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Host Cell Actin Assembly Is Necessary and Likely To Provide the Propulsive Force for Intracellular Movement of *Listeria monocytogenes*

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***Listeria monocytogenes* is able to escape from the phagolysosome and grow within the host cell cytoplasm. By 3 h after initiation of infection, actin filaments begin to concentrate at one end of the bacterium. Polarization of F-actin is associated with intracellular bacterial movement, long projections of actin filaments forming directly behind the moving bacteria. New actin monomers are added to the region of the projection in proximity to the bacterium. The rate of new actin filament growth correlates closely with the speed of bacterial migration. This actin structure is anchored within the cytoplasm, serving as a fixed platform for directional expansion of the actin filament network. The actin projection progressively lengthens as the bacterium migrates. Cytochalasin blocks both elongation of the projection and bacterial movement but does not result in complete depolymerization of the bacterially induced actin structure, residual actin and α-actinin persisting in proximity to one end of the bacterium. Bacteria initially migrate within the cortical cytoplasm but later move to the peripheral membrane, where they form filopodiumlike structures which pivot and undulate in the extracellular medium. In the filopodia, bacteria are occasionally seen to abruptly change direction, turn 180°, and move back into the medullary region of the host cell. All filopodium movement ceases once the bacterium containing the F-actin projection returns to the cortical cytoplasm. These results indicate that host cell actin polymerization is necessary for intracellular migration of listeriae and suggest that directional actin assembly may in fact generate the propulsive force for bacterial and filopodial movement.**

Cell biologists as well as microbiologists and infectious disease specialists have recently become intrigued by the unusual life style of the gram-positive bacterium *Listeria monocytogenes*. *L. monocytogenes* first gains entry by host cell phagocytosis. Within 30 min of being ingested by the host cell, the bacterium dissolves the phagolysosomal membrane and escapes into the cytoplasm, where it multiplies rapidly (3, 4, 10, 21). Investigations with NBD-phallacidin have shown that within approximately 30 min of escaping from the phagolysosome, this bacterium becomes surrounded by actin filaments (1, 7, 15). Electron micrographs reveal an extensive network of short actin filaments fully surrounding the bacterium (7, 21).

After growth in the cytoplasm for approximately 2.5 h, the location of the actin filaments changes, F-actin concentrating at one end of the bacterium (1, 7, 21), forming projections of up to 40 μm in length (1). Video microscopy of live infected cells has shown that formation of these actin filament projections is associated with bacterial movement, actin filaments assembling behind the bacteria as they migrate through the cytoplasm (1). The rates of actin assembly and bacterial migration are rapid, reaching speeds of up to 1.4 μm/s (1). The bacteria migrate to the peripheral cytoplasm into filopodiumlike structures (1, 7, 21) which may be phagocytosed by adjacent cells (7, 10, 21). This mechanism would allow listeriae to spread from cell to cell without coming in contact with the host's extracellular defenses.

Several lines of evidence suggest that the two actin morphologies represent unique stages in *Listeria* pathogenesis. Treatment with the antibiotic chloramphenicol has demonstrated that de novo bacterial protein synthesis is required for the initial induction of actin assembly but not for the subsequent remodeling of actin filaments to form polar projections (1, 17). In addition, a *Listeria* mutant (M117 Imt$^-$) has been discovered which is incapable of producing an actin projection and remains stuck in the first actin stage. This bacterium becomes trapped within its surrounding actin filament network and is unable to spread from cell to cell (6). The low virulence of this mutant emphasizes the importance of polar actin filament formation and bacterial intracellular movement in the pathogenesis of *Listeria* infection.

Experiments with cytochalasin D, an agent which blocks actin filament assembly, lend further support to these suppositions. When cytochalasin D is added to live infected cells, bacterially associated polar actin assembly and bacterial movement cease (1) and cell-to-cell spread by listeriae is prevented (7, 15, 21). Myosin II might provide the force required for bacterial intracellular movement. This protein, however, has not been found to be associated with the intracellular bacteria or with actin filament projections (1, 7). Actin polymerization, therefore, is the most likely candidate for providing the propulsive forces required to generate listerial movement.

Investigations of *Listeria*-induced actin polymerization should provide additional clues as to the exact role that actin assembly plays in cell motility. Does actin simply form the latticework which fills in empty spaces as membranes protrude to form lamellapodia and pseudopods, or can actin filament assembly actually generate the forces required for cellular movements? Using the PtK2 epithelial cell line microinjected with fluorescent probes for actin, we have

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attempted to answer this important question by examining in more detail the kinetics of the actin filament assembly associated with listerial movement. We have found that new actin monomers are added to filaments only at the bacterium-actin interface. The actin filament projection is not pulled along by the moving bacterium but remains stationary, as if tethered within the host cell cytoplasm, acting as a stable platform for further forward expansion of the actin filament network. The actin filament projections lengthen at the same rate as bacterial movement. In addition, we have found that bacteria with actin projections are able to induce the formation of undulating filopodia in the periphery of host cells. These findings strongly support our hypothesis that directional host cell actin filament assembly is necessary for *L. monocytogenes* intracellular migration and suggest that actin assembly may, in fact, generate the force for bacterial movement.

(These findings were previously presented in preliminary form [12].)

MATERIALS AND METHODS

Bacterial strains and growth conditions. Experiments were performed primarily with strain 10403S, a virulent strain (9) which belongs to serotype 1, is resistant to streptomycin at 1 mg/ml, and has a 50% lethal dose level for mice of 3×10^4 organisms. In one series of experiments, the nonmotile mutant strain DPL-9881, a Tn*916* mutant of 10403S (kindly provided by Daniel A. Portnoy, University of Pennsylvania, Philadelphia, Pa.), was used. This virulent strain fails to migrate on soft agar plates or to exhibit motility when observed under phase microscopy. Bacteria were grown in suspension in brain heart infusion (BHI) broth (Difco Laboratories Inc., Detroit, Mich.) at 30°C.

Tissue culture cells. The kangaroo rat kidney epithelial cell line PtK2 was obtained from the American Type Culture Collection and grown on glass coverslips (22 by 22 mm) in 35-mm culture dishes as described previously (11).

Infection. Listeriae were grown overnight in BHI broth at 30°C to a density of 2×10^9/ml, washed once with tissue culture medium, and then added to the PtK2 cells at a final concentration of 10^7/ml. PtK2 cells were washed twice in antibiotic-free tissue culture medium prior to infection. Samples were incubated for 1 h at 37°C, washed twice with antibiotic-free medium, and incubated for a further 30 min, at which time a final concentration of 5 μg of gentamicin per ml was added to the medium. This concentration of gentamicin killed all extracellular listeriae but did not harm intracellular listeriae (4, 9). This treatment was required to prevent any organisms which escaped from the cells from growing in the medium.

Fluorescent labeling of actin, α-actinin, tropomyosin, and myosin I. G-actin prepared from rabbit skeletal muscle was gel filtered, α-actinin was prepared from chicken gizzard smooth muscle as described previously, and tropomyosin was purified from skeletal muscle (11, 13). All three proteins were then conjugated with lissamine rhodamine sulfonyl chloride (13). These fluorescently labeled proteins were microinjected into individual adherent cells by a pressure injection system as previously described by Sanger et al. (11). Coverslips were then removed from the culture dish and mounted on a glass slide with a drop of medium containing 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) and sealed with Vaseline for microscopic observation of live cells. Cells were maintained at 37°C throughout each experiment. In some experiments, cells were fixed with 3.7% formaldehyde in phosphate-buffered saline for a minimum of 10 min at 25°C. To stain F-actin, fixed cells were permeabilized with 0.1% Nonidet P-40 and then incubated in 1.7×10^{-7} M NBD-phallacidin or bodipy-phallacidin (Molecular Probes, Eugene, Ore.) for 10 min at 37°C.

Cytochalasin D treatment. Coverslips containing PtK2 cells microinjected with rhodamine-α-actinin were mounted with a partial seal of Vaseline to allow medium to be perfused through the cell preparation. Approximately 5 volumes of medium containing cytochalasin D (0.5 μg/ml) were perfused through the coverslip chamber, and a similar volume of control medium was later perfused through to wash out the cytochalasin.

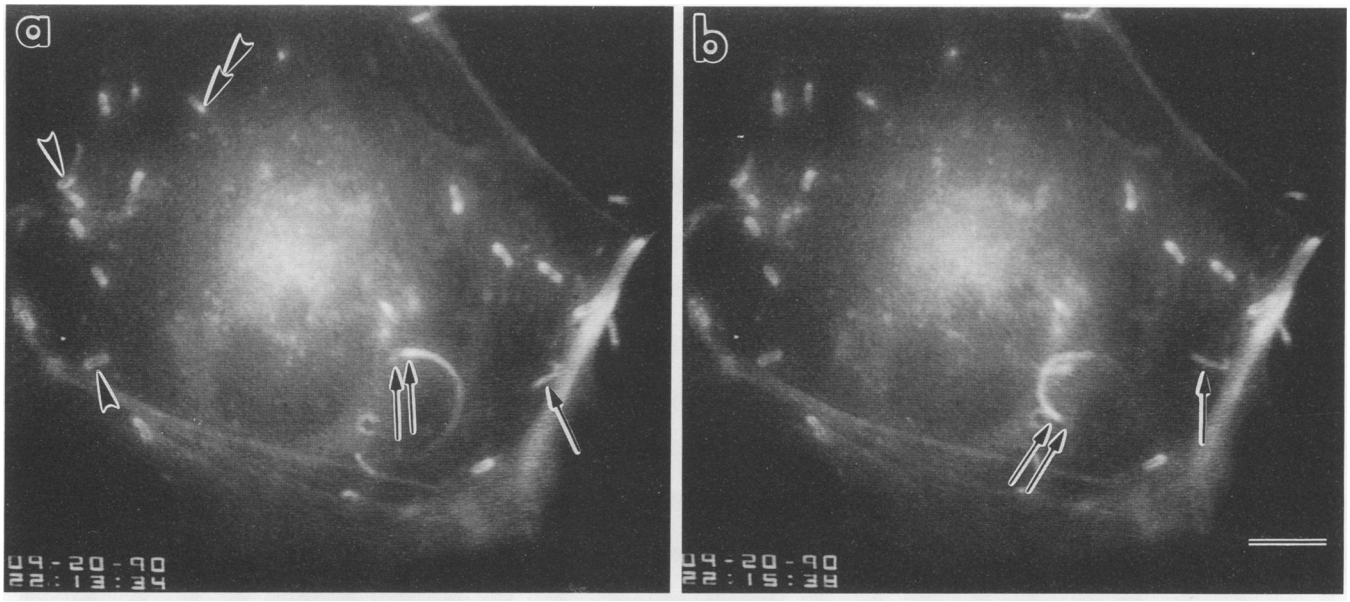
Microscopy and image processing. Living cells were observed with epifluorescence on an Olympus Vanox photomicroscope with a SIT camera (Dage-MIT, Michigan City, Ind.) coupled to an image processing system (Image-I/AT, Media, Pa.). Images were subjected to frame summation and then photographed with a 35-mm camera from the video monitor. To determine the half-life of actin projections, the relative fluorescence intensity was measured at different locations on the tail with a fixed circular template (brightness function; Image-I/AT) at various times. The fluorescence intensity of an identical area adjacent to the projection within the cell was measured and subtracted from these values.

RESULTS

Time-lapse video microscopy of intracellular listeriae. Figure 1a and b shows the two actin morphologies associated with listeriae grown in living PtK2 cells. A long actin filament projection extends from one end of the moving bacterium. The projection extends from the end of the bacterium opposite the direction of movement. The bacteria generally move at speeds of 0.04 to 0.12 μm/s (see Fig. 8B), with some bacteria moving as fast as 0.4 μm/s in PtK2 cells. We have found that the mean rate of bacterial movement varies markedly from day to day, mean rates varying from 0.05 to 0.25 μm/s on different days. Motile bacteria are always associated with projections. Such polar structures are never observed in association with stationary bacteria. The half-life of the intensity of fluorescent actin in the actin projection was 78 ± 2.6 s (standard deviation [SD], $n = 3$). Figure 1c illustrates the decay rate at three locations in an actin projection. Measurements at all locations in the tail demonstrate nearly identical half-lives. Under the conditions of our experiments, photobleaching represented only 3 to 8% of the decay over the period of our experiments.

Bacteria that are stationary are fully surrounded by actin. In some instances, the actin is brighter at one end of the bacterium (Fig. 1a). The increase in F-actin concentration at one end of the bacterium is seen before the onset of movement. In pairs of bacteria, presumably recently divided, one of the two cells occasionally is seen to form a projection and move away from its stationary partner (Fig. 2). In other cases, both daughter cells assemble actin processes at their distal ends and move apart (data not shown).

Figure 1a also illustrates the two types of listerial movement observed in living PtK2 cells. First, there are the medullary and cortical movements associated with the projections of fluorescent contractile proteins. Second, in some instances, the listeriae are propelled from the cortical region, forming filopodiumlike structures which pivot at their base (Fig. 1a and b and 2a to e). The majority of bacteria continue



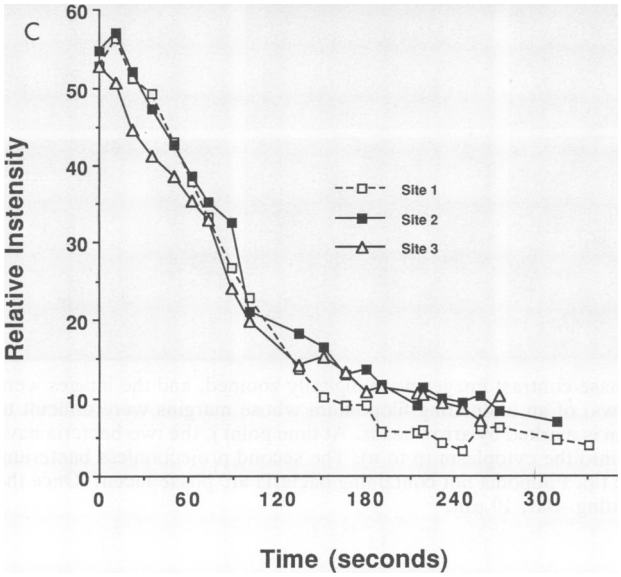


FIG. 1. (a and b) Listeriae photographed at two time points, 2 min apart, in the cytoplasm of a PtK2 cell injected with rhodamine-labeled monomeric actin. Four hours after initiation of infection, the live cell was injected with fluorescent actin monomers. The actin assembles around stationary listeriae (arrowheads). In some stationary bacteria, the fluorescence of the actin probe is brighter at one end of the bacterium (double arrowheads). This polar staining is unaffected by changing the plane of focus. The fluorescent monomers also incorporate into the projection behind a motile bacterium (double arrows). This bacterium is moving in an arc within the cytoplasm. The single arrows point to a bacterium and its actin filaments inside a small filopodium that projects out of the cell and pivots near the edge of the cell. Bar, 10 μm. (c) Graph of fluorescence intensity at three locations in an actin projection versus time. The fluorescence intensity of three sites in the actin projection shown in panel a (double arrows) was measured at different times and compared with background intensity. The half-lives of the fluorescence intensity were 79 s at site 1, 80 s at site 2, and 75 s at site 3.

to move peripherally, forming progressively longer membrane projections that move sinuously in the extracellular medium. Occasionally, however, a bacterium inside a filopodium turns 180°C (Fig. 2e to j) and moves back into the medullary region of the host cell (Fig. 2j to o). Once this motile bacterium leaves the filopodium, all filopodial movement ceases (Fig. 2o to u). The filopodium in Fig. 2 contains two bacteria as a result of bacterial division within the filopodium (Fig. 2a to e). One of the bacteria moves back into the cell, while the other remains stationary and fails to form a phase-dense actin projection during the period of observation. Similar sudden changes in the direction of bacterial movement have also been seen when a single bacterium is present in the filopodium.

Figure 3 shows video time-lapse microscopy of a living PtK2 cell infected with *L. monocytogenes* for 4 h and then microinjected with α-actinin. Because of the flattened shape of adherent PtK2 cells, the F-actin projections remain within a single focal plain throughout the time course of the experiment. As the bacterium moves rapidly within the host cell, the actin projection appears to be fixed or anchored in the host cell cytoplasm. Bacterial movement is clearly seen to be associated with progressive elongation of these projections, suggesting that actin polymerization may be pushing the bacteria through the cytoplasm.

As the actin processes elongate, the fluorescence intensity of the α-actinin remains brightest in the region of the projection closest to the bacterium. This fluorescence gradually dissipates in the more distal regions of the actin processes. The increased fluorescence intensity proximal to the bacteria is also seen with phalloidin staining of the projections (Fig. 4) and following microinjection of fluorescent actin. *Listeria*-induced actin assembly does not visibly affect stress fibers during the first 4 h of infection (Fig. 4). In fact, on occasion, host cell stress fibers appear to alter the migration path of the intracellular bacterium (Fig. 5).

Video microscopy of a mutant immotile in agar. The finding that the actin projections remain fixed in the cytoplasm and progressively lengthen suggests that host cell actin rather than the bacterium's own motile machinery is providing the force for intracellular bacterial movement. Comparisons of the wild-type bacteria's intracellular migration rate with that of a mutant, strain DPL-9881, which fails to migrate in agar

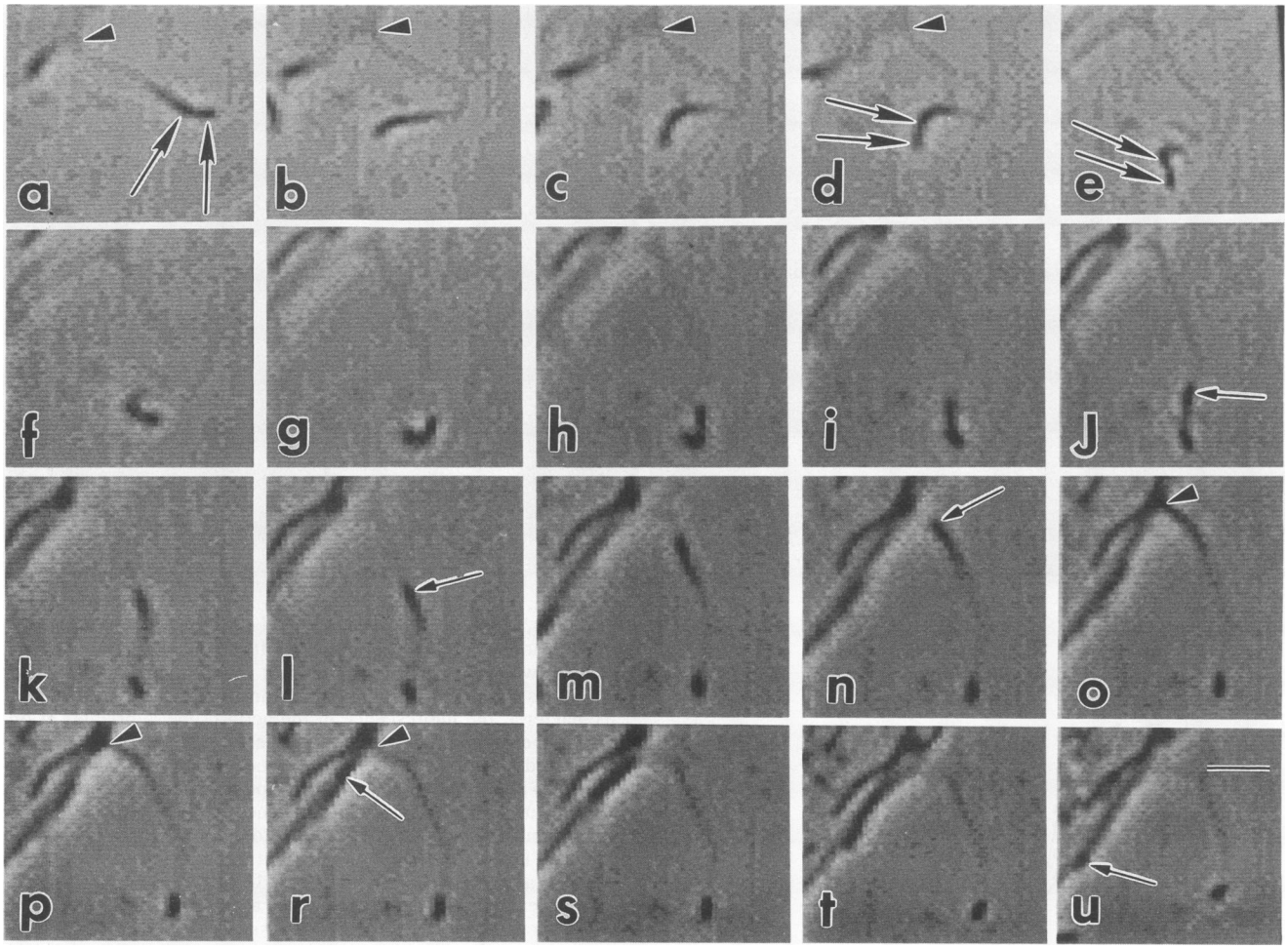


FIG. 2. Time-lapse sequence of bacteria moving inside a filopodium. Phase-contrast images were digitally zoomed, and the images were enhanced to emphasize the phase density of two bacteria in the tip (arrows) of an undulating filopodium whose margins were difficult to resolve clearly. The junction of the base of the filopodium and the cytoplasm is marked by arrowheads. At time point j, the two bacteria have separated, and one moves down the filopodium (j to o, small arrows) and into the cytoplasm (p to u). The second projectionless bacterium remains in the tip of the filopodium, causing an area of phase density at the tip. Filopodia not containing bacteria are phase lucent. Once the first bacterium begins moving toward the cell, the filopodium stops undulating. Bar, 10 μm.

or tumble in liquid medium, also support this conclusion (Fig. 5). The rate (0.1 to 0.2 μm/s) and character of intracellular movement of this mutant were the same as those of the wild type. The time-lapse sequence in Fig. 5 further reinforces our conclusion that the actin filament projections are fixed in the cytoplasm rather than dragged by the bacterium. The arrowhead points to a bend in the actin process which persists despite continued migration of the bacterium and lengthening of the projection. If the bacterium were producing a pulling tension, such bends might be expected to straighten.

Video microscopy of intracellular listeriae exposed to cytochalasin D. When a moving bacterium with an actin projection (Fig. 6a to d) is exposed to cytochalasin D (0.5 μg/ml), forward motion stops within 2 min of perfusion of the drug. Continued exposure to this agent results in the gradual disappearance of the actin process (Fig. 6e to h) until, by 20 min, filamentous actin is seen only in proximity to the bacterium (Fig. 6h). This cytochalasin D-resistant cluster of F-actin is seen in cells treated with cytochalasin for up to 4 h (1). Upon removal of cytochalasin D, new filament growth is seen directly behind the bacterium as it again begins to move (Fig. 6i to l). Actin assembly begins in the region between the residual F-actin and the bacterium. As the actin filament process lengthens, the bacterium moves away from the residual F-actin. The cytochalasin D-resistant F-actin cluster is not dragged along with the moving bacterium but instead remains fixed within the cytoplasm as the actin filament projection progressively lengthens (Fig. 6i to l).

Sites of contractile protein incorporation in bacterial projections. To determine more accurately the site of actin monomer incorporation into the projections, infected PtK2 cells were microinjected with rhodamine-labeled monomeric actin and fixed at different times. The slides were also stained with bodipy-phalloidin to map out the total length of F-actin in the bacterial projections. As shown in Fig. 7, when cells are fixed within 30 s, rhodamine-labeled actin is found only in the most proximal 2 to 4 μm of the projections directly adjacent to the motile bacteria (Fig. 7a to d). At the

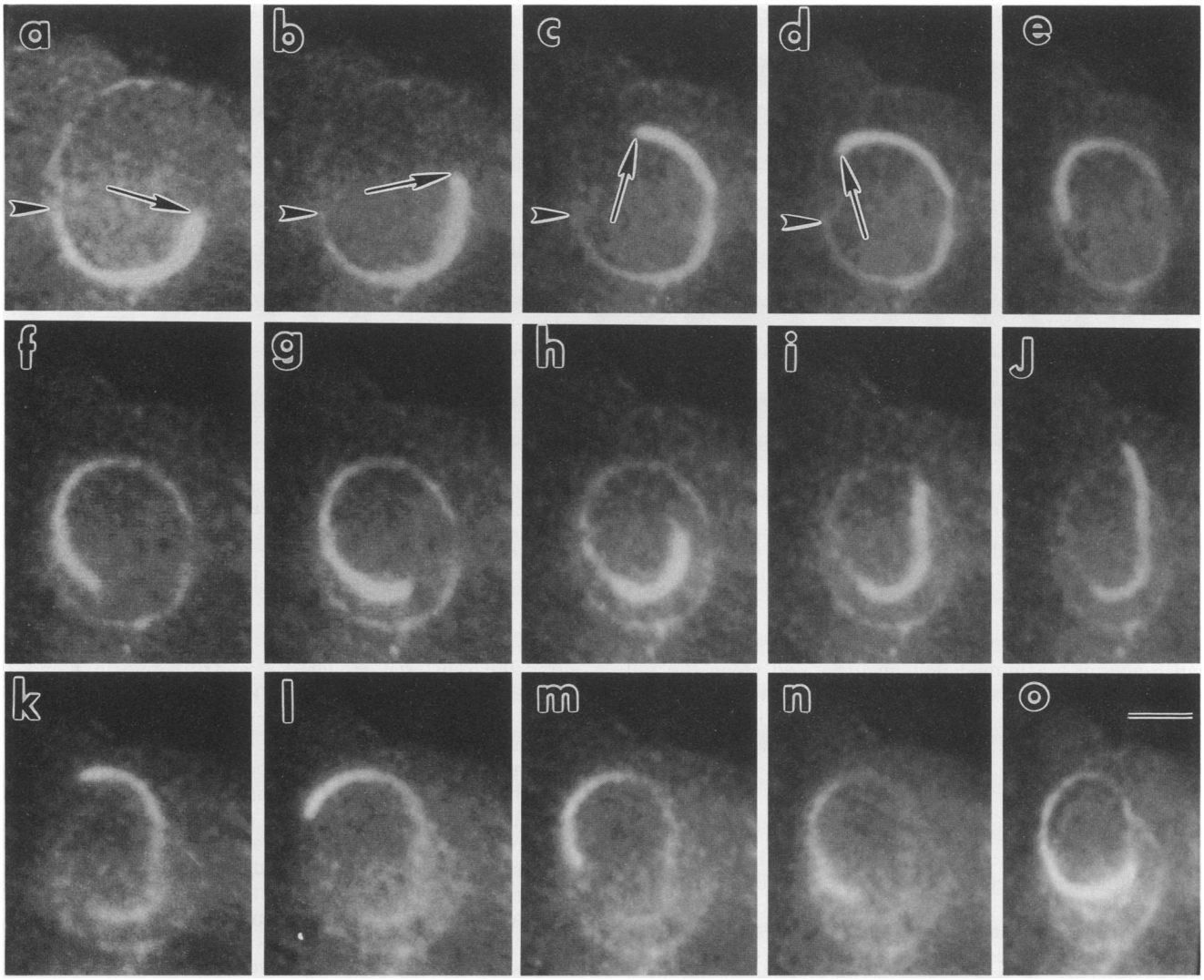


FIG. 3. Time-lapse fluorescent images of a single intracellular bacterium migrating through the cytoplasm of a PtK2 cell. The cell was infected for 4 h and then microinjected with fluorescently labeled α-actinin as described previously (1). As shown in Fig. 4, the region of the projection closest to the bacterium has the brightest fluorescence. The actin process does not move with the bacterium. Note the fixed position of the distal region of the projection in frames a to d (arrowheads). The actin process lengthens as the bacterium moves forward. The fluorescence of the distal end of the projection decreases over time, suggesting disassembly of the tail. The disassembly of the distal end of the tail proceeds more slowly than the rate of assembly at the proximal end (see Fig. 1b). Time interval between frames, approximately 25 s. Bar, 10 μm.

same time, nonmotile bacteria are fully surrounded by the microinjected actin. In contrast to single nonmotile bacteria, dividing bacteria do not incorporate actin in a zone corresponding to the region of fission (Fig. 7a). After 2 to 3 min, the rhodamine-actin is incorporated into the most proximal 5 to 8 μm of the projection (Fig. 7e and f). When the microinjected actin is allowed to incorporate for 5 min before fixation, the fluorescent probe is present along the entire length of the projection (data not shown).

The total length of each actin filament projection was compared with the length of incorporated rhodamine-actin monomers. The two lengths demonstrated a positive linear correlation (Fig. 8A). Because the rate of individual bacterial movement varied markedly during the time of observation (Fig. 8B), comparisons of the rate of bacterial movement prior to microinjection of monomers demonstrated only a rough correlation with the rate of rhodamine-actin incorporation (data not shown). The speeds of seven bacteria within a single cell were measured several times prior to microinjection of actin monomers and compared with the rates of actin monomer incorporation (Formalin-fixed 33 s after microinjection). Within this cell, the mean rate of bacterial movement (0.048 ± 0.023 μm/s [SD], n = 24; range, 0.003 to 0.080 μm/s) was nearly identical to the mean rate of actin monomer assembly (0.051 ± 0.014 μm/s [SD], n = 7; range, 0.036 to 0.080 μm/s).

Microinjection of fluorescently labeled tropomyosin and α-actinin followed by fixation at 30 s, 2 min, and 5 min results in a very different pattern of staining than obtained with monomeric actin. Tropomyosin (Fig. 9, 30-s time point)

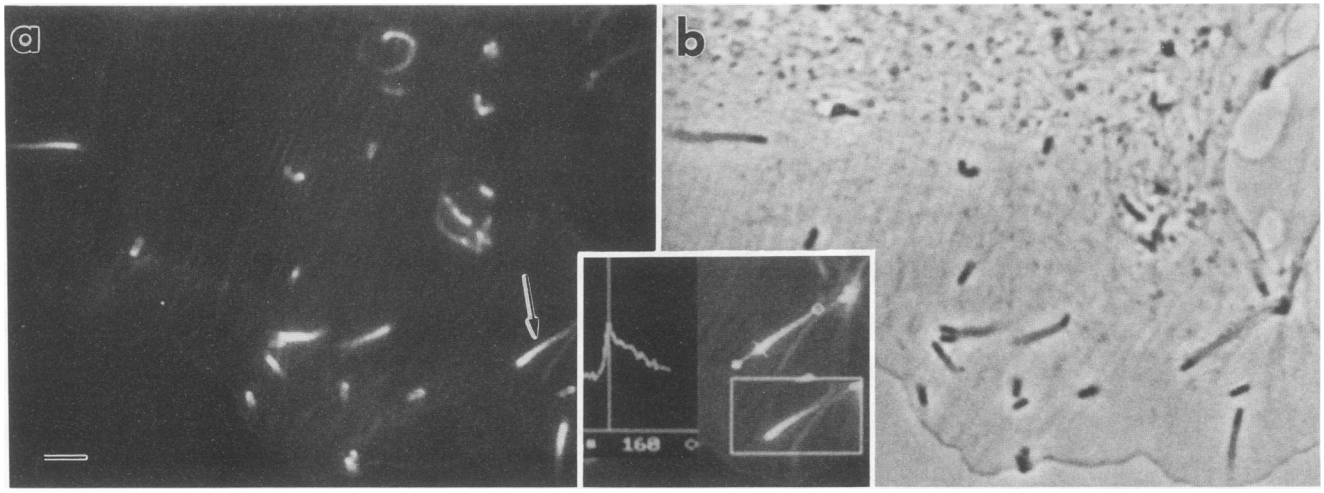


FIG. 4. Fluorescent (a) and phase-contrast (b) images of a *Listeria*-infected PtK2 cell fixed and stained with bodipy-phalloidin. The bacteria and some F-actin projections can be seen with phase-contrast microscopy. The fluorescence intensity of the F-actin in the actin projections is highest about 1 μm behind the motile bacteria. The inset illustrates the relative fluorescence intensity along one actin process (arrow in a). The intensity shown in the graph was measured along the axis of the projection, with the vertical intercept marking the peak of fluorescence, corresponding to the small hash mark on the scan line. The actin projection is shown without the scan line inside the rectangle. It is shown with the superimposed scan line above the rectangle. The pictures were taken 4 h after initiation of the infection. Note the normal appearance of the stress fibers. Bar, 10 μm.

and α-actinin are immediately incorporated along the full length of the projections. Unlike monomeric actin and α-actinin, tropomyosin is not incorporated into the short actin filaments surrounding the stationary bacteria (Fig. 9, arrowheads).

DISCUSSION

These studies of actin polymerization in PtK2 cells infected with *L. monocytogenes* rekindle the debate over the exact role that actin assembly plays in force generation. Studies of the acrosomal process of Thyone sperm suggest that actin assembly could generate the forces required for cell protrusions. In that system, actin filament assembly is rapid, with filaments elongating at 10 μm/s from their barbed ends, resulting in the formation of an acrosomal process which protrudes from the sperm and penetrates the Thyone egg (18, 20). However, it is also possible that osmotic changes in activated sperm induce membrane extensions and that these extensions are then stabilized by elongation of actin filaments (8, 19). In contrast to the Thyone sperm model, the *Listeria*-induced actin filaments form at many sites within the cytoplasm. In addition, migrating listeriae can rapidly reverse direction in host cell filopodia. Although we cannot totally exclude an osmotic mechanism, these observations make such a mechanism for bacterial migration and filopodium formation less likely.

L. monocytogenes provides another model for examining the role of actin assembly in force generation. Based on our previous preliminary studies (1), we hypothesized that actin filament assembly might be producing the propulsive force for bacterial movement. In order for this to be the case, (i) the bacterial F-actin projection must remain anchored in the cytoplasm and progressively lengthen as the bacterium moves forward, (ii) incorporation of actin monomers would be expected to take place at the bacterium-actin projection interface, (iii) the rate of actin monomer assembly into filaments should equal the rate of bacterial migration, (iv) inhibition of actin assembly would be expected to block intracellular listerial movement, and (v) myosin I and II contractile proteins, which are known to generate movements of 1 μm/s in some systems, should not be found concentrated near the bacteria.

With the exception of requirement v, our findings support all of these suppositions. The apparent absence of myosin II from the projections (1, 7) rules out force production by a conventional actin-myosin interaction. We cannot totally exclude a role for myosin I, but it is difficult to envision myosin I interacting with the bacteria and filaments of actin that project from one bacterial end.

Using time-lapse video microscopy, we have now clearly documented that bacterial movement is associated with progressive lengthening of the actin filament projection. Our studies with cytochalasin D demonstrate that the projection remains fixed in the cytoplasm during movement. Using NBD-phallacidin as well as fluorescently labeled α-actinin, we have found that *Listeria*-infected cells treated with cytochalasin D have a persistent F-actin cluster at one end, which can serve as a reference point. When cytochalasin D is removed from infected cells and the bacteria begin to move, these F-actin clusters remain fixed in the cytoplasm, actin filaments assembling between the clusters and the bacteria. A gap does not appear between the clusters and the fluorescent projections as would be expected if these actin structures were moving with the bacteria. The F-actin projections, on the contrary, remain anchored in the cytoplasm as they lengthen. Using a photoactivated fluorescent actin probe, Theriot et al. have also recently documented that the projections remain stationary once formed (16).

If the actin filaments are anchored in the cytoplasm, then in order for the projections to push the bacteria forward, actin filament elongation would have to take place at the end of the projections adjacent to the bacteria. The results obtained after microinjection of live infected cells with rhodamine-labeled actin monomers document this expectation. Actin monomers are incorporated only at the proximal end of the projection near the moving bacteria.

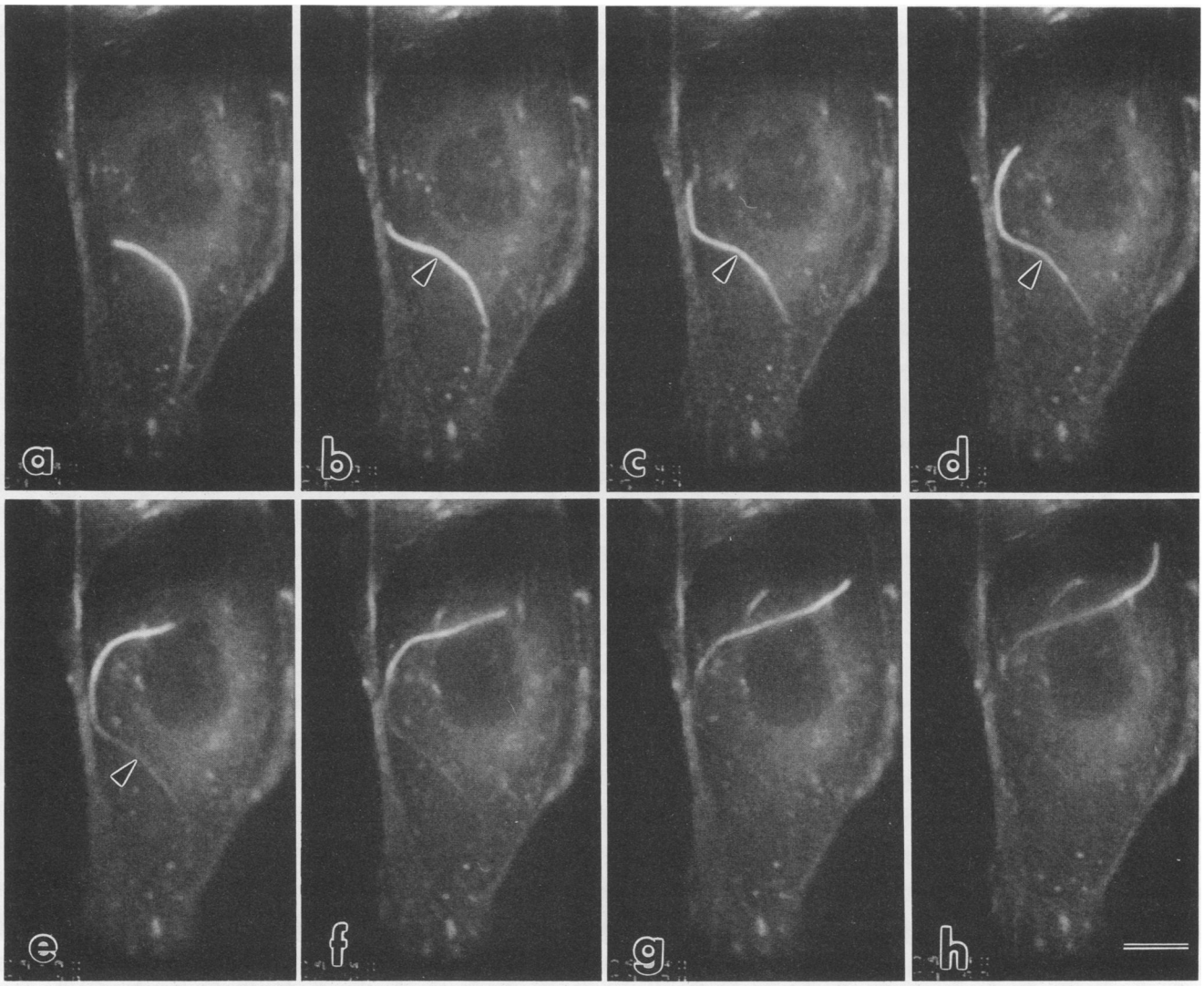


FIG. 5. Time-lapse sequence of a PtK2 cell that was infected with a mutant *Listeria* strain which fails to migrate through agar or tumble in growth medium. Cells were previously microinjected with fluorescently labeled α-actinin. The rate and character of bacterial migration and actin filament projection formation were identical to those found for the wild type. The bacterium can be seen to move in an arc in the cytoplasm (a) until it abuts a stress fiber at the cell periphery (b). It then moves parallel to the stress fiber (c) and curves back toward the middle of the cell (d to h). Note how the shape of the "older" part of the actin projection is maintained as new protein is incorporated at the forward region. Arrowheads point to a bend in the projection that persists (b to e) until disassembly occurs in that region. Time interval between frames, approximately 30 s. The half-life of the fluorescence intensity (70 to 80 s) was the same as in the wild-type infection. Bar, 10 μm.

One possible explanation for this finding is that assembly of new actin filaments is stimulated by a nucleation activity. The bacterium alone is incapable of directly stimulating purified actin assembly (15, 17), and therefore it is unlikely that listeriae are continually synthesizing and releasing an actin-nucleating activity. The ability of this organism to move at undiminished speeds for hours in the presence of chloramphenicol, a drug which inhibits listerial protein synthesis (12a), further supports this conclusion. Polycationic beads placed on the outer surface of growth cone cells induce similar intracellular actin projections as well as rates of bead movement similar to those found for listeriae, proving that active protein synthesis is unnecessary for this process (2, 14). It is likely that once listeriae escape into the cytoplasm of host cells, they express a surface component. Recently, an outer cell wall protein, ActA, has been identified which is required for *Listeria*-induced actin assembly (5). Once expressed on the bacterial surface, this component could sequentially activate host cell nucleating protein molecules as the bacterium moves through the cytoplasm. Each activated host cell nucleating molecule would be expected to stimulate the production of a single actin filament. The ends of these newly formed filaments may become capped after the filaments reach a certain length. These filaments could then be cross-linked by various actin-binding proteins (e.g., α-actinin).

Actin monomer incorporation at a single region of the actin projection might also be explained if the projections consisted of a parallel array of actin filaments which extended the full length of the projection and whose barbed

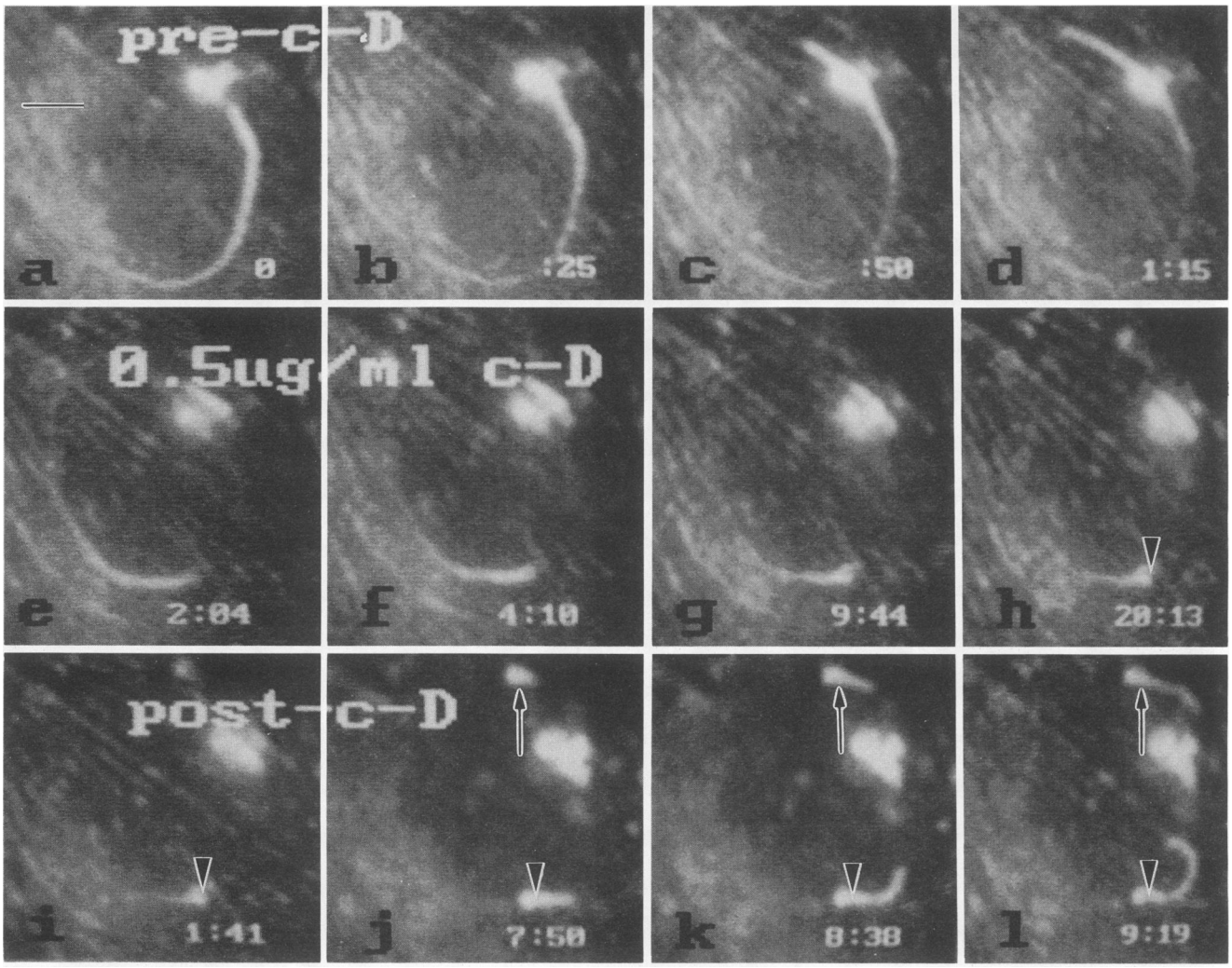


FIG. 6. Time-lapse fluorescent images of a single intracellular bacterium before (a to d), during (e to h), and after (i to l) treatment with cytochalasin D (c-D). Cells were microinjected with labeled α-actinin and infected for 4 h. The time of addition of cytochalasin D (0.5 μg/ml, final concentration) was 2:04 min (e), and exposure ended at 20:15 min (h). Note the gradual dissolution of the fluorescent projection with continued exposure to cytochalasin D (e to h). On video microscopy, the stress fibers were seen to fragment soon after addition of cytochalasin D, followed by more gradual depolymerization. After 20 min, a bright fluorescent aggregate (arrowhead) was seen at one end of the bacterium (h). At this time, cytochalasin D was washed out of the cells; within approximately 7 min, actin filament assembly and bacterial movement resumed. The numbers on images i to l represent the time (in minutes) after cytochalasin D removal. The fluorescent aggregate (arrowhead) remained fixed within the cytoplasm, new actin filaments forming between this aggregate and the bacterium. The F-actin process lengthened progressively as the bacterium moved forward. A second bacterium illustrating the same type of F-actin projection formation can be seen at the top of images i to l (arrows). An additional fluorescent cluster is seen in the upper right-hand region of the cell both before and after cytochalasin D treatment. This cluster represents a residual collection of α-actin at the microinjection site. Bar, 10 μm.

ends were all in close proximity to the bacterium. The published ultrastructural data (21), however, do not support this possibility. The projection is composed of short actin filaments, about 0.2 μm in length, with many of their barbed ends pointing towards the bacterium (21).

The rate of new actin monomer incorporation into the projections in our pulse experiments approximates the rate of bacterial movement. Because fluorescent actin monomers microinjected into cells produce a diffuse fluorescence blush, it is not possible to continuously measure actin monomer incorporation in live cells. Host cells must be fixed and permeabilized to remove unincorporated monomers before the actin projections can be visualized. Variations in the speed of each organism as it encounters stress fibers and other structural impediments (1) have made it impossible to directly correlate the rate of movement with the rate of actin monomer incorporation in an individual bacterium. The mean rate of bacterial movement within a single cell before injection of actin monomer, however, was found to be nearly identical to the mean rate of actin monomer growth. In addition, we were able to estimate individual bacterial speeds by measuring the lengths of phalloidin-stained actin projections. Since the half-life of fluorescent actin remains constant, the total length of each actin filament projection would be expected to correlate directly with the rate of bacterial movement (the faster the bacterial movement, the longer the expected actin projection). Theriot et al. have documented this expectation, demonstrating a linear corre-

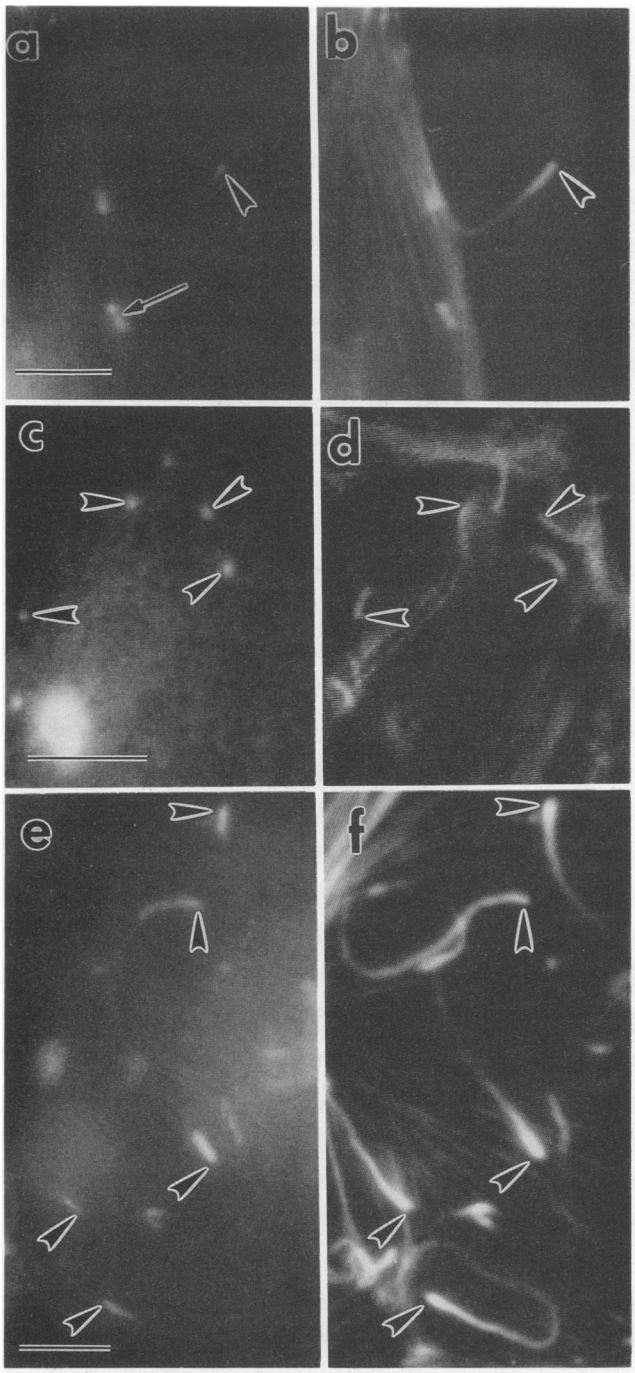


FIG. 7. Short-term incorporation of actin in cells injected with rhodamine-labeled monomeric actin. PtK2 cells were injected with actin, fixed, and then counterstained with bodipy-phalloidin. Cells fixed 30 s after injection show rhodamine-actin incorporation (a) extending approximately 2 μm along the actin tail of a bacterium located in a filopodium extension (arrowhead). The phalloidin-stained image (b) indicates the full extent of F-actin behind the bacterium and in the cell. The injected actin also polymerized around nonmotile bacteria (arrow). Images of a cell fixed at 30 s (c and d) demonstrate bacteria (arrowheads) within the cytoplasm that also have incorporated actin at the bacterium-actin interface (rhodamine-actin in c, bodipy-phalloidin in d). A cell (e and f) fixed 1 min after microinjection shows actin incorporation extending 3 to 8 μm behind the bacteria along projections that are up to 32 μm in length. Bar, 10 μm.

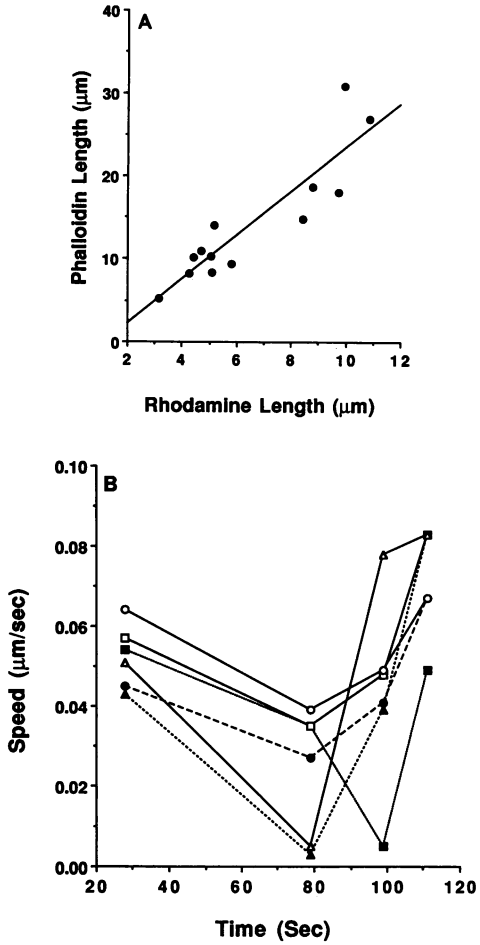


FIG. 8. (A) Length of incorporation of rhodamine-actin versus total length of bacterial actin projections (bodipy-phalloidin stain). The cell was fixed 90 s after microinjection of rhodamine-actin monomers and counterstained with bodipy-phalloidin. The lengths of rhodamine- and phalloidin-stained projections were determined for 13 bacteria in a single infected PtK2 cell. A linear relationship between the length of the rhodamine label and total actin projection length (phalloidin staining) was observed. The rhodamine elongation rates for the bacteria in this cell varied from 0.035 to 0.12 μm/s (mean, 0.07 μm/s). (B) Rate of movement of six bacteria within an infected PtK2 cell. Conditions were identical to those in panel A, but the live cells were observed under phase microscopy to determine the rates of bacterial movement. For each bacterium, the speed of migration varied markedly (e.g., for bacterium 6 [△] it was 0.005 to 0.083 μm/s). This experiment was performed on a different day than that shown in panel A. The mean rate of bacterial movement varied considerably from day to day (see Results). Each symbol represents a separate bacterium.

lation between bacterial speed and actin projection length (16). The length of a bodipy-phalloidin-stained projection can therefore be used to infer the rate of bacterial movement. Comparisons of the full length of each actin projection with the length of rhodamine-actin incorporation has yielded a positive linear correlation for each bacterium studied. These observations provide strong evidence that the rate of actin monomer incorporation is the same as the rate of bacterial movement.

The rapid incorporation of α-actinin and tropomyosin throughout the length of the actin filament projections suggests that uptake of actin monomers at the bacterium-actin

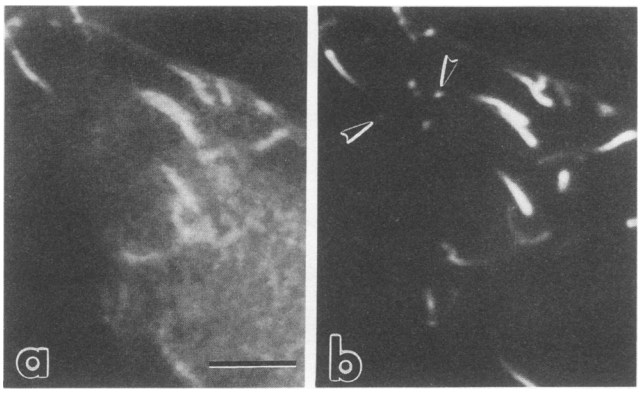


FIG. 9. Incorporation of fluorescently labeled tropomyosin. Cells were fixed 30 s after injection of rhodamine-labeled tropomyosin (a) and counterstained with bodipy-phalloidin (b). Tropomyosin was found along the full length of all actin tails but was not found around a group of immotile bacteria (arrowheads, b). Bar, 10 μm.

filament interface is not a generalized phenomenon seen with all contractile proteins but is an actin-specific reaction. These findings may be applicable to other cytoplasmic actin bundles, such as stress fibers, cleavage furrows, and myofibrils. Actin similarly may incorporate only at the barbed ends embedded in attachment plaques, dense bodies, and Z-bands, but this has not yet been shown.

Incorporation of tropomyosin throughout the actin filaments may make them more rigid. Binding of α-actinin to the sides of actin filaments may serve to cross-link these filaments and may explain the persistence of the tightly associated actin filament projections. Furthermore, the presence of these and other actin-binding proteins may prevent the exchange of cytoplasmic actin with actin bound in the projections. The assembly of a rigid structure would be expected to provide a stable platform which could allow directional actin network expansion to push the bacterium forward.

The inhibition of actin filament projection formation and bacterial movement by cytochalasin D further emphasizes the importance of actin filament assembly for intracellular listerial motility. In addition to preventing new actin from being incorporated into these polar actin filament structures, cytochalasin treatment led to the disassembly of the projections. Complete disassembly did not occur, however; a remnant of material that stained with NBD-phallacidin (1) and also incorporated α-actinin (present study) persisted adjacent to the bacteria, suggesting the presence of actin filaments. This area of persistent staining is in the same location as a fine fibrillar material found in electron micrographs of listeriae grown within cytochalasin D-treated cells (17). The material detected in the electron micrographs appears identical to the actin-containing matrix which has been observed in a number of uninfected cells treated with cytochalasins (22).

Finally, the formation of motile filopodia by listeriae further supports the role of actin assembly in cell motility. As long as a bacterium containing F-actin at one end is present in the filopodium, active filopodial movement is observed; however, if the actin projection-containing bacterium leaves the filopodium, all movement ceases.

All evidence to date suggests that listeriae conscript the host's own contractile system to move within the host cell cytoplasm. Previous investigators have called the polar actin structures associated with listerial movement "tails" (1, 16, 21). Our findings, however, indicate that these actin structures are not true tails but rather fixed platforms against which newly growing actin filaments can push to provide the directional forces required for bacterial movement (Fig. 10). The present studies indicate that host cell actin polymerization is necessary for listerial intracellular migration and suggest that actin assembly may, in fact, generate the propulsive force for movement as well as the formation and the undulations of filopodiumlike membrane protrusions in nonmuscle host cells.

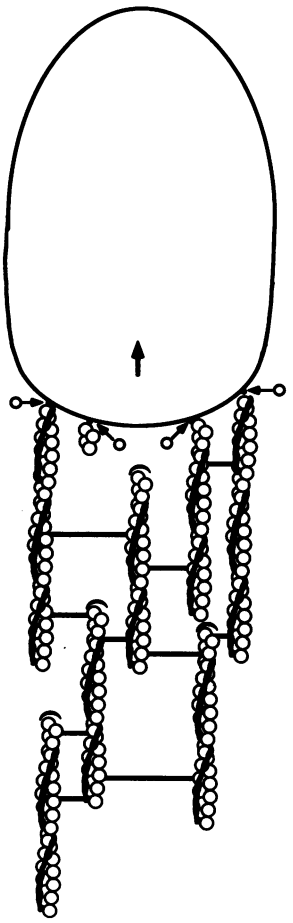


FIG. 10. Schematic depiction of listeriae producing a filamentous actin platform. The bacterial surface stimulates a host cell actin nucleating activity, which causes actin monomers (open circles) to assemble into filaments at the bacterium-actin projection interface. The ends of actin filaments are subsequently blocked by barbed-end capping proteins (half circles). These actin structures are anchored into the cytoplasm, allowing them to form a stable platform. α-Actinin links the actin filaments to each other (horizontal straight lines), while tropomyosin binds along the filament sides (twisting dark vertical lines). As the bacterium induces new actin filaments to form, the network is prevented from expanding in the direction of the platform. Expansile forces therefore are directed toward the bacterium, pushing it forward.

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