Enteropathogenic *E. coli*, *Salmonella*, and *Shigella*: Masters of Host Cell Cytoskeletal Exploitation

Danika L. Goosney, Derek G. Knoechel, and B. Brett Finlay
University of British Columbia, Vancouver, British Columbia, Canada

Bacterial pathogens have evolved numerous strategies to exploit their host's cellular processes so that they can survive and persist. Often, a bacterium must adhere very tightly to the cells and mediate its effects extracellularly, or it must find a way to invade the host's cells and survive intracellularly. In either case, the pathogen hijacks the host's cytoskeleton. The cytoskeleton provides a flexible framework for the cell and is involved in mediating numerous cellular functions, from cell shape and structure to programmed cell death. Altering the host cytoskeleton is crucial for mediating pathogen adherence, invasion, and intracellular locomotion. We highlight recent advances in the pathogenesis of enteropathogenic *Escherichia coli, Salmonella* Typhimurium, and *Shigella flexneri*. Each illustrates how bacterial pathogens can exert dramatic effects on the host cytoskeleton.

Enteropathogenic *Escherichia coli* (EPEC): A Model for Studying Bacterial Attachment and Effacement

Pathogenic *E. coli* strains remain a leading cause of severe and persistent infant diarrhea in developing countries. Although EPEC is recognized as a major diarrheal pathogen, until recently our understanding of how it causes disease lagged behind that of other pathogenic *E. coli*, such as enterotoxigenic *E. coli* or enteroinvasive *E. coli*.

EPEC is one of a class of pathogens identified as causing attaching and effacing (A/E) lesions on intestinal cells (1). A/E pathogens typically reside on a pedestal on the surface of the host epithelial cell and ultimately cause severe disruption of the microvilli brush border (Figure 1A). Other pathogens displaying similar histopathologic features include *Hafnia alvei*, *Citrobacter rodentium* (formerly *C. freundii* biotype 4280), and enterohemorrhagic *E. coli,* the causative agent of hemolytic uremic syndrome.

Address for correspondence: B. Brett Finlay, Biotechnology Laboratory and the Departments of Microbiology and Immunology and Biochemistry and Molecular Biology, University of British Columbia, Vancouver, British Columbia, Canada V6T 1Z3; fax: 604-822-9830; e-mail: bfinlay@unixg.ubc.ca.

Bacterial Factors Involved in EPEC-Induced A/E Lesion Formation

The interactions between EPEC and host cells have been divided into three stages. Initial adherence to cultured epithelial cells is mediated by the formation of type IV fimbriae known as bundle forming pili (BFP) (2). While not essential for forming the characteristic A/E lesions, initial adherence helps bring the bacteria in close contact with the host cell. BFP mediate bacterial-bacterial interactions in a human intestinal organ culture model (3).

All the genes necessary for the formation of A/E lesions by EPEC are contained within a 35-kbp pathogenicity island termed the locus of enterocyte effacement (LEE) (Figure 1B) (4,5). These include the *esps* (*E. coli*-secreted protein), *escs* (*E. coli* secretion), *sep* (secretion of *E. coli* proteins), *eae* (*E. coli* attaching and effacing that encodes intimin), and *tir* (translocated intimin receptor) genes (6).

The second stage of EPEC pathogenesis involves the secretion of bacterial proteins, some into the host cell, including EspA, EspB, and EspD (7,8). The expression of these proteins is maximal at the host body temperature (9) and at conditions similar to those found in the gastrointestinal tract (10), which implies that they may be involved in virulence. The translocation of these proteins is essential for

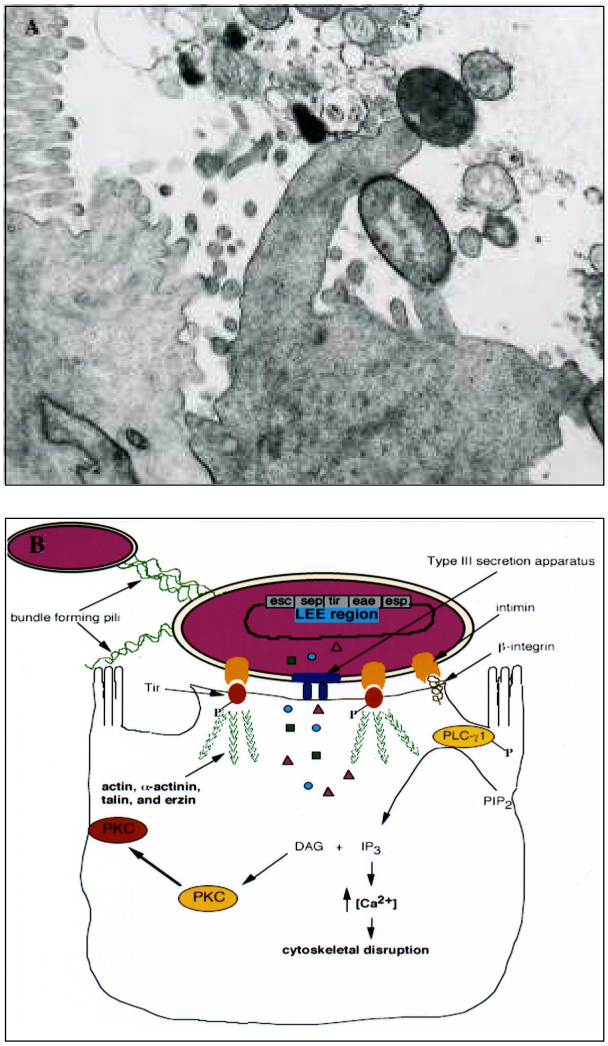


Figure 1. A. Transmission electron micrograph of an A/E lesion formed by rabbit enteropathogenic *Escherichia coli* (EPEC) infecting rabbit intestinal epithelial cells (micrograph provided by Dr. Ursula Heczko, Biotechnology Laboratory, University of British Columbia). B. Effects of EPEC infection on host intestinal epithelial cells. EPEC initially adheres to the host cell by its bundle-forming pili, which also mediate bacterial aggregation. Following initial attachment, EPEC secretes several virulence factors by a type III-secretion system. Signal transduction events occur within the host, including activation of phospholipase C (PLC) and protein kinase C (PKC), inositol triphosphate (IP_3) fluxes, and Ca^{2+} release from internal stores. The bacterium intimately adheres to the cell by secreting its own receptor, Tir, into the host and binding to it with its outer membrane ligand, intimin. Intimin can also bind ß1-integrins. Several cytoskeletal proteins are recruited to the site of EPEC attachment, including actin, α-actinin, talin, and ezrin. Cytoskeletal rearrangements occur following Tir-intimin binding, resulting in the formation of a pedestal-like structure upon which the pathogen resides.

activating a number of signal transduction pathways (7), although their precise role in pathogenesis is not well defined. EspA makes filamentous appendages outside the bacterium and may be part of the translocation machinery involved in delivering other virulence proteins (11). EspB is translocated into the host cytosol and membrane, where it may effect changes in the host cell's signaling pathways (12). All of these effector proteins are secreted by a type-III secretion system encoded by the *esc* and *sep* genes (6). Type-III secretion systems also play an important role in other gram-negative pathogenic bacteria such as *Yersinia*, enabling virulence factors to be translocated directly from the bacterial cytoplasm to the host-cell membrane or cytoplasm (13).

The third stage of EPEC infection is characterized as intimate attachment with the host cell. Intimin, a 94-kDa outer membrane protein encoded by the *eae* gene (14), binds to a 90-kDa tyrosine phosphorylated protein in the host membrane (15). This receptor, originally thought to be a host protein, has recently been found to be of bacterial origin and has been designated as the translocated intimin receptor (Tir) (16). As the name suggests, Tir is translocated from the bacterial cell into the host membrane, where it becomes phosphorylated on one or more tyrosine residues and functions as a receptor for its binding partner, intimin. The resultant tight association is accompanied by the formation of actin pedestals up to 10 µm in length (15). Purified intimin also binds ß1 integrins, which suggests that intimin may be binding more than one receptor on the epithelial cell (17). Although integrins are not present on the apical surface of enterocytes, they are located on the apical surface of microfold cells found in Peyer's patches along the intestinal lumen (18).

Host-Cell Factors Involved in A/E Formation

The host cell undergoes a number of changes during infection by EPEC (Figure 1B). The most striking change in the cellular structure of the host cell is the formation of characteristic actin pedestals. Within 3 hours of infection by EPEC, host-cell actin, α-actinin, talin, erzin, and villin accumulate directly under the bacteria (19,20). The latter four cytoskeletal components are involved in cross-linking of actin microfilaments. Localized actin accumulation is so distinct that it forms the basis of an in vitro diagnostic test for EPEC, which uses fluorescein-tagged phalloidin to detect actin accumulation within infected cells (21). The actin pedestals are not static; instead they lengthen and shorten, resulting in apparent movement of EPEC along the host-cell surface (20). The pedestals resemble microvilli in the distribution of actin and villin (20). Microtubule and intermediate filament structures are not affected by EPEC virulence factors (19).

Intracellular calcium levels also seem to play a role in EPEC pathogenesis. EPEC-infected HEp-2 cells show significant elevation of intracellular calcium levels (22), and buffering of these levels can prevent or delay the formation of A/E lesions (23). Increases in intracellular calcium levels can result in the depolymerization of actin by villin (a calcium-dependent microvillus protein) and a breakdown of the host cytoskeleton not unlike that seen in EPEC-infected cells (24). Inositol triphosphate (IP_3) is involved in the release of Ca^{2+} from intracellular stores, and increased levels of IP_3 (25) and inositol phosphate fluxes (26) have been observed in EPEC-infected cells. EPEC interactions with PLC-γ1 HeLa epithelial cells activate a number of proteins, including phospholipase C-γ1 (PLC-γ1) (27). Phosphorylation of PLC-γ1 leads to the IP_3 and Ca^{2+} fluxes mentioned above, underscoring the importance of this signaling event. Cytosolic protein kinase C also gets activated upon EPEC infection and translocates to the plasma membrane (28).

Despite the dramatic changes induced by EPEC in the cytoskeleton, there appears to be little involvement of the Rho family of small GTP-binding proteins normally involved in cytoskeletal rearrangements (29). Inhibition of Rho, Rac, and Cdc42 by compactin and *Clostridium difficile* ToxB, as well as dominant negative alleles, had no effect on pedestal formation by EPEC, which suggests that this pathogen uses a nontraditional mechanism to rearrange actin.

Salmonella Typhimurium: A Model for Studying Bacterial Invasion

S. Typhimurium is a gram-negative bacterium that causes a variety of diseases, from gastroenteritis in humans to typhoid fever in mice. *S.* Typhimurium infections are contracted by oral ingestion and penetration into the intestinal epithelium before induction of systemic (invasive) disease. Invasion into the host intestinal cells results in dramatic morphologic changes to the cell that are due to exploitation of the host cytoskeleton.

Once in close contact with the epithelium, *Salmonella* induces degeneration of enterocyte microvilli (30). Loss in microvillar structure is followed by profound membrane ruffling localized to the area of bacterial-host cell contact (Figure 2A) (29-31). Membrane ruffling is accompanied by profuse macropinocytosis, which leads to the internalization of bacteria into the host cells (32). The entire process occurs within minutes and when completed, *Salmonella* resides within membrane-bound vesicles, and the cytoskeleton returns to its normal distribution (33).

Bacterial Factors Involved in *Salmonella* Invasion

Salmonella entry into nonphagocytic epithelial cells requires several chromosomal genes (*inv/spa*) clustered in a pathogenicity island termed SPI1 (*Salmonella* pathogenicity island 1) (34). Like EPEC, SPI1 encodes a type III-secretion system and several potential virulence factors secreted by this machinery. The type III-secretion system is activated upon host-cell contact and allows export of virulence determinants directly into the host cell, where they effect bacterial uptake (35,36). Recently, SptP, a bacterial protein encoded within SPI1, has been shown to be translocated into the host epithelial cell, where it modulates the host actin cytoskeleton through its tyrosine phosphatase activity (37) (Figure 2B). Disruption of a critical Cys residue in the catalytic domain of SptP results in loss of phosphatase activity (38). It is hypothesized that SptP may function in disrupting host actin stress fibers, thereby facilitating membrane ruffling and subsequent bacterial uptake into host cells.

Other bacterial factors are not encoded next to the secretion apparatus but instead on the genome of a cryptic bacteriophage found in the *Salmonella* chromosome. Recently, a virulence factor encoded within this genome, SopE, has been shown to be required for efficient bacterial entry into host cells (39). SopE requires the type III-secretion system to be translocated into the host cell, where it can directly stimulate actin cytoskeletal rearrangements. It acts as a guanidine exchange factor for members of the

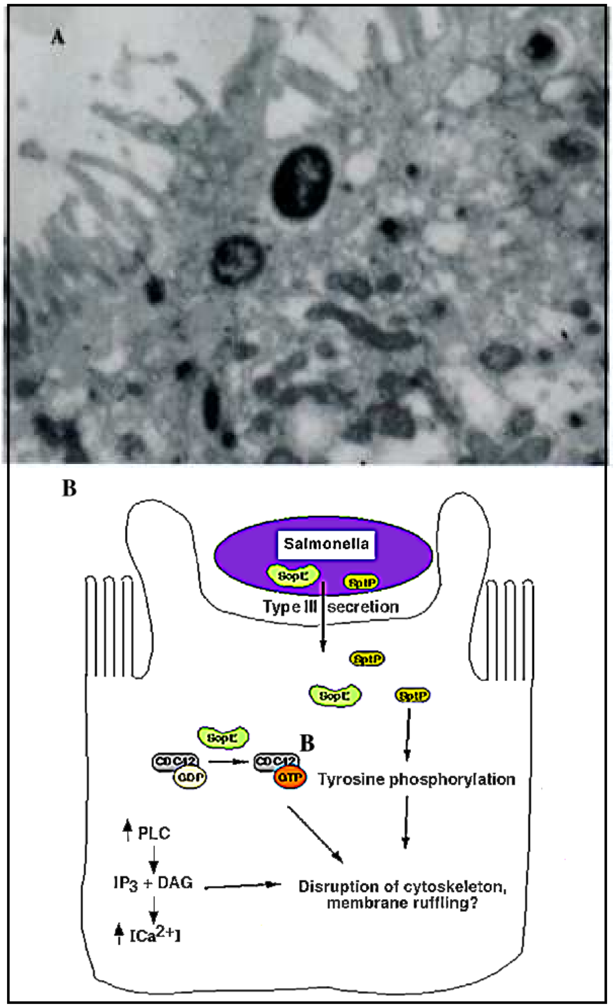


Figure 2. A. Transmission electron micrograph of *Salmonella*-induced membrane ruffling in polarized Caco-2 epithelial cells. B. *Salmonella* invasion into host epithelial cells. *Salmonella* secrete virulence proteins, including SopE and SptP, into host cells by the type III-secretion system. SopE functions as a guanidine exchange factor for small GTP-binding proteins, probably mediating the exchange of GDP for GTP on a Rho subfamily member, CDC42. SptP is a tyrosine phosphatase required for invasion, probably by disrupting the cytoskeleton. Invasion also stimulates phospholipase C (PLC) activity, leading to inositol triphosphate (IP_3) and Ca^{2+} fluxes, which in turn may be involved in cytoskeletal rearrangements leading to membrane ruffling and *Salmonella* internalization.

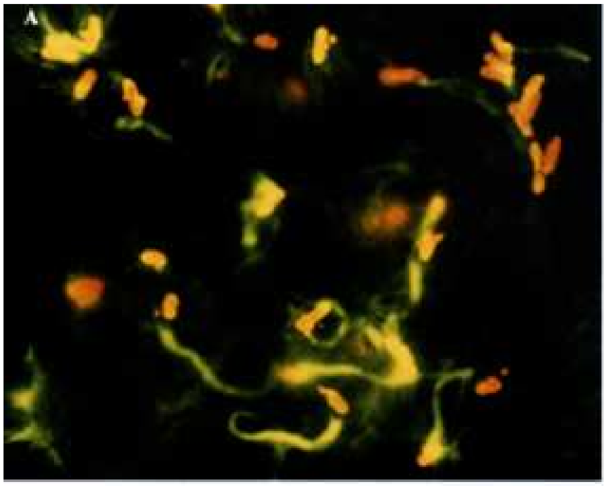
Rho subfamily of small GTPases. *sopE* mutants exhibit less extensive actin cytoskeletal rearrangements upon entry into epithelial cells than do wild-type *Salmonella* (40). This discovery clearly illustrates how pathogens (which contain no primary sequence homology with host proteins) can craftily subvert the host's own signaling machinery within the cell by mimicking host proteins.

Host Factors Involved in *Salmonella* Invasion

The massive restructuring of the host cytoskeletal components during *Salmonella* entry requires many host factors. A Rho subfamily member, Cdc42, is needed for mediating bacterial uptake through membrane ruffling (41). It is believed that the guanidine exchange activity of SopE is responsible for the stimulation of Cdc42 in the host. The pathogen also activates host PLC upon bacterial contact, leading to the production of two second messengers, which further initiate signaling events (42). As a consequence, the host cell's Ca^{2+} levels are altered to trigger cytoskeletal rearrangements resulting in *Salmonella* invasion. Although EPEC and *Salmonella* use some of the same signaling components (PLC, Ca^{2+} fluxes), the cytoskeletal changes induced in the host cell by each pathogen are quite different. This could be the result of different upstream or downstream effectors in the signaling pathway. Several cytoskeletal components involved in invasion have been identified. These include α-actinin, tropomyosin, ezrin, and talin (19). The specific roles of these proteins in *Salmonella* invasion are not defined.

Shigella flexneri: A Model for Intracellular Motility

S. flexneri, a gram-negative bacillus that causes bacillary dysentery in humans, directs its own uptake into the colonic mucosa through membrane ruffling and macropinocytosis in a manner similar to *Salmonella* uptake (43,44). After engulfment, the pathogen is surrounded by a membrane-bound vacuole within the host. Unlike *Salmonella*, however, *Shigella* rapidly lyses the surrounding vacuole and is released into the cytosol, where it grows and divides (45). Once the microbe has escaped from the vacuole, it quickly becomes coated with filamentous actin and ultimately forms an actin tail at one pole of the bacterium (Figure 3A) (46,47). This actin polymerization propels the bacterium through the cytoplasm at speeds reaching 0.4 µm/sec (48). When the pathogen reaches the plasma membrane of the cell, it forms a long protrusion



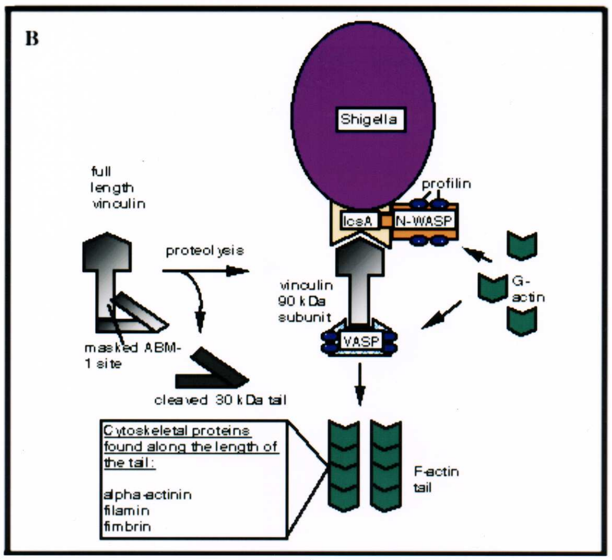


Figure 3. A. Immunofluorescence micrograph showing *Shigella* (red) propelling itself through the cytoplasm by polymerizing actin (green) (Philippe Sansonetti, Institut Pasteur, reprinted with permission from Trends in Microbiology, 1996). B. *Shigella*-mediated cytoskeletal rearrangements. The outer membrane protein, IcsA, is sufficient to drive actin-based motility of *Shigella*. IcsA directly binds two proteins, vinculin and neural-Wiskott-Aldrich Syndrome protein (N-WASP). Vinculin undergoes proteolysis within the host cell upon *Shigella* infection, producing a 90-kDa fragment that can bind to IcsA and to the vasodilator-stimulated phosphoprotein (VASP). VASP in turn can recruit profilin to the bacterial surface, which can provide actin for tail construction. N-WASP binding of IcsA can also recruit profilin to the bacterial surface and may be another means of obtaining monomeric actin for tail formation and subsequent bacterial motility.

into the neighboring cell, which subsequently internalizes the microbe (49). The bacterium again breaks out of the vacuole, thereby starting a new cycle of infection in a new host cell (50). This process allows *Shigella* to move from cell to cell without ever contacting the extracellular milieu.

Bacterial Factors Involved in *Shigella* Motility

Analysis of mutants deficient in intracellular motility and cell-to-cell spread has identified a bacterial gene, *icsA*, necessary for *Shigella* locomotion (46,51,52). IcsA (also called VirG) is a 120-kDa outer membrane protein that hydrolyzes ATP and is localized to one pole of the bacterium, at the junction between the microbe and the actin tail (Figure 3B) (53). IcsA expression on the surface of *Shigella* is sufficient to direct actin-based motility (54,55). In fact, *E. coli* expressing IcsA can synthesize actin tails in cytoplasmic extracts (54,55).

During infection, IcsA is also detected as a 95-kDa amino-terminal fragment of the 120-kDa full-length protein (53). This proteolytic cleavage of IcsA is due to a bacterial protease, SopA (IcsP) (56,57). Cleavage is required for polarized distribution of IcsA on the bacterial surface and for proper actin-based motility of *Shigella* in infected cells (56-58).

Host Factors Involved in *Shigella* Motility

IcsA expression on the *Shigella* surface promotes rapid accumulation of actin around the bacterium. Following bacterial division and IcsA polarization, actin tails begin to form on one end of the bacterium. Several host cytoskeletal proteins are involved in tail formation, including α-actinin (48), filamin (59), fimbrin (59), vasodilator-stimulated phosphoprotein (VASP) (60), vinculin (49,61), and neural-Wiskott-Aldrich syndrome protein (N-WASP) (63). Of these proteins, only vinculin and N-WASP are able to directly bind IcsA (61,62).

Shigella infection results in the cleavage of intact vinculin (120 kDa) to produce a 90-kDa fragment (63). This proteolysis unmasks an actin-based motility 1 site on vinculin, which contains a polyproline region capable of binding VASP. VASP recruitment to the bacterial surface in turn allows the recruitment of other cytoskeletal proteins, such as actin and profilin, and forms the basis of an actin-based motor for *Shigella* movement.

Recently, N-WASP was shown to be required for *Shigella* motility (62); like vinculin, it can bind IcsA directly. It is possible that N-WASP, in addition to VASP, can recruit profilin and actin to the surface of *Shigella*, thereby mediating actin polymerization. Furthermore, N-WASP contains an actin depolymerization factor/cofilin homologous region, which could be used for severing actin filaments at the pointed ends and increasing the monomeric actin concentration. The precise mechanisms involved in *Shigella*-driven actin polymerization, however, are unclear.

Conclusions

Bacterial pathogens have evolved several mechanisms to hijack host-cell signaling machinery and disrupt the cytoskeleton. EPEC mediates its effects on the host cell from the cellular surface. It secretes its own receptor, Tir, into the host and then binds intimately to it by its outer membrane protein, intimin. Tir-intimin binding results in a dramatic reorganization of the cytoskeleton to form the pedestal upon which EPEC resides. *Salmonella*, on the other hand, actively invades intestinal epithelial cells by inducing membrane ruffling and macropinocytosis. Invasion is dependent on the secretion of virulence proteins, including SptP and SopE, into the host cell, and mediates its effects on the host from within a membrane-bound vesicle. *Shigella* is also an invasive pathogen but lyses the phagocytic vacuole and initiates intracellular actin-based locomotion to spread from cell to cell in the cytoplasm. This motility is dependent on the bacterial outer membrane protein IcsA, which recruits several actin-associated proteins to the bacterial surface. Despite the outward differences between each mode of pathogenesis, EPEC, *Salmonella*, and *Shigella* have effectively managed to subvert the host cytoskeleton for their own purposes and cause substantial diarrheal disease.

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Danika Goosney is a Ph.D. candidate in Dr. B. Brett Finlay's laboratory in the Department of Microbiology and Immunology and the Biotechnology Laboratory at the University of British Columbia. She is currently investigating EPEC-induced cytoskeletal rearrangements in cultured epithelial cells.

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