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Shigella flexneri surface protein IcsA is sufficient to direct actin-based motility

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ABSTRACT ***Shigella flexneri* is a Gram-negative bacterial pathogen that can grow directly in the cytoplasm of infected host cells and uses a form of actin-based motility for intra- and intercellular spread. Moving intracellular bacteria are associated with a polarized "comet tail" composed of actin filaments. IcsA, a 120-kDa outer membrane protein necessary for actin-based motility, is located at a single pole on the surface of the organism, at the junction with the actin tail. Here, we demonstrate that stable expression of IcsA on the surface of *Escherichia coli* is sufficient to allow actin-dependent movement of *E. coli* in cytoplasmic extracts, at rates comparable to the movement of *S. flexneri* in infected cells. Thus, IcsA is the sole *Shigella*-specific factor required for actin-based motility. Continuous protein synthesis and polarized distribution of the protein are not necessary for actin tail formation or movement. *Listeria monocytogenes* is an unrelated bacterial pathogen that exhibits similar actin-based intracytoplasmic motility. Actin filament dynamics in the comet tails associated with the two different organisms are essentially identical, which indicates that they have independently evolved mechanisms to interact with the same components of the host cytoskeleton.**

The pathogenesis of diarrhea induced by *Shigella* species involves bacterial invasion and spread through the colonic mucosa. The bacterium enters the colonic epithelial cell by parasite-directed phagocytosis and rapidly escapes the phagocytic vacuole. Within the cell cytoplasm, the bacteria continue to grow and divide, and move rapidly through the cell (1). Moving bacteria are associated with polarized "comet tails" rich in filaments of the host cytoskeletal protein actin (2), and movement is inhibited by the actin-depolymerizing drug cytochalasin D (3). Bacteria spread from one infected cell into uninfected neighbors through membrane-bound protrusions containing one or two bacteria at the tip associated with actin tails (4–6). Actin-dependent movement is necessary for efficient spread of the organisms through tissue culture monolayers and for virulence in infected animals (7).

Analysis of mutants deficient in cell-to-cell spread has indicated that the protein product of the *Shigella flexneri icsA* (*virG*) gene is necessary for actin-based motility (2, 8, 9). IcsA, a 120-kDa outer membrane protein, hydrolyzes ATP and is located at a single pole on the surface of the organism, at the junction with the actin tail (10). A cleaved form of the protein is also secreted by the bacteria (10, 11). The expression of IcsA is growth-phase dependent and is highest when bacteria are dividing rapidly; similarly, actin-dependent movement of *S. flexneri* inside cells is most often initiated when the bacteria are in the process of dividing (12). These correlations suggest that IcsA may be directly involved in the interaction with the host cell cytoskeleton.

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Listeria monocytogenes is an unrelated Gram-positive intracytoplasmic bacterial pathogen that also forms actin tails and moves rapidly inside host cells (13–15). A variety of studies of actin dynamics in the comet tails associated with *L. monocytogenes* have provided information about its mechanism of motility. Short actin filaments are nucleated at or recruited to the bacterial surface and are rapidly elongated at their barbed ends (16). The growing filaments are incorporated into a tail structure that is stationary in the cytoplasm (17), when they become coated and crosslinked by actin-binding proteins. The rate of bacterial movement is simply equal to the rate of actin filament growth at the front end (17, 18). ActA, a surface protein of *L. monocytogenes*, is essential to actin-based motility (19, 20) and is polarized on the bacterial surface (21). Despite these similarities to *S. flexneri* IcsA, there are no significant regions of sequence similarity between the two proteins (19). It has generally been assumed that *L. monocytogenes* and *S. flexneri* employ similar mechanisms for motility, though little is known about actin filament dynamics in tails associated with *S. flexneri* and direct comparisons have not been performed.

In this report we demonstrate that *Escherichia coli* cells expressing IcsA exhibit actin-based motility in cytoplasmic extracts, in the absence of other *S. flexneri* gene products. Normal motility does not require the polar localization of IcsA, its growth-phase regulation, or its cleavage. Furthermore, we have determined that the spatial distribution and turnover rates of actin filaments in the comet tails of *E. coli* expressing IcsA are very similar to those associated with *L. monocytogenes*, in support of the hypothesis that *S. flexneri* and *L. monocytogenes* employ essentially identical means of movement through host cells.

MATERIALS AND METHODS

Bacterial Strains, Media, and Plasmids. *S. flexneri* wild-type serotype 5 strain M90T (22) carries virulence plasmid pWR100, which includes *icsA* and other genes involved in virulence. *E. coli* strains MC1061 [F⁻ *hsdR mcrB araD139 Δ(araABC–leu)7679 galU galK Δ(lac)X74 rpsL thi*] (23) and YK4104 (F⁻ *araD139 lacU169 rpsL thi pyrC46 gyrA thyA his flaD*) (24), which lacks flagella, were used as recipients for the *ompT::km* allele from *E. coli* strain AD202 [*araD139 relA1 thi rpsL150 flbB5301 Δ(lac)U169 deoC1 ptsF25 ompT::km*] (25). The *ompT::km* allele from AD202 was introduced by P1*vir* transduction into MC1061, to give MBG263, and into YK4104, to give MBG264. Bacteria were grown in Luria broth or tryptic soy broth. Ampicillin was added to a final concentration of 100 μg/ml where appropriate. Plasmid pHS3199 contains *icsA* cloned into pBR322 (26). Genetic procedures, including generalized transduction and transformation of bacterial strains, were performed according to standard methods (27).

Protein Preparation and Analysis. Whole-cell (28) and supernatant (29) protein extracts were prepared as described. The relative amounts of IcsA expressed by different strains were estimated from the relative intensities of the signals produced on Western blots. Western blot analysis was per-

formed as described, using an affinity-purified rabbit IcsA antiserum (10), with subsequent visualization by enhanced chemiluminescence (ECL; Amersham). The samples of supernatant protein extract loaded onto the gel were prepared from ≈20 times the culture volume compared with the samples of whole-cell protein extract loaded onto the same gel.

Motility Assays. Meiotically arrested cytoplasmic extracts of *Xenopus laevis* eggs were prepared (30). Bacteria were grown to midlogarithmic phase (OD_{600} of 0.2–0.3) in Luria broth with ampicillin (100 μg/ml) where appropriate, pelleted by brief centrifugation in a tabletop microcentrifuge, and suspended in XB (*Xenopus* extract buffer; ref. 31). For observations using formaldehyde-fixed bacteria, freshly grown bacteria were suspended in PBS (phosphate-buffered saline) containing 1% EM-grade formaldehyde (Ted Pella, Redding, CA), rotated at room temperature for 10–15 min, pelleted and rinsed three times in PBS, and then suspended in XB. Fixed bacteria were stored at 4°C. Ten microliters of extract was mixed with 1 μl of resuspended bacteria in XB and incubated on ice for 15 min. One microliter was then removed and squashed between a microscope slide and a 22-mm square coverslip. For observations using fluorescent actin as a tracer, 1 μl of rabbit skeletal muscle actin (0.2 mg/ml in XB) covalently labeled with tetramethylrhodamine-iodoacetamide (Molecular Probes) (32) was added to the 10 μl of extract prior to the addition of bacteria.

All observations and recordings were performed on a Nikon Diaphot-300 inverted microscope with phase-contrast and epifluorescence optics. Phase-contrast images were recorded with a Hamamatsu (Middlesex, NJ) Newvicon camera; fluorescence images were recorded by using standard rhodamine and fluorescein filter sets (Chroma Technologies, Brattleboro, VT) and a silicon-intensified tube (SIT) camera (Hamamatsu). Images were averaged and recorded with METAMORPH (Universal Imaging, Media, PA) and stored on an optical disc (Panasonic) or as digital files. Fluorescence intensity profiles and actin filament half-lives were determined as described (30).

Immunofluorescence. Expression of IcsA on the surface of bacterial strains was assessed by labeling of bacteria with affinity-purified rabbit IcsA antibodies and rhodamine-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch) (10).

For IcsA immunofluorescent labeling of live bacteria in extracts, the affinity-purified polyclonal antibodies were covalently labeled with fluorescein. Immunoglobulin ≈0.2 mg/ml in 50 mM sodium phosphate (pH 8.3) was mixed with a 200-fold molar excess of 5-carboxyfluorescein-*N*-hydroxysuccinimide ester (Molecular Probes) at room temperature for 30 min. Unreacted dye was removed by gel filtration. Freshly grown bacteria were harvested and mixed with labeled antibody at 50 μg/ml in PBS with 1% bovine serum albumin for 15 min at room temperature, pelleted and rinsed three times in PBS, and finally suspended in XB for addition to extract.

RESULTS

Expression of IcsA in *E. coli*. To determine whether IcsA is the only *Shigella* protein necessary for actin-based motility, we have expressed IcsA in *E. coli* in the absence of other *Shigella* gene products. Because most *E. coli* strains express an OmpT protease that cleaves IcsA from the bacterial surface (33), we constructed the *ompT*$^-$ strain MBG263 by P1*vir* transduction of a known *ompT::km* allele (25) into *E. coli* K-12 strain MC1061. In addition, we constructed the *ompT*$^-$ nonflagellated strain MBG264 by transduction of *ompT::km* into K-12 strain YK4104 (24). These K-12 strains were chosen because in *Shigella* an intact lipopolysaccharide core is necessary for proper surface presentation of IcsA (34).

icsA from *S. flexneri* serotype 5 wild-type strain M90T (22) on pHS3199 (26) was transformed into MBG263 and MBG264. In contrast to its unipolar distribution on wild-type *S. flexneri*, IcsA is distributed circumferentially on the surface of MBG263(pHS3199) (Fig. 1) and MBG264(pHS3199) (data not shown). Each of the *E. coli* strains expresses an amount of IcsA comparable to or slightly less than *S. flexneri* M90T (Fig. 2, lanes 1–3). Further, IcsA expressed from pHS3199 in either an *icsA*$^-$ derivative of M90T or a derivative of M90T that has been cured of the virulence plasmid localizes to one pole on the bacterial surface (data not shown). Thus, the circumferential distribution of IcsA on strains MBG263(pHS3199) and MBG264(pHS3199) is not due to overexpression of IcsA from the plasmid and resultant supersaturation of specific polar localization mechanisms. The expression of IcsA on the surface is not growth-phase dependent, as it is in *S. flexneri* (data not shown; ref. 12). Therefore, the polar localization and temporal regulation of IcsA in *S. flexneri* appear to be determined by *Shigella* factors that are absent in MBG263(pHS3199) and MBG264(pHS3199).

A 95-kDa cleaved form of IcsA is secreted by *S. flexneri* (10, 11). In infected host cells, secreted IcsA is associated with the entire length of the actin-rich comet tail, and it has been suggested that this association might be involved in actin tail formation (10). MBG263(pHS3199) and MBG264(pHS3199) do not secrete detectable levels of the cleaved form of IcsA, although they do secrete low levels of apparently full-length protein (Fig. 2, lanes 4–6). Thus, cleavage of IcsA in *S. flexneri* also seems to be mediated by *Shigella* factors that are absent in MBG263(pHS3199) and MBG264(pHS3199).

Actin-Based Motility of *E. coli* Expressing IcsA. We tested whether *E. coli* cells expressing IcsA were capable of performing actin-based motility by mixing the bacteria with concentrated cytoplasmic extracts from the eggs of the clawed frog *X. laevis*. These cytoplasmic extracts have previously been shown to support normal actin-based motility of the unrelated Gram-positive intracellular bacterial pathogen *L. monocytogenes* (30). To prevent bacterial flagellar motility of MBG263

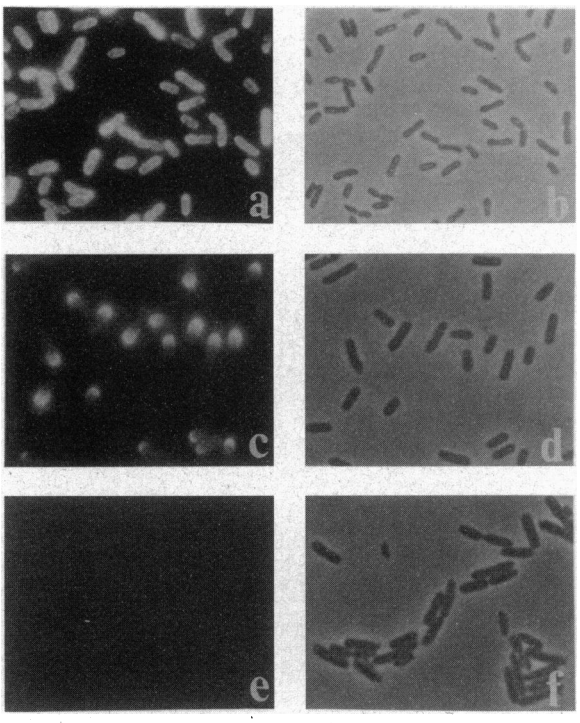

FIG. 1. Distribution of IcsA on the surfaces of *E. coli* MBG263(pHS3199) and wild-type *S. flexneri* M90T. Indirect immunofluorescent labeling with IcsA antiserum (*a*, *c*, and *e*) and corresponding fields by phase-contrast (*b*, *d*, and *f*, respectively) are shown. (*a* and *b*) MBG263(pHS3199). (*c* and *d*) M90T. (*e* and *f*) MBG263 (without the plasmid). MBG264(pHS3199) and MBG264 were labeled similarly to MBG263(pHS3199) and MBG263, respectively (data not shown).

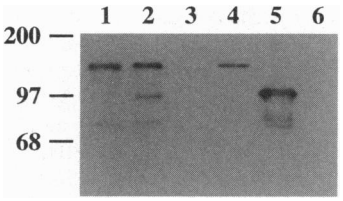

FIG. 2. Expression of IcsA in *E. coli* MBG263(pHS3199) and wild-type *S. flexneri* M90T. Western blot analysis, performed with IcsA antiserum, of whole-cell protein extracts of MBG263 carrying pHS3199 (which encodes IcsA) (lane 1), M90T (lane 2), and MBG263 (lane 3) and of supernatant protein extracts of MBG263(pHS3199) (lane 4), M90T (lane 5), and MBG263 (lane 6). Approximately equivalent amounts of comparable protein preparations were loaded in lanes 1–3, as well as in lanes 4–6. Samples of supernatant protein were prepared from ≈20 times the culture volume used to prepare the samples of whole-cell protein. Apparent molecular masses of standard proteins run in parallel are indicated in kilodaltons at left. MBG264(pHS3199) expressed a similar amount of IcsA in whole-cell protein extracts and less IcsA in supernatant protein extracts when compared with MBG263(pHS3199), both at the same apparent molecular mass as for IcsA of MBG263(pHS3199) (data not shown).

(pHS3199) from interfering with the association with actin, we killed the bacteria by treatment with 1% formaldehyde for 10–15 min. Formaldehyde-fixed MBG263(pHS3199) cells do not exhibit any flagellar motility and do not form colonies when plated on solid medium. However, they are capable of actin-based motility in cytoplasmic extracts. In a typical preparation, >80% of the bacteria in a cytoplasmic extract were associated with actin filaments, as assayed by accumulation of fluorescently labeled rabbit skeletal muscle actin added to the extract as a tracer. Although many actin-associated bacteria moved in small circles without forming typical actin comet tails, 10–30% of the bacteria did form tails and move unidirectionally (Fig. 3). The bacteria associated with comet tails moved at rates up to ≈30 μm/min, with an average rate of 12.9 ± 7.8 μm/min (mean ± SD, n = 47). This is essentially identical to the rate of live *S. flexneri* moving inside PtK2 epithelial tissue culture cells (12.0 ± 6.3 μm/min, n = 110).

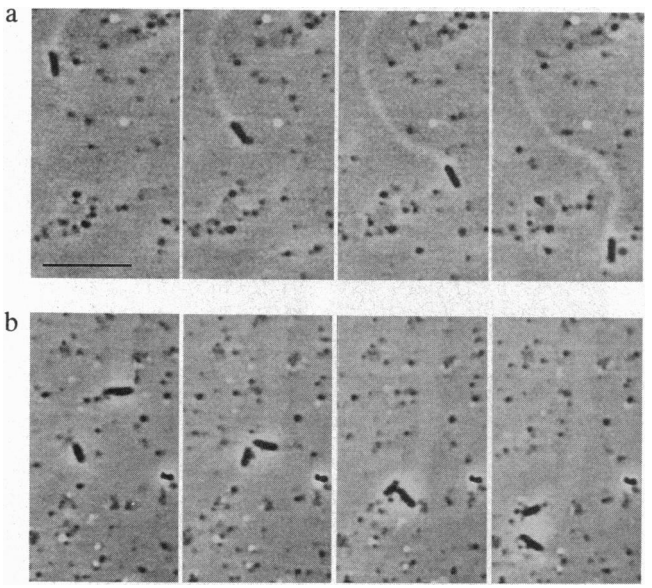

FIG. 3. Actin-based movement of formaldehyde-fixed MBG263-(pHS3199) in *Xenopus* egg cytoplasmic extract. (*a*) Phase-contrast time-lapse images showing movement parallel to the long axis of the bacterium. The actin-rich comet tail is visible as a phase-lucent streak behind the bacterium. Frames are separated by 20-sec intervals. (Bar = 10 μm.) (*b*) Movement perpendicular to the long axis. Frames are separated by 2-min intervals.

Actin-based movement of live or formaldehyde-fixed nonflagellated MBG264(pHS3199) bacteria in *Xenopus* egg extract was similar to the movement of formaldehyde-fixed MBG263(pHS3199). Neither MBG263 nor MBG264 lacking pHS3199 formed actin clouds or tails in cytoplasmic extracts. Surprisingly, *S. flexneri* wild-type strain M90T did not form actin clouds or tails in extracts that were able to support motility of *E. coli* expressing IcsA (data not shown).

Observation of fluorescently labeled actin incorporated into the comet tails of MBG263(pHS3199) moving in cytoplasmic extracts revealed a pronounced gradient of actin filament density through the tail, such that the filament density was highest closest to the bacterium and decreased exponentially toward the tail's distal tip (Fig. 4 *a* and *b*). Average filament half-life could be indirectly measured by calculating the rate of decrease of fluorescence intensity over time in a region of the tail fixed in space. The fluorescence decrease over time approximated exponential decay, regardless of the initial distance from the bacterium (Fig. 4*c*), suggesting that filaments in the tail were stochastically lost by depolymerization throughout the tail, as has previously been demonstrated for

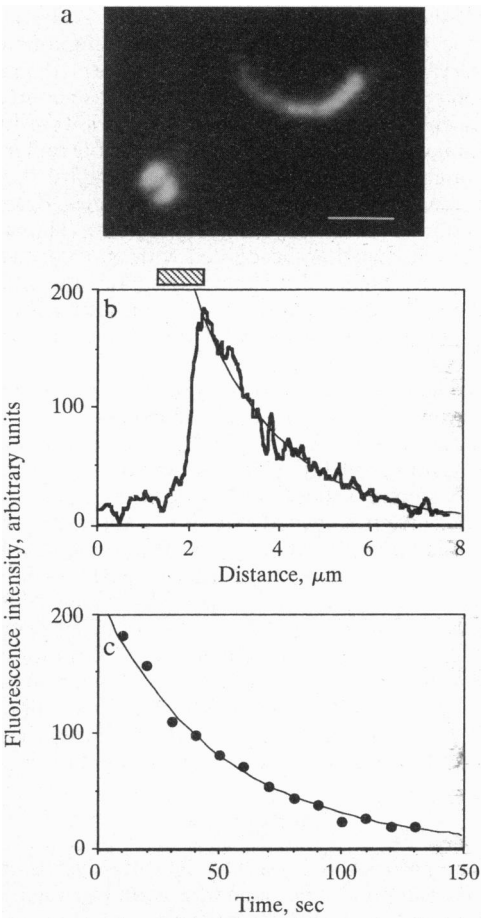

FIG. 4. Actin filament dynamics in MBG263(pHS3199) comet tails. (*a*) Rhodamine-labeled actin associated with formaldehyde-fixed MBG263(pHS3199) in *Xenopus* egg cytoplasmic extract. Actin filaments form either symmetrical clouds (lower left) or polarized comet tails (upper right). (Bar = 5 μm.) (*b*) Fluorescence intensity profile through a comet tail. The position of the bacterium is indicated by the hatched box. The thin line shows an exponential curve fitted to the intensity profile. (*c*) Actin filament turnover in a comet tail. Fluorescence intensity was measured every 10 sec in a 4 × 4-pixel box fixed in space. The decrease in fluorescence intensity represents depolymerization of rhodamine-labeled actin filaments. The curve drawn is the best exponential fit. The average actin filament half-life in this tail was 35 sec.

L. monocytogenes (17, 30). The average half-life of filaments in the tails was 38 ± 16 seconds (mean ± SD, $n = 12$).

Distribution of IcsA on Moving Bacteria. *S. flexneri* bacteria inside infected cells invariably move parallel to their long axis (unpublished observations), with the IcsA-rich pole trailing and forming the base of the actin tail (12). MBG263(pHS3199) and MBG264(pHS3199), which express IcsA circumferentially, move more erratically. The majority of bacteria in cytoplasmic extracts move in small circles or spirals, and the minority of bacteria associated with well-polarized actin tails are capable of moving either parallel to (Fig. 3*a*) or perpendicular to (Fig. 3*b*) their long axis, over a similar range of rates. Bacteria moving rapidly with actin tails were not observed to change directions.

We wished to determine whether these variations in actin-based motility of *E. coli* expressing IcsA might be due to variable redistribution of the protein in the bacterial outer membrane once the bacteria were mixed with cytoplasmic extract. An affinity-purified polyclonal antibody preparation was covalently labeled with fluorescein and mixed with live or formaldehyde-fixed bacteria prior to their addition to cytoplasmic extract. As expected, the covalently labeled antibodies were localized primarily to one pole on live or formaldehyde-fixed *S. flexneri* and were uniformly distributed on the surface of MBG263(pHS3199) and MBG264(pHS3199) (data not shown). When *E. coli* cells expressing IcsA were added to cytoplasmic extracts, the labeled antibody remained uniformly circumferentially distributed on the surface of the bacteria for at least 4 hr regardless of whether the individual bacteria were associated with uniform clouds of actin filaments or with polarized tails (Fig. 5). The result was similar with MBG263-(pHS3199) and MBG264(pHS3199), whether the bacteria were living, fixed with formaldehyde prior to addition of labeled antibodies, or fixed with formaldehyde after addition of labeled antibodies (data not shown). Thus, polarized localization of IcsA is not necessary for comet tail formation or for unidirectional motility, although it may contribute to the efficient establishment of the direction of movement.

DISCUSSION

The product of *icsA* (*virG*) is the only *S. flexneri* protein that has been directly implicated in intracytoplasmic actin-based motility (2, 8, 9). In this report, we have demonstrated that IcsA is not only necessary but also sufficient to direct the actin-based motility of a heterologous bacterium. Since MBG263 and MBG264 lacking *icsA* do not accumulate actin clouds or form actin tails in *Xenopus* egg extracts, it seems unlikely that *E. coli* factors are directly involved in the actin-based bacterial motility of our constructs. However, we cannot yet rule out the possibility that other bacterial products present in both *S. flexneri* and *E. coli* are acting in concert with IcsA to generate movement. Secretion of the cleaved form of IcsA (10, 11) is not necessary for tail formation. Furthermore, continuous synthesis of IcsA by a live bacterium is unnecessary for normal actin-based motility; this is consistent with our prior observation that inhibition of bacterial protein synthesis in *S. flexneri*-infected cells does not disrupt movement (12). We conclude that the critical role of IcsA in *S. flexneri* motility occurs only at the bacterial surface, while other processes necessary for tail formation, including actin filament crosslinking and regulation of filament turnover, are directed by host cell factors. It is likely, therefore, that the primary function of IcsA in bacterial actin-based motility is to generate nucleation sites for actin filament growth and/or to catalyze rapid elongation of actin filaments near the bacterial surface.

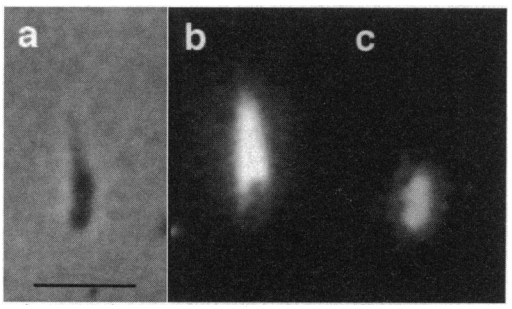

FIG. 5. Distribution of IcsA on MBG264(pHS3199) moving in a *Xenopus* egg extract. (*a*) Phase-contrast. (*b*) Rhodamine-actin. (*c*) Fluorescein-conjugated IcsA antiserum. IcsA density is uniform, and not detectably higher at the junction with the actin tail. (Bar = 5 μm.)

Surprisingly, unipolar localization of IcsA is not necessary for comet tail formation by *E. coli*. In *S. flexneri*, the unipolar localization of IcsA is established at bacterial division, so that only the old pole has a high concentration of the protein, and the new pole (created from the septation zone) is essentially devoid of IcsA (10). Inside infected cells, the bacteria that have just completed division invariably move with the new pole leading (unpublished observations), and consequently the actin-rich comet tail is always associated with the IcsA-rich pole. In the case of *E. coli* expressing IcsA circumferentially, the actin-based motility is not so uniformly directional, and bacteria may move perpendicular to their long axis or in small spirals, although bacteria moving rapidly in association with long comet tails can persist in their direction of movement for many minutes. It appears that, once initiated, movement is positively reinforced. This is probably because actin filaments in the comet tail are typically crosslinked to one another by host actin-filament-bundling proteins (5), and therefore new filaments growing near the existing comet tail will become incorporated into the force-generating structure whereas filaments growing elsewhere will not. We do not know whether the initial formation of the comet tails associated with MBG263(pHS3199) in cytoplasmic extracts is due to transient nonuniform IcsA distribution that we could not detect in our labeling experiments or due to random fluctuations in actin filament density around the surface of the bacteria. We conclude that the major function of unipolar IcsA expression is to aid in the efficient establishment of unidirectional movement, although IcsA polarity is not strictly necessary for polarized comet-tail formation and does not appear to contribute to the maintenance of the polar actin-rich structure once it has formed.

The actin-based motility of *L. monocytogenes*, a Gram-positive organism unrelated to the Gram-negative *S. flexneri*, has been more widely studied with respect to actin filament dynamics in the comet tails (13, 15, 17, 18, 30, 35). It has been shown that actin filaments in the comet tail are elongated only at the front of the tail, adjacent to the bacterial surface (18), and that, once formed, the filaments of the tail remain stationary in the host cell cytoplasm as the bacterium moves forward (17). Actin filament depolymerization occurs at a uniform rapid rate throughout the tail (17, 35). These factors result in a distribution of actin filament density that approximates exponential decay in both space and time (17).

It has been widely assumed that *L. monocytogenes* and *S. flexneri* move by similar mechanisms, although equivalent studies of actin filament dynamics had not been performed for moving intracellular *S. flexneri*. Here we have reported quantitative measurements of actin filament dynamics in tails generated in association with the *S. flexneri* IcsA protein. We have shown that the actin filament density distribution in tails associated with MBG263(pHS3199) moving in cytoplasmic extracts is indeed approximately exponential and that filament turnover in the tail occurs uniformly by stochastic filament loss. Further, the rate of actin filament turnover is similar to that in tails associated with *L. monocytogenes* (17, 30, 35). These observations are consistent with the model that actin

filament polymerization occurs only adjacent to the bacterial surface in the case of *S. flexneri* movement as well. Although we have not formally shown that actin filaments in the tails are stationary, we have observed that characteristic bends and nonuniformities in tails are fixed in space, as has been described for *L. monocytogenes* tails (15). Our observations provide strong support for the hypothesis that steady-state host cytoskeletal dynamics, rather than specific bacterially directed regulation, control the tail architecture and behavior and that this behavior is essentially identical between *S. flexneri* and *L. monocytogenes*. The role of the bacteria in this model is to trigger a process that is inherent to the steady-state regulation of actin filament dynamics in the infected host cells.

This process can apparently be triggered in several different ways. The *L. monocytogenes* ActA protein, which is both necessary (19, 20) and sufficient (37) for its actin-based motility, has no primary sequence homology with IcsA. Further, under some conditions polycationic beads sprinkled on the dorsal surface of motile cells can induce the formation of structures like comet tails by some signaling mechanism through the plasma membrane, and the beads then move rapidly on the cell surface, imitating moving intracellular bacteria (36). *S. flexneri, L. monocytogenes*, and the polycationic beads may cause accumulation of one or more cytoskeletal proteins that catalyze this form of actin-based motility, though they may each operate by initiating separate steps of a regulatory cascade. The next steps in understanding the mechanism of actin-based motility of *S. flexneri* will involve defining the functional domains of IcsA and identifying the host proteins that are essential for actin-based motility and/or associate directly with IcsA. The advances that we have reported in this work, including reconstitution of IcsA-based motility in a heterologous bacterium and its function in a cell-free cytoplasmic extract, will facilitate biochemical and molecular biological experiments to characterize the functional domains of IcsA and determine which host cell factors are involved in this interesting form of actin-based motility.

Note Added in Proof. C. Kocks, J. B. Marchand, E. Gouin, H. d'Hauteville, P. J. Sansonetti, M. F. Carlier, and P. Cossart (personal communication) have independently reported expression of IosA in *E. coli* and reconstitution of motility in cytoplasmic extracts.

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