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Report

Rickettsia Actin-Based Motility Occurs in Distinct Phases Mediated by Different Actin Nucleators

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Summary

Many intracellular bacterial pathogens undergo actin-based motility to promote cell-cell spread during infection [1]. For each pathogen, motility was assumed to be driven by a single actin polymerization pathway. Curiously, spotted fever group *Rickettsia* differ from other pathogens in possessing two actin-polymerizing proteins. RickA, an activator of the host Arp2/3 complex, was initially proposed to drive motility [2, 3]. Sca2, a mimic of host formins [4, 5], was later shown to be required for motility [6]. Whether and how their activities are coordinated has remained unclear. Here, we show that each protein directs an independent mode of *Rickettsia parkeri* motility at different times during infection. Early after invasion, motility is slow and meandering, generating short, curved actin tails that are enriched with Arp2/3 complex and cofilin. Early motility requires RickA and Arp2/3 complex and is correlated with transient RickA localization to the bacterial pole. Later in infection, motility is faster and directionally persistent, resulting in long, straight actin tails. Late motility is independent of Arp2/3 complex and RickA and requires Sca2, which accumulates at the bacterial pole. Both motility pathways facilitate cell-to-cell spread. The ability to exploit two actin assembly pathways may allow *Rickettsia* to establish an intracellular niche and spread between diverse cells throughout a prolonged infection.

Results and Discussion

Rickettsia Motility Occurs in Two Phases with Distinct Movement Parameters

Pathogens that undergo actin-based motility (ABM), including *Listeria monocytogenes* and *Shigella flexneri*, were assumed to deploy one protein that harnesses a single actin polymerization pathway, resulting in a mechanistically uniform mode of motility throughout infection [7]. However, spotted fever group (SFG) *Rickettsia* differ from *L. monocytogenes* and *S. flexneri* in having a slower doubling time (8–12 hr versus 40–60 min) and longer persistence time in host cells (120 hr or more versus 8–24 hr) [8, 9]. Previous studies examined *Rickettsia* ABM primarily at 24–48 hr postinfection (hpi) [10–12], with one report of rare motility at 30 min postinfection (mpi) [10]. These reports did not, however, quantify movement parameters or molecular requirements throughout infection. We sought to determine how ABM progresses during infection with *Rickettsia parkeri*, an emerging SFG pathogen that causes eschar-associated rickettsiosis in humans [13].

We investigated the appearance of actin tails at various times after infection of human microvascular endothelial (HMEC-1) cells. Early after invasion (15–30 mpi), actin tails were predominantly short or curved (Figures 1A and 1B). Most bacteria with actin tails were intracellular even at 15 mpi (see Figure S1A available online), with a small extracellular fraction that may have been invading or escaping from host cells. At intermediate times postinfection (2–12 hpi), few tails were observed (Figure 1A). At later times (24–48 hpi), tails were more frequent and were predominantly long (Figures 1A and 1B). This suggests that *R. parkeri* transitions through an early motile phase prior to bacterial replication, an intermediate phase with infrequent motility, and a late motile phase after replication commences.

To discern whether the parameters of movement change over time, we observed motility of individual *R. parkeri* early (15–60 mpi) and late (48 hpi) after infection of HMEC-1 cells (Movies S1 and S2; Figure S1B and S1C) and compared these with the parameters of *L. monocytogenes* motility (8–12 hpi) (Movie S3; Figure S1D). Early *R. parkeri* motility was slower and produced shorter actin tails in comparison with late *R. parkeri* and *L. monocytogenes* motility (Figures 1C and 1D). Tail length and speed were well correlated for both early *R. parkeri* (R^2 = 0.69) and *L. monocytogenes* motility (R^2 = 0.78) (Figure 1E), similar to previous observations for *L. monocytogenes* [17]. However, there was little correlation for late *R. parkeri* motility (R^2 = 0.30), as seen previously [18]. We also measured path curvature by calculating movement efficiency (displacement / distance traveled) and the average cos($\Delta\theta$) ($\Delta\theta$ = change in tangent angle between track segments) (Figures 1F and 1G) over 60 s. The median values for both curvature measures were close to 1 for late *R. parkeri* motility, indicating straighter trajectories. Both measurements were significantly different for early *R. parkeri* motility, reflecting more curved trajectories similar to those of *L. monocytogenes*. Thus, early and late *R. parkeri* motility differ in key parameters, suggesting that each phase is driven by a distinct actin polymerization mechanism.

Different Host Proteins Are Recruited and Required for Early and Late Motility

The observation that early *R. parkeri* motility resembles *L. monocytogenes* motility, which requires the host Arp2/3 complex [7], suggested that differences between early versus late *R. parkeri* motility result from differential utilization of proteins involved in Arp2/3-dependent actin network assembly and disassembly. The recruitment of the Arp2/3 complex to *R. parkeri* actin tails was examined in HMEC-1 cells expressing the mCherry-tagged ARPC5 subunit. mCherry-ARPC5 localized intensely to early *R. parkeri* actin tails in both live and fixed

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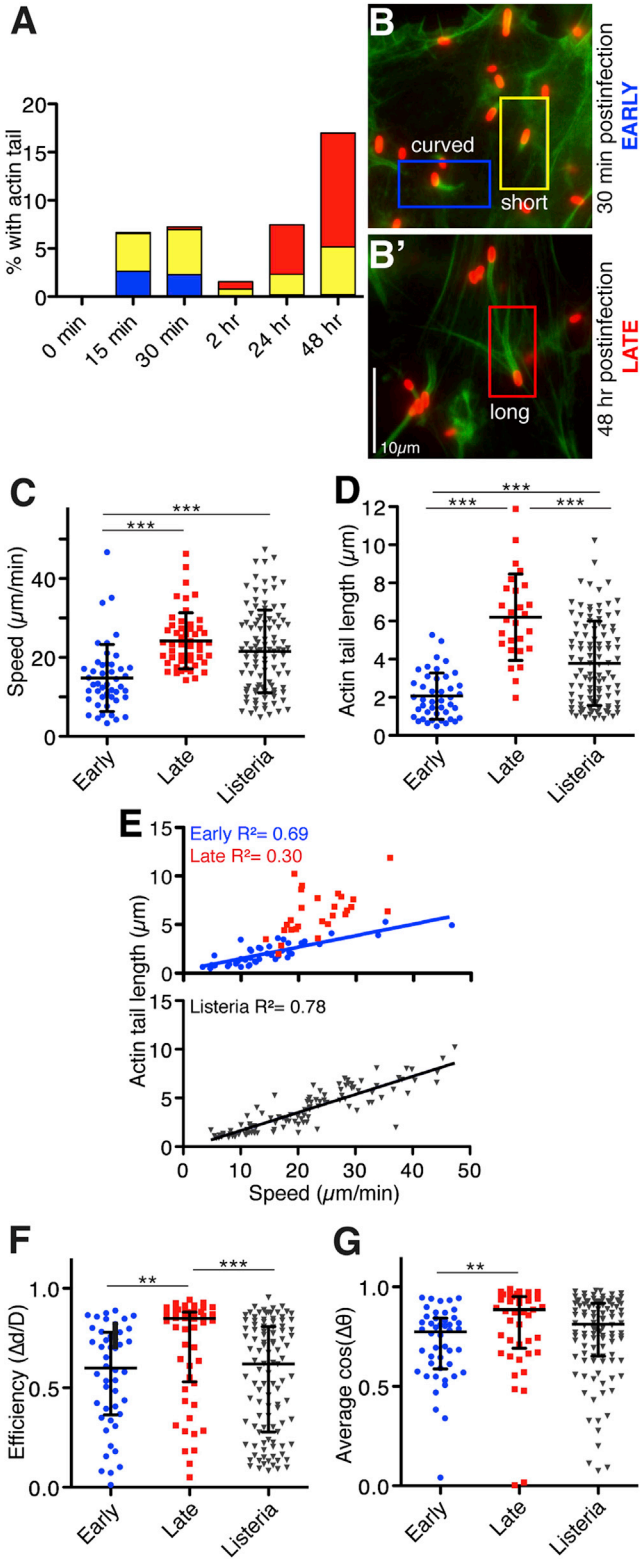

Figure 1. *R. parkeri* Motility Occurs in Early and Late Phases with Distinct Movement Characteristics

(A) Graph depicting the percentage of *R. parkeri* with actin tails that are curved (blue; >90° bend in tail), short (yellow; <2 bacteria lengths), or long (red; >2 bacteria lengths) in HMEC-1 cells infected synchronously for the indicated times as described previously [14]. Results are the average from three independent experiments performed in duplicate.

cells (Figures 2A and 2C), similar to Arp2/3 complex localization in *L. monocytogenes* tails [20, 21]. In fixed cells, the Arp3 subunit of native Arp2/3 complex was also observed in early tails (Figure S2A). In contrast, ARPC5 and Arp3 intensities were indistinguishable from controls in late *R. parkeri* actin tails (Figures 2B and 2C; Figure S2B), consistent with previous findings [12, 18, 22]. We also examined the localization of the actin-severing and -depolymerizing protein cofilin, which is enriched in cellular actin networks containing Arp2/3 [23]. In HMEC-1 cells expressing cofilin tagged with EGFP, EGFP-cofilin intensity was significantly higher in early *R. parkeri* actin tails compared with late tails, and intensity in both tail types was significantly higher than control intensity (Figures 2D–2F; Figure S2C). Earlier qualitative observations also found cofilin to be more abundant in *L. monocytogenes* actin tails than in late *R. parkeri* tails [12, 18]. The distinct composition of actin cytoskeletal proteins in early and late *R. parkeri* actin tails likely reflects differences in the mechanisms of actin assembly and dynamics.

We tested for differences in the functional requirement for host Arp2/3 complex in early versus late *R. parkeri* ABM by treating infected HMEC-1 and COS7 cells with the small-molecule Arp2/3 inhibitor CK869 [24], the inactive control compound CK312, or DMSO alone (Figures 2G–2I; Figure S2). Early actin tail formation (30 mpi) was significantly reduced upon 15 min treatment with inhibitor relative to controls (Figure 2G; Figure S2D). In contrast, late motility (48 hpi) was unaffected by 30 min or 24 hr treatment with Arp2/3 inhibitor (Figures 2H and 2I; Figure S2E). Thus, the molecular mechanisms of actin nucleation during early and late motility are distinct, with early motility exhibiting an Arp2/3-dependent mechanism similar to that used by *L. monocytogenes* [7].

Early Motility Requires RickA and Late Motility Requires Sca2

Differences in the requirement for host Arp2/3 complex in early versus late *R. parkeri* ABM suggested that the bacterial actin-polymerizing proteins RickA and Sca2 might also contribute differentially to each motility phase. Using the pMW1650/*himar1* transposon system [25, 26] in *R. parkeri*, we isolated transposon insertion mutations in the *rickA* or

(B) *R. parkeri*-infected cells were fixed at the indicated times and stained with anti-*Rickettsia* antibody (red) and for actin with Alexa Fluor 488-phalloidin (green).

(C–G) Bacterial motility parameters in HMEC-1 cells expressing Lifeact-EGFP and infected with *R. parkeri* expressing mCherry [15] early (12–60 min postinfection [mpi]) or late (48 hr postinfection [hpi]), or HMEC-1 cells expressing Lifeact-mCherry and infected with *L. monocytogenes* strain 10403S expressing GFP [16] at 8–12 hpi.

(C and D) Speed of movement (C) and actin tail length (D) for *R. parkeri* early (blue) and late (red), and for *L. monocytogenes* (gray). Data are mean ± SD, ***$p < 0.001$ by ANOVA with Bonferroni's multiple comparison test, from three separate experiments, with tracking and speed data in 60 s intervals for each bacterium.

(E) Relationship between average speed and actin tail length for each bacterium, with best-fit linear regression.

(F) Average efficiency of movement, calculated by dividing the total xy displacement by the total distance moved over 60 s for each bacterium.

(G) Path straightness for each bacterium, calculated by averaging cosines of the change in tangent angle between adjacent track segments ($\Delta\theta$) over 60 s of movement. For (F) and (G), we analyzed variation around the medians due to the non-Gaussian distribution of the data. Data are median ± interquartile range, **$p < 0.01$, ***$p < 0.001$ by Kruskal-Wallis test with Dunn's multiple comparison test.

See also Figure S1 and Movies S1, S2, and S3.

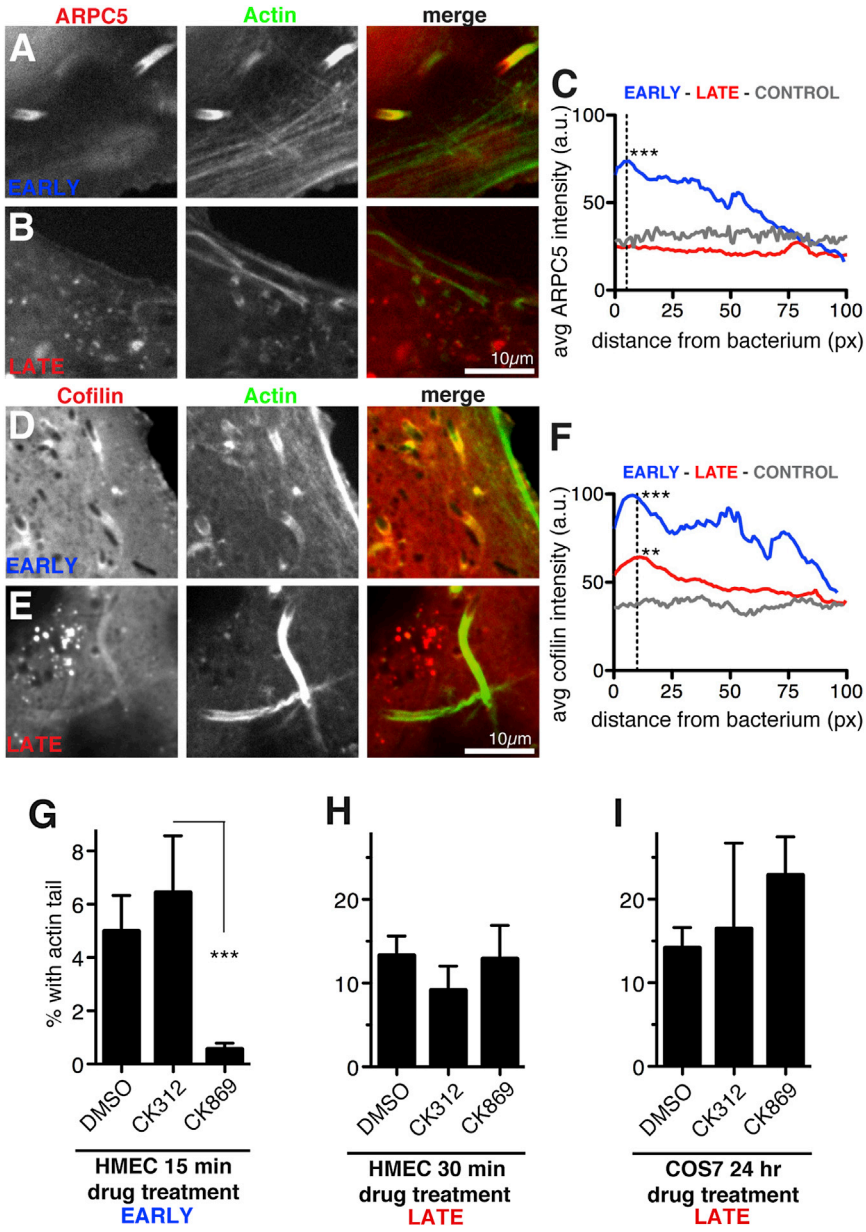

Figure 2. Different Host Proteins Are Recruited and Required for Early and Late Motility

(A, B, D, and E) Confocal micrographs of live HMEC-1 cells stably expressing Lifeact-3×BFP (green) infected with *R. parkeri* for 30 mpi (early) or 48 hpi (late). Cells also expressed mCherry-ARPC5 (red; A and B) or EGFP-cofilin (D and E; red) [19].

(C and F) Average intensities of line scans along the first 100 pixels (~6.5 μm) of actin tails proximal to bacteria, or control lines in various areas of transfected cells (a.u., arbitrary units). Intensity was measured for 26–36 tails in snapshots of multiple live cells after identical rolling circle background correction using ImageJ. Asterisks indicate significantly different average peak intensities (at 5 pixels for C and 10 pixels for F) compared with controls, with $^{**}p < 0.01$ or $^{***}p < 0.001$ based on ANOVA with Bonferroni's multiple comparison test.

(G) Percent of all bacteria with an actin tail in HMEC-1 cells infected with *R. parkeri* for 15 min to allow invasion and then treated with 1% DMSO or 100 μM inactive control (CK312) or Arp2/3 inhibitor (CK869) for 15 mpi before fixation at 30 mpi.

(H) HMEC-1 cells infected with *R. parkeri* for 48 hr and then treated with 1% DMSO or 100 μM CK312 or CK869 for 30 min before fixation.

(I) COS7 cells infected with *R. parkeri* for 24 hr and then treated with 1% DMSO or 100 μM CK312 or CK869 for an additional 24 hr (with fresh media plus inhibitors exchanged at 32 hr, 40 hr, and 48 hr). For (G)–(I), cells were stained with Alexa Fluor 488-phalloidin and anti-*Rickettsia* antibody, and 5–10 random fields of view were imaged. Results represent the mean ± SD of two (G) or three (H and I) independent experiments performed in duplicate; $^{***}p < 0.001$ by ANOVA with Bonferroni's multiple comparison test.

See also Figure S2.

sca2 genes (Figures S3A and S3B). The *sca2::tn* mutation causes the expression of a truncated 50 kDa protein, whereas the *rickA::tn* mutation eliminates detectable expression of RickA (Figure S3C). Neither mutation deleteriously affected the growth of *R. parkeri* (Figure S3D). Importantly, the *rickA::tn* mutant was completely defective in early tail formation (Figures 3A and 3B) but showed no defect in late tail formation (Figures 3C and 3D). Conversely, the *sca2::tn* mutant was completely deficient in late tail formation (Figures 3C and 3D; similar to the *sca2::tn* mutant in *R. rickettsii* [6]) and exhibited a slight defect in early tail formation (Figures 3A and 3B), perhaps reflecting a small subset of early ABM driven by Sca2. Thus, the two phases of *Rickettsia* motility are mechanistically independent, with RickA and Arp2/3 complex required for early motility, and Sca2 alone required for late motility.

We next investigated the function of RickA and Sca2 in *R. parkeri* cell-cell spread. The efficiency of spread at 24 hpi was determined by measuring the size of infectious foci in A549 cell monolayers, and at 5 days postinfection (dpi) by measuring the size of plaques in Vero cell monolayers. At 24 hpi, both the *rickA::tn* and *sca2::tn* mutants exhibited significantly smaller foci than wild-type, although the *sca2::tn* mutant phenotype was more severe (Figures 3E and 3F). At 5 dpi, however, only the *sca2::tn* mutant had significantly smaller plaques than wild-type (Figures 3G and 3H), consistent with small plaques formed by a *R. rickettsii sca2* mutant [6]. Thus, both RickA and Sca2 are important for efficient spread kinetics early in infection, whereas Sca2 is primarily important for spread later in infection.

A Transition between Phases Is Mediated by Changes in RickA and Sca2 Localization

For *L. monocytogenes* and *S. flexneri*, polar localization of the bacterial actin-polymerizing proteins ActA or IcsA is important for efficient ABM [27]. Previous studies reported diffuse localization of RickA [2, 15] and polar localization of Sca2 [4] late in infection. We hypothesized that changes in the localization or abundance of RickA and Sca2 might coordinate their activities in the two phases of motility. Surface-accessible protein

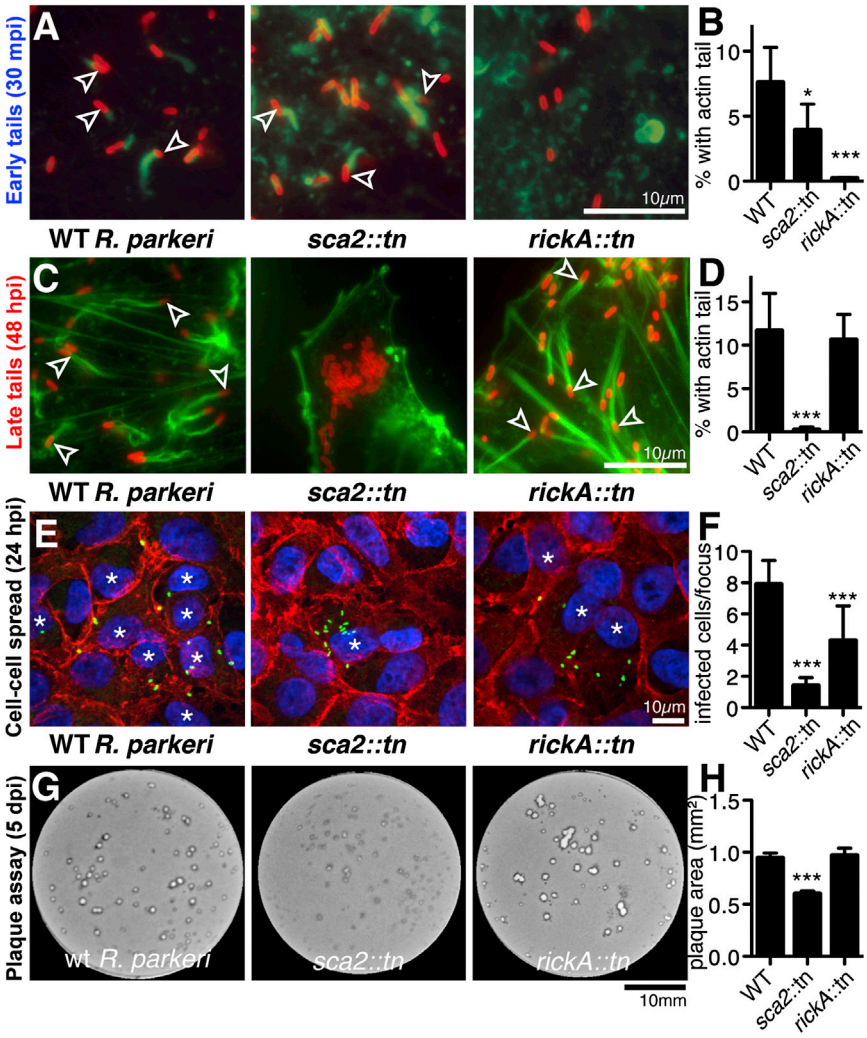

Figure 3. Early Actin-Based Motility Requires RickA, and Late Motility Requires Sca2

(A and C) HMEC-1 cells infected with wild-type or mutant *R. parkeri* for 30 min (A) or 48 hr (C), stained with Alexa Fluor 488-phalloidin to label actin and anti-*Rickettsia* antibody. Arrowheads indicate bacteria with an actin tail.

(B and D) Percentage of wild-type or mutant *R. parkeri* with actin tails in HMEC-1 cells infected for 30 min (B) or 48 hr (D). Data are mean ± SD of five random fields of view in each of three independent experiments performed in duplicate.

(E) Infectious foci formed by wild-type or mutant *R. parkeri* in A549 cells, stained with DAPI (blue), anti-β-catenin antibody (red), and anti-*Rickettsia* antibody (green). Asterisks indicate infected cells.

(F) Number of infected cells per focus for strains in (E). Data are mean ± SD for ten foci.

(G) Plaques formed by wild-type or mutant *R. parkeri* in Vero cells 5 days postinfection (dpi).

(H) Plaque area for strains in (G). Data are mean ± SEM of data from three independent experiments. *p < 0.05, ***p < 0.001 for mean versus wild-type values by ANOVA with Bonferroni's multiple comparison test.

See also Figure S3.

localization was determined in a synchronized infection of HMEC-1 cells (Figure 4; Table S1). At 30 mpi, 97% of bacteria with actin tails exhibited robust RickA staining at the actin-polymerizing pole (Figures 4A and 4C). In bacteria expressing FLAG-tagged RickA [15], the tagged protein was also polar at 30 mpi (Figure S2A). In contrast, 96% of bacteria with early actin tails lacked Sca2 staining or exhibited weak and diffuse Sca2 (Figures 4D and 4F). At 8 or 48 hpi, 80%–93% of bacteria with actin tails showed intense staining of Sca2 at the actin-polymerizing pole (Figures 4E and 4F). However, more than 90% of bacteria with late tails lacked RickA staining or had diffusely distributed or dispersed punctate RickA (Figures 4B and 4C). Thus, there was excellent correspondence of polar RickA localization with early tail formation, and polar Sca2 localization with late tail formation. We also quantified RickA and Sca2 protein levels by western blotting relative to the control outer membrane protein OmpA but did not observe statistically significant changes in their abundance (Figures S4A and S4B). Thus, if protein abundance does vary, it does so at the level of individual bacteria rather than the whole-population level. The correspondence between polar localization of each protein and formation of actin tails at each time point suggests that sequential polar localization of RickA followed by Sca2 regulates the transition between early and late motility.

Models and Functions for Dual Motility Phases

Our results indicate that *R. parkeri* ABM proceeds in mechanistically independent phases. Early motility is driven by RickA and the host Arp2/3 complex, whereas late motility is driven by Sca2. The *rickA* and *sca2* genes are highly similar in nearly all SFG *Rickettsia*, suggesting that biphasic motility is conserved among these species. However, the typhus group species *R. typhi*, which forms short actin tails [10], lacks *rickA* [28] and has a divergent *sca2* [4], suggesting that it may undergo a single motility phase driven by a distinct mechanism. For SFG *Rickettsia*, a transition between phases is correlated with changes in RickA and Sca2 localization. Such changes could be attributed to temporal control over secretion, with RickA being secreted during or just following invasion and Sca2 being secreted and localized during bacterial replication, as seen for *S. flexneri* IcsA and other autotransporter proteins [29]. RickA and Sca2 localization and function might also be regulated by posttranslational modification. Changes in motility behavior and surface protein localization may define key stages of *Rickettsia* infection and suggest that *Rickettsia* may undergo previously unappreciated developmental switches. It is also notable that early RickA-Arp2/3 and late Sca2-formin-like actin polymerization pathways result in fundamentally different movement parameters. This suggests that actin networks generated by different actin nucleators result in biophysically distinct forces and movements. Further study of *Rickettsia* motility may reveal how different actin nucleation pathways impact the movement of pathogens as well as host cell organelles and structures.

The execution of two ABM phases distinguishes SFG *Rickettsia* from other motile bacterial pathogens, which are thought to undergo a single mode of motility [1], although *S. flexneri* protrusion formation and spread are enhanced by

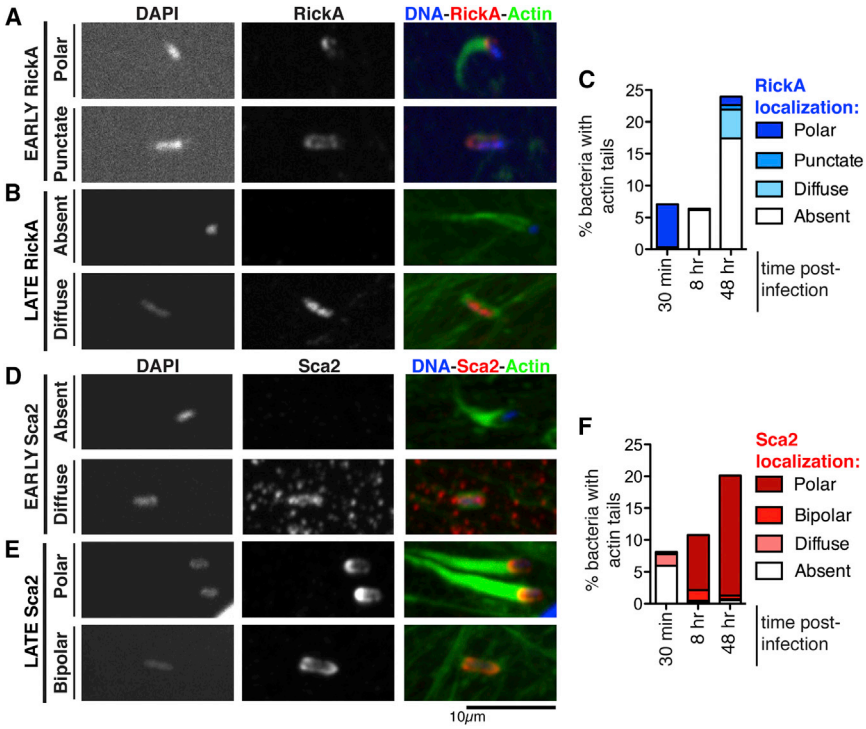

Figure 4. Transition between Early and Late Motility Is Accompanied by Changes in the Localization of RickA and Sca2

(A, B, D, and E) Localization of RickA using anti-RickA antibody (A and B) [3] or Sca2 using anti-Sca2 antibody (D and E) [4] in HMEC-1 cells infected with wild-type *R. parkeri* for 30 min (early) or 48 hr (late). Cells were fixed and stained for immunofluorescence microscopy with DAPI to visualize DNA and Alexa Fluor 488-phalloidin to visualize actin. Time postinfection and localization patterns are noted at left.

(C and F) The percentage of bacteria with the indicated RickA or Sca2 localization patterns was scored for 400–1,200 bacteria over two independent experiments. Localization patterns correspond to those shown in (A), (B), (D), and (E), and only bacteria also having an actin tail are shown.

See also Figure S4 and Table S1.

the host formin Dia1 [30]. Why did SFG *Rickettsia* evolve to separately exploit both RickA-Arp2/3 and Sca2-formin-like pathways? Our results suggest that one function of both early and late ABM is to promote cell-cell spread. RickA-driven early motility might promote efficient spread kinetics from previously infected cells before bacterial replication, as has been observed for vaccinia virus [31]. Sca2-mediated late motility appears to play a crucial role in spread once bacterial replication has commenced. The newly developed mouse model of *R. parkeri* infection [32] will ultimately be useful for illuminating how each motility mechanism enhances spread between the diverse cell types of the mammalian host. A second central function of actin polymerization is to modulate bacterial targeting by the host cell autophagy pathway. For *L. monocytogenes*, binding and recruitment of the Arp2/3 complex is crucial for evading autophagy [33]. For *S. flexneri*, in contrast, association with actin appears to promote autophagy [34, 35]. For *Rickettsia*, however, the role of actin in autophagic targeting is poorly understood [34], and differential recruitment of the Arp2/3 complex and actin early and late in *Rickettsia* infection may modulate autophagy to adapt *Rickettsia* to their long residence time in host cells. Thus, understanding why SFG *Rickettsia* exploit two actin polymerization pathways will reveal diverse evolutionary strategies by which pathogens exploit actin to establish an intracellular niche and avoid degradation.

Supplemental Information

Supplemental Information includes four figures, one table, Supplemental Experimental Procedures, and three Movies and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2013.11.025.

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