Actin-dependent movement of bacterial pathogens

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Abstract | *Listeria*, *Rickettsia*, *Burkholderia*, *Shigella* and *Mycobacterium* species subvert cellular actin dynamics to facilitate their movement within the host cytosol and to infect neighbouring cells while evading host immune surveillance and promoting their intracellular survival. 'Attaching and effacing' *Escherichia coli* do not enter host cells but attach intimately to the cell surface, inducing motile actin-rich pedestals, the function of which is currently unclear. The molecular basis of actin-based motility of these bacterial pathogens reveals novel insights about bacterial pathogenesis and fundamental host-cell pathways.

Lamellipodia
Sheet-like protrusions of the plasma membrane associated with exploratory cell movements and cell motility. Composed of orthogonal, highly crosslinked actin arrays. The Rho GTPase Rac has a crucial role in lamellipodia formation through activation of NPFs.

Filopodia
Protrusions of the plasma membrane with finger-like morphology reaching 200 µm in length. Composed of dynamic bundles of actin filaments. Associated with rapid membrane remodelling, transient matrix adhesions and cell motility. Cdc42 has a crucial role in initiating filopodia formation.

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After invasion of host cells, some members of the genera *Listeria*, *Shigella*, *Rickettsia*, *Mycobacterium* and *Burkholderia* gain access to the cytosol by lysis of the entry vesicle. Once in the cytosol, these organisms are propelled by polymerization of the eukaryotic cytoskeletal protein actin (BOX 1) at the surface of one pole of the bacterium. Intracellular motile bacteria can reach speeds of 3 to 87 µm per minute, depending on the cell type and the pathogen. Bacterial motility can lead to the formation of host-cell-membrane protrusions tipped by intracellular bacteria that project into adjacent cells. These protrusions can be engulfed and lysed by neighbouring cells by a process known as paracytophagy, which is facilitated by bacterial proteins. Paracytophagy results in spreading of infection from one cell to the next in the absence of immune surveillance[1,2]. Actin-based motility can facilitate intracellular survival in other ways. For instance, rapid actin-based motility might assist *Listeria monocytogenes* in avoiding recognition by the ubiquitin degradation system[3]. The central role of actin-based motility in bacterial virulence is reflected in the marked attenuation of defined non-motile mutants of *L. monocytogenes*[4], *Shigella flexneri*[5–7] and *Burkholderia pseudomallei* (M.P.S., E.E.G. & G.J. Bancroft, unpublished results). As such, bacterial factors that control actin assembly are often key determinants of virulence and attractive targets for intervention.

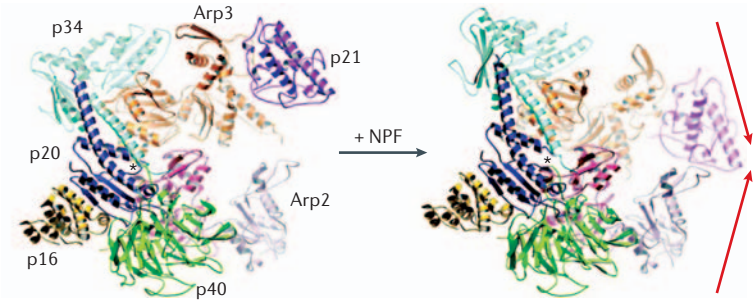
This field has been described previously, most recently by Gouin *et al.*[8] In this review, we describe the most recent advances in the understanding of the mechanisms of intracellular motility of both *B. pseudomallei* and *Mycobacterium marinum*. This review also focuses on the group of 'attaching and effacing' (AE) *Escherichia coli* that do not enter host cells, but attach intimately to the surface of enterocytes and form motile and undulating actin-rich structures termed pedestals, possibly facilitating spread to adjacent cells in the intestinal tract (FIG. 1). Recent studies reviewed below have revealed differences in the mechanisms by which AE pathogens recruit and activate cellular components to elicit pedestal formation.

Mechanisms of actin-based motility

The interaction between the eukaryotic-cell plasma membrane and the underlying actin cytoskeleton has a central role in cell motility, morphogenesis and phagocytosis. However, because it is difficult to dissect such interactions at the molecular level, little is known about the factors that participate in the nucleation of actin filaments at these sites. The formation of actin-enriched structures by bacterial pathogens provides a convenient model to identify cellular factors that are recruited to sites of actin assembly and to dissect their role in actin dynamics. The actin tails formed by *Listeria*, *Shigella* and *M. marinum*, and the actin-rich arrays in the pedestal structures induced by AE *E. coli*, are composed of short, crosslinked actin filaments that rapidly turn over, reminiscent of those at the leading edge of lamellipodia of motile cells. By contrast, the actin tails formed by spotted-fever-group *Rickettsia* species are composed of long, unbranched actin filaments that are similar to those found in filopodia. Identification and structure–function analysis of the bacterial factors that mediate actin-based motility of these pathogens has offered fascinating insights into the protein–protein interactions and signalling pathways that lead to lamellipodia and filopodia formation.

Many of the bacterial factors that are necessary for intracellular motility are expressed at the pole of the

Box 1 | **Regulation of actin polymerization**



Actin is one of the most abundant proteins in eukaryotic cells and exists in two forms, ATP-bound monomeric (G) actin and ADP-bound filamentous (F) actin. Filaments are assembled by the reversible polymerization of monomers at the fast-growing barbed (plus) end rather than the pointed (minus) end. A key protein that regulates polarized growth is profilin, which binds G-actin and funnels monomers to the barbed end of filaments. Polymerization requires ATP hydrolysis and is tightly regulated by monomer- and filament-binding proteins that also maintain the free monomer pool and mediate capping, crosslinking, bundling or severing of actin filaments[91]. An initial nucleation step creates free barbed ends by uncapping or severing of filaments or *de novo* nucleation of monomers. However, actin nucleation from free monomers is kinetically unfavourable and has to be stimulated by cellular factors. Actin-nucleating factors include formins[92], *Drosophila* Spire[93] and the Arp (actin-related protein) 2/3 complex[94].

The best characterized of these is the highly conserved seven-subunit Arp2/3 complex, which comprises two Arp proteins and five additional protein subunits; p16, p20, p21, p34 and p40 (see the figure). The Arp2/3 complex requires activation by proteins known as nucleation-promoting factors (NPFs) such as Wiskott–Aldrich syndrome protein (WASP)-family members. The Arp2 and Arp3 subunits are structurally related to actin, and conformational changes induced by NPFs might allow these subunits to mimic barbed ends to serve as a template for polymerization. Arp2/3 can also crosslink newly formed filaments into Y-branched orthogonal arrays, creating actin networks. NPFs often share conserved domains, including proline-rich regions, WASP homology domain-2 (WH2) and central and acidic (CA) domains. Proline-rich regions recruit profilin and many regulatory proteins, whereas WH2 and CA domains respectively bind monomeric actin or the Arp2/3 complex.

In eukaryotic cells, the NPFs WASP and neural (N)-WASP are in an autoinhibited conformation, which is relieved by the binding of the Rho family GTPase Cdc42 and phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P_2). This results in Arp2/3 activation and initiation of actin polymerization.

The intracellular bacteria discussed in this review either mimic NPFs or NPF activators to initiate actin polymerization at their surfaces in the cytosol. However, pedestal-inducing enteropathogenic *E. coli* stimulate N-WASP-dependent actin polymerization at the plasma membrane through a Cdc42-independent mechanism. This is facilitated by the host-cell adaptor protein Nck, which can substitute functionally for Cdc42 in the activation of N-WASP at tyrosine-phosphorylated Nck-binding sites[95].

The figure shows a ribbon diagram of the atomic structure of the bovine Arp2/3 complex. The inactive form of Arp2/3 is shown on the left. The model proposed by Robinson *et al.*[96] for the active form of the Arp2/3 complex involves a conformational change that brings Arp2 (shown in grey) and Arp3 (shown in orange) subunits together (indicated by red arrows) so that they mimic the barbed ends of actin filaments and might serve as a template for actin polymerization. Figure reproduced with permission from REF. 96 © (2001) American Association for the Advancement of Science.

bacterium from which the actin-rich tail forms (for example, *Listeria* ActA[9], *Shigella* IcsA[10] and *Burkholderia* BimA[11]) and are also expressed at high levels within the host cytosol (*Listeria* ActA[12], *Shigella* IcsA[13] and *Mycobacterium* motility factor[14]). In some instances, expression of the bacterial factor has been shown to be growth-phase dependent (*Shigella* IcsA[15]).

The strategies used by intracellular bacterial pathogens to induce polar actin polymerization, and those used by AE *E. coli* to elicit pedestal formation at the plasma membrane, involve the recruitment and activation of the cellular actin-related protein (Arp)2/3 complex. However, pathogens that show actin-based motility can be separated into two groups depending on whether or not they mimic a cellular nucleation-promoting factor (NPF) (for example, *Listeria* ActA and *Rickettsia* RickA) or whether they require the recruitment of a cellular NPF to the bacterial surface to promote Arp2/3-mediated motility (for example, *Shigella* IcsA and AE *E. coli* Tir) (TABLE 1).

Facultative intracellular pathogen
A pathogen that can replicate either within the host cell or as a cell-free unit.

Pathogens expressing NPF mimics

Listeria monocytogenes. *L. monocytogenes* is a Gram-positive facultative intracellular pathogen. Infections are frequently acquired through the oral route and can result in gastroenteritis and severe systemic sequelae, including meningitis, septicaemia and miscarriage. *L. monocytogenes* induces its own uptake into non-phagocytic cells, replicates in the host-cell cytoplasm and spreads from cell to cell by actin-based motility. Identification and characterization of the bacterial and cellular factors that are required for actin-based motility of *L. monocytogenes* have contributed greatly to the understanding of cellular actin dynamics. Most notably, *Listeria* research has led to the characterization of the cellular Arp2/3 complex as a key actin-nucleating component[16,17] and has opened the way to studying actin-filament assembly in the absence of the plasma membrane using *in vitro* reconstitution systems.

The *L. monocytogenes* factor that mediates actin-based motility is the product of the *actA* gene[4,18]. ActA is encoded in the lecithinase operon, upstream of a gene

Wiskott–Aldrich syndrome protein
(WASP). The prototypic member of a family of proteins that stimulate the low intrinsic nucleation activity of the Arp2/3 complex.

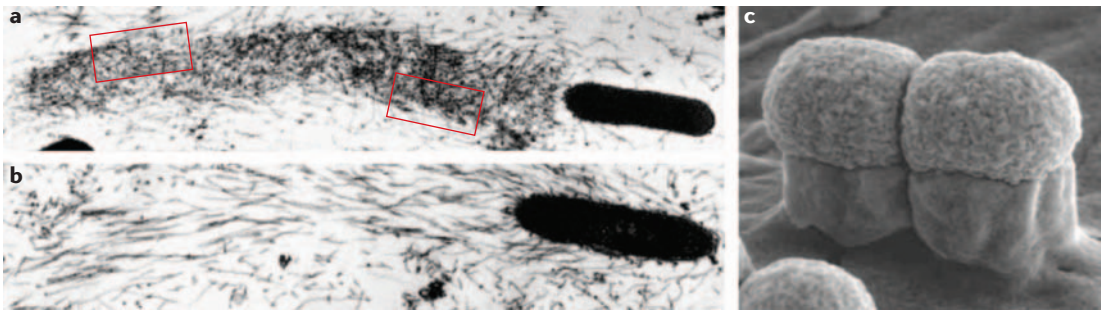


Figure 1 | **Interaction of bacterial pathogens with actin.** The figure shows transmission electron micrographs of myosin-S1-decorated actin tails that are induced by the intracellular pathogens *Listeria monocytogenes* (**a**) and *Rickettsia conorii* (**b**). In *R. conorii* tails, actin filaments are long and arranged in parallel. In *L. monocytogenes*, filaments are short and highly crosslinked, displaying characteristic Y-branched structures. The density of the filaments is higher closer to the bacterium compared with the rest of the tail (boxed regions). Part **c** shows a scanning electron micrograph of actin-rich pedestals induced by enteropathogenic *Escherichia coli*. Parts **a** and **b** are reproduced with permission from REF. 34 © (1999) The Company of Biologists Ltd. Part **c** is reproduced with permission from REF. 97 © (2004) John Wiley & Sons Ltd.

that encodes a phospholipase (PlcB) that is required for lysis of membrane protrusions surrounding bacteria during intercellular spread[19,20]. ActA is both necessary and sufficient for actin-based motility, as attachment of ActA to *Streptococcus pneumoniae* and expression of ActA in the related, but non-pathogenic, *Listeria innocua* conferred actin-based motility on these otherwise non-motile bacteria in *Xenopus* oocyte extracts[21,22]. Furthermore, ActA-coated beads can polymerize actin and undergo motility in a cell-free system in the absence of other bacterial factors[23]. *In vitro* reconstitution experiments have shown that ActA can promote actin-based motility of coated beads in cell-free extracts that contain only purified Arp2/3, CapZ, cofilin and ATP, but does not require Wiskott–Aldrich syndrome protein (WASP)-family members[24–26]. As ActA shares similar domains with WASP-family members, and *L. monocytogenes* forms normal actin tails in neural-WASP (N-WASP)$^{-/-}$ cells[27],

Table 1 | **Actin-based motility of intracellular bacterial pathogens**

	L. monocytogenes	*R. conorii/ rickettsii*	*B. pseudomallei*	*S. flexneri*	*M. marinum*
Bacterial factor required	ActA	RickA	BimA	IcsA	Unknown
Polar localization of the factor?	Yes	Unclear, surface exposed	Yes	Yes	Unknown
Actin-tail morphology	Short, highly crosslinked filaments	Long filaments in parallel, bundled in protrusions	Unknown	Short, highly crosslinked filaments	Short, highly crosslinked filaments
Rates of movement	10–87 μm per minute	5–8 μm per minute	Unknown	3–26 μm per minute	11 μm per minute
Homologues identified in other species	Homologue identified in *L. ivanovii*[98,99], complements *L. monocytogenes actA* mutant[99]	Genes encoding RickA homologues identified in all SFG *Rickettsia* species, absent from typhus group[38,39]	BimA homologue identified in pathogenic *B. mallei* and the non-pathogenic *B. thailandensis*[51]	Close homologues in all virulent strains of *S. dysenteriae*, *S. flexneri*, *S. boydii*, *S. sonnei* and in EI *E. coli*[5]	None described to date
Mechanism of tail formation	ActA functions as an Arp2/3-dependent NPF (WASP) mimic, activating Arp2/3 *in vitro* without WASP-family members[17,24]	Bacterial factor functions as an Arp2/3-dependent NPF (WASP) mimic, activating Arp2/3 *in vitro* without WASP. Overexpression of WCA domain inhibits motility[38,39]	N-WASP-independent, probably an NPF (WASP) mimic, involvement of Arp2/3 not clear, BimA does not interact with Arp2/3 or activate Arp2/3-dependent actin polymerization *in vitro*[11]	Arp2/3 and N-WASP-dependent, Cdc42 mimic	Largely unknown but probably NPF (WASP) and Arp2/3 dependent
Role in cell-to-cell spread?	Yes, *actA* mutant does not form plaques[18]	Unknown	Unknown	Yes, *icsA* mutants do not form infective foci in cell culture[5,6]	Unknown
Role in virulence?	Yes[4]	Unknown	Yes (G.J. Bancroft, E.E.G. & M.P.S., unpublished results)	Yes[5–7]	Unknown

Arp2/3, actin-related protein-2/3; *B. mallei*, *Burkholderia mallei*; *B. pseudomallei*, *Burkholderia pseudomallei*; *B. thailandensis*, *Burkholderia thailandensis*; EI *E. coli*, enteroinvasive *Escherichia coli*; *L. ivanovii*, *Listeria ivanovii*; *L. monocytogenes*, *Listeria monocytogenes*; *M. marinum*, *Mycobacterium marinum*; NPF, nucleation-promoting factor; N-WASP, neural WASP; *R. conorii*, *Rickettsia conorii*; *R. rickettsii*, *Rickettsia rickettsii*; *S. boydii*, *Shigella boydii*; *S. dysenteriae*, *Shigella dysenteriae*; *S. flexneri*, *Shigella flexneri*; *S. sonnei*, *Shigella sonnei*; SFG, spotted fever group; WCA, WH2, central and acidic; WASP, Wiskott–Aldrich syndrome protein.

Src homology-3 (SH3) domain
A small module of ~50 amino-acid residues found in proteins that interact with proline-rich motifs.

Focal adhesion sites
Adhesions by which cells attach to the underlying substrate. Many structural, cytoskeletal and signalling proteins are concentrated in these structures.

Stress fibres
Contractile filaments involved in the maintenance of cell shape that support the motile apparatus of the cell. At least one end is anchored by a focal adhesion site.

Central and acidic (CA) domain
Forms part of a larger domain called a WCA domain. This region of WASP-family proteins stimulates the Arp2/3 complex.

WASP homology-2 (WH2) domain
WASP homology-2 domain motifs are composed of approximately 35 amino acids and are conserved in cellular proteins that recruit actin monomers.

it is thought that ActA functions as an NPF, directly activating the Arp2/3 complex by mimicking the activity of WASP.

In infected cells, immunocytochemistry has shown that the ActA protein is asymmetrically distributed on the bacterial surface, and is localized at the site of actin-tail formation[9]. The mature ActA protein is expressed on the bacterial surface as a protein comprising 610 amino acids with three functional domains (FIG. 2). The N-terminal domain, composed of the first 233 amino-acid residues, contains all the necessary residues for motility in cultured cells and cell-free extracts[28,29] and can stimulate the activity of the Arp2/3 complex *in vitro*[17]. The central domain (amino acids 234–394), which contains proline/glutamic-acid-rich repeats reminiscent of Src homology-3 (SH3) domains, is not essential for motility, but deletion of this region shortens actin tails in infected cells and decreases the velocity of movement in *Xenopus* oocyte extracts[28]. Indeed, the number of proline-rich repeats in this region has been correlated with the velocity of movement, with each contributing to ~2.5 μm per minute[30]. The hydrophobic C-terminal portion of ActA probably constitutes a membrane anchor[18], which tethers ActA to the bacterial surface after it secretion. Several host-cell proteins have been localized to the actin tails of *L. monocytogenes* in infected cells. Their importance in motility has been extensively reviewed elsewhere and is summarized in Supplementary information S1 (table).

The proline-rich region and C-terminal portion of ActA shows significant sequence similarity with zyxin, an actin-binding protein that is associated with focal adhesion sites and stress fibres[31]. Interestingly, although ActA mimics WASP-family proteins in its activation of Arp2/3-dependent actin assembly in host cells, ActA and zyxin also harbour similar Arp2/3-independent actin-polymerization activities *in vitro*[32]. From *in vitro* reconstitution experiments, it has been proposed that *Listeria* propulsion in host cells might involve an initial Arp2/3-dependent nucleation step followed by an Arp2/3-independent tail-elongation phase that requires the actin-filament-bundling protein fascin[33].

Rickettsia *species.* *Rickettsia* are Gram-negative obligate intracellular bacteria that cause arthropod-borne diseases of humans, including typhus (*Rickettsia prowazekii* and *Rickettsia typhi*) and spotted-fevers (*Rickettsia conorii*, *Rickettsia rickettsii*, *Rickettsia sibirica* and *Rickettsia montana*). *Rickettsia* species of the spotted-fever group show actin-based motility in the cytosol and nucleus of host cells at 5–8 μm per minute and are capable of intercellular spread[34–37].

Comparison of the complete genome sequence of *R. conorii* with that of *R. prowazekii*, which cannot use actin-based motility, identified a 2-kb *R. conorii*-specific region that encodes a predicted protein of 517 amino acids known as RickA[38,39]. RickA contains a central proline-rich domain and a C-terminal WH2 domain followed by a region with homology to the central and acidic (CA) domains of WASP-family proteins, including an amphipathic helix predicted to bind the Arp2/3 complex[39] (FIG. 2). The RickA protein lacks predicted signal sequences for secretion or obvious hydrophobic domains that might serve as a membrane anchor. Separate analysis of the *R. rickettsii* genome for WASP-like proteins identified a 494-amino-acid RickA homologue[39]. RickA homologues are also found in the genomes of *R. sibirica* and *R. montana* and share the same general organization, although there are differences in the number and sequence of proline-rich repeats and C-terminal WASP homology-2 (WH2) domains. RickA proteins from *R. conorii*, *R. rickettsii* and *R. sibirica* have a single WH2 domain similar to WASP, whereas *R. montana* RickA

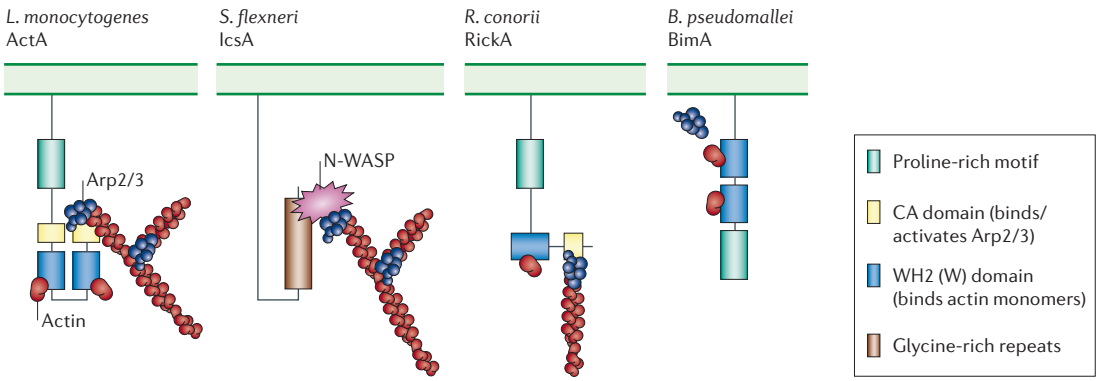


Figure 2 | **Mechanisms of intracellular bacterial actin polymerization.** Schematic diagram depicting the proposed mechanisms by which intracellular pathogens recruit and activate actin-related protein-2/3 (Arp2/3)-dependent actin polymerization at the bacterial surface. *Listeria monocytogenes* ActA directly interacts with, and activates, the Arp2/3 complex. The Wiskott–Aldrich syndrome protein (WASP) homology-2 (WH2) domain binds to actin monomers, and the central and acidic (CA) regions bind to, and activate, Arp2/3. *Shigella flexneri* IcsA functions as a Cdc42 mimic, recruiting neural (N-)WASP to the bacterial surface through a glycine-rich region, which in turn recruits and activates the Arp2/3 complex. *Rickettsia conorii* RickA interacts with, and directly activates, the Arp2/3 complex in a manner similar to that of ActA; however, *Rickettsia*-induced actin tails lack the Y-branches seen in *Listeria*-induced tails, and other bacterial or cellular factors might organize the actin filaments into unbranched arrays. The structure of *Burkholderia pseudomallei*-induced actin tails and the molecular mechanism by which they form are unclear. The schematic reflects the tertiary structure of the proteins.

has two WH2 domains similar to N-WASP[39]. Recent findings that the *rickA* gene of the non-motile and non-pathogenic spotted-fever-group organism *Rickettsia peacockii* is disrupted by a naturally occurring transposon also support a role for RickA in *Rickettsia* motility[40].

The overall structures of the identified RickA proteins indicate that they can act as NPF mimics. Indeed, purified *R. conorii* and *R. rickettsii* RickA polymerize pyrene-actin monomers *in vitro* in both an Arp2/3- and concentration-dependent manner without requiring a cellular NPF[38,39]. Expression of the C-terminal CA domain of RickA, which recruits and activates the Arp2/3 complex *in vitro*, impairs actin-based motility of *R. conorii* in Hep-2 cells[38,39]. Therefore, RickA recruits the Arp2/3 complex to the bacterial surface and seems to function as a WASP mimic to initiate Arp2/3-dependent actin polymerization.

A striking distinction between the actin tails formed by *R. rickettsii* and those of other intracellular pathogens is that they comprise several distinct bundles of long unbranched F-actin filaments[41] (FIG. 1b). *R. rickettsii* tails are distinct from those of *R. conorii*, which tend not to be bundled[34]. Consistent with the lack of Y-branched actin filaments in *Rickettsia* tails, the Arp2/3 complex is absent from the length of the tails in *Rickettsia*-infected cells[34,41,42] and localizes only to the bacteria–tail interface of motile *Rickettsia*[38].

Although *Rickettsia* actin tails are unbranched, purified *R. conorii* and *R. rickettsii* RickA can stimulate the Y-branching activity of the Arp2/3 complex *in vitro*, and Y-branches can readily be detected around *R. rickettsii* RickA-coated beads in *Xenopus* oocyte extracts[38,39]. This implies that, whereas RickA has a role in forming the actin tails, other bacterial or host factors might organize the actin filaments into the unbranched arrays seen in *Rickettsia*-infected cells. The actin-binding protein vasodilator-stimulated phosphoprotein (VASP) interacts with *Rickettsia* tails and this might influence actin-tail morphology, as VASP is thought to compete with capping proteins at the barbed ends of actin filaments, thereby increasing their length[43]. Given that Arp3 is localized only at the bacteria–tail interface during intracellular motility, it is possible that RickA initiates actin assembly by stimulating the nucleating activity of the Arp2/3 complex. Sustained motility might then involve elongation of the barbed end of tail filaments by an Arp2/3-independent mechanism, as recently suggested for *Listeria*[33]. The extent to which Arp2/3 mediates continuous actin polymerization in tails, or promotes an initial nucleation step followed by Arp2/3-independent elongation of actin filaments, might vary between the organisms described and requires further study. It has been reported that *Listeria* propulsion in host cells might involve an initial Arp2/3-dependent nucleation step followed by an Arp2/3-independent tail-elongation phase, as once motility had been initiated in an Arp2/3 dependent manner *in vitro*, propulsion could be maintained following inhibition of Arp2/3 solely in the presence of actin and fascin[33].

The role of RickA in intracellular motility, cell-to-cell spread and virulence of spotted-fever-group *Rickettsia* has yet to be proven, as genetic systems to manipulate these pathogens are unavailable. However, *in vitro* depletion and reconstitution experiments have confirmed that *R. rickettsii* RickA can promote the movement of coated beads in cell-free extracts in an Arp2/3-dependent manner. Furthermore, targeting of a chimeric RickA–CAAX protein to the inner surface of the plasma membrane of eukaryotic cells induces the formation of filopodia-like structures[38], distinct from the lamellipodia-like structures observed on expression of similarly targeted ActA[44]. RickA proteins might therefore serve as useful tools to dissect filopodia formation, and it will be of interest to determine if host proteins thought to contribute to filopodia assembly are required for actin-based motility of *Rickettsia*.

It is presently unclear whether differences in the sequence of RickA homologues from other spotted-fever-group *Rickettsia* species leads to recruitment of distinct factors or activation of the actin-nucleating and Y-branching activities of the Arp2/3 complex to differing extents. Of the typhus-group *Rickettsia* species, only *R. typhi* has been reported to show erratic actin-based motility, forming 1–3-μm straight or hooked actin tails that are shorter than those induced by *R. conorii* or *R. rickettsii*, although the rate of *R. typhi* movement is the same as *R. rickettsii*[36,37,41]. This is surprising, as the actin-tail length of *L. monocytogenes* has been correlated with velocity of actin-based motility[45]. Interestingly, analysis of the complete genome sequence of *R. typhi* failed to detect a homologue of RickA, indicating that it might use a distinct mechanism of actin-based motility[46].

Burkholderia pseudomallei. *B. pseudomallei* is a Gram-negative facultative intracellular pathogen that causes melioidosis, an invasive disease that is endemic in subtropical areas. The invasion of non-phagocytic cells by *B. pseudomallei* is dependent on a type III secretion system. After internalization, *B. pseudomallei* lyses the endocytic vacuole, forms actin tails in the cytosol and can spread from cell to cell[47]. Uniquely, *B. pseudomallei* can also induce cell fusion[48], although the role of actin-based motility in this process is unknown.

Analysis of the complete genome sequence of *B. pseudomallei* identified a putative type V secreted protein, known as BimA, which contains proline-rich motifs and WH2-like domains and which shares homology at the C terminus with the *Yersinia* type V secreted adhesin YadA. The finding that actin-based motility of *B. pseudomallei* in murine macrophages was abolished by *bimA* mutation and could be restored by transient expression of the gene *in trans* confirmed that BimA is necessary for *B. pseudomallei* motility[11] (FIG. 3). The following observations indicate that BimA might function as an NPF mimic to induce actin-based motility in infected cells. First, the transient expression of BimA results in F-actin clustering that is reminiscent of that seen on WASP overexpression[11]. Second, WASP-family members are not required for BimA-mediated actin polymerization *in vitro*[11]. Third, N-WASP is not recruited to the surface of intracellular *B. pseudomallei*[49] and, last, motility of *B. pseudomallei* is supported by N-WASP$^{-/-}$ fibroblasts[49].

CAAX motif
A motif that is post-translationally modified by farnesylation at the cysteine residue and that results in localization to the inner leaflet of the plasma membrane.

Type III secretion system
A transmembrane-spanning 'needle complex' involved in the secretion of two classes of proteins, translocators and effectors.

Type V secreted protein
A protein translocated across the outer membrane of Gram-negative bacteria through a transmembrane pore formed by a self-encoded β-barrel structure.

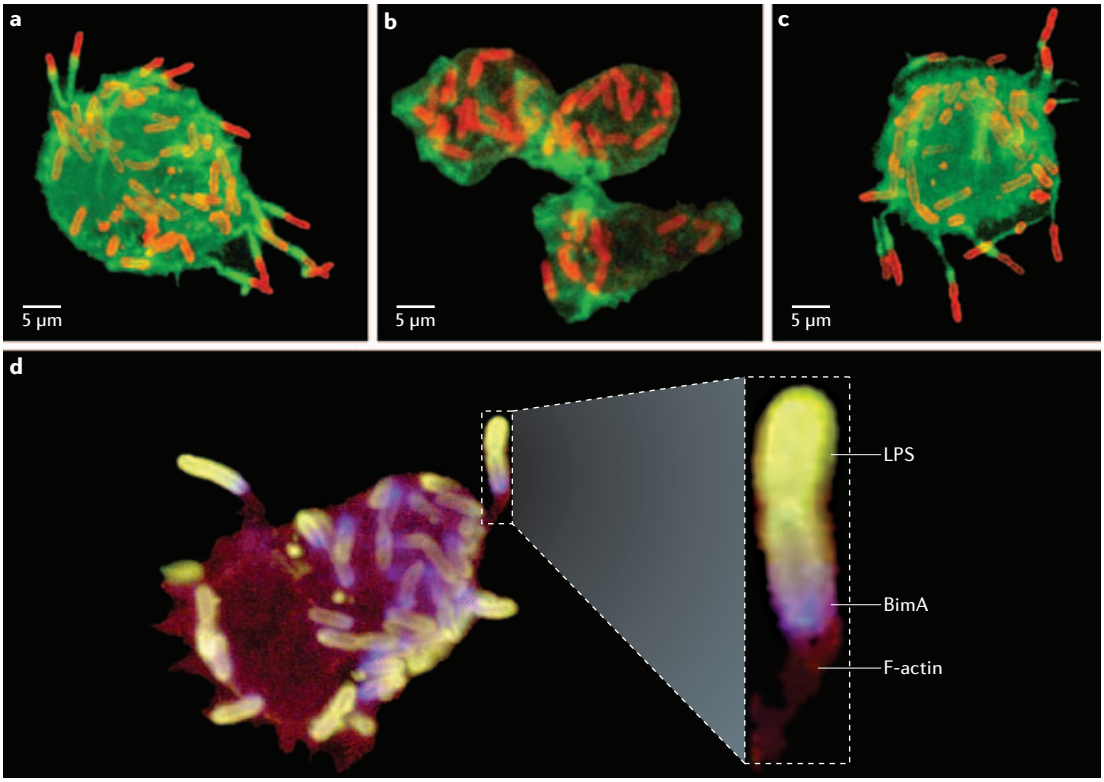


Figure 3 | **Actin-based motility of *Burkholderia pseudomallei*.** Confocal micrographs showing actin-based motility displayed by *B. pseudomallei* in J774.2 macrophage-like cells (**a**), which is abolished on mutation of the *bimA* gene (**b**) and restored by expression of the cloned *bimA* gene *in trans* (**c**). Bacteria are shown in red, F-actin in green. BimA (blue) is located at the bacterial surface (yellow) at the pole at which actin polymerization occurs (red) (**d**). Scale bars in **a**–**c** represent 5 µm. Figures reproduced with permission from REF. 11 © (2005) Blackwell Publishing.

By using green-fluorescent-protein fusions and immunocytochemistry, Arp2/3-complex components have been shown to localize throughout *B. pseudomallei*-induced actin tails[49]. However, the role of the Arp2/3 complex in intracellular motility of *B. pseudomallei* is unclear, as overexpression of the WCA (WH2, central and acidic) domain of Scar1, which sequesters cellular Arp2/3 and inhibits actin-tail formation by *S. flexneri*, *L. monocytogenes* and *R. conorii*, does not interfere with actin-based motility of *B. pseudomallei*[49]. A purified but truncated BimA protein was reported to bind monomeric actin in a concentration-dependent manner and to weakly stimulate actin polymerization *in vitro* in an Arp2/3-independent manner[11]. These data imply that BimA alone might be sufficient for intracellular motility of *B. pseudomallei*. However, several post-translational modifications of BimA are possible and it is unclear if these, or bacterial cofactors, are needed for full activity. Taken together, these observations indicate that actin-based motility of *B. pseudomallei* occurs by a mechanism which is distinct to that used by other intracellular pathogens with respect to Arp2/3 dependence, but which might involve NPF mimicry.

The related *Burkholderia* species *Burkholderia mallei* and *Burkholderia thailandensis* have BimA homologues[50]. *B. mallei* and *B. thailandensis* are capable of actin-based motility in murine macrophage-like cells, and the BimA homologues of these bacteria bind actin *in vitro* and can restore actin-based motility of a *B. pseudomallei* *bimA* mutant[51]. However, the N termini of the BimA proteins of the three species differ in the number of WH2 domains, sequence of proline-rich motifs and the presence of a CA domain, which indicates that they might initiate actin assembly by distinct mechanisms. Consistent with the presence of a unique CA domain, only *B. thailandensis* BimA has been found to sequester Arp3 from cell extracts *in vitro*[51].

Pathogens that rely on cellular NPFs

Shigella *species*. *Shigella* species are Gram-negative facultative intracellular pathogens that cause acute gastroenteritis in humans. The bacterium invades colonic epithelial cells in a type-III-secretion-system-dependent manner, escaping into the cytosol by lysis of the phagosomal membrane[52]. Within the cytosol, *Shigella* induces the formation of polar actin-rich tails[53]. Motile bacteria form membrane-bound protrusions that are engulfed by adjacent cells, and cell-to-cell spread is facilitated by the bacterial protein IcsB[1,54–57].

The gene that is essential for cell-to-cell spread of *Shigella* (*icsA*, initially annotated as *virG*) is encoded by the large virulence plasmid[5]. An *icsA* insertion mutant efficiently entered and replicated in cultured cells but was unable to spread from cell to cell or cause disease in a murine model[5]. The *icsA* gene of *S. flexneri* was localized to a 4-kb fragment that putatively encodes a single protein, and homologous sequences were detected in other *Shigella*

and enteroinvasive *E. coli* (EIEC) strains[5,58]. The *icsA* gene is highly conserved in the sequenced genomes of *Shigella dysenteriae* and *Shigella boydii*; however, most studies have focused on the product of the *S. flexneri icsA* gene. Characterization of the *S. flexneri icsA* locus revealed that the gene product is a 1,102-amino-acid outer-membrane protein with an apparent molecular weight of 120 kDa[6,58] that belongs to a family of Gram-negative bacterial proteins secreted through the type V pathway[59].

IcsA is both necessary and sufficient for actin-based motility of *S. flexneri*. Early attempts to reconstitute actin-based motility of *Shigella* in cell-free extracts were unsuccessful, as IcsA is only expressed at levels that are sufficient to induce actin polymerization in the host-cell cytoplasm[13]. However, overexpression of IcsA in non-motile *E. coli* conferred actin-based motility in *Xenopus* oocyte extracts[21], and beads coated with IcsA protein show actin-based motility in cell-free extracts in an Arp2/3-dependent manner[10,21]. IcsA has no homology to *Listeria* ActA or other known bacterial or cellular actin nucleators. The sequences that are required to assemble actin *in vitro* have been localized to amino acids 53–508 (REF. 60) but do not contain any obvious domain structures or motifs known to recruit actin-associated proteins. Several cellular actin-associated proteins have been localized to *Shigella* tails. Their importance in motility has been extensively reviewed and is summarized in Supplementary information S1 (table).

Unlike *Listeria* ActA, which is an NPF, IcsA relies on the recruitment of a cellular NPF (N-WASP) for actin-tail formation. IcsA interacts directly with N-WASP *in vitro* and *in vivo* through a glycine-rich region, and depletion and reconstitution experiments in *Xenopus* oocyte extracts and cell-free systems have shown that N-WASP is essential for IcsA-mediated actin-tail assembly[25,61,62]. Consistent with this key role, *Shigella* does not form actin tails in N-WASP$^{-/-}$ cells[27], and overexpression of a dominant-negative form of N-WASP markedly inhibits actin-tail formation by intracellular *S. flexneri*[61]. Domain-swap experiments have indicated that IcsA specifically interacts with N-WASP rather than with other WASP-family proteins through its N-terminal pleckstrin homology domain and calmodulin-binding IQ motif[62].

It has been proposed that once IcsA has recruited N-WASP to the bacterial surface, it mimics N-WASP activation by Cdc42 (REF. 60). There are conflicting reports of the association of Cdc42 with *Shigella* tails[63,64]. An N-WASP protein that lacks a CRIB domain and cannot interact with Cdc42 can rescue the actin-based motility defect of *Shigella* in N-WASP$^{-/-}$ cells, indicating that Cdc42 is not required for motility[27]. Furthermore, *Shigella* intracellular motility is unaffected by the clostridial toxin TcdB-10463, which inhibits the activity of the Rho-family GTPases of which Cdc42 is a member[65]. Taken together with the fact that Cdc42 is not required to reconstitute motility of *E. coli* IcsA in a cell-free system[25], it is considered that IcsA mimics Cdc42 activation of N-WASP at the bacterial surface, probably by inducing conformational changes in N-WASP that release it from its autoinhibited conformation. This allows the association of N-WASP with the Arp2/3 complex. However, it should be noted that a key role for Abl-family kinases in the activation of N-WASP by phosphorylation has recently been described, and that this tyrosine-kinase family is indispensable for *Shigella* motility[66]. It is possible therefore that IcsA acts as an Abl-kinase mimic rather than a Cdc42 mimic.

Mycobacterium marinum. The Gram-positive bacterium *M. marinum* causes a systemic tuberculosis-like disease in fish and amphibians, and pulmonary and cutaneous lesions in humans. The bacteria persist in macrophages *in vivo* and induce granuloma formation. *M. marinum* was recently reported to escape endosomes and spread from cell to cell. Cytoplasmic *M. marinum* induces actin tails in fish and murine macrophages, as well as murine cell-free extracts, promoting movement at up to 11 μm per minute[14]. As with *Listeria* ActA, *Shigella* IcsA and *Burkholderia* BimA proteins, expression of the *M. marinum* factor(s) that promotes actin-based motility is induced intracellularly, as broth-cultured bacteria were non-motile in cell-free extracts but bacteria isolated from infected cells were motile[14].

Ultrastructural analysis of F-actin tails formed by *M. marinum* revealed a branched pattern, consistent with the presence of Arp2/3-complex components throughout the tails[14]. WASP is recruited to the bacterial pole at which actin assembly occurs, and it is likely that this initiates Arp2/3-dependent actin polymerization[14]. Intracellular motility of *M. marinum* is reduced in WASP$^{-/-}$ cells and almost totally abolished in N-WASP$^{-/-}$ cells[67]. Motility could be restored by expression of WASP and N-WASP but not by expression of another WASP-family member WAVE, indicating a crucial role for these WASP-family NPFs in motility[67]. *M. marinum* motility has also been shown to be independent of upstream signalling molecules of WASP-family members[67]. By requiring WASP or N-WASP for actin-tail formation, *M. marinum* might use a mechanism similar to that of *Shigella* IcsA. Vasodilator-stimulated phosphoprotein (VASP) is present throughout *M. marinum*-induced actin tails, probably as a result of the interaction of VASP with F-actin[14]. In this regard, *M. marinum*-induced actin tails are similar to those formed by *Shigella*, but differ from those of *Listeria*, as ActA directly recruits VASP to the bacteria–actin interface[68] (see Supplementary information S1 (table)). The bacterial factor(s) required for actin-based motility of *M. marinum* awaits identification, and it remains unclear whether other pathogenic mycobacteria share the ability to form actin tails, or what role actin-based motility has in the pathogenesis of *M. marinum* infections.

***Attaching and effacing* Escherichia coli.** In contrast to the bacterial pathogens discussed above, certain diarrhoeagenic *E. coli* pathotypes can be propelled by actin polymerization without entering host cells. Enteropathogenic *E. coli* (EPEC) and enterohaemorrhagic *E. coli* (EHEC) induce attaching and effacing lesions on intestinal epithelia, which are characterized by intimate bacterial adherence to enterocytes on actin-rich 'pedestals' and

Pleckstrin homology domain
These domains share little sequence conservation but all have a common fold involved in lipid binding and targeting of proteins to the plasma membrane.

CRIB domain
Cdc42/Rac interactive binding (CRIB) domains bind Cdc42 and other Rho-family GTPases.

WAVE
WASP-family verprolin homologous (WAVE)/Scar proteins are members of the WASP family of Arp2/3 activators that lack the domains required for Rho GTPase binding. As such, they are thought to be regulated in a different manner to other WASP-family members.

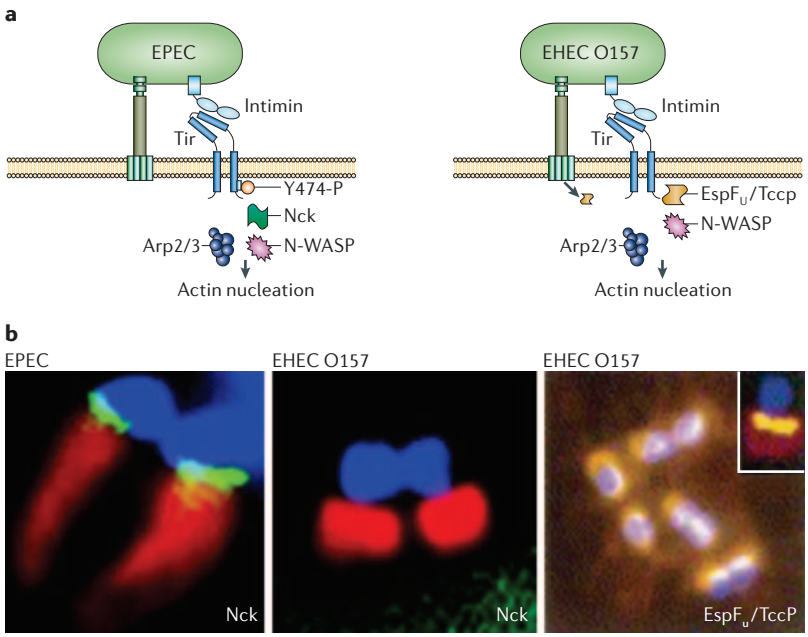


Figure 4 | **Mechanisms of actin pedestal formation by enteropathogenic *Escherichia coli* (EPEC) O127:H6 and enterohaemorrhagic *E. coli* (EHEC) O157:H7.** **a** | Translocated intimin receptor (Tir) is injected into the enterocyte and inserts into the plasma membrane. The Tir extracellular domain binds to the bacterial outer-membrane protein intimin. Tyrosine residue 474 (Y474) in the cytosolic C-terminal domain of Tir is phosphorylated, and the 12-amino-acid motif that includes Y474 recruits the host-cell adaptor protein Nck which, in turn, recruits neural Wiskott–Aldrich syndrome protein (N-WASP). EHEC O157:H7 Tir lacks the crucial Y474 residue and is not tyrosine-phosphorylated in host cells. The type III secreted protein (EspF$_U$/TccP) is translocated from EHEC O157:H7 into host cells, where it associates with both Tir and N-WASP and stimulates actin assembly. Non-O157 EHEC strains share the C-terminal 12-amino-acid phosphopeptide that is typical of EPEC O127:H6 Tir and that is required for Nck recruitment. **b** | The host adaptor protein Nck (green) localizes to the actin pedestal structures (red) formed by EPEC (blue, left panel) but not EHEC (blue, middle panel). **c** | By contrast, the type III secreted factor EspF$_U$/TccP (yellow) located at the tip of an actin pedestal (red) couples N-WASP to the EHEC O157:H7 Tir protein under adherent bacteria (blue). Part **b** is reproduced with permission from REF. 83 © (2003) Elsevier. Part **c** is reproduced with permission from REF. 87 © (2004) Elsevier.

localized destruction of microvilli[69] (FIG. 1). Pedestals induced by EPEC O127:H6 are not static and propel the bacteria from the cell surface, moving at up to 4.2 μm per minute[70], possibly to facilitate spread of the bacteria to adjacent enterocytes. EPEC pedestals can reach lengths of 10 μm and tend to be longer and more electron-dense than those induced by EHEC O157:H7 (REF. 71).

Actin nucleation under adherent bacteria requires injection of the translocated intimin receptor (Tir) into enterocytes through a type III secretion system. Tir inserts into the host-cell plasma membrane so that its central extracellular domain functions as a receptor for the bacterial outer-membrane protein intimin[72]. This interaction is necessary for optimal actin assembly[73,74], which is both Arp2/3- and N-WASP dependent. Pedestals do not form in N-WASP$^{-/-}$ cells despite adherence being unaffected, and can be restored by expression of full-length N-WASP or N-WASP mutants unable to bind Cdc42, indicating that Cdc42 is not required for pedestal formation[27].

Actin assembly by EPEC O127:H6 Tir requires phosphorylation of tyrosine residue 474 (Y474) in the cytosolic C-terminal domain of Tir[75] (FIG. 4). Insertion of Tir into enterocyte membranes, and subsequent tyrosine phosphorylation, has been detected during infection of mice with the murine AE pathogen *Citrobacter rodentium* and is essential for actin nucleation, at least *in vitro*[76]. Y474 is part of a 12-amino-acid motif that recruits the host-cell adaptor protein Nck, which in turn recruits N-WASP[77–79] (FIG. 4). EPEC O127:H6 Tir also recruits the host adaptor proteins Grb2 and CrkII, as well as other actin-regulatory proteins, focal-adhesion proteins and lipid-raft-associated proteins[80]. A peptide from EPEC O127:H6 Tir comprising the 12-amino-acid motif that contains phosphorylated Y474 can induce Nck clustering and actin assembly in *Xenopus* oocyte extracts[78], and is conserved in the vaccinia-virus A36R protein, which mediates Nck and Grb2 recruitment and N-WASP–Arp2/3-mediated motility of virions at the plasma membrane[81,82]. Recent evidence indicates that EPEC O127:H6 Tir can also stimulate actin polymerization by a Nck-independent mechanism involving Tir oligomerization and phosphorylation of Y454 and Y474 (REFS 73,78).

Studies of EHEC O157:H7 Tir reveal that it recruits most of the same host factors as EPEC O127:H6 Tir, with the exception of the adaptor proteins Nck, CrkII and Grb2 (REF. 83) (FIG. 4). This correlates with the observation that EHEC O157:H7 Tir lacks the crucial Y474 residue and is not tyrosine-phosphorylated in host cells[84,85]. Furthermore, the motif required for Nck recruitment is not conserved in Tir from EHEC O157:H7 and O157:H$^-$, although it is found in EHEC serotypes O26:H$^-$ and O111:H$^-$ (REF. 86). Recent studies have shown that a proline-rich type III secreted protein (EspF$_U$/TccP) is required to recruit N-WASP, α-actinin and Arp3 beneath adherent EHEC O157:H7 for subsequent actin assembly[87,88] (FIG. 4). EspF$_U$/TccP is translocated into host cells by the bacteria, associates with both Tir and N-WASP and stimulates actin assembly in the presence of purified Arp2/3 (REFS 87,88). EHEC O157:H7 Tir cannot restore the ability of EPEC O127: H6 or *C. rodentium tir* mutants to form pedestals on cultured cells[76,88,89]. This correlates with the absence of EspF$_U$/TccP in these bacteria and reflects the key role of EspF$_U$/TccP in coupling EHEC O157:H7 Tir to the N-WASP–Arp2/3 pathway.

The role of actin-based motility of AE *E. coli* in pathogenesis remains unclear. When EspF$_U$/TccP is mutated, intimin–Tir-mediated adherence of EHEC O157:H7 and subsequent colonization in calves or sheep is unaffected, whereas *in vitro* pedestal formation is impaired[90]. Furthermore, a *C. rodentium tir* mutant complemented with a non-phosphorylated Y471F variant of Tir showed no defect in intestinal colonization in mice[76]. These data imply that the role of Tir in actin assembly can be uncoupled from its role in intimin-mediated adherence *in vivo*; however, pedestals could still be detected under an adherent EHEC O157:H7 *espF$_U$/tccP* mutant in calves[90] and a *C. rodentium* Tir Y471F mutant in mice[76], indicating that actin assembly might occur by distinct mechanisms *in vivo*.

Adaptor proteins
Proteins involved in signalling cascades that lack any intrinsic enzymatic activities. They mediate protein–protein interactions to form diverse protein complexes.

Concluding remarks

Analysis of bacterial actin-based motility has revealed novel insights into both pathogen biology and the control of actin assembly in eukaryotic cells. It provides convenient models to study the formation of lamellipodia at the leading edge of motile cells (*Listeria* and *Shigella*) and filopodia (*Rickettsia*), and can even be reconstituted *in vitro* using purified components. This allows the minimal requirements for actin assembly to be defined and enables the involvement of regulatory factors in actin-tail formation, Y-branching, force generation and directionality to be assessed, although how *in vitro* assays relate to motility within the tightly packed milieu of the host-cell cytosol is unknown. The ability to generate knockout cell lines and animals or to deplete selected factors by use of small inhibitory RNA molecules (siRNA) will further define the role of bacterial and cellular cofactors *in vivo*. It will be of particular interest to determine how the Y-branched actin arrays induced by RickA *in vitro* are reorganized into the long unbranched filaments and bundles seen in *Rickettsia*-infected cells and filopodia. The structural basis of activation of Arp2/3 and endogenous NPFs by bacterial and cellular factors is poorly understood, and co-crystallization of such complexes and the development of specific inhibitors would aid understanding of the molecular events leading to actin assembly.

The availability of complete bacterial genome sequences has facilitated identification of new factors that activate actin assembly, including *Rickettsia* RickA and *Burkholderia* BimA, and families of homologues of these proteins are emerging in related species that might stimulate actin dynamics in distinct ways. The sequences of such factors indicate that diverse post-translational modifications are possible, and it will be of interest to determine what role this might have in regulating their activity, polar localization and in bacterial virulence. Mutation of *Listeria actA* and *Shigella icsA* has proven to be an effective way of generating live-attenuated vaccine strains that persist for long enough to induce protective immunity but lack the ability to spread to deeper tissues. This knowledge might be useful in tackling emerging intracellular pathogens for which vaccines are not yet available, such as *B. pseudomallei* and *B. mallei*. Furthermore, screening of pharmacological agents for their ability to selectively interfere with actin assembly by pathogens might yield novel treatments for infections as well as other human diseases.

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