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A comparative study of the actin-based motilities of the pathogenic bacteria *Listeria monocytogenes*, *Shigella flexneri* and *Rickettsia conorii*

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SUMMARY

Listeria monocytogenes*, *Shigella flexneri*, and *Rickettsia conorii* are three bacterial pathogens that are able to polymerize actin into 'comet tail' structures and move within the cytosol of infected cells. The actin-based motilities of *L. monocytogenes* and *S. flexneri* are known to require the bacterial proteins ActA and IcsA, respectively, and several mammalian cytoskeleton proteins including the Arp2/3 complex and VASP (vasodilator-stimulated phosphoprotein) for *L. monocytogenes* and vinculin and N-WASP (the neural Wiskott-Aldrich syndrome protein) for *S. flexneri*. In contrast, little is known about the motility of *R. conorii*. In the present study, we have analysed the actin-based motility of this bacterium in comparison to that of *L. monocytogenes* and *S. flexneri*. *Rickettsia* moved at least three times more slowly than *Listeria* and *Shigella* in both infected cells and *Xenopus laevis* egg extracts. Decoration of actin with the S1 subfragment of myosin in infected cells showed that the comet tails of *Rickettsia* have a structure strikingly different from those of *L. monocytogenes* or *S. flexneri*. In *Listeria* and *Shigella* tails, actin filaments form a branching network while *Rickettsia* tails display longer and not cross-linked actin filaments. Immunofluorescence studies revealed that the two host proteins, VASP and α-actinin colocalized with actin in the tails of *Rickettsia* but neither the Arp2/3 complex which we detected in the *Shigella* actin tails, nor N-WASP, were detected in *Rickettsia* actin tails. Taken together, these results suggest that *R. conorii* may use a different mechanism of actin polymerization.

Key words: *Rickettsia, Listeria, Shigella*, Actin assembly, Motility, Myosin S1

INTRODUCTION

A group of unrelated pathogenic microorganisms including several bacteria (*Listeria, Shigella* and *Rickettsia*) and one virus (Vaccinia) have independently developed an apparently similar actin-based mechanism that is essential for their intercellular spread (Lasa et al., 1998; Dramsi and Cossart, 1998). *L. monocytogenes* is a gram positive bacterium, responsible for food-borne infections leading to severe septicaemia, meningo-encephalitis and abortion in pregnant women (Gray and Killinger, 1966). The gram negative bacterium *S. flexneri* is the etiological agent of bacillary dysentery among higher primates and humans (Labrec et al., 1964). *L. monocytogenes* and *S. flexneri* are facultative intracellular pathogens. The gram negative bacterium *R. conorii* and its closely related species *R. rickettsii*, are obligate intracellular parasites, responsible for spotted fevers (Raoult and Roux, 1997; Winkler, 1990).

Rickettsia, Listeria and *Shigella* grow and replicate within the host cytoplasm and behave similarly in infected tissue-cultured cells with an apparently identical infectious cycle. After entry into the cell and escape from the phagocytic vacuole, bacteria, free in the host cytoplasm, recruit and polymerize host actin around them. This actin coat then rearranges into a tail located at one pole of the bacterium. The continuous process of actin assembly propels the bacteria inside the cytosol (Heinzen et al., 1993; Mounier et al., 1990; Teysseire et al., 1992; Tilney and Portnoy, 1989). As shown for *Listeria*, actin polymerization takes place at the posterior end of the bacterium and the actin tail remains stationary in the cytoplasm while the bacteria move away (Theriot et al., 1992). Electron microscopy studies, mainly performed in *L. monocytogenes*-infected cells, have enabled the analysis of the structural organization of the actin tail. The intracytoplasmic *Listeria* tails are composed of short, cross-linked actin filaments, with their fast growing barbed ends oriented towards the bacteria (Tilney et al., 1990, 1992a,b; Tilney and Portnoy, 1989). In contrast, tails within cell membrane protrusions were shown to contain long actin filaments (Sechi et al., 1997).

Genetic approches have led to the identification of two bacterial genes involved in this process, *actA* for *L. monocytogenes* (Domann et al., 1992; Kocks et al., 1992) and *icsA* (*virG*) for *S. flexneri* (Bernardini et al., 1989; Lett et al.,

1989; Makino et al., 1986). These two genes are necessary and sufficient to induce actin assembly and movement in cell free extracts (Goldberg and Theriot, 1995; Kocks et al., 1995). The bacterial proteins ActA and IcsA do not share any sequence homology, but share a common property, i.e. they both are asymmetrically distributed on the bacterial surface and colocalize with the site of actin assembly in infected cells. This polar distribution would play a key role in actin-tail formation and movement (Goldberg et al., 1993; Kocks et al., 1993). Regions in ActA and IcsA involved in the actin polymerization process have been identified. ActA is a 610 amino-acid protein consisting of three domains. The N-terminal domain (ActA-N, amino acids 1-233) contains all the necessary elements for motility (Lasa et al., 1995, 1997). The central domain (ActA-P, amino acids 234-394) made of repeats rich in proline residues is not absolutely essential but stimulates movement. The C-terminal domain (ActA-C, amino acids 395-585) is involved in anchoring ActA to the bacterial membrane (Lasa et al., 1995, 1997). IcsA is an outer membrane protein of 1102 amino acids consisting of two domains, IcsAα and IcsAβ. The IcsAβ autotransporter domain (residues 759-1102) allows translocation across the outer membrane of the amino-terminal IcsAα domain (residue 53-758) (Suzuki and Sasakawa, 1995). The IcsAα domain contains two regions critical for the actin polymerization process, a glycine-rich repeat domain (residues 103-319) and the 320-508 domain (Suzuki and Sasakawa, 1995). No intrinsic polymerization activity has been detected for ActA or IcsA and there is no indication that ActA and IcsA can bind directly to actin. These observations have led to the proposal that ActA and IcsA may be modified inside cells or recruit some cellular factors. Several cytoskeletal proteins have been localized to the tails by immunofluorescence (Lasa et al., 1998). They include the Arp2/3 complex, VASP, α-actinin, cofilin, ezrin, N-WASP and CapZ. Their function has only been addressed in the case of α-actinin, cofilin and the Arp2/3 complex for *L. monocytogenes* (Carlier et al., 1997; Dold et al., 1994; Rosenblatt et al., 1997; Welch et al., 1997b, 1998; Chakraborty et al., 1995) and N-WASP and vinculin for *S. flexneri* (Laine et al., 1997; Suzuki et al., 1996, 1998). The Arp2/3 complex was shown to cooperate with ActA to nucleate actin assembly at the bacterial surface (Welch et al., 1998) but no direct interaction with ActA has been reported. In contrast, VASP and its homologue MENA were shown to bind directly to ActA in vitro (Gertler et al., 1996) and to colocalize with the front of the actin tails in infected cells. However, deletion of the VASP binding region in ActA does not abolish actin polymerization (Lasa et al., 1995, 1997; Niebuhr et al., 1997), suggesting that VASP is not essential for the process.

In the case of *Shigella*, vinculin binds to IcsA and is recruited to the *Shigella* tails. Its role in the actin polymerization process is currently controversial (Goldberg, 1997; Kadurugamuwa et al., 1991; Suzuki et al., 1996; Zeile et al., 1996). N-WASP recruitment by IcsA seems to be essential for actin polymerization (Suzuki et al., 1998).

The original reports concerning the actin-based motility of *R. conorii* and *R. rickettsii* have shown that the actin tails appear formed of filaments longer than those of *Listeria* and *Shigella* and that actin polymerization requires the de novo synthesis of one or more rickettsial proteins (Heinzen et al., 1993; Teysseire et al., 1992). Moreover, treatment of infected cells with cytochalasin D has established a correlation between actin polymerization, movement and spreading but the lack of genetics tools has hampered the isolation of gene(s) responsible for this process. In this paper, we examined in details the intracellular motility of *Rickettsia*. We measured the speed of movement in mammalian cells and in cytosolic extracts from *Xenopus* eggs. To analyse the structure of the comet tails, we used electron microscopy including labeling with myosin S1. In addition, we performed immunofluorescence studies to examine whether cellular proteins known to be involved in movement of *Listeria* or *Shigella* were also recruited by *Rickettsia*. In all these studies, we compared *Rickettsia* to *Listeria* and *Shigella*.

MATERIALS AND METHODS

Cell lines, bacterials strains, rickettsial culture and purification

The HEp-2 epithelial-like human cell line (ATCC CCL23), the HeLa cell line (ATCC CCL 2) and the Vero cell line (ATCC CRL1587) were cultured in DMEM medium (Gibco-BRL) supplemented with 2 mM glutamine (Gibco-BRL), 1% non-essential amino acids (Gibco-BRL) and 10% fetal calf serum at 37°C in 10% CO_2. Bacterial strains used were *L. monocytogenes* LO28 serotype 1/2c (Vicente et al., 1985), *S. flexneri* wild-type serotype 5 strain M90T (Sansonetti et al., 1982) and *R. conorii* (VR141 from American Type Culture Collection). *L. monocytogenes* and *S. flexneri* were grown in BHI broth (Difco Laboratories) and in Trypticase Soy broth (Diagnostics Pasteur), respectively. *R. conorii* was grown in Vero cells with DMEM medium (Gibco-BRL) supplemented with 5% fetal calf serum at 32°C in an atmosphere of 5% of CO_2. *Rickettsia* were harvested by disruption of heavily infected Vero cells, with glass beads, followed by centrifugation at 12,000 rpm for 40 minutes. Bacterial pellets were resuspended in BHI broth and stored in aliquots at –80°C.

Antibodies

The anti-cofilin and anti-Arp3 specific polyclonal antibodies were produced against synthetic peptides derived from the conserved C-terminal sequences of cofilin and Arp3, respectively, and affinity purified as previously described (David et al., 1998). Polyclonal antibodies against VASP (Reinhard et al., 1992), capZ (Schafer et al., 1996) and ezrin (Andreoli et al., 1994) were generously provided by U. Walter, J. A. Cooper and P. Mangeat, respectively. Monoclonal antibody against α-actinin was purchased from Sigma (BM-75.2). The anti-*Rickettsia* R47 antibody was obtained by immunizing New Zealand rabbits with formalin-killed bacteria *R. conorii* purified from infected yolk sac. Animals were boosted six and twelve weeks after inoculation and bled ten days after the last challenge.

Double fluorescence labelling and electron microscopy of infected cells

Vero cells were infected with *L. monocytogenes* as described (Kocks et al., 1993; Lasa et al., 1995) with a multiplicity of infection (MOI) of 60 bacteria per cell. HeLa cells were infected by *Shigella* M90T strain as previously described (Adam et al., 1996) at a MOI of 100. Overnight cultures of *Shigella* were diluted in tryptic soy broth and grown to mid-exponential phase, centrifuged at 5000 ***g*** for 5 minutes, washed and resuspended in MEM, 50 mM Hepes, pH 7.5, to bring the suspension to a concentration of 10^8 bacteria/ml. HeLa cells were washed three times with MEM, then overlaid with the respective bacterial suspensions. Bacteria were centrifuged over cells for 10 minutes at 800 ***g***. Dishes were then set on top of a 37°C waterbath for 30 minutes, washed with MEM five times and incubated with MEM, 50 mM Hepes, pH 7.5, with 50 μg/ml of gentamicin for a further 1 hour at 37°C. Vero cells were infected with *R. conorii* prepared as described above, and grown for 36-48 hours at 32°C in 5% CO_2. For immunofluorescence staining, infected cells were permeabilized with 0.2% Triton X-100 in 0.1 M MES, pH 7.4, 1 mM $MgCl_2$, 1 mM EGTA, 4% PEG 6000, and then fixed in 3.5%

paraformaldehyde in PBS or cells were fixed in 3.5% paraformaldehyde in PBS and then permeabilized either with 0.4% Triton X-100 in PBS or methanol. Incubation with antibodies were performed as previously described (Kocks et al., 1993). F-actin was visualized using FITC-phalloidin (Sigma, 0.1 mg/ml) or bodipy-phallacidin (Molecular Probes, Eugene, OR). Preparations were examined with a confocal laser scanning microscope (Zeiss Axiophot or Wild Leitz microscope).

Analysis of motility of *L. monocytogenes*, *S. flexneri* and *R. conorii*

Interphasic extracts of *Xenopus laevis* eggs were prepared as described (Marchand et al., 1995) *L. monocytogenes* were prepared for motility assays as described (Lasa et al., 1995, 1997; Marchand et al., 1995). *R. conorii* were freshly prepared from infected cells before the motility assay. Briefly, Vero cells heavily infected with bacteria were harvested after glass bead treatment, centrifuged at 3000 rpm to eliminate cells debris, and free *Rickettsiae* were pelleted by 10,000 ***g*** centrifugation. The bacterial pellet was resuspended in XB buffer.

Preparations were observed with a Zeiss Axiovert 135 microscope equipped with a CCD 54058 camera (Hamamatsu) which allowed simultaneous visualization of bacteria by phase contrast, and actin tails by fluorescence. Analysis of movement of bacteria was performed as described (Lasa et al., 1997).

Purification of myosin and subfragment 1

Rabbit skeletal muscle (385 g) was finely cut, and suspended in 2 liters of an extraction solution containing 0.5 M KCl and 50 mM $KHCO_3$ for 10 minutes. All manipulations were performed at 4°C. After centrifugation at 2500 ***g*** for 10 minutes, the supernatant was kept and the pellet was resuspended in 2 liters of the same extraction solution. The pH of the two supernatants was adjusted to 6.8 and 14 volumes of cold water were added. The solution was centrifuged at 7000 ***g*** for 10 minutes and the pellet was washed with a solution containing 15 mM KCl, 5 mM KH_2PO_4 and 2.5 mM K_2HPO_4 (myosin is insoluble in this solution). After centrifugation, the pellet was dissolved in 0.5 M KCl, 32.5 mM K_2HPO_4, 17.5 mM KH_2PO_4 and 1 mM EDTA. Myosin was purified by ammonium sulfate fractionation. The precipitate obtained at 31% saturation was discarded, and the supernatant brought to 37% saturation. The resulting precipitate was dissolved in 130 mM K_2HPO_4, 15 mM KH_2PO_4 and 10 mM EDTA (pH 7.8) and was dialyzed against this solution. Myosin was stored in 50% glycerol at –20°C.

Subfragment-1 was prepared from myosin at 10 mg/ml (in 10 mM KH_2PO_4, 120 mM NaCl, 1 mM EDTA and 0.1% β-mercaptoethanol at pH 7.0) by chymotrypsin digestion: α-chymotrypsin (Worthington Biochemical Corporation) at 0.1 mg/ml for 10 minutes at 25°C. Digestion was stopped by addition of phenylmethylsulfonyl fluoride (PMSF) at a final concentration of 0.5 mM and all manipulations were then carried out at 4°C. After dialysis against 10 mM K_2HPO_4, 40 mM NaCl, 1 mM EDTA and 0.1% β-mercaptoethanol to reduce the ionic strength, undigested myosin and myosin rods were removed by centrifugation at 17,000 ***g*** for 30 minutes. The proteins in the supernatant were precipitated by ammonium sulfate at 60% saturation. The pellet was dissolved in 10 mM MOPS (3-[N-morpholino]propanesulfonic acid) at pH 7.0, 0.2 mM DTT (dithiothreitol), 0.01% NaN_3 and dialyzed against this buffer. Proteins were applied to a HiTrapTM ion exchange column (HiTrapTM SP 5 ml, Pharmacia Biotech) equilibrated in the same buffer, and the subfragment-1 species was eluted between 120 and 200 mM with NaCl. Subfragment-1 fractions were pooled, precipitated by ammonium sulphate at 60% saturation and the pellet was dissolved in 100 mM phosphate buffer pH 6.8. The solution was stored at –20°C.

Electron microscopy

For electron microscopy, HEp2 cells were seeded in 35 mm diameter Petri dishes 24 hours before infection, infected as described above and in situ fixation, staining, embedding and further processing were carried out as described (Tilney and Portnoy, 1989). Sections of infected cells were examined using Philips CM12 and Jeol 1010 electron microscopes. For decoration with subfragment 1 of myosin (S1), all steps were performed in situ in Petri dishes. Infected cells were permeabilized with 1% Triton X-100, in 3 mM $MgCl_2$, 50 mM phosphate buffer at pH 6.8 for 10 minutes at 4°C. Detergent solution was decanted and replaced with 0.1 M phosphate buffer, pH 6.8, at 4°C. Cells were first incubated with 5 mg/ml S1 in 0.1 M phosphate buffer (pH 6.8) for 10 minutes at 4°C, and then for 20 minutes at room temperature on an oscillating table. Cells were subsequently washed in 0.1 M phosphate buffer for 20 minutes to remove unbound S1. Fixation was performed at room temperature in 1% glutaraldehyde with 2% tannic acid and 0.05 M phosphate buffer at pH 6.8 for 30 minutes. Cells were washed three times for 10 minutes in 0.1 M phosphate buffer, pH 6.8, at room temperature, postfixed in 1% OsO_4 in 0.1 M phosphate buffer, pH 6.3, for 30 minutes at 4°C, rinsed 3 times for 5 minutes in distilled water, stained overnight with 0.5% uranyl acetate, dehydrated in solutions containing increasing concentrations of ethanol and finally embedded in epon. Thin sections were mounted on uncoated grids, stained with uranyl acetate and lead citrate and examined on JEOL 1010 or Philips CM12 electron microscopes.

RESULTS

Actin-based movement of *R. conorii* in epithelial cells and *Xenopus* extracts

Double staining of *Rickettsia*-infected cells performed at different time points after infection, in Vero and HEp2 cells revealed that actin tails could be observed 24 hours post-

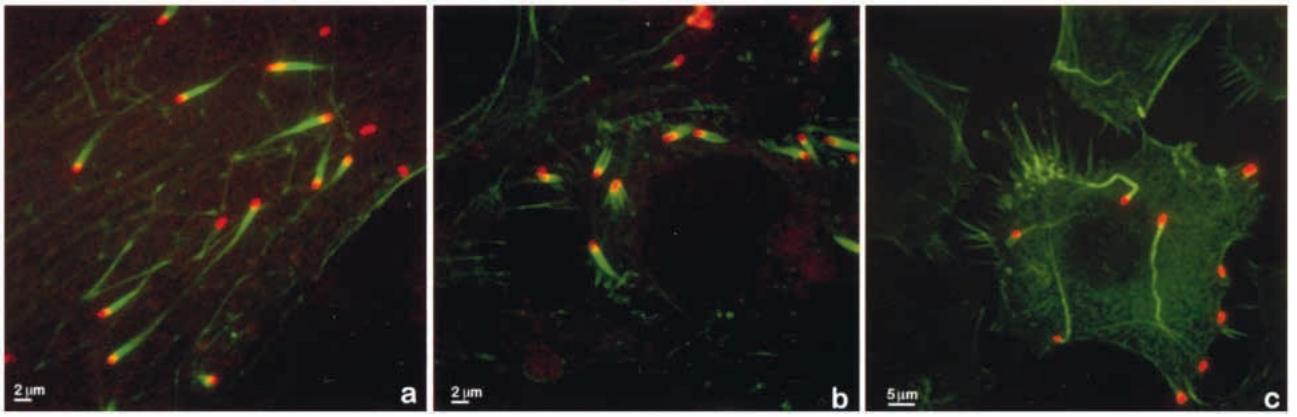

Fig. 1. Confocal laser scanning images of Vero cells (a,b) and HEp2 cells (c) infected with *R. conorii*. Bacteria were localized by immunofluorescence with specific antibodies and actin with FITC-phalloidin.

infection in both cells lines. Protrusions containing bacteria were observed 48 hours post-infection in both types of cells. In Vero cells, the tails appeared short (3-5 μm), straight, hollow in the center and seemed to originate from the stress fibers (Fig. 1a and b). In HEp2 cells (Fig. 1c), the comets were longer (30-40 μm), and 'zigzagging'. With these data, we were then in a position to analyse the different stages of the infectious process by electron microscopy, using the procedure originally described by Tilney and Portnoy (1989). Fig. 2 displays the successive steps of the intracellular cycle of *R. conorii*: engulfment of the bacteria (2a), formation of a membrane-bound vacuole (2b), actin polymerization with formation of an actin cloud (2c) followed by rearrangement of F-actin in tails (2d), formation of a protrusion (2e,f), and generation of a two-membrane vacuole (2g), which will be lysed to allow release of the bacteria. These results confirmed that *R. conorii* has an infectious cycle similar to those of *L. monocytogenes* and *S. flexneri.*

We measured the rate of movement of *R. conorii* in Vero cells as well as that of *L. monocytogenes* and *S. flexneri* (Fig. 3 and Table 1). *R. conorii* moved at a rate of 8 μm/minute (n=75, s.d.=1) (3B), whereas the speed of movement for *L. monocytogenes* was 22 μm/minute (n=90, s.d.=5) (3A), in agreement with previous results (Dabiri et al., 1990; Lasa and Cossart, 1996; Sanger et al., 1992; Tilney and Portnoy, 1989). The rate of movement for *S. flexneri* was 26 μm/minute (n=90, s.d.=5), a value higher than that reported for PtK_2 cells (3-10 μm/minute) (Zeile et al., 1996). These differences could be explained by differences in cell types used and in times of infection before recording. We also evaluated the speed of movement in *Xenopus laevis* egg extracts. *R. conorii* moved in the extracts at a relatively low speed, i.e. 2 μm/minute (n=20, s.d.=0.2) compared to *L. monocytogenes* which moved at an average speed of 13 μm/minute (n=54, s.d.=5) in agreement with previously reported values (Kocks et al., 1995; Marchand et al., 1995; Theriot et al., 1994) *S.*

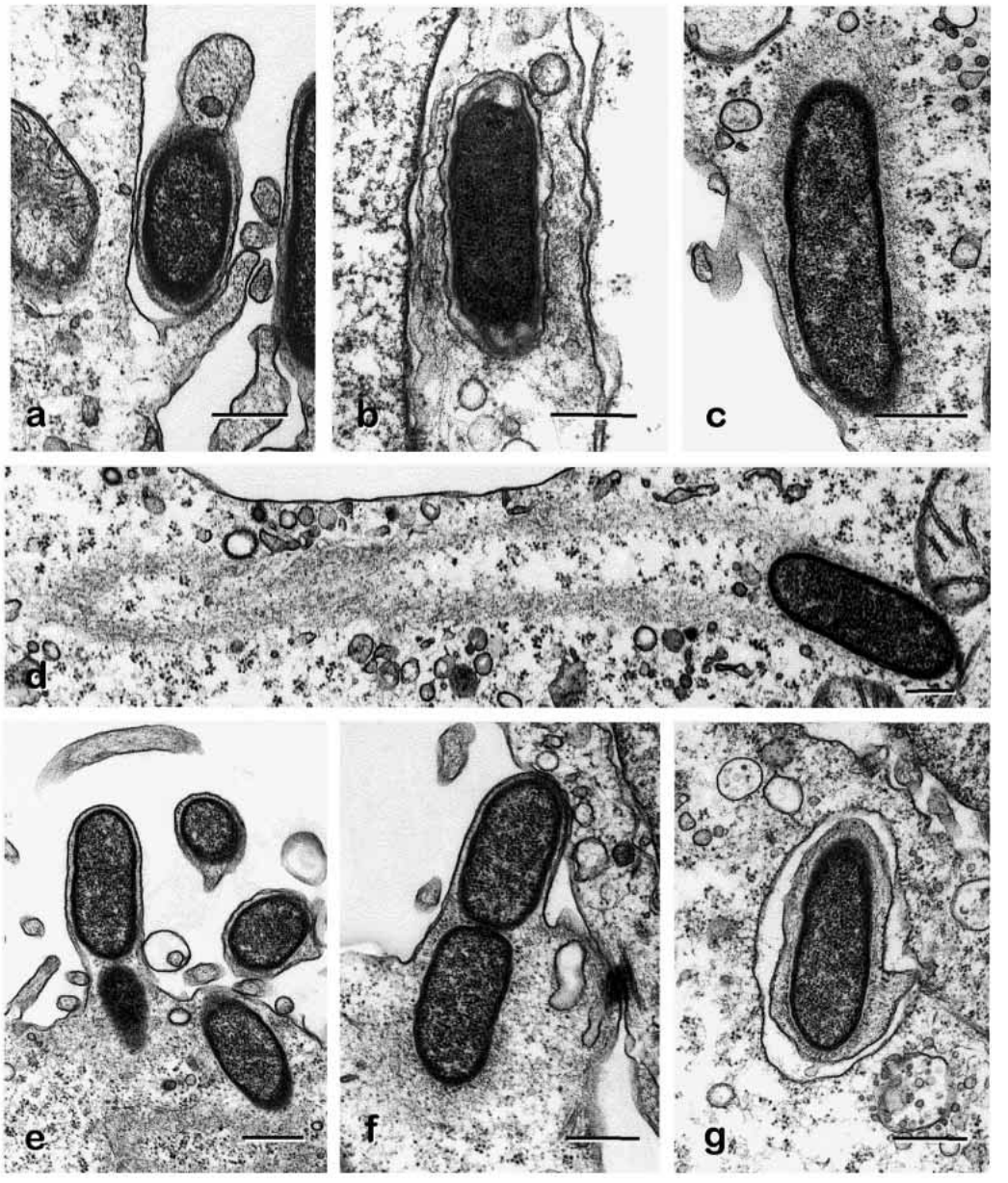

Fig. 2. Thin sections of HEp2 cells infected with *R. conorii* 48 hours postinfection.
(a) Engulfment of the bacterium by the cell. (b) Bacterium inside a vacuole. (c) Bacterium surrounded by a layer of actin filaments. (d) Bacterium associated with a comet tail of actin. (e,f) Bacteria into cell surface protrusions invading neighboring cells. (g) Bacterium within a two membrane vacuole. Bars: (a,b,c,e,f,g) 0.5 μm; (d) 0.2 μm.

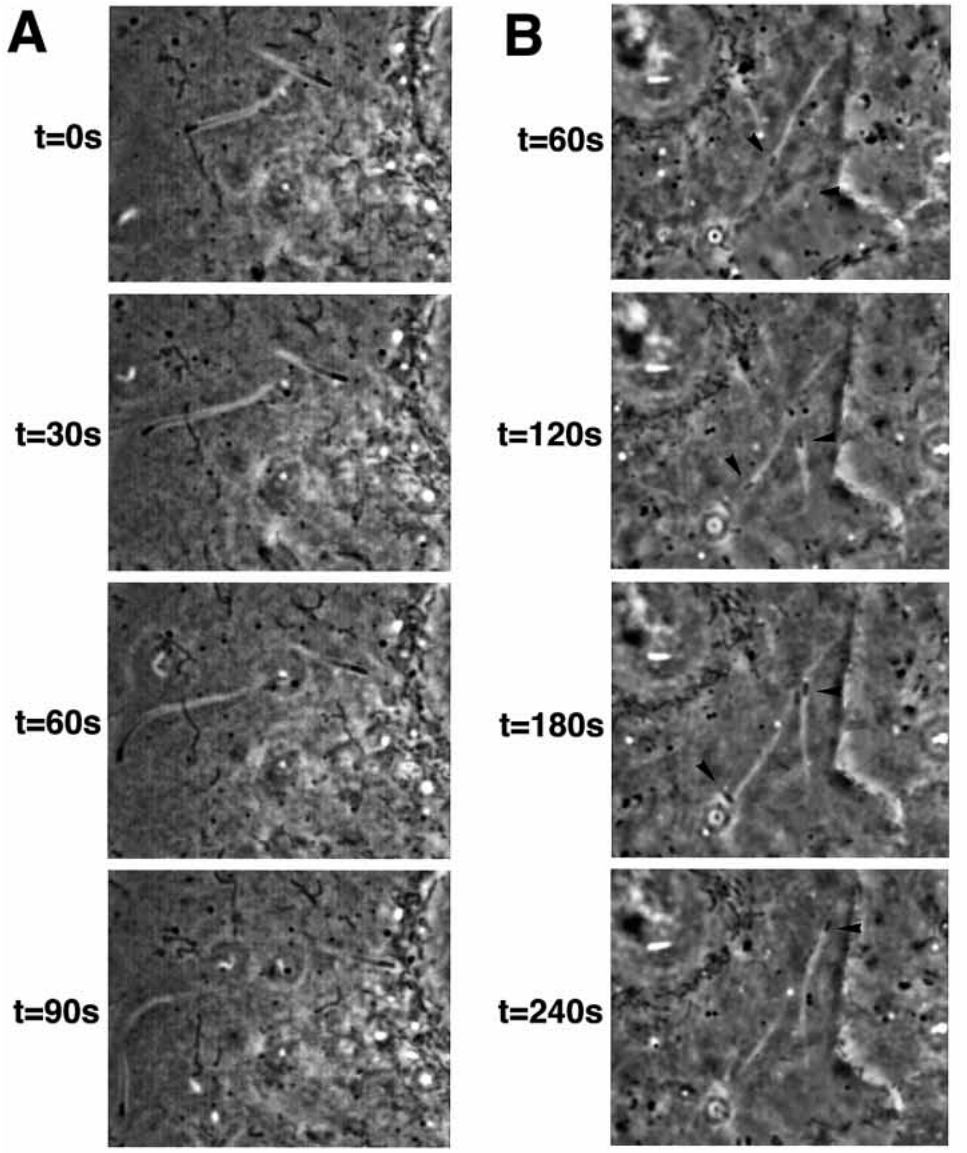

Fig. 3. Movement of *L. monocytogenes* (A) and *R. conorii* (B) in Vero cells. The tails of bacteria were visualized by phase-contrast microscopy and movement recorded by videomicroscopy. Fields were observed every 30 seconds (A) or 60 seconds (B).

flexneri was unable to move in extracts as previously observed (Kocks et al., 1995).

Characteristics of the *R. conorii* comet tails: comparison with tails of *L. monocytogenes* and *S. flexneri*

We studied by electron microscopy the actin tails and the protrusions of *R. conorii* in comparison with those of *L. monocytogenes and S. flexneri*. Thin sections of infected cells were processed by the technique originally described by Tilney et al. (1992a,b). Fig. 4a,b,c illustrates that the actin tails of *R. conorii*, *L. monocytogenes* and *S. flexneri* appeared different.

Table 1. Rate of movement in infected cells and cell free extracts

	L. monocytogenes	*S. flexneri*	*R. conorii*
Vero cells	22±5 μm/min	26±5 μm/min	8±1 μm/min
Xenopus laevis egg extracts	13±5 μm/min	ND	2.0±0.2 μm/min

For *R. conorii*, the electron-dense fibrillar material corresponding to actin filaments was mostly present along both sides of the bacteria and virtually absent from both extremities. For *L. monocytogenes*, the filaments were located along both sides of the bacterium and at one pole of the bacterium but in many instances, as in Fig. 4b, the pole close to the tail was also free, in agreement with previous observations (Kocks et al., 1995; Tilney et al., 1992a). For *S. flexneri*, the fibrillar material was mainly at one pole.

To examine the structure of the actin tails in more detail, we used the technique of myosin S1 decoration (Figs 5 and 6). We first measured the length of the comet tails in HEp2 cells. Tail length varied from 4 to 6 μm (mean value of 5 μm) for *R. conorii*, 4 to 12 μm (with a mean value of 5 μm) for *L. monocytogenes* and 5 to 15 μm (mean value of 7 μm) for *S. flexneri*. The mean width of the *R. conorii*, *S. flexneri* and *L. monocytogenes* tails were 1.5, 0.7 and 1 μm, respectively. We then examined the filaments. In all cases, they were oriented with the barbed ends towards the pole of the bacterium, as observed by Tilney et al. (1992a,b) for *L. monocytogenes*.

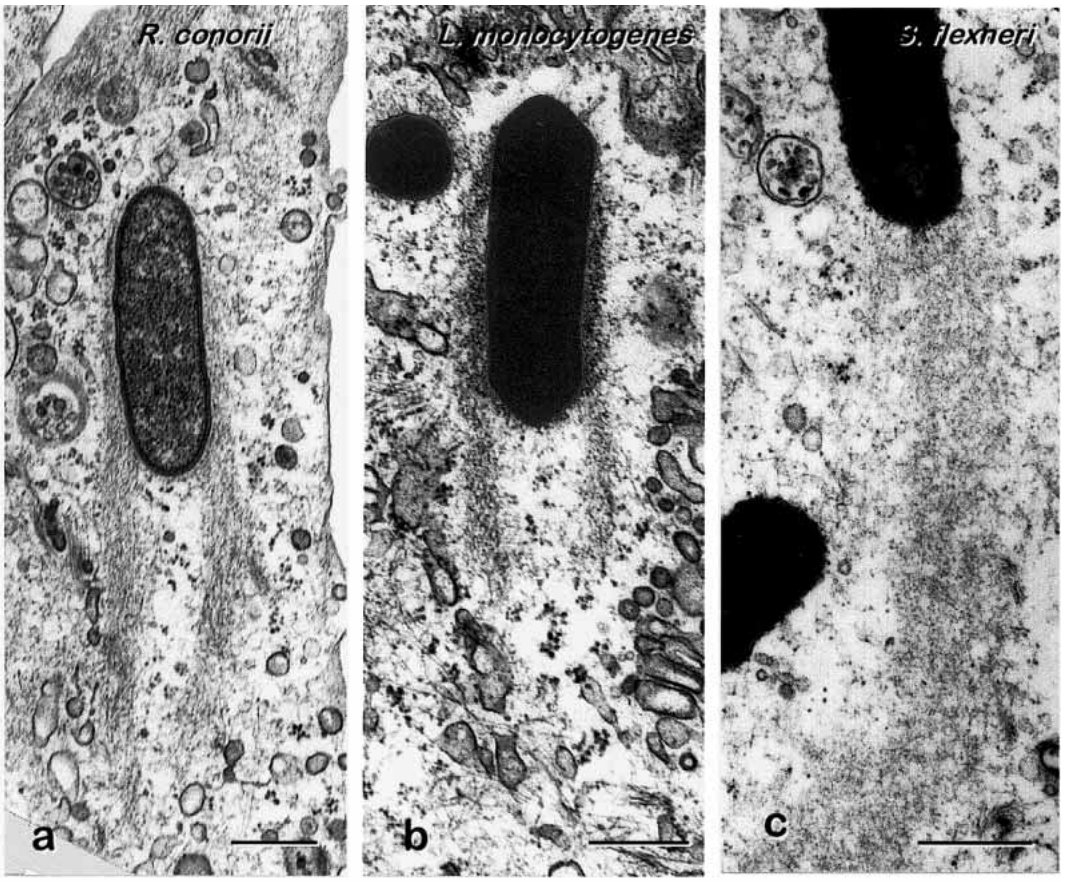

Fig. 4. Ultra thin sections of intracytoplasmic *R. conorii* (a), *L. monocytogenes* (b) and *S. flexneri* (c) in HEp2 cells. For fixation, cells were infected for 48h with *R. conorii*, 5 hours for *L. monocytogenes* and 1.5 hours for *S. flexneri*. Bars: 0.5 μm.

In *R. conorii* tails (Figs 5 and 6a), actin filaments were strikingly long (0.3 to 3 μm) and nearly parallel. Their density was low (~20 filaments per section at a distance of 1 μm from the bacterial pole) and identical all along the tail. The barbed ends of the filaments appeared attached to the bacterial surface. In *L. monocytogenes* and *S. flexneri* tails (Fig. 6b,e), the density of the filaments was higher in the vicinity of the bacterium, compared to the rest of the tail. At 1 μm from the bacterial pole, filament number was too high to be evaluated. Filaments were short, ranging from 0.1 to 1 μm, with most filaments having a length of about 0.1 μm. Filaments appeared crosslinked and forming a branching network. In the case of *Listeria* but not for *Shigella*, longer filaments ranging from 0.3 to 1.2 μm were observed along the sides of the tails.

Rickettsia protrusions were shorter than those of *Listeria* and *Shigella* (Fig. 7a,b). The parallel filaments observed in *Rickettsia* intracytoplasmic tails appeared to form tight bundles in the protrusions. In *Listeria* and *Shigella* protrusions, as shown in (Fig. 7c,d), actin filaments were similar to those in the intracytoplasmic tails, i.e. with a higher density at the front of the tail but they were more bundled towards the end of the tail, at the base of the protrusion.

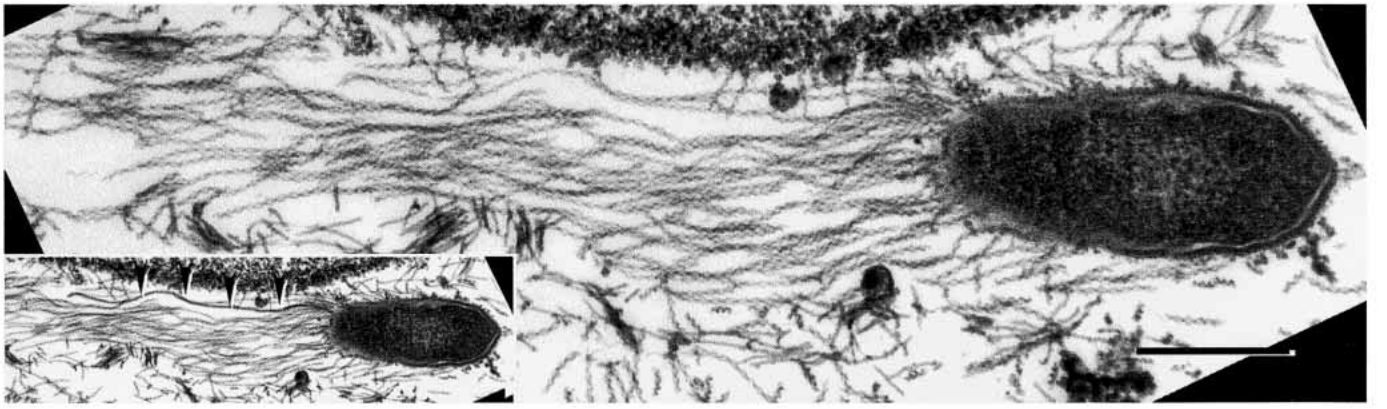

Fig. 5. Electron microscopy of myosin S1 decorated *R. conorii* actin tail. Inset, a long actin filament has been darkened and pinpointed by arrowheads. Bar, 0.5 μm.

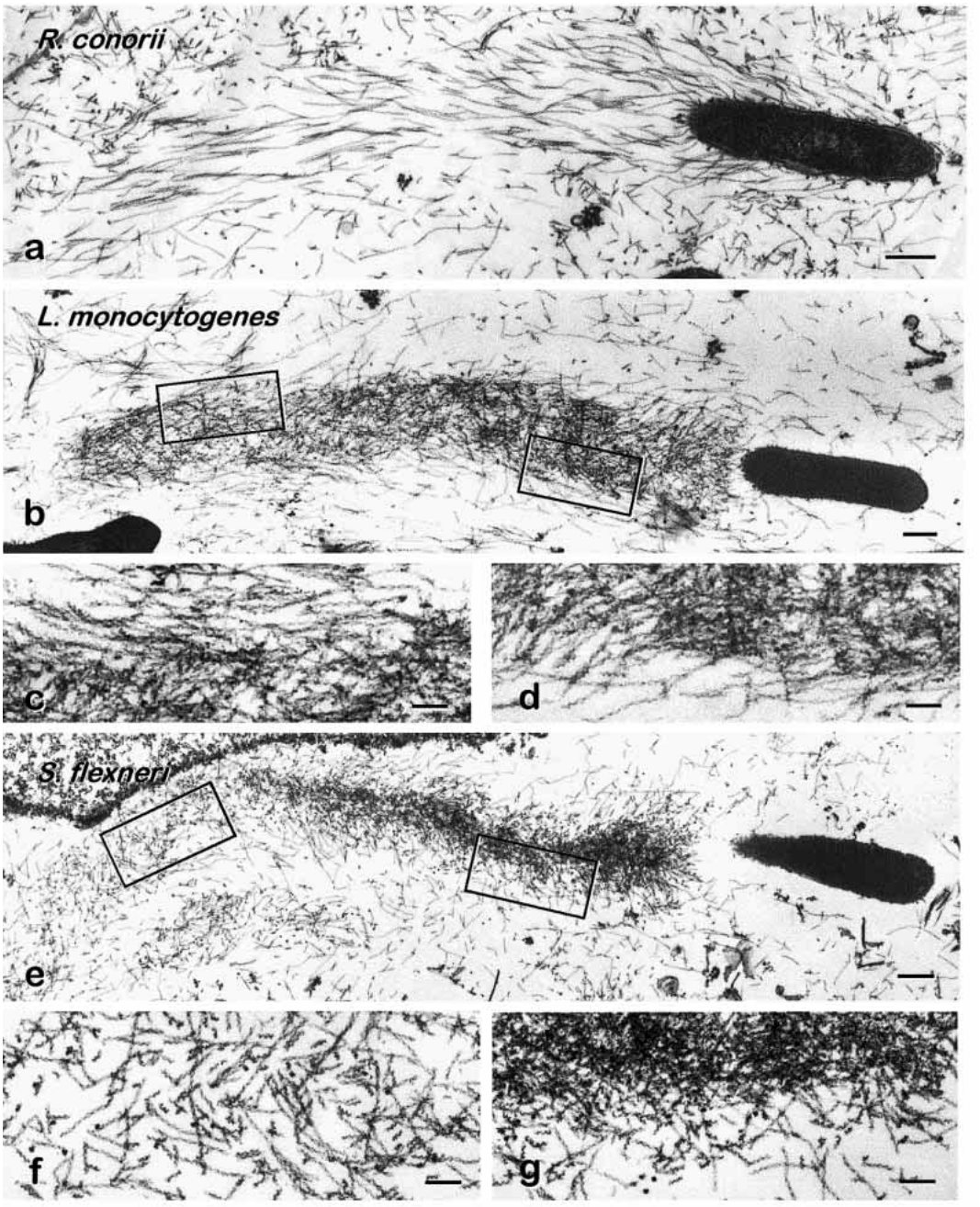

Fig. 6. Electron microscopy of myosin S1 decorated actin tails of *R. conorii* (a), *L. monocytogenes* (b) and *S. flexneri* (e) in HEp2 cells. The two boxes in b and e are shown at higher magnification in c and d, and in f and g, respectively. Bars: (a,b) 0.2 μm; (e) 0.4 μm; (c,d,f,g) 50 nm.

Localization of actin-binding proteins in actin-comet tails

Localization of actin-binding proteins was studied by immunofluorescence (Fig. 8). Results are summarized in Table 2. We detected VASP throughout the *Rickettsia* tails. As previously reported, it also colocalized with actin in *Shigella* tails and was concentrated at the front of the *Listeria* tails (Chakraborty et al., 1995). α-Actinin colocalized with actin in cytoplasmic tails of *Rickettsia*, *Listeria* and *Shigella* and in *Rickettsia* and *Shigella* protrusions. It was absent from *Listeria* protrusions as previously reported (Sechi et al., 1997). Ezrin was not detected in *Rickettsia* tails or protrusions. As reported by Sechi et al. (1997), it was present in *L. monocytogenes* protrusions and absent in intracellular *Listeria* tails. In *Shigella* intracellular tails and protrusions, ezrin was barely detectable. Arp3 colocalized with *Listeria* tails as previously described (Welch et al., 1998) and with *Shigella* tails, in contrast to preliminary results (Laine et al., 1997). Arp3 was not detected in *Rickettsia* actin tails. Cofilin and capZ colocalized with actin in *Listeria* tails as reported (David et al., 1998) and in *Shigella*

Table 2. Actin binding proteins in *R. conorii*, *L. monocytogenes* and *S. flexneri* actin tails

	Listeria	*Shigella*	*Rickettsia*
VASP	+	+	+
α-Actinin	+	+	+
Ezrin	+	+	–
Arp3	+	+	–
Cofilin	+	+	–
capZ	+	+	–

tails, but were not detected in *Rickettsia* tails. As reported, vinculin and N-WASP were detected in *Shigella* but not in *Listeria* tails (Suzuki et al., 1998; Temm-Grove et al., 1994). These proteins were also absent in *Rickettsia* tails.

DISCUSSION

In this report, we have compared the actin-based motilities of *Rickettsia*, *Listeria and Shigella* by use of video-microscopy, immunofluorescence and electron microscopy including S1 myosin decoration. Our results suggest that for actin motility, *Rickettsia* may use an original mechanism different from that used by the two other bacterial species.

R. conorii has an infectious cycle similar to those of *L. monocytogenes* and *S. flexneri*. It enters non-phagocytic cells and gets internalized into a phagosome. After lysis of this vacuole, *Rickettsia*, free in the cytosol, recruit cellular actin, move intracellularly and spread from cell to cell after formation of a protrusion and a two-membrane vacuole. In contrast to *Shigella* and *Listeria* whose actin tails appear 2-3 hours after infection, *Rickettsia* actin tails only appear after 24-36 hours of infection. This longer lag time necessary for movement initiation may be related to the doubling time of *Rickettsia* which is about 8-9 hours compared to 1 hour for *Listeria* and 40 minutes for *Shigella*. This interpretation would be in agreement with the observation that de novo synthesis of rickettsial proteins is required for movement (Heinzen et al., 1993).

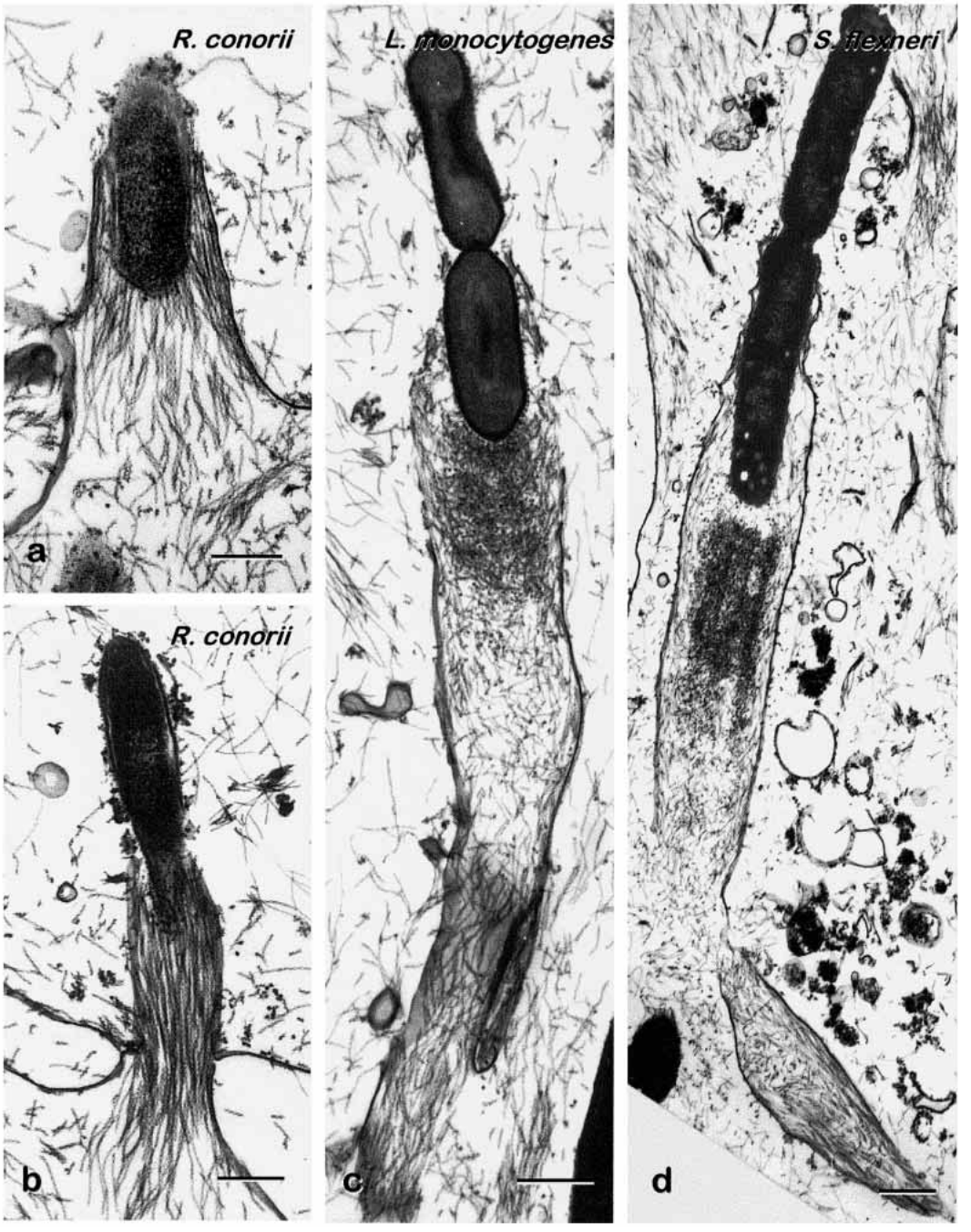

Fig. 7. Electron microscopy of S1 myosin decorated actin tails of *R. conorii* (a,b), *L. monocytogenes* (c) and *S. flexneri* (d) inside Hep2 cell protrusions. Bars, 0.5 μm.

We have measured the speed of *R. conorii* in mammalian cells and *Xenopus* egg extracts. Whereas *S. flexneri* is unable to move in *Xenopus* egg extracts, *R. conorii* can move at 2 μm/minute, a speed six times slower than that of *L. monocytogenes*. In Vero cells, the speed of *R. conorii* was 8 μm/minute, a speed three times slower than those of *L. monocytogenes* and *S. flexneri*. Since for *Listeria*, a direct correlation between tail length and motility had been demonstrated in cell-extracts (Theriot et al., 1992), we had anticipated that *Rickettsia* and *Listeria* which have similar tail length in Vero cells would move at similar speeds. It is not the case. These differences are probably be due to intrinsic

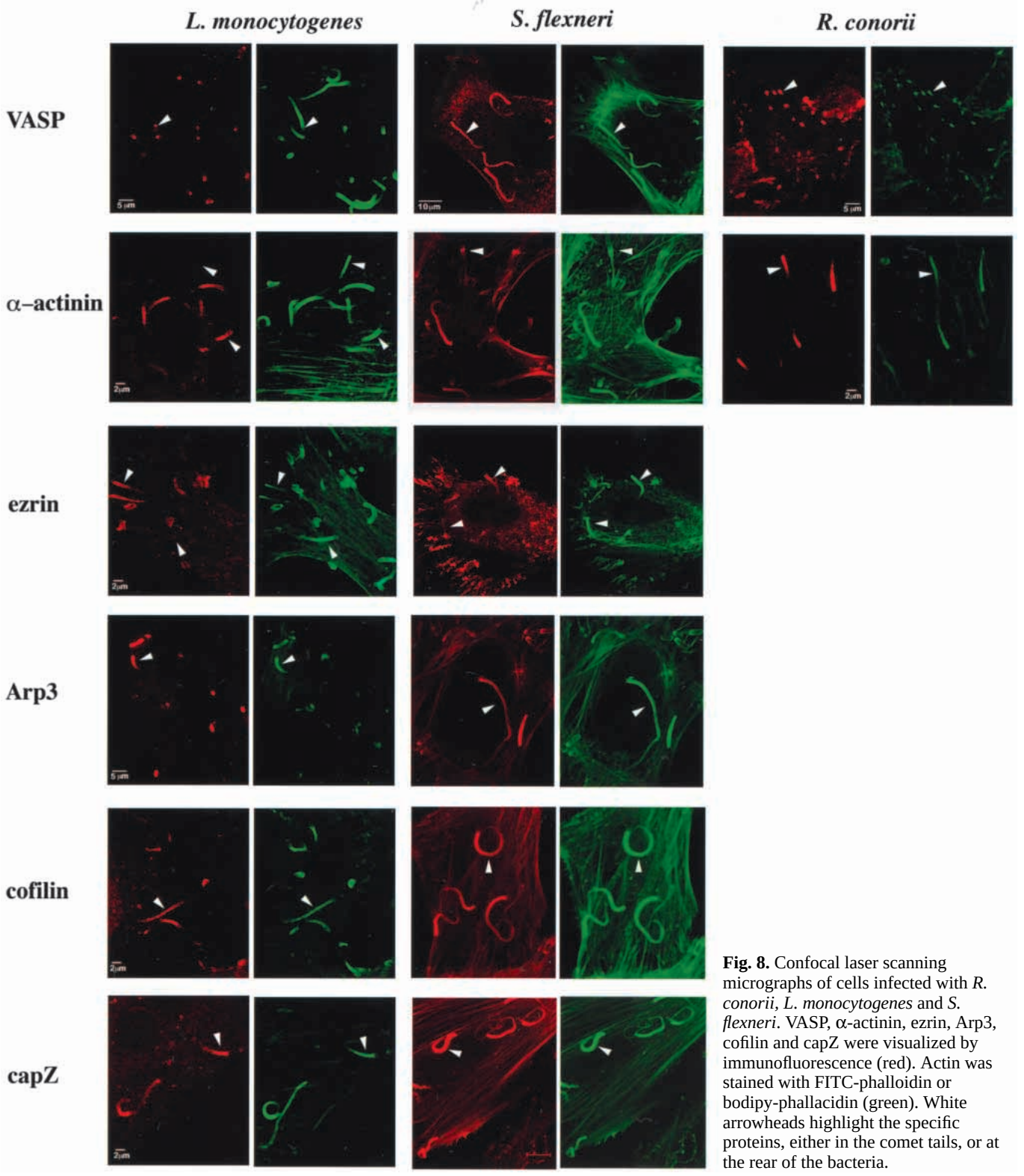

Fig. 8. Confocal laser scanning micrographs of cells infected with *R. conorii*, *L. monocytogenes* and *S. flexneri*. VASP, α-actinin, ezrin, Arp3, cofilin and capZ were visualized by immunofluorescence (red). Actin was stained with FITC-phalloidin or bodipy-phallacidin (green). White arrowheads highlight the specific proteins, either in the comet tails, or at the rear of the bacteria.

differences in structural and dynamic properties of *Rickettsia* and *Listeria* tails affecting both the polymerization and the depolymerization process (see below).

We have used S1 decoration to analyse the position, orientation and length of actin filaments in the actin tails of the three bacterial species. Actin filaments in the protrusions containing the bacteria were also examined. For *Rickettsia*, most filaments appeared anchored to the bacterial body, in a more or less parallel fashion and with an average length of 3 μm. In the protrusions, they formed a tight bundle. In the case of intracytoplasmic *Listeria* and *Shigella* tails, filaments were more rarely attached to the bacterial surface but we do not exclude that this difference may be due to the technique used. For both *Shigella* and *Listeria*, filaments were short (0.1 to 0.2 μm) and randomly oriented, with a higher density in the front of the tail forming a sort of fan as previously observed (Tilney and Portnoy, 1989). In the case of *Listeria*, long filaments (0.3 to 1.2 μm) were detected along the sides of the tail. In *Shigella* and *Listeria* protrusions, filament organization was similar to that of intracellular tails but at the base of the protrusion, filaments appeared more bundled. A recent report has described long actin filaments in *Listeria* protrusions isolated from a cell line where protrusions are particularly long (Sechi et al., 1997). Our present study indicates that actin tails in protrusions may represent a situation different from intracytoplasmic tails. In addition, tail organization may vary according to the cell type used for infection. Taken together, *Shigella* and *Listeria* actin tails seem rather similar, although *Shigella* does not display the longer filaments on the sides of the comet. In contrast, *Rickettsia* tails are organized differently, suggesting a different polymerization process. It is possible that because actin filaments in *Rickettsia* tails are more tightly attached to the bacterial body, they are less accessible to actin monomers for further polymerization, explaining the slow movement of *Rickettsia*. The filaments may also be less accessible to capping proteins preventing their dissociation and appearance of new nucleation sites, leading to a mechanism more related to treadmilling.

Several cytoskeletal proteins have been detected in the *Listeria* or *Shigella* actin tails but their role in actin-based motility has only been established for a few of them (Lasa et al., 1998). The only protein shown to physically interact with ActA is VASP (Chakraborty et al., 1995) or its homologue Mena (Gertler et al., 1996), while IcsA interacts with vinculin and N-WASP (Laine et al., 1997; Suzuki et al., 1996, 1998). VASP colocalizes with the front of the *Listeria* tails. In *Shigella*, it is present all along the tail but no direct interaction with IcsA has been reported. Since vinculin is known to interact with VASP (Brindle et al., 1996; Reinhard et al., 1996), it has been proposed that vinculin could play the role of an adaptor between IcsA and VASP (Laine et al., 1997). In the case of *Rickettsia*, VASP is also present all along the tails but vinculin and N-WASP are not detected. These data suggest that *Rickettsia* behave differently from *Listeria* and *Shigella*. Whether *Rickettsia* express a protein able to interact directly with VASP, or use another adaptor, is unknown. Alternatively, it is possible that the amount of vinculin present in the *Rickettsia* tails is below the detection level.

Recently, the role of the Arp2/3 complex in actin-based motility has received a lot of attention and a breakthrough in the field has been brought by Welch et al. (1998) who showed that this complex in conjunction with ActA and even with only the N-terminal part of ActA can efficiently polymerize actin. In *Listeria*-infected cells, this complex is localized around bacteria before tail formation and in actin tails in locomoting bacteria (David et al., 1998; Welch et al., 1997a,b). In *Shigella*, in contrast to preliminary observations (Laine et al., 1997), we have detected the Arp2/3 complex in the actin tails. In *Rickettsia* tails, this complex was undetectable. Interestingly, the Arp2/3 complex can initiate actin polymerization, and remain associated with the pointed ends of the nascent actin filaments. Moreover it can associate with filamentous actin generating a branched network with new filaments oriented at 70° with respect to the first filament (Mullins et al., 1998a,b). That the Arp2/3 complex was undetectable in *Rickettsia* actin tails seems to correlate with the absence of branching in *Rickettsia* tails. The branching capacity of Arp2/3 was proposed to be responsible for the explosion of actin polymerization in *Listeria* actin tails as well as at the leading edge of moving cells. We propose that the slow rate of movement of *Rickettsia* may at least in part be due to the absence of Arp2/3.

Cofilin has been demonstrated to increase the dynamics of the actin polymerization process by stimulating depolymerization at the pointed ends and providing actin monomers for polymerization (Carlier et al., 1997; Theriot, 1997). The severing activity of cofilin may also contribute to the dynamics of actin polymerization (Theriot, 1997). Cofilin is present in *Listeria* actin tails (David et al., 1998). We have shown here that it is also present in *Shigella* tails and absent in *Rickettsia* tails. These results are also in agreement with the slower movement of *Rickettsia*. It remains, however, possible that the lack of detection of cofilin is due to its low concentration in the actin tails.

α-Actinin is critical for *Listeria* motility (Dold et al., 1994). We confirmed that this protein is present in intracellular *Listeria* tails but absent in *Listeria* protrusions (Sechi et al., 1997). It is present in *Shigella* and *Rickettsia* tails. α-Actinin is a cross-linking protein and it has been proposed that cross-linking of actin filaments provide the bacteria with a rigid platform from which they can start moving (Sanger et al., 1992). α-Actinin has two actin binding sites which may link actin filaments with a flexible spacer of about 50 nm and thus form loose bundles, a situation which is found in *Rickettsia* tails.

We have recently identified the barbed end capping protein capZ in *Listeria* tails (David et al., 1998). This protein is also present in *Shigella* tails reinforcing the similarities between *Listeria* and *Shigella* tail structures. CapZ was undetectable in *Rickettsia* tails. The small number of actin filaments might be responsible for this lack of detection.

Taken together, our data suggest that the actin assembly process in *R. conorii* is different from that of *Listeria* and *Shigella*. Although we did not compare *R. conorii* with vaccinia virus in this study, it is to be noted that the S1 decoration images reported by Cudmore et al. (1996), are very similar to ours in the case of *Listeria* and *Shigella*, with branched network of actin filaments. Actin polymerizing pathogens would thus fall in two categories: the first including *Listeria*, *Shigella* and vaccinia virus, the second *Rickettsia*. For *Listeria* and *Shigella*, the bacterial genes involved in the process have been identified (Bernardini et al., 1989; Domann

et al., 1992; Kocks et al., 1992; Lett et al., 1989; Makino et al., 1986). We have thus tested whether homologous genes are present in *R. conorii* but our attempts to detect *actA* or *icsA* homologues in *Rickettsia* DNA have failed, even when using very low stringency hybridization conditions. The DNA sequence of another *Rickettsia*, *R. prowazekii* (Andersson et al., 1998), has recently been reported but we could not detect an *actA* or *icsA* homologue in this genome. The nature of the rickettsial factor involved in actin-based motility thus remains to be established. For *Listeria* and *Shigella*, *actA* and *icsA* are well established virulence genes. Mutations in *actA* or *icsA* clearly attenuate virulence (Bernardini et al., 1989; Brundage et al., 1993; Kocks et al., 1992). For *Rickettsia*, whether actin-based motility represents a true virulence factor remains elusive. Indeed, *Rickettsia* species are divided into two groups, the typhus group (TG) that includes the highly virulent *R. prowazekii* and the spotted fever group (SFG) to which *R. conorii* and *R. rickettsii* belong. *R. prowazekii* resides inside the cytosol but does not polymerize actin (Heinzen et al., 1993). In same TG group, the pathogenic species *R. typhi* polymerizes actin albeit very inefficiently. In contrast, all SFG *Rickettsia* appear to polymerize actin, but some are not pathogenic. For example, *R. montana* is not pathogenic although able to polymerize actin. It will thus be of the highest interest to decipher what is the critical role of actin polymerization during infection with these different rickettsial species.

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