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Directional Actin Polymerization Associated with Spotted Fever Group Rickettsia Infection of Vero Cells

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Members of the spotted fever group (SFG) of rickettsiae spread rapidly from cell to cell by an unknown mechanism(s). Staining of *Rickettsia rickettsii*-infected Vero cells with rhodamine phalloidin demonstrated unique actin filaments associated with one pole of intracellular rickettsiae. F-actin tails greater than 70 μm in length were seen extending from rickettsiae. Treatment of infected cells with chloramphenicol eliminated rickettsia-associated F-actin tails, suggesting that de novo protein synthesis of one or more rickettsial proteins is required for tail formation. Rickettsiae were coated with F-actin as early as 15 min postinfection, and tail formation was detected by 30 min. A survey of virulent and avirulent species within the SFG rickettsiae demonstrated that all formed actin tails. Typhus group rickettsiae, which do not spread directly from cell to cell, lacked F-actin tails entirely or exhibited only very short tails. Transmission electron microscopy demonstrated fibrillar material in close association with *R. rickettsii* but not *Rickettsia prowazekii*. Biochemical evidence that actin polymerization plays a role in movement was provided by showing that transit of *R. rickettsii* from infected cells into the cell culture medium was inhibited by treatment of host cells with cytochalasin D. These data suggest that the cell-to-cell transmission of SFG rickettsiae may be aided by induction of actin polymerization in a fashion similar to that described for *Shigella flexneri* and *Listeria monocytogenes*.

Members of the genus *Rickettsia* are obligate intracellular bacteria that grow within the cytoplasm of the eucaryotic host cell (49). They are the etiologic agents of a variety of serious diseases in humans, including Rocky Mountain spotted fever, which is caused by *Rickettsia rickettsii*. Related members of the spotted fever group (SFG) include the rickettsial agents of Mediterranean spotted fever, Queensland tick typhus, North Asian tick typhus, and rickettsial pox as well as several nonpathogenic species. Other members of the genus *Rickettsia* are the etiologic agents of endemic and epidemic typhus (49).

In 1957, Schaechter et al. (30) reported intracellular movement of *R. rickettsii* within cultured rat fibroblasts. By using phase-contrast microscopy, they described rickettsiae in moving microfibrils which extended from the cell and occasionally released organisms into the extracellular medium. Subsequently, others have noted intracellular movements and rapid spread in cell culture of SFG (7, 18, 50) and scrub typhus group (TG) (12) organisms. Intercellular spread has been observed with *R. rickettsii* by 10 h postinfection, with most cells in culture infected within 48 h (50). The mechanism of intra- and intercellular movement is unknown.

Shigella flexneri (4, 23) and *Listeria monocytogenes* (16, 22, 37) are facultative intracytoplasmic pathogens that are capable of rapid cell-to-cell spread and plaque formation in cultured cell monolayers. Tilney and Portnoy (40) and Bernardini et al. (4), working with *L. monocytogenes* and *S. flexneri*, respectively, recently described parasite-induced directional actin assembly by intercellular organisms. It was suggested by both groups that this event drives intracellular movement and facilitates intercellular spread. Subsequent reports in both the *Listeria* (10, 11, 17, 19, 22, 28, 36–39) and *Shigella* (25, 44, 45) systems have since provided convincing evidence that recruitment and polymerization of host cell actin provide the actual mechanical force that allows both pathogens to move through the cytoplasm. It has been demonstrated for both pathogens that short actin filaments are unidirectionally added at one pole of the bacterium, eventually forming what is termed a comet tail (25, 28, 36–40). Contact between moving bacteria and the host plasma membrane occasionally results in the formation of parasite-containing pseudopodia that can be internalized by adjacent cells. Dissolution of the double membrane surrounding the bacterium allows the organism access to the cytoplasm of the newly encountered cell (29, 40). This method of intercellular spread is an elegant pathogenic mechanism that ensures spread to adjacent cells while allowing evasion of host immune responses.

In this report we demonstrate that members of the SFG rickettsiae similarly induce polymerization of actin in the form of polar projections (tails) and suggest that this polymerization plays a role in intracellular motility and cell-to-cell spread.

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MATERIALS AND METHODS

Rickettsiae. The rickettsiae used in this study were *R. rickettsii* R, Hlp, and Iowa, *R. montana* M/5-6, *R. canada* 2678, *R. conorii* Kenya tick typhus, *R. australis* NQTT, *R. parkeri* Maculatum, *R. prowazekii* Madrid E and Breinl, and *R. typhi* Wilmington (24). Rickettsiae were propagated in Vero cells with M199 medium and purified by Renografin density gradient centrifugation as described previously (15).

Infection of Vero cells. Twelve-millimeter coverslips were seeded with 2×10^5 Vero (African green monkey kidney) cells and cultivated overnight at 37°C in RPMI medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented

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with 10% fetal bovine serum (Hyclone Laboratories, Logan, Utah) and 20 μg of gentamicin (Whittaker Bioproducts, Walkersville, Md.) per ml. Rickettsiae suspended in 3.7% brain heart infusion broth (Difco Laboratories, Detroit, Mich.) were used to infect monolayers at a multiplicity of infection of 0.1 to 1.0 for 45 min. The cells were then washed once with M199 medium (GIBCO) supplemented with 2% fetal bovine serum. Fresh M199 medium plus 2% fetal bovine serum was then added, and incubation was continued at 34°C.

Chloramphenicol was utilized as an inhibitor of rickettsial protein synthesis at a final concentration of 20 μg/ml. Emetine was employed as an inhibitor of eucaryotic protein synthesis at a final concentration of 2 μg/ml (15). Both inhibitors were purchased from Sigma Chemical Co., St. Louis, Mo.

Dual fluorescence staining of rickettsiae and F-actin. All fixation and staining procedures were carried out at room temperature. Infected cells on coverslips were fixed and permeabilized essentially as described by Clerc and Sansonettii (8). Cells were then washed three times in phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin (BSA; mixtures, PBSA), and rickettsiae were labelled by indirect immunofluorescence. *R. rickettsii* R and Hlp were incubated for 20 min with a 1:100 dilution in PBSA of the monoclonal antibody 13-2, which is directed against the rOmpB protein (1, 3). Coverslips were then washed three times with PBSA, and rickettsiae were stained by incubating coverslips for 20 min with a 1:100 dilution of an anti-mouse immunoglobulin G fluorescein conjugate (Sigma). *R. typhi* and *R. canada* were stained similarly by employing a polyclonal rabbit antiserum generated against formalin-fixed organisms and an anti-rabbit fluorescein conjugate (Zymed Laboratories, Inc., South San Francisco, Calif.) at 1:100 in PBSA. After rickettsiae were stained, coverslips were washed three times in PBSA and F-actin was stained by being incubated with rhodamine phalloidin (Molecular Probes, Eugene, Oreg.) at 10 U/ml for 20 min. Coverslips were then washed three times with PBS, blotted dry, and viewed with a Zeiss fluorescence microscope. Color photography was performed with Ektachrome 400hc film (Eastman Kodak Co., Rochester, N.Y.).

Electron microscopy. Infected cells were grown in 60-mm Permanox dishes (Nunc, Inc., Naperville, Ill.). Cell membranes were extracted, and actin structures were fixed in situ by using fixatives containing ruthenium red essentially as described by Tilney et al. (38). Immunogold labelling modified this procedure and employed the actin-specific monoclonal antibody C4 (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) that was directly conjugated to 10-nm gold colloid (Janssen Pharmaceutica, Beerse, Belgium) by a previously described method (27). After detergent extraction and washing of infected cells as described above (38), cells were fixed for 1 h at 4°C with 2.5% glutaraldehyde–0.075 M cacodylate buffer (pH 7.4), washed two times with 0.05 M cacodylate buffer (pH 7.4), and blocked with 1% BSA in 0.05 M cacodylate buffer (pH 7.4) for 10 min. Anti-actin gold conjugate was then added to a final concentration of 10 μg/ml, and incubation was carried out overnight at 4°C. Cells were then washed three times for 1 h in 0.05 M cacodylate buffer (pH 7.4). Cells were then fixed, postfixed, and stained as described previously (38). Embedding and processing of all samples were performed by previously described methods (26). Controls for nonspecific adsorption of gold were performed with a BSA-gold conjugate. Photography was done with a Hitachi HU-11E-1 electron microscope at 75 kV and SO 163 electron image film (Eastman Kodak).

Rickettsial release assay. Six-well plates (35 mm per well; Flow Laboratories, Inc., McLean, Va.) were seeded overnight with Vero cells to 50% confluency. Cells were infected with rickettsiae as described previously (48) at a multiplicity of infection of 0.3 and incubated for 48 h. The medium was then aspirated, and cell sheets were washed five times with M199 medium. Three milliliters of fresh M199 medium containing 0, 0.25, or 0.5 μg of cytochalasin D (Sigma) per ml was then added to the appropriate well. An aliquot of medium (0.1 ml) was then removed from each well, serially diluted, and plaqued in triplicate as described previously (48). Additional aliquots were drawn and plaque assays were conducted every 30 min for 3 h, with an additional aliquot drawn and plaqued at 4 h. Datum points were calculated as the average of the three plaque assays.

RESULTS

Association of F-actin with intracellular rickettsiae. Members of the SFG rickettsiae spread rapidly from cell to cell in cultured cell monolayers (50). *R. rickettsii*-infected Vero cells were stained with rhodamine phalloidin 1 to 3 days after infection to determine if there was any association between host F (filamentous)-actin and intracellular rickettsiae. Phalloidin is a bicyclic peptide that binds F-actin and not G (globular)-actin (13). Figure 1 depicts infected cells in which F-actin has been stained with rhodamine phalloidin and rickettsiae have been stained by indirect immunofluorescence. Observation through a rhodamine filter system allows visualization of F-actin (Fig. 1A). Prominent actin stress fibers were visible as parallel arrays of F-actin bundles. Also evident were intensely staining, wavy F-actin structures that appeared distinct from stress fibers. The same field observed through a fluorescein filter system allows visualization of rickettsiae that stain apple green (Fig. 1B). Because of bleed-through in the fluorescein filter system, rhodamine-phalloidin-stained F-actin appears yellow. The wavy F-actin structures visible in Fig. 1A were articulated with one pole of *R. rickettsii*, giving the appearance of an actin tail. Some rickettsial actin tails were seen extending to great lengths, often exceeding 70 μm. Rickettsiae were frequently observed in clusters, seemingly acting in concert to promote the formation of a single, short, stocky tail. In some instances, rickettsiae were observed apparently exiting cells while still tethered to their actin tail (Fig. 1C). Rickettsia-associated actin polymerization was not restricted to Vero cells as similar events were seen with infected Chinese hamster ovary cells. Infected cells treated with antitubulin mouse monoclonal antibody did not demonstrate an association of rickettsiae with microtubules (data not shown).

We next determined whether avirulent strains of *R. rickettsii* induced formation of actin tails. The Hlp strain of *R. rickettsii*, which is avirulent for guinea pigs (2), is shown in Fig. 1D. F-actin tails associated with the Hlp strain were indistinguishable from those of the virulent R strain. The avirulent Iowa strain (9) of *R. rickettsii* and avirulent *R. montana* and *R. parkeri* also induced tail formation. Two additional virulent SFG organisms, namely *R. conorii*, the causative agent of Mediterranean spotted fever, and *R. australis*, the agent of Queensland tick typhus, were found to have F-actin tail associations (data not shown).

In contrast to SFG rickettsiae, members of the TG accumulate to extraordinary numbers (>800) within infected cells, and intercellular spread occurs only upon lysis of the

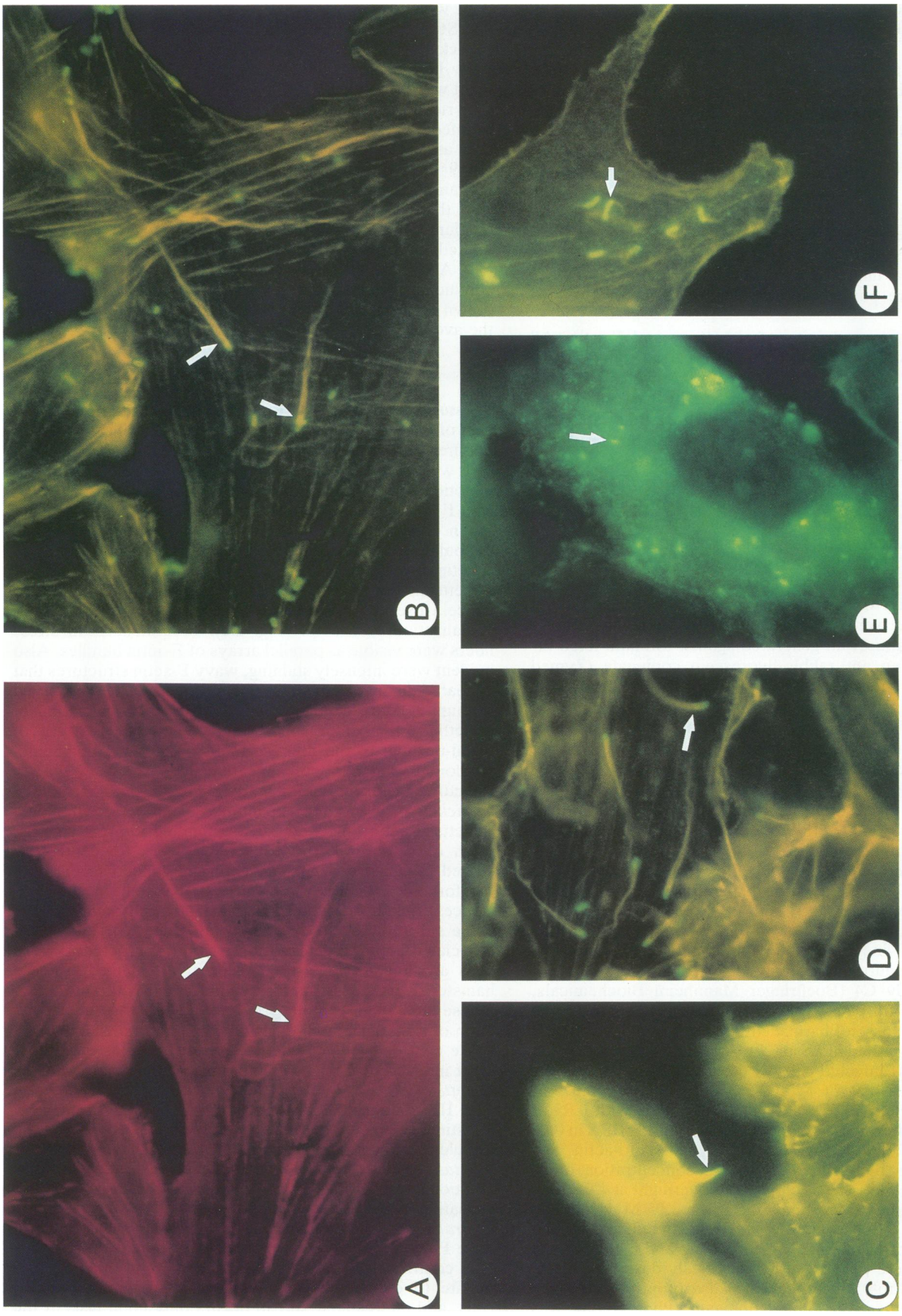


FIG. 1. Dual fluorescent staining of rickettsiae and F-actin in infected Vero cells. F-actin was stained with rhodamine phalloidin, and rickettsiae were stained by indirect immunofluorescence. (A) Doubly stained Vero cells infected for 23 h with virulent *R. rickettsii* R strain showing rhodamine-phalloidin-stained F-actin. Arrows indicate F-actin structures that are distinct from stress fibers. (B) Same field as that in panel A, but showing fluorescein-labeled *R. rickettsii* R (apple green) and F-actin (yellow). Note the colocalization of F-actin fibrils with one pole of rickettsia, giving the appearance of a tail. (C) *R. rickettsii* R at 68 h postinfection, possibly in the process of exiting the host cell while still associated with actin. (D) Avirulent *R. rickettsii* Hlp with actin tails. (E) *R. canada* lacking F-actin tails. (F) *R. typhi* with short, hook-shaped F-actin tails. Arrows identify a typical organism(s) in panels B to F. All panels are of equal magnification. Bar in panel A, 10 μm.

TABLE 1. Association of actin tails with members of the genus *Rickettsia*

Group	Species	Strain	Actin tail
SFG	*R. rickettsii*	R	+
		Hlp	+
		Iowa	+
	R. conorii	KTT	+
	R. montana	M/5-6	+
	R. australis	NQTT	+
	R. parkeri	Maculatum	+
TG	*R. prowazekii*	Madrid E	−
		Breinl	−
	R. typhi	Wilmington	+/−[a]
	R. canada	2678	−

[a] +/− indicates very short actin tails that are dissimilar to the long tails produced by the SFG.

host cells (50, 51). Neither the avirulent Madrid E strain nor the virulent Breinl strain of *R. prowazekii*, the causative agent of epidemic typhus, exhibited actin association. A similar observation was made for avirulent *R. canada* (Fig. 1E). However, *R. typhi*, the etiological agent of endemic typhus, did display polar F-actin projections but of a morphology different from those associated with SFG organisms (Fig. 1F). F-actin tails were very short and hook-shaped, never exceeding a few micrometers in length. The association of F-actin tails with SFG and TG rickettsiae is summarized in Table 1.

Time course and requirement for rickettsial protein synthesis. Dual fluorescence staining of F-actin and rickettsiae was performed at different time points postinfection to determine how quickly actin tails develop. Figure 2A shows *R. rickettsii* that is intensely stained with rhodamine phalloidin at 15 min after infection, suggesting that one of the early events in actin tail formation is the coating of the organism with short actin filaments. Actual tail formation was witnessed as early as 30 min postinfection (Fig. 2B). To determine whether de novo rickettsial protein synthesis was required for tail formation, infected cells were treated with 20 μg of chloramphenicol per ml 24 h prior to fixation and staining. This treatment eliminated all rickettsial actin tails, signifying that one or more rickettsial proteins are necessary to induce actin tail formation (Fig. 2C). A noticeable effect on F-actin tails could be seen as early as 3 h after addition of the antibiotic, at which time they appeared more tangled and disorganized. A complete lack of tails was noted after 14 h in the presence of chloramphenicol (data not shown). When infected cells were treated with emetine, a potent inhibitor of eucaryotic protein synthesis, at 2 μg/ml, no difference in tail formation was observed (Fig. 2D). Thus, de novo host protein synthesis does not seem to be required for rickettsia-induced actin tail formation. Figure 2D also illustrates the helical twisting and bilateral attachment to rickettsiae of some actin tails.

Electron microscopy. We employed a fixation procedure described by Tilney et al. (38) that includes ruthenium red as an F-actin stabilizer. Electron micrographs of *R. rickettsii*-infected Vero cells demonstrated fibrillar material in parallel arrays articulating with one pole of the bacterium in a bilateral fashion (Fig. 3A and B). We believe this material corresponds to F-actin tail appendages observed with rhodamine phalloidin staining and that it is likely bundles of actin filaments. These cross sections suggest that the actin filaments constituting the tail may be cross-linked in a cylindrical fashion, with the middle being hollow. This would be similar to the actin tails of both *L. monocytogenes* (38) and *S. flexneri* (25). Occasionally, this material appeared contiguous with the outer membrane of rickettsiae. Daughter cells resulting from a recent binary fission event revealed that only one cell was associated with the majority of fibrillar material (Fig. 3C). This is in keeping with observations made with fluorescence microscopy in which only one pole of septating cells had an F-actin tail. One characteristic distinguishing SFG rickettsiae from TG rickettsiae is occasional intranuclear growth (50). Figure 3D shows *R. rickettsii* residing in the nucleus but devoid of fibrillar material. In agreement with the results of phalloidin staining, *R. prowazekii* displayed no associations with fibrillar material (Fig. 3E).

To confirm the actin component of the fibrillar material, immunogold labelling was performed with a gold-conjugated monoclonal antibody directed against a highly conserved epitope of actin. Gold particles were concentrated on the polar fibrillar material associated with intracellular *R. rickettsii* (Fig. 3F), thereby verifying actin as a constituent.

Inhibition of release of rickettsiae into the extracellular medium by cytochalasin D. The small size of rickettsiae make direct observation of their intracellular movement difficult to record; therefore, we used an indirect assay to demonstrate a role for actin polymerization in the exit of rickettsiae. Treatment of infected cells with cytochalasin D, a powerful inhibitor of actin polymerization, caused a reduction in rickettsial transit into the extracellular medium (Fig. 4). The inhibitory effect of cytochalasin D was readily detectable by 1 h after its addition. At this point, there was little or no exit of rickettsiae from infected cells into the extracellular medium as assessed by the numbers of plaque-forming rickettsiae in the medium. At 4 h, the medium without cytochalasin D had 2.5- and 2.9-fold more free rickettsia than the medium containing 0.5 and 0.25 μg of cytochalasin D per ml, respectively. This assay could not be conducted reliably after 4 h because of the detachment of infected cells from the substratum. The degree of inhibition of rickettsial exit by cytochalasin D may be artificially low in this assay. Entry of *R. prowazekii* is known to occur through a microfilament-dependent process (47). Thus, significant reentry of organisms (i.e., PFU) into cells not treated with cytochalasin D may have occurred during the assay period. This assay also does not distinguish between actin-mediated release of rickettsiae into the medium and that directly into an adjacent cell. We assume that actin-based motility is as likely to lead to extrusion of an organism into the extracellular space as it is into a neighboring cell. Nonetheless, these data suggest a requirement for actin polymerization in the exit of *R. rickettsii*.

DISCUSSION

Adaptations allowing rickettsial spread from cell to cell certainly represent a major mechanism of pathogenesis. We have demonstrated that SFG rickettsiae induce directional actin polymerization in the form of a polar tail that is seen as early as 30 min after infection. Moreover, cytochalasin D inhibits the transit of organisms from infected cells into the extracellular medium. Taken together, these observations suggest a mechanism of movement based on parasite-induced directional actin assembly similar to that described for *S. flexneri* and *L. monocytogenes*. The absence of actin-based motility in TG rickettsiae may help explain the different growth phenotypes between this group and SFG organ-

isms. *R. prowazekii* replicates to very large numbers (>800) within chicken embryo fibroblasts (CEF) with little cytopathic effect (32, 51). Infection of adjacent cells occurs only upon lysis of the host when a lethal rickettsial load is presumably reached (32, 51). Plaques produced by TG rickettsiae in CEF monolayers are approximately one-half the size of those produced by SFG rickettsiae and take approximately 3 days longer to develop (48). In contrast to the spread of *R. prowazekii*, the spread of *R. rickettsii* in CEF monolayers has been documented as early as 10 h after infection (50). *R. rickettsii* organisms appear more toxic to cells; rarely are more than 100 organisms observed per cell, and cytopathological changes are observed (for example, dilation of the rough endoplasmic reticulum) as early as 3 days after infection (31). Because the numbers of SFG rickettsiae that are cytotoxic are small compared with those of TG rickettsiae, an actin-based motility system may be advantageous to help escape the autolytic effects of the dying cell. Conversely, continual bidirectional movement of SFG rickettsiae through the plasma membrane may be deleterious to the host and actually contribute to the cytotoxicity of the agent (33, 46).

Avirulent *R. montana* and *R. parkeri* and avirulent strains of *R. rickettsii* (Hlp and Iowa) both induced formation of F-actin tails indistinguishable from those associated with the virulent R strain of *R. rickettsii*. Thus, the basis of avirulence for these strains is not an inability to induce actin polymerization, and other factors must contribute to the virulence of rickettsiae. It has been demonstrated with *S. flexneri* (4, 20) and *L. monocytogenes* (11, 17, 19) that mutants that are unable to spread by inducing actin polymerization have significantly reduced virulence in their respective animal models. *R. typhi* F-actin tails were quite different from those observed for SFG rickettsiae. Morphologically, they were hook-shaped and only a few micrometers in length. Perhaps *R. typhi* fails to synthesize a protein or produces a nonfunctional form of a protein that is required for true F-actin tail formation and actin-based movement. Like *R. prowazekii* and *R. canada*, which lacked F-actin tails altogether, *R. typhi* does not display the dramatic spread in cell culture associated with SFG organisms (50, 51).

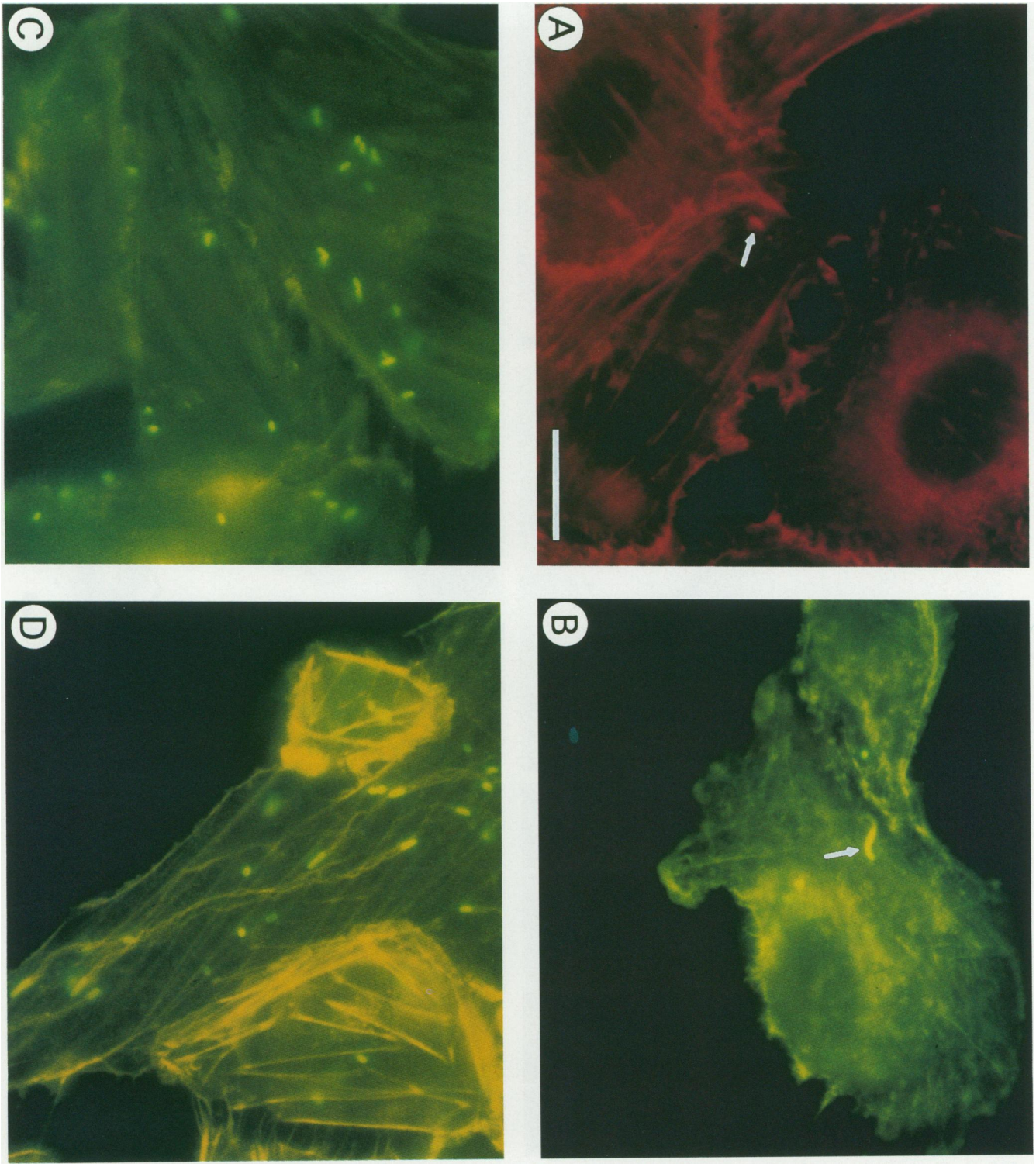
Electron micrographs of *R. rickettsii*-infected Vero cells demonstrated rickettsiae with a polar association of parallel arrays of fibrillar material. We believe that this material is composed of bundles of actin filaments and that it corresponds to the F-actin tails identified by rhodamine phalloidin staining. An actin component of these fibrillar structures is supported by anti-actin immunogold labelling. These structures are reminiscent of actin bundles that give the microvilli of brush border cells their characteristic shape (5).

Previous electron microscopy studies of rickettsiae have consistently demonstrated an electron-lucent zone surrounding the organism (31–34). It has been proposed that this clear zone is indicative of a rickettsia-produced microcapsular layer that is poorly preserved during fixation (34). Treatment of infected cells with ruthenium red or antirickettsial antibodies stabilized components of the halozone and demonstrated that it contained rickettsial antigens (34). Todd et al. (41–43), employing high-voltage electron microscopy with *R. rickettsii*-infected cells, were able to delineate fine fibrillar structures within the halozone that occasionally appeared contiguous with the bacterial outer membrane. It was suggested that these fibrils were microfilaments and that they may play a role in intracellular movements of rickettsiae. In our preparations, a halozone was not detected around *R. rickettsii*. Rather, most organisms had fibrillar material in close contact with the outer membrane that was frequently seen as polar projections. The fixation procedure used in this report employed ruthenium red to protect F-actin against oxidative fragmentation by osmium tetroxide (OsO_4) (35, 38). We suspect that the putative microcapsular layer of *R. rickettsii* described in previous reports (31, 34) may be composed of actin filaments in conjunction with host and/or rickettsial proteins that are detectable only when adequately stabilized.

Intranuclear *R. rickettsii* displayed no obvious F-actin associations by phalloidin staining or by electron microscopy. Indeed, the mechanism of entry into the nucleus is unknown. Some have suggested that rickettsiae are simply trapped in this compartment as a consequence of nuclear division (43). An alternative hypothesis is that motile rickettsiae directly penetrate the nuclear membrane. The nucleus contains actin that is biochemically distinct from cytoplasmic actin (6), but it is unknown which actin-binding proteins are present. Rickettsiae may interact less efficiently with nuclear actin; thus, the organisms may be stuck but continue to grow in the nucleus as a microcolony.

L. monocytogenes can move through the cytoplasm of PtK2 (kangaroo rat kidney epithelial) cells at speeds approaching 0.4 μm/s (10). The propulsive force for movement is provided by incorporation of short actin filaments (~0.2 μm in length) at the bacterial surface into polar F-actin projections (28, 36, 37, 40). Nucleation and filament assembly occur only at the bacterial surface, and the filaments are always oriented with their barbed ends pointed towards the bacterium (37, 40). As the bacterium moves through the cytoplasm, the actin tail remains stationary (10, 28, 36). By using caged actin, Theriot et al. (36) calculated a turnover rate of 33 s for the short actin filaments that are cross-linked to form the tail. This study and another (28) concluded that tail length is directly related to the rate of movement; bacteria with the longest tails are polymerizing actin and, consequently, moving the fastest. If one assumes a similar half-life for the actin filaments that constitute rickettsial tails, then these organisms would be moving at a faster rate than *L. monocytogenes* since tails in excess of 70 μm are frequently seen. *Listeria* tails longer than 30 μm are rarely seen (10). Alternatively, actin filaments constituting rickettsial tails may be inherently more stable. Indeed, rhodamine phalloidin staining of infected cells treated with 0.5 μg of cytochalasin D for 4 h prior to fixation showed that while F-actin was primarily in pools and most rickettsial tails were gone, an organism with a long tail was occasionally observed (data not shown). This is unlike *L. monocytogenes*, in which no tails are seen after exposure for 20 min to the same concentration of cytochalasin D (29). Tilney et al. (38)

FIG. 2. Time course and treatments with specific inhibitors of rickettsial and host protein synthesis in *R. rickettsii* R-infected Vero cells. (A) Cells infected for 15 min and stained with rhodamine phalloidin. The arrow indicates an organism coated with F-actin. (B) A cell infected for 30 min and doubly stained for F-actin and rickettsiae, showing *R. rickettsii* R with a short actin tail (arrow). (C) Cells infected for 44 h and then treated for 24 h with 20 μg of chloramphenicol per ml. No actin tails were seen. (D) Cells infected for 44 h and then treated for 24 h with 2 μg of emetine per ml. No effect on tail formation was seen. All panels are of equal magnification. Bar in panel A, 10 μm.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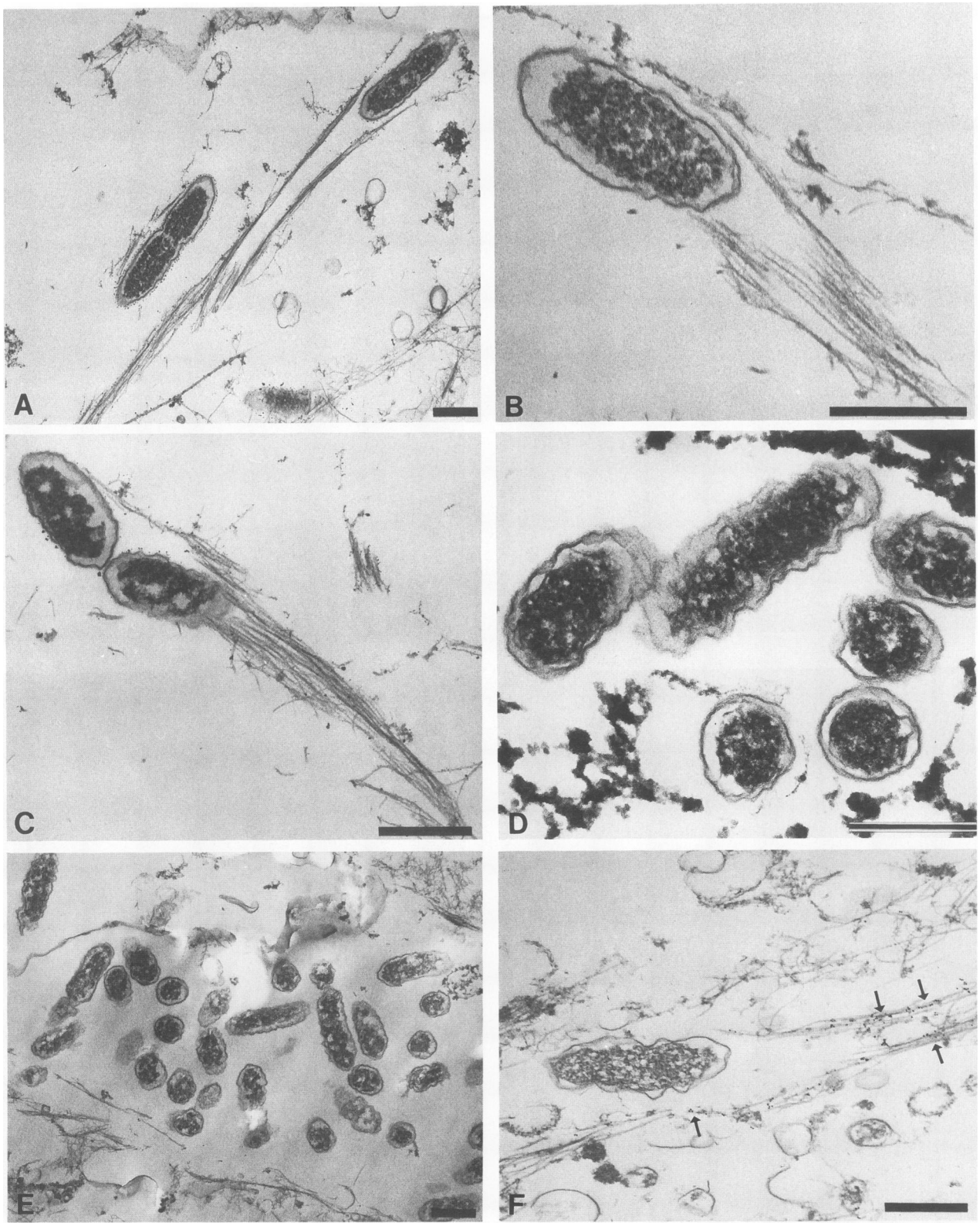

FIG. 3. Electron micrographs of Vero cells infected with rickettsiae for 72 h. (A to D) Sections of Vero cells infected with *R. rickettsii* R showing a bilateral association of bundles of fibrillar material (A), fibrillar material at high magnification (B), a recent binary fission event in which most of the fibrillar material is associated with one daughter cell (C), and intranuclear organisms with no fibrillar associations (D); (E) *R. prowazekii* Madrid E cells that are devoid of fibrillar material; (F) anti-actin immunogold labelling of *R. rickettsii*-infected Vero cells. Gold particles are concentrated on polar fibrillar material (arrows). Gold-conjugated BSA did not label this material. Bars, 0.5 μm.

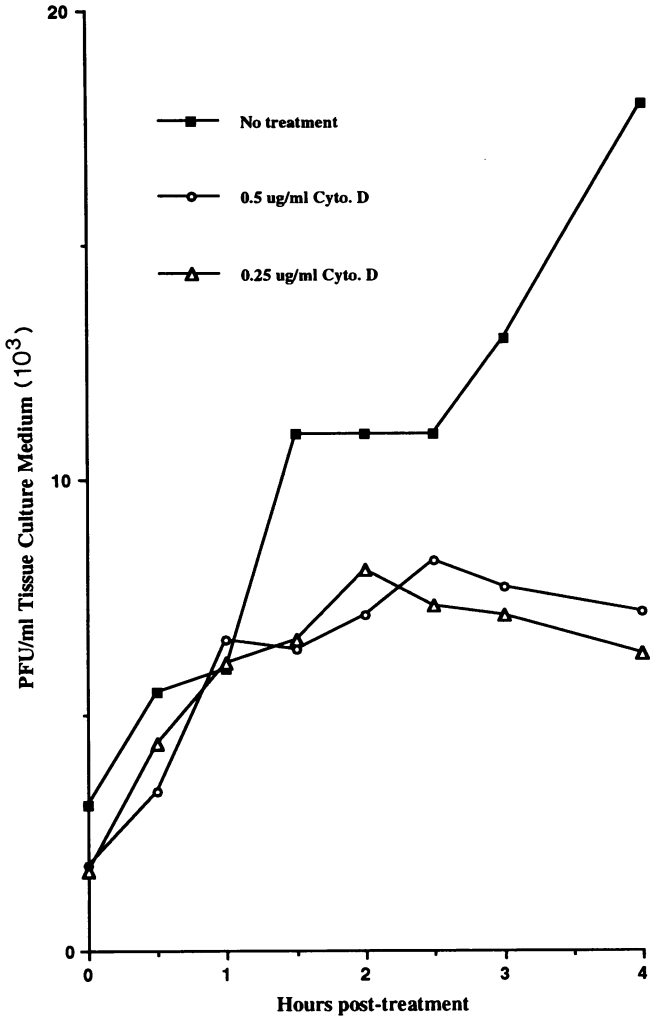

FIG. 4. Inhibition of release of *R. rickettsii* R from infected Vero cells by cytochalasin D. Infected monolayers in a six-well plate were infected for 48 h and then washed five times with culture medium. After the final wash, fresh medium was added with or without cytochalasin D. Aliquots of 0.1 ml were then drawn from each well every 30 min for 3 h and again at 4 h and assayed for PFU.

suggest that the length of the listerial tail is also directly related to the length of time the organism has spent in contact with the cytoplasm. Bacteria with older surfaces have longer tails. Only one newly formed daughter cell retains a tail appendage, while the other must reinitiate tail formation, possibly because new nucleating material must be synthesized and presented in the septated region (38). Rickettsial division occurs on average every 8 h (50, 51). Thus, the lengths of rickettsial F-actin tails may also be a consequence of their slow doubling time. The mechanism of rickettsial movement by actin polymerization is unknown, but a simple interpretation of the data consistent with more detailed studies with *S. flexneri* and *L. monocytogenes* is that the F-actin tail represents a structural scaffold to which new building blocks in the form of G-actin or small F-actin units are continually added at the tail-bacterium interface, thereby pushing the organism forward.

Progress has been made in identifying *Listeria* and *Shigella* proteins necessary for actin-based movement. In *L. monocytogenes*, the *actA* gene product is essential for actin polymerization and bacterial spread (11, 17). ActA is a 90-kDa cell surface protein (11, 17) whose deduced amino acid sequence shows a proline-rich repetitive region (11, 17) and limited homology to the cytoskeletal protein vinculin (11). The *icsA* (*virG*) gene of *S. flexneri* encodes a 120-kDa outer membrane protein that is required for actin assembly (4, 20). Despite having similar roles in promoting actin polymerization, ActA and IcsA do not display any significant homology (4, 11, 17).

Since de novo synthesis of rickettsial protein is required for actin polymerization, it is reasonable to suspect that at least one rickettsial outer membrane protein may bind monomer actin, or a host actin-binding protein, and that this complex might act as a nucleus for actin filamentation. Many actin-binding proteins have repetitive domains (21). Indeed, all SFG rickettsiae, with the possible exception of *R. australis*, synthesize a major outer membrane protein of unknown function, designated rOmpA, that contains a large region of tandem repeats (3, 14). TG rickettsiae lack the rOmpA protein and do not form F-actin tails or produce truncated tails. Whether the rOmpA interacts with actin or actin-binding proteins is speculative, but the apparent correlation of the presence of this protein with actin-based movement suggests that it may warrant further study.

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ADDENDUM IN PROOF

Since the manuscript was submitted, Teysseire et al. (N. Teysseire, C. Chiche-Portiche, and D. Raoult, Res. Microbiol. **134**:821–829, 1992) have reported rhodamine-phalloidin staining of *R. conorii* and *R. typhi* actin tails.

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