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Host Actin Polymerization Tunes the Cell Division Cycle of an Intracellular Pathogen

Graphical Abstract

Highlights
- Division is faster and non-dividing elongation is slower when Lm are intracellular
- Actin polymerization rebalances Lm cell division cycle
- Cell division cycle rebalancing increases Lm propensity to form actin tails

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In Brief
Siegrist et al. use a chemical labeling strategy to show that host actin polymerization rebalances the cell division cycle of intracellular Listeria monocytogenes (Lm). This skews the bacterial population toward shorter cells that are more likely to form actin tails, which, in turn, are critical for motility and virulence.

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Host Actin Polymerization Tunes the Cell Division Cycle of an Intracellular Pathogen

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SUMMARY

Growth and division are two of the most fundamental capabilities of a bacterial cell. While they are well described for model organisms growing in broth culture, very little is known about the cell division cycle of bacteria replicating in more complex environments. Using a D-alanine reporter strategy, we found that intracellular Listeria monocytogenes (Lm) spend a smaller proportion of their cell cycle dividing compared to Lm growing in broth culture. This alteration to the cell division cycle is independent of bacterial doubling time. Instead, polymerization of host-derived actin at the bacterial cell surface extends the non-dividing elongation period and compresses the division period. By decreasing the relative proportion of dividing Lm, actin polymerization biases the population toward cells with the highest propensity to form actin tails. Thus, there is a positive-feedback loop between the Lm cell division cycle and a physical interaction with the host cytoskeleton.

INTRODUCTION

Growth and division are well defined for model bacteria replicating in broth culture. These organisms rely on proteins like the actin homolog MreB and tubulin homolog FtsZ to organize elongation and division, respectively (Bi and Lutkenhaus, 1991; Jones et al., 2001). One role of these proteins is to provide a scaffold for enzymes that synthesize, remodel, and degrade peptidoglycan (PG), a biopolymer that forms a protective meshwork around the cell (de Boer, 2010; White and Gober, 2012). The dimensions of rod-shaped bacteria such as Escherichia coli and Bacillus subtilis are initially determined by MreB, FtsZ, and other cytoskeletal proteins and fixed in place by the rigid cell wall (Typas et al., 2012; Young, 2010).

However, the vast majority of bacterial species are unlikely to fit this paradigm neatly because of differences in shape, mode of growth, or envelope structure. Moreover, growth and division have been primarily studied under defined conditions that may or may not recapitulate replication in the natural environment. There is increasing evidence that bacteria respond to the external milieu by adjusting their growth or division (Deghelt et al., 2014; Frirdich and Gaynor, 2013; Justice et al., 2008). Such alterations are often apparent in obvious changes to the size and shape of the cell, since inhibiting division can cause a bacterium to filament and inhibiting elongation can result in very short or even spherical cells (Young, 2006). Even for species that are not known to undergo clear morphological changes, transcriptional and mutant data suggest that the requirements for both cell wall-acting enzymes and cytoskeletal proteins change according to the environment (Boneca, 2005; Camejo et al., 2009; Frirdich and Gaynor, 2013).

Defining the impact of environmental perturbations on the bacterial cell division cycle is not straightforward. Alterations in growth and division may not necessarily manifest in changes to colony-forming units (CFUs), the most common measurement of bacterial replication outside of broth culture (Crimmins and Isberg, 2012; Helaine and Holden, 2013; Manina and McKinney, 2013). Enumeration of the total bacterial burden can undercount quiescent organisms as well as those that are growing but not dividing. The traditional standard for assessing whether bacteria are in the process of septating is electron microscopy, but this is laborious and poorly suited to screening a multiplicity of bacterial strains or conditions. Finally, fluorescent protein fusions can, in
some cases, disrupt the processes that they report (Meniche et al., 2014; Swulius and Jensen, 2012).

Previously, we developed a chemical approach for probing the cell wall of bacteria replicating in broth or in host cells (Siegrist et al., 2013). Here, we have adapted this method to assess the growth and division of the Gram-positive pathogen Listeria monocytogenes (Lm). By comparing PG labeling under different environmental conditions and in various genetic backgrounds, we discovered that the balance between Lm division and non-dividing elongation shifts during infection. Host actin polymerization at the bacterial cell surface accelerates cell division and primes the intracellular population for subsequent rounds of actin tail formation.

RESULTS

D-Alanine Reporter Marks the Lm Cell Division Cycle

Live Lm within macrophages incorporate D-alanine derivatives into their PG (Siegrist et al., 2013), which can be detected by bio-orthogonal covalent reaction with a complementary fluorescent probe (Figure 1A). Although the precise mechanism is not yet known (Cava et al., 2011), several lines of evidence suggest that alkylene-D-alanine (alkDala) incorporates preferentially into nascent Lm PG. Specifically, alkDala incorporation occurs at the fifth position of the muropeptide, is acutely sensitive to fosfomycin but not to penicillin, and yields signal that colocalizes with that of fluorescent vancomycin (Siegrist et al., 2013). Like E. coli and B. subtilis, Lm inserts new PG along the sidewall during dispersive elongation and at the septum during division (Bruck et al., 2011; Rafelski and Theriot, 2006). After a long alkDala incubation (approximately one to one and a half generations) followed by reaction with an azide-functionalized fluorophore (az-488), we observed clear septal labeling but dim sidewall signal in Lm replicating in broth or in macrophage-like J774 cells (Figure 1B); faint sidewall labeling likely reflects carboxypeptidase activity (Siegrist et al., 2013). We also noticed fluorescence at the bacterial poles. This was puzzling because Lm are not known to grow from that part of the cell.

We characterized the alkDala labeling patterns (Supplemental Experimental Procedures) by nucleoid status and cell length (Figures 1C and S1A), membrane and propidium iodide staining (Figure S1B), and timing (Figures 1D and S1C). Together these data implied that septal and diffuse fluorescence following a short alkDala incubation mark Lm that are in the division or non-dividing elongation phases, respectively, while polar labeling occurs on newly divided cells. Importantly, the proportion of bacteria with any septal labeling over time. The data are the sum of the orange and blue lines from (D). Error bars represent 95% confidence intervals and median lengths (n = 206) were recorded for strains growing in BHI or J774 were incubated in alkDala for 5 or 30 min (broth) or 15 or 60 min (J774) and processed as in (B). The proportions of the population with any septal labeling over time. The data are the sum of the orange and blue lines from (D). Error bars represent 95% confidence intervals and median lengths (n = 206) were recorded for Lm growing in BHI broth, left, or in J774 cells, right, were incubated in alkDala for 30 min or 1 hr, respectively. The cultures were fixed and reacted with az-488. Arrows point to examples of septal (s) and polar (p) labeling. Image on the right is the same as in Figure S2C, top.

(C) Lm in BHI were incubated in alkDala for 5 min and processed as in (B). Bacteria were also labeled with DAPI to visualize DNA. The nucleoid status (n = 483) ± 95% confidence intervals and median lengths (n = 206) were recorded for Lm with diffuse, septal, or polar alkDala staining. The nucleoid status was significantly different between the groups at p < 0.005, chi-square test, and the cell lengths were significantly different at p < 0.005, Kruskal-Wallis test.

Figure 1. D-Alanine Reporters Reveal that Lm in Host Cells Spend Less Time Dividing and More Time Elongating without Dividing Than Lm in Broth

(A) Schematic of D-alanine reporter labeling. Bacteria in broth or in host cells are incubated in the presence of alkylene-D-alanine (alkDala). The reporter incorporates into peptidoglycan and can be detected by copper-catalyzed azide-alkyne cycloaddition (CuAAC) with an azide-fluorophore (az-488).

(B) Lm growing in BHI broth, left, or in J774 cells, right, were incubated in alkDala for 30 min or 1 hr, respectively. The cultures were fixed and reacted with az-488. Arrows point to examples of septal (s) and polar (p) labeling. Image on the right is the same as in Figure S2C, top.

(C) Lm in BHI were incubated in alkDala for 5 min and processed as in (B). Bacteria were also labeled with DAPI to visualize DNA. The nucleoid status (n = 483) ± 95% confidence intervals and median lengths (n = 206) were recorded for Lm with diffuse, septal, or polar alkDala staining. The nucleoid status was significantly different between the groups at p < 0.005, chi-square test, and the cell lengths were significantly different at p < 0.005, Kruskal-Wallis test.

(G) Estimates of division and non-dividing elongation time periods. The proportions from (F) were multiplied by the corresponding doubling times (Table S1).
**Lm in Host Cells Spend Less Time Dividing and More Time Elongating without Dividing Than Lm in Broth**

Although we previously found similar PG labeling patterns for Lm growing in BHI broth or in J774 cells, the bacteria appeared to have a lower incidence of septal fluorescence when intracellular (Siegrist et al., 2013) (Figure 1B). Indeed, ~60% of Lm growing in broth had septal labeling compared to ~30% of Lm in host cells (Figure 1F). Notably, the latter corresponds well to an earlier estimate derived from electron microscopy (Tilney et al., 1992). The decreased frequency of septal fluorescence suggested that a smaller proportion of Lm were actively dividing in the intracellular milieu.

In *E. coli* and *B. subtilis*, the frequency of FtsZ rings in a population is inversely proportional to generation time (Den Blaauwen et al., 1999; Weart and Levin, 2003). Given that FtsZ ring assembly is closely followed by septal PG synthesis (Den Blaauwen et al., 1999), we considered whether the decreased frequency of septal PG labeling in host cells could be explained by an increase in doubling time. We tested this by estimating the length of the division and non-dividing elongation periods for Lm replicating in different environments. Assuming that every cell in the population is replicating, the relative fraction of cells that contain septal or non-septal (diffuse or polar) labeling is directly proportional to the duration of division or non-dividing elongation, respectively. We therefore calculated the time spent by Lm in these periods by multiplying the doubling times (Table S1) by the observed proportions of bacteria with septal or non-septal labeling (Figure 1F). We verified our estimate for division time in broth by performing a pulse chase experiment and measuring the rate of septal-to-polar conversion over time (Figure S1F). Compared to Lm in BHI, those in J774 cells took ~10 fewer minutes for division and ~16 more minutes for non-dividing elongation (Figure 1G). AlkDala incubation itself did not significantly change the doubling time (Table S1), and estimates were constant over a range of amino acid concentrations (Figure S1E).

In contrast to the robust D-amino acid incorporation that takes place during *B. subtilis* stationary phase (Caparro´ s et al., 1992; Cava et al., 2011; Fura et al., 2014; Lam et al., 2009), very little modification of Lm PG by D-alanine reporters occurs in the absence of bacterial growth (Siegrist et al., 2013). Nearly all of the bacteria detectable by immunostaining in J774 cells were also labeled by alkDala, suggesting that most Lm were actively replicating. The shorter division times and longer non-dividing elongation times of intracellular Lm thus suggested that factors in addition to growth rate impact the cell division cycle of Lm during infection.

**PrfA Activation Prolongs Non-dividing Elongation and Shortens Division of Intracellular Lm**

We sought to determine whether cues from the host modulate the Lm cell division cycle. During infection, the Lm transcription factor PrfA becomes activated and induces expression of many virulence factors (de las Heras et al., 2011). Although PrfA is generally inactive when Lm are growing in broth, in vivo state can be recapitated in vitro by prfA* mutants, which express constitutively active alleles (de las Heras et al., 2011; Miner et al., 2008), or by wild-type SLCC-5764, an Lm strain isolate that naturally carries an active prfA allele (Gründling et al., 2003; Leimeister-Wächter et al., 1989). To determine whether PrfA activation might contribute to changes in growth or division, we first compared PG labeling in broth of wild-type 10403S and SLCC-5764 to *prfA* mutant strains. PrfA activation did not appreciably alter PG structure (Figure S2A; Table S2). However, the proportion of bacteria with septal labeling decreased when PrfA was activated in 10403S and increased when PrfA was inactivated in SLCC-5764 (Figures 2B and S2B). Reciprocally, septal labeling was observed in a larger proportion of the *ΔprfA* population (engineered to express low levels of the LLO toxin to permit phagosomal escape and growth in the cytosol [Birmingham et al., 2008]) during infection (Figures 2A and S2C). The difference in septal labeling was not preserved when the strains were growing in broth (Figure S2D), consistent with the absence of PrfA activation under this condition. These data suggested that PrfA activation decreases the proportion of bacteria in the process of division.

The decreased frequency of septal labeling in host cells compared to broth was the result of changes in both the division and non-dividing elongation periods (Figures 1F and 1G). We found that division time was unchanged but non-dividing elongation time increased when PrfA was activated in broth culture (Figure 2D). By contrast, both the division and non-dividing elongation periods were reduced when PrfA was activated intracellularly (Figure 2C). Therefore, PrfA de-activation in the host cell results in a cell division period that resembles that of Lm in broth.

**Host Actin Polymerization Prolongs Non-dividing Elongation and Shortens Division of Intracellular Lm**

Our findings implied that alterations to the bacterial cell division cycle during infection might require an interaction with the host. We focused on a potential role for ActA, a PrfA-controlled cellular surface protein that is required for in vivo survival because it nucleates host actin to form tails that propel the bacterium from cell to cell (de las Heras et al., 2011; Kocks et al., 1992). Of note, host actin polymerization can correct intracellular cell division for a septation-deficient mutant (Alonzo et al., 2011).

To test whether the ActA-actin interaction might regulate the normal cell division cycle, we first compared the PG labeling of *ΔactA* to wild-type 10403S. Loss of the gene increased the proportion of the population with septal labeling in J774 cells, kangaroo epithelial PtK2 cells, and C57BL/6 bone marrow-derived macrophages (BMM) (Figures 2B, 3B, and S2F), which, in turn, corresponded to lengthened division periods and shortened non-dividing growth periods (Figures 2C and 3C). To investigate whether the effect of ActA on the cell division cycle was specific to the host environment, we compared broth PG labeling of Lm overexpressing ActA to wild-type organisms that, because of the absence of PrfA activation, do not normally express the protein. We found that ActA expression under this condition had the opposite effect on septal labeling compared to loss of the protein in J774 cells (Figures 2A and 2B). Deletion of *actA* did not impact broth labeling (Figure S2D). As with the PrfA-dependent change in broth culture, but unlike the PrfA- and ActA-dependent change in host cells, the altered cell division cycle of Lm overexpressing ActA in broth could be explained by an extended non-dividing elongation period (Figure 2D). These findings suggested that ActA helps to rebalance the Lm cell division cycle when the bacteria are intracellular.
Because ActA nucleates host actin polymerization, we next tested whether this function was important for the Lm division or non-dividing elongation during infection. We found that post-invasion inhibition of actin polymerization by cytochalasin D increased the proportion of Lm with septal labeling within J774 cells (Figure 2A). This change in labeling reflected a compressed non-dividing elongation period and extended division period (Figure 2C). The inhibitor had no effect on the cell division cycle of Lm growing in broth (Figure S2E). Moreover, its impact on intracellular bacteria was not additive with the actA deletion (Figure S2F), confirming that the perturbations were in the same pathway. Finally, an actA allele (actA 146-150) that is expressed at normal levels but unable to nucleate actin polymerization (Lauer et al., 2001) had a comparable effect to either actA deletion or cytochalasin D treatment (Figures 2A and 2C). Taken together, these results implicate host actin polymerization as one mechanism by which intracellular Lm alters its cell division cycle.

The intensity of alkDala-derived fluorescence for Lm with septal labeling within J774 Lm increased the proportion of freshly divided bacteria. This change in labeling reflected a compressed non-dividing elongation period and extended division period (Figures 2A and 2C). To test the relationship between division and replication time more broadly, we assessed septal PG

Enhanced Cell Division by Actin Polymerization Occurs over a Range of Doubling Times in Different Host Cells

Our data suggested that the effect of actin polymerization on Lm division frequency in J774 cells was independent of doubling time (Figures 2A and 2C). To test the relationship between division and replication time more broadly, we assessed septal PG
Peptidoglycan Hydrolysis Compresses Cell Division in the Absence of Actin Polymerization

An Lm mutant lacking the NamA (MurA) PG hydrolase forms chains and is defective for actin tail formation at early stages of cellular infection but replicates as single cells with actin tails at later time points (Alonzo et al., 2011). Cytochalasin D treatment abrogates this phenotype, suggesting that actin polymerization can rescue NamA septation under certain circumstances. We found that NamA was also defective for flagellar motility but that, unlike actin-based motility, the impairment was not rescued at later time points (Figure S3A). There was no change in division or non-dividing elongation frequency in a mutant lacking flagella (Figures S3A and S3B). These data suggest that cell division cycle alterations are not the result of motion per se and that actin polymerization exerts a specific stress to shorten division and extend non-dividing elongation.

The observation that NamA chains are disrupted by actin polymerization (Alonzo et al., 2011) implied that the effects of actin polymerization and PG hydrolysis on cell division might be partially interchangeable. To test this idea, we first established a system for manipulating PG hydrolysis under broth and host cell conditions by lysozyme (Supplemental Experimental Procedures). We then compared the cell division cycles of Lm in the presence or absence of actin polymerization, lysozyme, or both (Figure 3B). Lysozyme treatment restored division of Lm growing in broth or in host cells (Supplemental Figure S3A). No change was observed in Lm growing in broth (Supplemental Figure S3A). The enzyme did not impact non-dividing elongation in a consistent manner (Figures S3A and S3B). These data suggest that cell division cycle alterations are not the result of PG hydrolysis.

Intracellular Cell Division Cycle Rebalancing Increases the Proportion of Short Cells with Unipolar ActA

Lm localizes ActA de novo along the length of the bacterium but excludes the protein from the division site (Kocks et al., 1993; Rafelski and Theriot, 2006). ActA polarization is dependent on cell division and PG dynamics (Kocks et al., 1993; Pilgrim et al., 2003; Rafelski and Theriot, 2006). Actin tail formation, in turn, depends on the underlying ActA distribution as well as

Figure 3. Cell Division Compression by Actin Polymerization Occurs over a Range of Doubling Times in Different Host Cells and Can Be Recapitulated by PG Hydrolysis

(A) Lm replicating in broth or host cells were incubated in alkDala for various amounts of time and processed as in Figure 1B. Closed diamonds, wild-type or complemented Lm growing in broth (gray) or host cells (colored). Open diamonds, Lm with altered actA or prfA or treated with cytochalasin D (cytoD). Blue, J774; orange, BMM; green, PtK2. 1, wild-type 10403S; 2, prfA*10403S; 3, 10403S + actA; 4, prfA*SLCC-5764; 5, wild-type SLCC-5764; 6, JactA 10403S; 7, wild-type 10403S + cytoD; 8, actA 146-150 10403S; 9, wild-type EGDe + cytoD; 10, JprfA + hly 10403S; 11, wild-type EGDe; 12, wild-type 10403S; 13, JactA + actA 10403S; 14, wild-type 10403S + cytoD; 15, JactA 10403S; 16, wild-type 10403S; 17, JactA 10403S; 18, wild-type 10403S + cytoD; 19, wild-type 10403S. (B and C) Lm growing in PtK2 were incubated in alkDala for various amounts of time and processed as in Figure 1B. Closed diamonds, wild-type or treated with cytochalasin D (cytoD). Blue, J774; orange, BMM; green, PtK2. 1, wild-type 10403S; 2, prfA*10403S; 3, 10403S + actA; 4, prfA*SLCC-5764; 5, wild-type SLCC-5764; 6, JactA 10403S; 7, wild-type 10403S + cytoD; 8, actA 146-150 10403S; 9, wild-type EGDe + cytoD; 10, JprfA + hly 10403S; 11, wild-type EGDe; 12, wild-type 10403S; 13, JactA + actA 10403S; 14, wild-type 10403S + cytoD; 15, JactA 10403S; 16, wild-type 10403S; 17, JactA 10403S; 18, wild-type 10403S + cytoD; 19, wild-type 10403S. (B and C) Lm growing in PtK2 were incubated in alkDala for 20 min and processed as in Figure 1B. Lysozyme (LZ) was added to the tissue culture medium from 1.5 to 4.5 hr. The proportions of the population with septal or non-septal labeling were tallied from n = 189–1,912 per strain per condition in (B), and estimates of division and non-dividing elongation periods in (C) were calculated as in Figure 1G.

Labeling and doubling times of different strains of Lm growing in broth and host cells. We measured these parameters within the fastest replication period supported by each condition. PrfA activation or ActA overexpression in broth culture decreased division frequency in a manner proportional to the doubling time (Figure 3A, compare closed to open gray diamonds). However, strains lacking these gene products or treated with cytochalasin D in J774 cells clustered separately from wild-type or complemented Lm (Figure 3A, compare closed to open blue diamonds). This pattern was also true for bacteria replicating in BMM (orange) and PtK2 cells (green). Across a range of doubling times, intracellular prfA and actA mutants and wild-type Lm treated with cytochalasin D had septal labeling frequencies that were more similar to strains growing in broth culture than to untreated wild-type in host cells (Figure 3A, p < 0.05, two-tailed Mann-Whitney test). Thus, the cell division cycle changes induced by host actin polymerization are a common feature of intracellular replication and are independent of doubling time.

ActA protects Lm against autophagy (Birmingham et al., 2007; Yoshikawa et al., 2009a, 2009b), a host degradative pathway that limits the growth of many cytosolic pathogens (Gomes and Dikic, 2014). The relationship between intracellular division frequency and the ActA-actin interaction was preserved in the absence of ATG5, a critical mediator of autophagy (Gomes and Dikic, 2014) (Figure S2F), indicating that autophagy was not responsible for the effect of host actin polymerization on the Lm cell division cycle. These results also discount the possibility of contributions from other ATG5-dependent processes such as septin cages (Mostowy et al., 2010).

Peptidoglycan Hydrolysis Compresses Cell Division in the Absence of Actin Polymerization

An Lm mutant lacking the NamA (MurA) PG hydrolase forms chains and is defective for actin tail formation at early stages of cellular infection but replicates as single cells with actin tails at late time points (Alonzo et al., 2011). Cytochalasin D treatment abrogates this phenotype, suggesting that actin polymerization can rescue NamA septation under certain circumstances. We found that NamA was also defective for flagellar motility but that, unlike actin-based motility, the impairment was not rescued at later time points (Figure S3A). There was no change in division or non-dividing elongation frequency in a mutant lacking flagella (Figures S3A and S3B). These data suggest that cell division cycle alterations are not the result of motion per se and that actin polymerization exerts a specific stress to shorten division and extend non-dividing elongation.

The observation that NamA chains are disrupted by actin polymerization (Alonzo et al., 2011) implied that the effects of actin polymerization and PG hydrolysis on cell division might be partially interchangeable. To test this idea, we first established a system for manipulating PG hydrolysis under broth and host cell conditions by lysozyme (Supplemental Experimental Procedures). We then compared the cell division cycles of Lm in the presence or absence of actin polymerization, lysozyme, or both (Figure 3B). Lysozyme treatment restored division and non-dividing elongation frequencies of ActA- or actin-deficient Lm to wild-type levels (Figure 3B). These changes were primarily the result of shortened division time; the enzyme did not impact non-dividing elongation in a consistent manner (Figure 3C). These data indicate that PG hydrolysis phenocopies the effect of actin polymerization on intracellular Lm cell division.

Intracellular Cell Division Cycle Rebalancing Increases the Proportion of Short Cells with Unipolar ActA

Lm localizes ActA de novo along the length of the bacterium but excludes the protein from the division site (Kocks et al., 1993; Rafelski and Theriot, 2006). ActA polarization is dependent on cell division and PG dynamics (Kocks et al., 1993; Pilgrim et al., 2003; Rafelski and Theriot, 2006). Actin tail formation, in turn, depends on the underlying ActA distribution as well as
bacterial size and shape. More specifically, actin tails form more readily on short cells with unipolar ActA than they do on long cells with bipolar ActA in an in vitro model of actin-based motility (Rafelski and Theriot, 2005).

Given that actin polymerization alters the cell division cycle, we hypothesized that it might also affect the localization of ActA on a population-wide basis. To test this idea, we first asked whether there was a correlation between cell division cycle period and ActA localization (Figures 4A and 4B) for intracellular Lm. We found a strong association between dividing bacteria and bipolar ActA, on the one hand, and newborn bacteria and unipolar ActA, on the other (Figure 4C). Next, we sought to determine whether there was a correlation between cell division cycle period and actin tail formation. We initially found no association between the presence of an actin tail and whether a bacterium was elongating without dividing, dividing, or newly divided (Figure 4C). We hypothesized that these steady-state proportions did not accurately reflect the propensity of a given sub-population to form actin tails because they represented a mix of bacteria: those with newly formed actin tails as well as those with older actin tails that were undergoing cycle(s) of growth and division (Figure 4B). To test this idea, we monitored cell division cycle stage and actin tail presence 20 min following washout of cytochalasin D. Since cytochalasin D inhibits actin polymerization,
actin tails observed after inhibitor removal should be of newer provenance than those observed during steady state. Under this condition, we found that dividing Lm were less likely to be associated with actin tails than those that were not dividing (Figure 4C). Newborn cells were the most likely to form tails (Figure 4C). These data confirm earlier in vitro findings (Rafelski and Theriot, 2005) that the cell division cycle stage of intracellular Lm correlates with ActA distribution and the ability to form actin tails.

PG and ActA co-staining demonstrated that dividing and newborn Lm have ActA distributions that are exclusively bipolar and unipolar, respectively (Figure 4C). Because actin polymerization impacts the cell division cycle, we hypothesized that it might also alter ActA localization on a population-wide basis. Cytochalasin D treatment of either EGDe or 10403S Lm modestly decreased the proportion of bacteria with unipolar ActA (Figure 4D). As well, a strain expressing the actA 146-150 allele (Lauer et al., 2001) had a lower proportion of the population with ActA at one pole (Figure 4D). Our data also indicated that dividing and recently divided Lm tended to be the longest and shortest cells of the population, respectively (Figures 1C and S1A). Since actin polymerization promotes bacterial division, we next asked whether it skews the cell-length distribution of the population. Inhibition of ActA-directed actin polymerization did not significantly alter the median cell length but modestly decreased the average by ~10% (Figure 4E). In aggregate, these results suggest that actin polymerization shifts the intracellular population structure of Lm in favor of shorter cells with unipolar ActA localization. Because the probability of actin tail formation is higher for this class of cell than for longer cells with bipolar ActA, biasing the population in this fashion should increase its overall propensity to form actin tails (Figure S4).

**DISCUSSION**

In one of the first descriptions of actin-based bacterial motility, Tilney and Portnoy commented that the “mat of filaments” surrounding Lm in the process of separation “elongate[d] away from the former division plane, as if trying to pull the Listeria apart” (Tilney and Portnoy, 1989). Other groups subsequently noted the importance of septation for promoting actin-based motility in both Lm and Shigella flexneri (Goldberg et al., 1993, 1994; Kocks et al., 1993; Pilgrim et al., 2003; Prévost et al., 1992; Rafelski and Theriot, 2005). We employed a D-alanine reporter to mark the cell division cycles of bacteria of different genetic backgrounds replicating in various host cells. We discovered that actin polymerization enhances division as well as slows non-dividing elongation. By increasing the proportion of short, newly divided Lm with unipolar ActA, host actin polymerization creates a positive-feedback loop that boosts the ability of the bacterial population to form actin tails.

We do not yet know the molecular mechanism(s) by which actin enhances Lm division and extends non-dividing elongation. Given that septation of a PG hydrolase mutant can be rescued by actin polymerization during infection (Alonzo et al., 2011) and that excess hydrolase can compensate for the loss of actin polymerization to shorten intracellular Lm division (Figures 3B and 3C), we speculate that Lm septation is directly or indirectly augmented by the mechanical stress associated with this process.

Bacterial cytoskeletal proteins such as MreB, FtsZ, and crescentin regulate bacterial growth and division and, therefore, morphogenesis (Wang and Sheaevitz, 2013). As well, prolonged physical constraints can alter cell growth and shape. Here, we show that an interaction between the bacterial cell surface and a cytoskeletal protein of mammalian origin can also influence the cell division cycle of an intracellular pathogen.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains**

Bacterial strains used in this study are listed in Table S3 and grown as indicated in Supplemental Experimental Procedures.

**PG Labeling**

PG was labeled in vitro and in host cells under similar conditions to those reported in Siegrist et al. (2013). Modifications are in Supplemental Experimental Procedures.

**Microscopy**

Microscopy was performed on fixed Lm as described (Siegrist et al., 2013). Details related to image processing and quantitation are in Supplemental Experimental Procedures.

**High-Performance Liquid Chromatography**

High-performance liquid chromatography (HPLC) analysis of unlabeled Lm PG was performed as described (Siegrist et al., 2013).

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures, four figures, and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.03.046.

**AUTHOR CONTRIBUTIONS**

M.S.S. conceived the project and acquired, analyzed, and interpreted data. M.S.S. and C.R.B. drafted the manuscript. A.K.A. analyzed the microscopy. A.E. and C.R.B. acquired, analyzed, and interpreted data. T.A.C. provided an unpublished analytical tool. S.A.W. acquired data. D.A.P. provided suggestions, strains, reagents, and protocols. All authors reviewed the manuscript.

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