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MINIREVIEW

Shigella Subversion of the Cellular Cytoskeleton: a Strategy for Epithelial Colonization

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INTRODUCTION

Shigella flexneri causes diarrheal disease, including bacillary dysentery, by invading the colonic mucosa, thereby triggering an intense acute inflammatory response with mucosal ulceration and abscess formation (18, 38). In vitro experiments carried out with various epithelial cell lines have shown that invasion by shigellae is a multistep process. It consists of entry into epithelial cells by induced phagocytosis, escape from the phagocytic vacuole, multiplication and spread within the epithelial cell cytoplasm, passage into adjacent epithelial cells by way of finger-like protrusions from the cell surface, and killing of the host cells (4, 11, 12, 18, 20, 25, 26, 41, 42). Several steps in the epithelial cell invasion process, including entry, intracellular spread, and passage into adjacent cells, involve the interaction of the bacteria with the host cell cytoskeleton (4, 6, 26, 41, 42). Invasion is additionally characterized in vivo by use of the Serény test (the capacity to produce keratoconjunctivitis in the guinea pig) (36) and by assay of experimental infection in ligated rabbit ileal loops (9). In this review, we will present what is known about the interaction of *S. flexneri* with the cellular cytoskeleton at the various stages of invasion.

ENTRY OF *S. FLEXNERI* INTO EPITHELIAL CELLS

Cell biology of the entry process. The cell biology of the entry process has been studied in in vitro systems using semiconfluent and confluent monolayers of mammalian cell lines. Early studies of *Shigella* invasion into Henle cells demonstrated that entry occurs by an energy-requiring endocytic process (12). In subsequent work (6), the presence of polymerized actin at the site of entry was demonstrated by labelling infected HeLa cells with 7-nitrobenz-2-oxa-1,3-diazole (NBD)-phallacidin, a fluorescent molecule that binds to polymerized, but not monomeric, actin (2) (Fig. 1). Short filaments of polymerized actin accumulate beneath the host cell cytoplasmic membrane at the site of bacterial entry. By measuring the ratio of monomeric to total actin present over time, it was demonstrated that a significant increase in the pool of polymerized actin occurs, beginning by 6 min after placement of the cells and overlying bacteria at temperatures permissive for entry, with a return to baseline by 12 min (6). Entry of *S. flexneri* into Henle cells is blocked by cytochalasins B and D, molecules that inhibit actin polymerization, further supporting the essential role of de novo actin polymerization in the entry process (12). It has also been demonstrated that myosin (37) accumulates at the site of *Shigella* entry into HeLa cells (6).

Location of the site of entry has been studied in in vitro systems using either semiconfluent or confluent monolayers of mammalian cell lines. In Caco-2 cells (24) and chicken embryo fibroblasts (42), penetration of *S. flexneri* occurs at the level of the cellular focal adhesion plaques. Adhesion plaques are structures in which converging filaments of intracellular actin and associated actin-binding proteins adhere to components of the extracellular matrix, most probably by way of integrins, a class of dimeric integral transmembrane proteins. Certain integrins have been identified as specific receptors for pathogenic bacteria: for example, the *Yersinia pseudotuberculosis* protein Inv binds to several β_1-containing integrins (15). These observations raise the possibility that a *Shigella* surface protein(s) recognizes an integrin receptor, but as yet, no such specific interaction has been demonstrated.

Further information on the site of entry has been obtained by examining *Shigella* invasion into polarized mammalian cells. Under these conditions, entry of *S. flexneri* occurs not through the apical surface of the cells but rather through exposed basolateral surfaces (24). On confluent monolayers, the paracellular junctions must be opened, allowing access of the bacteria to the basolateral surfaces, in order for invasion to occur (24). Whether specific receptors on the basolateral surfaces of these cell lines are recognized by the bacteria is not yet known. This process is in striking contrast to what is observed for salmonellae, which invade directly through the brush border of polarized cells after inducing disruption of the microvilli and cellular cytoskeletal rearrangements known as membrane ruffling (8, 38).

Inasmuch as these in vitro observations reflect the in vivo situation, they raise numerous questions about the site of entry of *S. flexneri* in the human intestine. Colonic epithelial cells and professional phagocytes, in particular intestinal macrophages, are perhaps the principal targets of *Shigella* invasion. Colonic epithelial cells are polarized nonphagocytic cells which early in their life cycle are located near the base of colonic crypts and are relatively undifferentiated, lacking a brush border. As they mature, they develop a microvillous brush border and migrate toward the tips of the colonic villi. While the presence of a brush border presents a physical barrier to cell invasion, it is unclear whether *S. flexneri* preferentially or exclusively invades epithelial cells at a particular stage of maturity. Another cell type present in the colonic epithelium is the M cell, which is an antigen-sampling cell that lacks a brush border and overlies submu-

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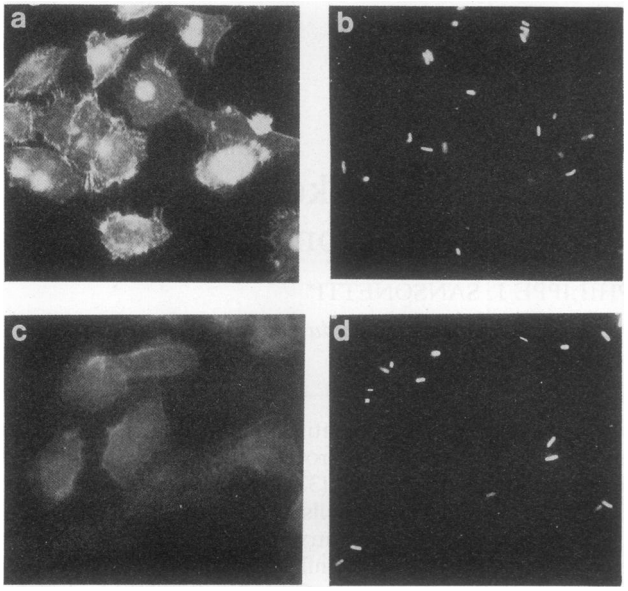

FIG. 1. Double fluorescence labeling of infected HeLa cells. Actin polymerization foci at sites of *S. flexneri* entry. (a and b) Invasive strain; (c and d) noninvasive mutant. Left panels correspond to NBD-phallacidin labeling of F-actin; right panels correspond to immunolabeled bacteria. Reprinted from reference 6.

cosal germinal centers (17). Given the inability of *S. flexneri* to invade through the intact brush border of mature polarized epithelial cells in vitro, the potential routes of entry of this organism in vivo may include (i) the apical surfaces of cells with disrupted brush borders; (ii) the apical surfaces of immature crypt cells or M cells, both of which lack brush borders; or (iii) the basolateral surfaces of enterocytes, accessed via the opening of intercellular junctions. Only a few inconclusive observations regarding the actual site of entry in vivo are available. Destruction of the brush border has been described for experimental infection of rabbit ileal loops (27). M cells are known to take up shigellae (44), but whether shigellae spread from M cells or M-cell-associated phagocytic cells into adjacent mucosal tissue is not yet clear. Further, based on in vitro observations described above, it seems likely that access to the basolateral surfaces will prove to be important.

Molecular biology of *Shigella* entry. All virulent shigellae carry a large 220-kb plasmid which carries several genes known to be essential to the entry process (30, 31). Virulence plasmids from different *Shigella* species are very similar (29). Molecular cloning analysis of the virulence plasmid of *S. flexneri* demonstrated that a 40-kb fragment was sufficient for entry (22). Subsequent transposon insertion mutagenesis of this region has lead to the definition of five distinct loci that contain genes necessary for *Shigella* pathogenesis (3). Among these is an 8-kb stretch, known as locus 2, that has been implicated in the entry process. Eight open reading frames have been identified in this region that encode proteins with molecular masses of 56, 14, 21, 17, 62, 41, 36, and 72 kDa (1, 3, 34, 35, 43). The last four of these open reading frames encode proteins recognized by sera from humans or monkeys convalescent from shigellosis and have been named IpaB (invasion plasmid antigen B), IpaC, IpaD, and IpaA, respectively, for their role in invasion (5, 13). Construction of *Shigella* strains that carry polar or nonpolar mutations in each of the genes encoding the Ipas has allowed the examination of each one's contribution to the invasive phenotype. A strain carrying a mutation in *ipaB*, *ipaC*, or *ipaD* is unable to enter semiconfluent HeLa cell monolayers (14, 23, 34). Each of these mutants adheres to the surface of HeLa cells, but none of them invokes the polymerization of host cellular actin beneath the sites of adherence. No further information is yet available on how each of the proteins implicated in the entry process interacts with cell surface structures or the cellular cytoskeleton, nor has any *Shigella* surface protein been identified as able to bind to a cellular receptor.

MOVEMENT OF *S. FLEXNERI* WITHIN AND BETWEEN EPITHELIAL CELLS

Movement of *S. flexneri* within epithelial cells. Within 15 min of entry into an epithelial cell, the bacterium escapes from its phagocytic vacuole and thereby enters the cytoplasmic compartment of the host cell (33). Immediately, short filaments of polymerized cytoplasmic actin accumulate at one extremity of the bacterium, as demonstrated by labelling of this material with NBD-phallacidin (4) (Fig. 2). The actin is bundled to form an actin-containing tail several microns in length behind the microorganism as it moves forward in spurts through the cytoplasm.

Several cytoskeletal proteins have been shown to be incorporated into the tail that trails the bacterium. In particular, it contains large amounts of polymerized actin (4). Pretreatment with cytochalasin D prevents both the accumulation of actin on the bacterial surface and the formation of the tail (4, 26), suggesting that de novo actin polymerization is required for these processes. As has been demonstrated for *Listeria monocytogenes*, the continuous addition of polymerized actin filaments to the tail at the junction of the tail with the bacterium is thought to motor the forward movement of the bacterium (39, 40).

In *S. flexneri*, the diameter of the actin tail immediately behind the bacterial body is slightly wider than that of the bacterium in a region known as zone A. At a short distance from the bacterial body in zone B, it becomes markedly narrowed (28). In immunolabelling experiments, it has been demonstrated that zone A contains the cellular actin-binding protein plastin (Fig. 3) (28). Plastin, an isoform of fimbrin, is unique in that each monomer contains two actin-binding sites located in close proximity to one another, so that when bound to actin, they form extremely tight actin bundles (21). Labelling with antiplastin serum is intense in zone A, rapidly diminishes at greater distances from the bacterium, and is absent around the remainder of the bacterial body (Fig. 3). This distribution of plastin, combined with its ability to form very tight actin bundles, suggests that it may cross-link actin adjacent to the bacterial body in a sphincter-like fashion and that sphincter-like contraction of the tail may contribute to the forward propulsion of the bacteria.

Other actin-associated proteins have also been identified within the tail. Antiserum to filamin, an actin gelation protein, weakly labels the full length of the tail, suggesting that small amounts of this protein are present (28). A monoclonal antibody to vinculin, one of several proteins that are involved in linking actin bundles to the plasma membrane at focal adhesion plaques (19), labels the entire length of protrusions, including fully around the bacteria at the protrusions' tips (16). Antiserum to bovine smooth muscle myosin does not recognize the bacterial tail (28). To what extent each of these actin-associated proteins is involved in

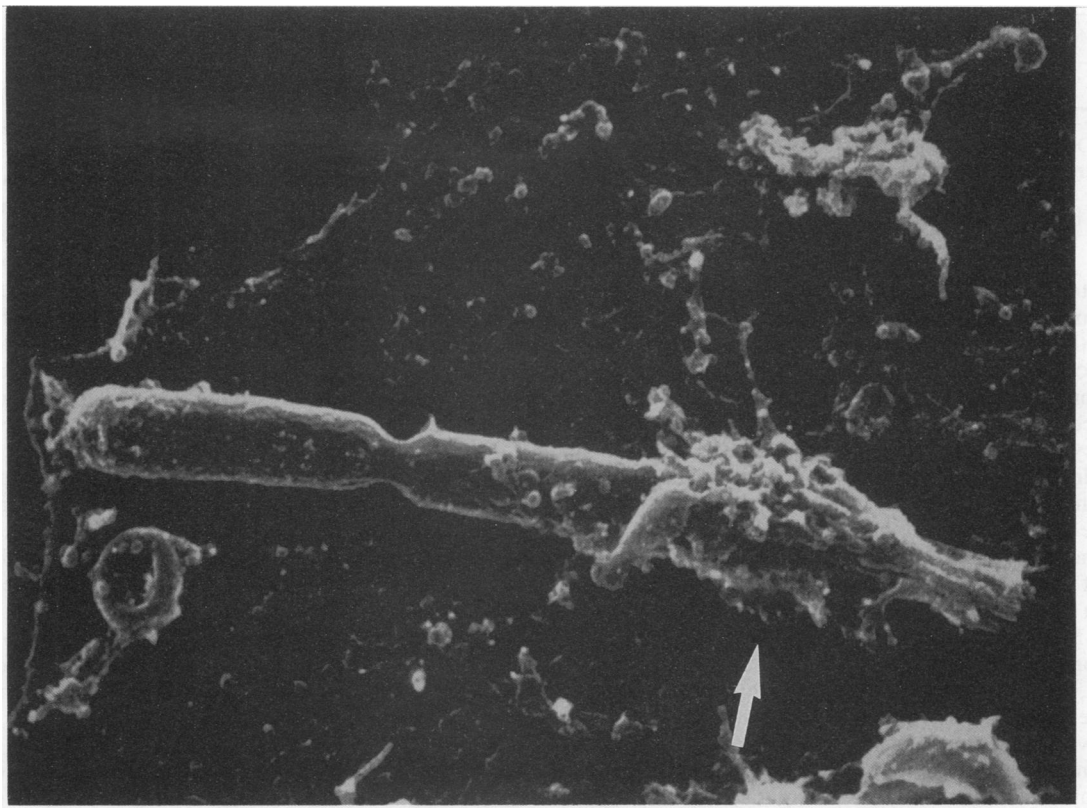

FIG. 2. Scanning electron microscopy of HeLa cell infected by *S. flexneri*. The cytoskeleton has been insolubilized, and the host cell membrane has been removed. A dividing bacterium is seen with tight bundles of actin filaments at one extremity (arrow). Bar, 1 μm. Reprinted from reference 28.

the mechanics of bacterial movement, or is merely associated with the tail because of an indirect association with the actin therein, is not yet clear. It seems reasonable that the bacteria would utilize at least some of the resources of the cellular cytoskeletal apparatus, probably in conjunction with certain bacterial elements, to motor itself through and between the host cells.

Consistent with this model, certain bacterial elements have been found to be involved in bacterial movement. The plasmid-encoded bacterial protein IcsA (VirG) has been

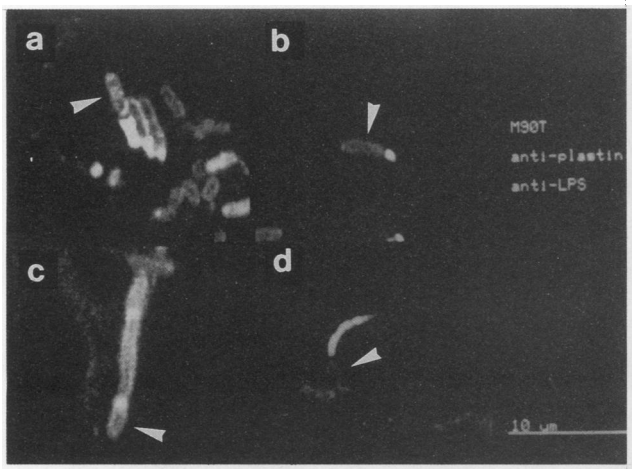

FIG. 3. Double immunofluorescence labeling of HeLa cells infected by *S. flexneri*. Panels are four preparations observed by confocal microscopy. Arrowheads point to immunolabeled bacteria. Note that bacteria are followed by a comet which is strongly labeled by an anti-fimbrin polyclonal serum. Reprinted from reference 28.

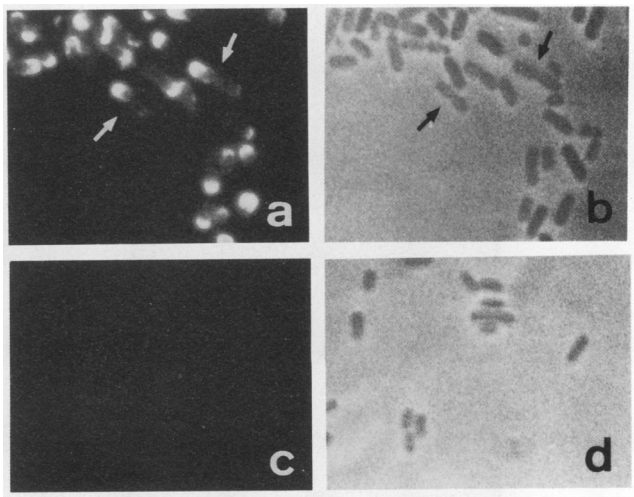

FIG. 4. Immunofluorescence labelling of *S. flexneri* with IcsA antiserum (a and c) and corresponding fields under phase-contrast light microscopy (b and d). (a and b) Wild-type strain M90T; arrows point to dividing bacteria. (c and d) *icsA* mutant SC560.

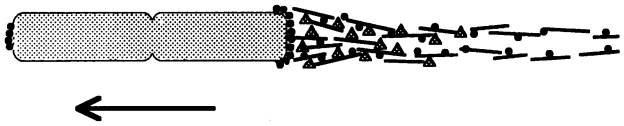

FIG. 5. Diagram representing an intracytoplasmic *Shigella* bacterium moving with an actin tail. Actin filaments are represented by thick lines, IcsA is represented by solid dots, and fimbrin is represented by checkered triangles.

demonstrated to be essential to intracellular movement and intercellular spread (4, 20, 41, 42). It is found as a 120-kDa protein in the outer membrane and is secreted as a 95-kDa protein in conjunction with carboxy-terminal cleavage (10). A strain containing a mutation in *icsA* does not accumulate polymerized actin on its surface, does not spread within the host cell cytoplasm but rather forms localized microcolonies, and does not spread into adjacent cells. Immunolabelling has demonstrated that the surface localization of IcsA is at one pole of both extracellular and intracellular shigellae. Thus, on moving intracellular bacteria, IcsA is located at the junction of the bacterium with the actin tail (Fig. 4) (10). Further, in infected HeLa cell monolayers, IcsA antiserum labels the full length of the actin tail, suggesting that the secreted form of IcsA is present within the tail (Fig. 5) (10). In addition, IcsA has been shown to bind and hydrolyze ATP (10). These data suggest that IcsA interacts directly with elements within the tail and that ATP hydrolysis by IcsA provides energy for some step in these processes (10).

A site on IcsA that can be phosphorylated by cyclic AMP-dependent protein kinase A has been identified (7). Mutagenesis of this site produces a mutant that spreads more rapidly than the wild-type strain in infections of HeLa cells, by an as yet undetermined mechanism. This observation suggests that phosphorylation of IcsA by a cellular kinase may slow down the motility process, thus serving as a potential defense mechanism of cells.

Movement of *S. flexneri* between epithelial cells. In order to spread from one cell to another, the bacterium forms a finger-like protrusion from the surface of the infected cell (Fig. 6). Around the site of exit of the protrusion at the cell surface, major rearrangement of the cytoskeleton occurs, with the formation of many tiny villosities (Fig. 6). The protrusion, with the bacterium at its tip, may elongate to as much as 20 μm. Within the protrusion, the bacterium is trailed by its actin tail, which can be seen to form a hollow cylinder. Those cellular and bacterial proteins shown to be associated with the actin tail trailing cytoplasmic bacteria are also associated with the actin tail within the protrusion. The tip of the protrusion penetrates the surface membrane of an adjacent cell and is then phagocytosed by the adjacent cell, thus placing the bacterium within a double membrane within the adjacent cell. The bacterium then lyses these membranes and is thereby released into the cytoplasm of this adjacent cell. Ninety-five percent of the protrusions contain a dividing bacterium or two bacteria at their tips, suggesting a correlation between the formation of protrusions and bacterial division (28).

As for the cellular factors necessary for the intercellular spread of bacteria, recent work has demonstrated that one of these factors is the cadherin L-CAM. In a mouse fibroblast cell line that does not produce cadherins (S180 cells), shigel-

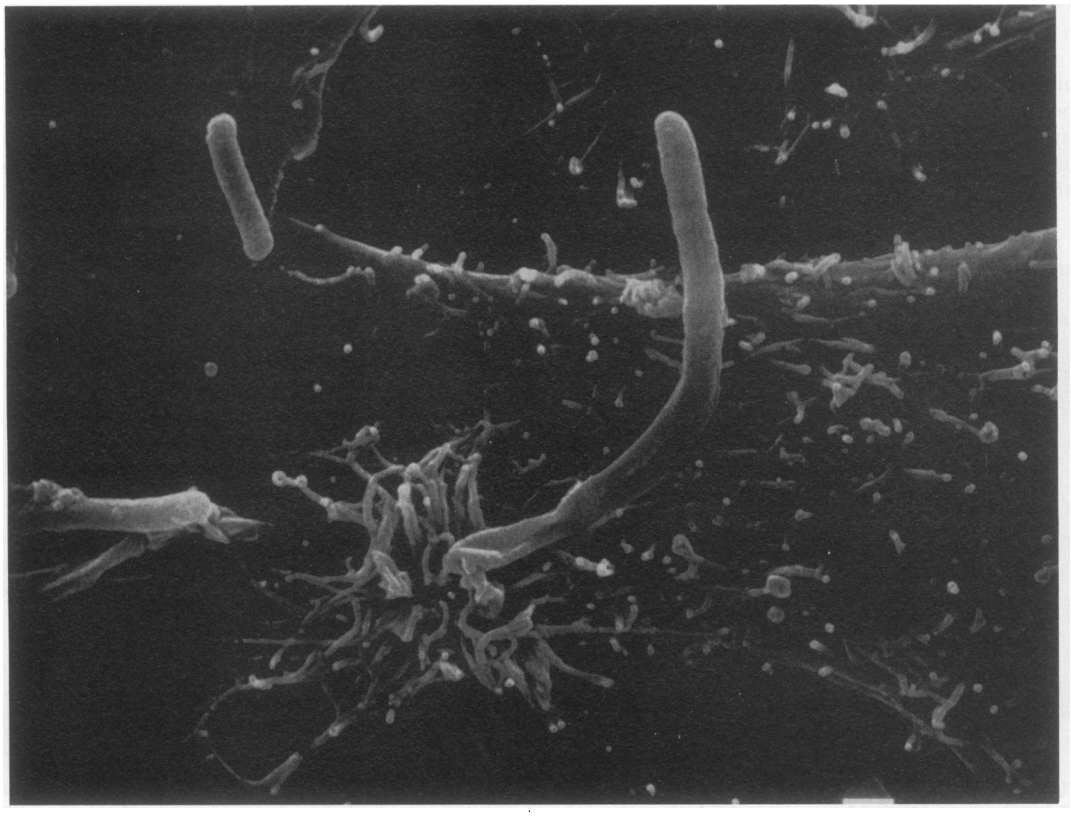

FIG. 6. Scanning electron microscopy of HeLa cell infected by *S. flexneri*. Note a long protrusion that is pushed outside by a moving microorganism. Bar, 1 μm.

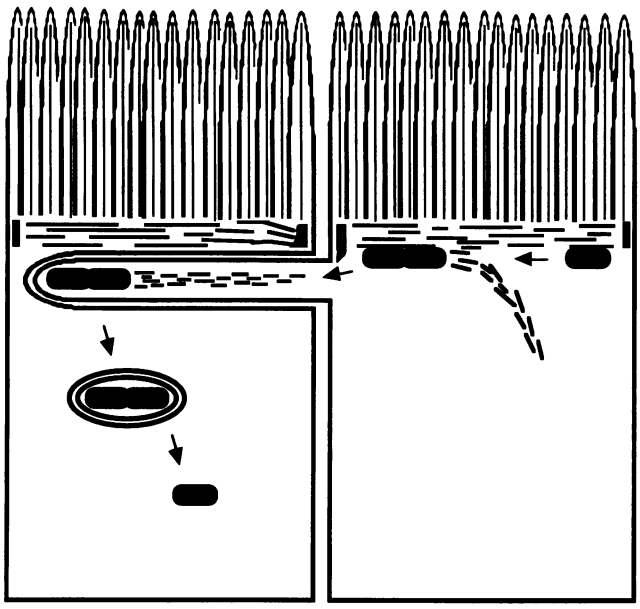

FIG. 7. Diagramatic overview of *Shigella* intracellular movement and spread in epithelium. Actin filaments are represented by short thick lines.

lae were unable to pass from cell to cell (32). Protrusions were formed, but they appeared excessively broad and flaccid and were not internalized by adjacent cells (32). When this cell line was stably transfected with L-CAM, shigellae were able to pass efficiently from cell to cell, the protrusions were narrow and straight, and they were systematically internalized by adjacent cells (32). It was further demonstrated in this study that several components of intermediate junctions, namely, α- and β-catenin, vinculin, and α-actinin, were associated with the actin tail within the protrusion, suggesting that the protrusion might form as an extension of this junction (32). Relatively little is known about other cellular factors essential to the intercellular spread of bacteria.

The *Shigella* gene *icsB* has been shown to be necessary for lysis of the two membranes that surround the bacterium following endocytosis of the protrusion by the recipient host cell (1). *icsB* is located on the virulence plasmid, approximately 1.5 kb upstream of the *ipa* genes. A strain containing a mutation in *icsB* forms protrusions similar to those of the wild type but is unable to lyse the double membrane, remaining trapped within it (1).

CONCLUSION

S. flexneri is unusual among pathogenic bacteria in the nature of its intimate interactions with the host cell. From the moment of entry into the host cell, it engages elements of the host cytoskeleton in each step of the pathogenic process (Fig. 7). Further, it avoids reexiting into the extracellular milieu, remaining instead in a relatively protected intracellular environment. The ability of *S. flexneri* to subvert the host cell cytoskeleton for its own purposes is an extraordinarily adept evolutionary adaptation to intracellular survival.

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