisms by which constituents of the T3SS and its effectors act, future research should also consider how we may exploit this knowledge for practical gain. Vaccines that target intimin, Tir, and translocon components show promise in control of EHEC O157 in cattle (reference 246); however, the extent of cross-protection against other EHEC strains is ill-defined. Inhibitors of Type III secretion have proven useful for “chemical genetics” to understand LEE function and regulation, but delivery of such molecules at the required sites, concentrations, and times to ablate T3SS activity poses a formidable challenge. Given the array of effectors dedicated to disarming the innate response, future research could consider the extent to which this mediates persistence in reservoirs and prevents development of effective adaptive immunity, as this may inform the design of live vaccines.

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