Short FtsZ filaments can drive asymmetric cell envelope constriction at the onset of bacterial cytokinesis

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Abstract

FtsZ, the bacterial homologue of eukaryotic tubulin, plays a central role in cell division in nearly all bacteria and many archaea. It forms filaments under the cytoplasmic membrane at the division site where, together with other proteins it recruits, it drives peptidoglycan synthesis and constricts the cell. Despite extensive study, the arrangement of FtsZ filaments and their role in division continue to be debated. Here, we apply electron cryotomography to image the native structure of intact dividing cells and show that constriction in a variety of Gram-negative bacterial cells, including *Proteus mirabilis* and *Caulobacter crescentus*, initiates asymmetrically, accompanied by asymmetric peptidoglycan incorporation and short FtsZ-like filament formation. These results show that a complete ring of FtsZ is not required for constriction and lead us to propose a model for FtsZ-driven division in which short dynamic FtsZ filaments can drive initial peptidoglycan synthesis and envelope constriction at the onset of cytokinesis, later increasing in length and number to encircle the division plane and complete constriction.

Keywords asymmetric division; bacterial cell division; *Caulobacter crescentus*; electron cryotomography; FtsZ

Subject Categories Cell Adhesion, Polarity & Cytoskeleton; Microbiology, Virology & Host Pathogen Interaction

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Introduction

Cytokinesis in nearly all prokaryotic cells is mediated by a filamentous structure at the division site of the cell. This structure, as first revealed by immunoelectron microscopy of *Escherichia coli*,

contains the eukaryotic tubulin homologue FtsZ (Bi & Lutkenhaus, 1991). FtsZ filaments serve as a scaffold to concentrate PG synthases (Egan & Vollmer, 2013) and recruit additional cell division proteins which together constrict the envelope, eventually dividing the cell (Adams & Errington, 2009; Erickson *et al*, 2010; Haeusser & Margolin, 2016). FtsZ is almost universally conserved throughout Bacteria (Margolin, 2005) and Euryarchaeota (Makarova *et al*, 2010).

Fluorescence microscopy studies have shown that FtsZ localizes to a ring-like volume at the division site (Ma *et al*, 1996; Sun & Margolin, 1998; Thanedar & Margolin, 2004) and is highly dynamic, with subunits exchanging in and out of the volume with a half-life of 9-s in *E. coli* (Anderson *et al*, 2004; Biteen *et al*, 2012). *In vitro* reconstitution experiments using liposomes have further shown that FtsZ alone can constrict membranes (Osawa *et al*, 2008, 2009), but many molecular details of the structures present *in vivo* remain unclear.

Electron cryotomography (ECT) can resolve individual FtsZ filaments in the cell directly (not relying on fluorescent tags). In 2007, our laboratory reported the first ECT of FtsZ filaments in intact *Caulobacter crescentus* cells. Reflecting the difficulty of early ECT, only seven wild-type cells were imaged in that study, and only five of these were in the process of constricting (one was pre-constriction and one post-constriction). The results revealed that FtsZ filaments are sometimes short and do not always form a closed ring (Li *et al*, 2007), supporting a model in which individual short filaments can generate constrictive force on the membrane by themselves (Lu *et al*, 2000). This model gained further support from several super-resolution fluorescence microscopy studies reporting that FtsZ filaments in dividing cells are dynamic and do not form continuous closed rings (Strauss *et al*, 2012; Buss *et al*, 2013, 2015).

ECT technology has advanced considerably since 2007, and a recent ECT study re-examined FtsZ arrangement in *C. crescentus* (Szwedziak *et al*, 2014). Utilizing a newly developed direct electron detector and choosing cells aligned with the tilt axis to minimize the

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effect of the “missing wedge” (Oikonomou & Jensen, 2016), Szwedziak *et al* imaged 20 dividing *C. crescentus* cells. Finding that FtsZ filaments bundle together to form a complete ring, the authors concluded that complete rings are required for constriction to begin, and proposed three possible mechanisms including one in which maximizing filament overlap via sliding drives constriction (Horger *et al*, 2008a,b; Lan *et al*, 2009). While more cells were imaged in this second ECT study than the first, the results shown were all from cells in the mid-constriction stage of division, leaving the arrangement of FtsZ filaments in other constriction stages unclear.

To develop a more complete picture of FtsZ-driven cell constriction, here we imaged a large number of dividing cells of several bacterial species by ECT. We found that in *Proteus mirabilis*, *C. crescentus*, *Halothiobacillus neapolitanus* c2, *Legionella pneumophila*, a *Belliella baltica*-related strain, *Thiomonas intermedia*, *Ralstonia eutropha*, and FtsZ-overexpressing *Salmonella enterica* spp. *enterica* minicells, initial constriction is asymmetric (starts on one side of the cell) and is accompanied by short FtsZ-like filaments on the constricting side. Fluorescence microscopy further revealed that a burst of peptidoglycan (PG) synthesis also frequently occurs on one side of *P. mirabilis* and *C. crescentus* cells. In *C. crescentus*, where the cells' natural curvature allows the sides to be distinguished, we observed that constriction, PG synthesis, and short FtsZ-like filaments all appear first on the outer curvature. Taken together, our observations support a general model in which cell envelope constriction and septal PG synthesis in the early stages of bacterial cytokinesis are driven by individual FtsZ filaments, and a complete ring is not required.

Results

Multiple bacterial species initiate constriction asymmetrically

Intrigued by the apparent contradiction in previous ECT studies of FtsZ in *Caulobacter crescentus* (Li *et al*, 2007; Szwedziak *et al*, 2014), we imaged a large number (49) of wild-type frozen-hydrated *C. crescentus* cells by ECT, targeting long (and therefore potentially dividing) cells. While *C. crescentus* is a well-studied model system for division and the thinness of its cells allows features such as filaments to be readily resolved by ECT, we found that its natural curvature complicated analysis of constriction (see below), so we also imaged 38 wild-type frozen-hydrated *Proteus mirabilis* cells, which have a relatively straight cell body. In addition, we searched the Caltech Tomography Database, a resource containing more than 15,000 tomographic 3D images of 88 different bacterial species (Ding *et al*, 2015), for cryotomograms of other dividing cells. In total, we identified 159 dividing cells, and noted that 47, from eight different species, exhibited indentations of the cell envelope on only one side of the cell, as if constriction had begun asymmetrically (Fig 1A and B). In each case, the indentation appeared near the middle of a long cell. Other cells of these same species were observed with deeper, bilateral constrictions (Fig EV1, note that examples from *P. mirabilis* and *C. crescentus* are shown in Figs 2 and 3).

To more quantitatively examine this phenomenon, we analyzed the *P. mirabilis* and *C. crescentus* cells in detail. For both species, we arranged the cell images in order of cytokinesis progression

based on the width ratio of the division plane to the cell body (W_{mid}/W , see Appendix Fig S1A for details). To identify constrictions of the cell envelope in an unbiased way, even in the inherently curved bacterial species *C. crescentus*, we wrote a Python program (available at <http://jensenlab.caltech.edu/software>) that characterizes constriction based on the length (L) of the indentation site and its radius of curvature (r) (see Appendix Fig S1B for details).

For *P. mirabilis*, the results of this classification are shown in Figs 2 and EV2A, with measurements listed in Appendix Table S1. On the basis of this classification, we divided the cells into four categories: pre-, early-, mid-, and late-constriction. “Pre-constriction” cells had no constrictions. The “early-constriction” class began with the first cell in the series with a constriction and ended with the last cell with only a single (one-sided) constriction. The “mid-constriction” class then extended until cells exhibited a deep furrow (later defined more specifically as the cells beyond which FtsZ-like filaments were no longer seen, see below). In the *P. mirabilis* data set, there were 10 pre-constriction cells. Among the 13 early-constriction stage cells (nos. 11–23), six exhibited an indentation on just one side. All 10 mid-constriction cells (nos. 24–33) exhibited constrictions on both sides of the cell body, and the five late-constriction cells (nos. 34–38) possessed deep furrows converging into a neck-like structure. Note that the unbiased quantitative ordering by width ratio likely failed to perfectly reflect internal states: gentle thinning in the center of cell #13 caused it to be classified with the early-constriction cells despite its lack of a detectable constriction, and the extra-deep one-sided furrow of cell #23 caused cell nos. 20–22 to be classed as early-constriction despite their bilateral constrictions. The objective ordering nevertheless made it clear that in many *P. mirabilis* cells constriction initiates asymmetrically.

The results for *C. crescentus* are shown in Figs 3 and EV2B, with measurements listed in Appendix Table S2. We observed nine cells (nos. 1–9) in the pre-constriction stage, 23 cells (nos. 10–32) in the early-constriction stage, 14 cells (nos. 33–46) in the mid-constriction stage, and three cells (nos. 47–49) in the late stage. Ten of the early-constriction cells were constricted on just one side (nos. 10, 12, 13, 14, 18, 19, 22, 25, 29, and 32), and in all but one, the constriction appeared on the outer curvature. The exception (#12) appears to be a false positive, as our software marked a constriction on the inner curvature, but the indentation is questionable and we would not in fact have marked it visually. Thus, constriction initiates asymmetrically on the outer curvature in *C. crescentus*. Together with the *P. mirabilis* data and the observations in the other six species shown in Fig 1, these results reveal that in multiple Gram-negative bacterial species, constriction can initiate on one side of the cell. Later in the process, constriction spreads to both sides (Figs 2, 3 and EV1), so that in some species like *C. crescentus*, late-constriction appears very nearly symmetric. In others, like the *Belliella baltica*-related strain, the asymmetry remains throughout division.

Peptidoglycan synthesis is also asymmetric at the onset of division

During division of rod-shaped cells that contain MreB and FtsZ, the dominant mode of peptidoglycan (PG) synthesis switches from MreB-dependent to FtsZ-dependent (Typas *et al*, 2011). It has been suggested that FtsZ filaments serve as scaffolds to organize PG synthetic enzymes and that FtsZ-mediated constriction is stabilized

Figure 1. Examples of eight different bacterial species that exhibit asymmetric early-constriction.

A Representative central slices of tomograms of eight different constricted cells are shown, arranged so that the asymmetric division site is on the right (indicated by white arrows). Scale bars, 100 nm.

B Table showing the numbers of cells observed constricting asymmetrically and symmetrically for each species.

by the construction of new PG (Egan & Vollmer, 2013). We therefore wondered whether PG synthesis in early-constriction is also asymmetric. To test this hypothesis, we tracked new PG synthesis in both *P. mirabilis* and *C. crescentus* by incorporation of a fluorescently labeled D-amino acid, 7-hydroxycoumarin-3-carboxylic acid-amino-D-alanine (HADA; Kuru et al, 2012).

We first tried a previously published protocol of 5-min HADA labeling, followed by washing and fixation (Kuru et al, 2012; Sundararajan et al, 2015). In an asynchronous culture of

C. crescentus labeled this way, we observed a strong band of fluorescence at the midcell in more than 60% of cells ($n > 200$). Whether this is MreB- or FtsZ-based is unclear, but the fact that bright labeling was only ever seen at midcell suggests that the midcell is the only location where new PG synthesis is highly concentrated. To increase the temporal resolution of staining, and perhaps capture more transient stages, we modified the protocol by labeling cells with HADA for only 1.5 min, immediately fixing and then washing. This resulted in half as many labeled cells (30%),

Figure 2. Atlas of dividing *P. mirabilis* cells imaged in this study.

Central slices of tomograms or projection images (marked by red asterisks) of *P. mirabilis* are sorted according to W_{mid}/W . Numbers and labels, as well as color scheme, correspond to Fig EV2A. White arrows denote indentations detected by the program on the indicated side. The three cells numbered 11, 26, and 31 correspond to cell 11 in Fig 5A and cells 26 and 31 in Fig 6A, respectively. The number of FtsZ-like filaments observed on each side of the division plane (left: right) is noted below the corresponding image. If no number is given, no filaments were observed, meaning either that there were none or that we could not resolve them. Scale bar, 300 nm.

again all at midcell, allowing us to sample cell cycle dynamics more finely.

Using this improved protocol, we labeled ~200 *C. crescentus* cells and arranged them in relative order approximating progression

through the cell cycle based on morphological features such as cell length and division plane diameter (Fig 4A). Fifty-five percent of the cells were unlabeled and between short and intermediate length, suggesting diffuse PG synthesis during elongation. ~5% of the cells

Figure 3. Atlas of all *Caulobacter crescentus* cells imaged in this study.

Central slices of tomograms of *C. crescentus* are sorted according to V_{mid}/W . Cells are arranged with the inner curvature on the left and the outer curvature on the right. Numbers and labels, as well as color scheme, correspond to Fig EV2B. White arrows denote indentations detected by the program. Measurements from the last three cells (47–49) are omitted. Pink arrows indicate filaments formed by CTP synthase (Ingerson-Mahar *et al.*, 2010). The six cells numbered 17, 24, 32, 34, 38, and 42 correspond to cell 32 in Fig 5B, cells 38 and 42 in Fig 6B, and cells 17, 24 and 34 in Fig EV4, respectively. The number of FtsZ-like filaments observed on each side of the division plane (left: right) is noted below the corresponding image. Scale bars, 100 nm.

Figure 4. Localization of new PG incorporation.

A, B Localization of new PG incorporation in different stages of *C. crescentus* (A) and *P. mirabilis* (B) cytokinesis. In each panel, the upper row shows the fluorescence (HADA) images of representative cells in different cell division stages overlaid with phase contrast images showing the cell profiles. The bottom rows show fluorescence profiles of the division plane of the indicated early-constriction and mid-constriction stage cells. The numbers in (A) and (B) are 1/10 values of the 8-bit gray values measured using the NIH ImageJ software. Scale bars, 1 μm .

exhibited a single bright fluorescent spot on one side of the cell, and ~25% of the cells exhibited a band of fluorescence all the way across midcell. The final, longest 15% of the cells were again unlabeled and were clearly in the final stages of division. Comparing the lengths of each of these classes of cells (Fig EV3A), it was observed

that the 5% of cells with the single bright spot on one side of midcell were intermediate in length between the short unlabeled cells and the cells with a complete fluorescent band across midcell. Among the cells with a single bright spot, 12 out of 13 of these spots appeared on the outer curvature of the cell.

Figure 5. Short FtsZ-like filaments accompany early asymmetric cell constriction.

A–E Short FtsZ-like filaments accompany early asymmetric cell constriction in *P. mirabilis* (A), *C. crescentus* (B), *H. neapolitanus* c2 (C), *L. pneumophila* (D), and *S. enterica* spp. *enterica* minicells (E). The two cells in (A) and (B) correspond to cells nos. 11 and 32 in Figs 2 and 3, respectively. A central x-y slice is shown at left. White arrows highlight the asymmetric constriction and indicate the plane of the cross-sectional x-z slice shown at right. The limits of visibility or apparent ends of continuous FtsZ-like filaments are marked by yellow arrows. OM, outer membrane; IM, inner membrane. Scale bars, 100 nm.

We next used a similar protocol to visualize division-related PG incorporation in *P. mirabilis* and found that a longer (7.5 min) labeling time gave the most interesting results. In 500 cells in three independent experiments, ~80% of the cells were unlabeled and short. One percent of the cells exhibited a single bright spot on one side of midcell, ~17% showed a uniform band at midcell, and the final ~3% of cells were again unlabeled, but clearly of a different class than the first because they were nearly twice as long and in the final stages of division (Figs 4B and EV3B). Again the small population of cells exhibiting HADA labeling on just one side of the division plane were intermediate in length between the short unlabeled cells and the cells with a complete fluorescent band across midcell (Fig EV3B). For both species, we think it unlikely that the asymmetric HADA labeling we observed is an artifact because (i) in both species it was only observed in cells of length consistent with an early-constriction stage, and (ii) in *C. crescentus*, nearly all cases were observed on the outer curvature. Together, these results suggest that the asymmetric early-constriction of bacterial cells reported above is accompanied by asymmetric PG synthesis.

Short FtsZ-like filaments accompany early asymmetric constriction

In many cases, we observed ~4-nm-wide filaments ~15–16 nm away from the inner membrane (distance measured from center-to-center) perpendicular to the long axis of the cell at midcell. Previous studies perturbing the abundance, turnover, or membrane tethering of the protein have already established that these are the characteristics of FtsZ filaments, at least in *C. crescentus* (Li et al, 2007; Szwedziak et al, 2014). Because it remains possible that any particular filament seen in a cell by ECT is not FtsZ, in the following we refer to the filaments as “FtsZ-like”, but we draw conclusions about FtsZ filaments in general based on the assumption that when FtsZ filaments were present in the cells, they were among those identified as “FtsZ-like.”

No FtsZ-like filaments were observed in the ten unconstricted *P. mirabilis* cells (Fig 2). FtsZ-like filaments were observed in three early-constriction stage cells. In two (nos. 11 and 14), a single short FtsZ-like filament was present on the same side as the unilateral indentation, and no filaments were seen on the opposite side (one example shown in Fig 5A). In the third cell (#15), indentations and a single FtsZ-like filament were seen on both sides. Within the 10 mid-constriction stage cells, FtsZ-like filaments were observed in five. In all five cases, the cells were constricted on both sides and FtsZ-like filaments were also seen on both sides, including both short and long filaments (two examples shown in Fig 6A). No FtsZ-like filaments were observed in the five late-constriction stage cells. While the relative straightness of *P. mirabilis* cells was an advantage in the constriction analysis described above, *P. mirabilis* cells were unfortunately quite thick for ECT, and so there may have been more filaments present than we detected.

Caulobacter crescentus cells are thinner, making it easier to resolve filaments. Again no FtsZ-like filaments were observed in the nine unconstricted *C. crescentus* cells (Fig 3). In the 23 early-constriction stage cells, FtsZ-like filaments were observed in 22. The numbers of filaments per cell ranged from 0 (cell #29) to 14 (cell

Figure 6. FtsZ-like filament localization in mid-constriction stages.

A–D FtsZ-like filament localization in mid-constriction stages of *P. mirabilis* (A), *C. crescentus* (B), *H. neapolitanus* c2 (C), and *S. enterica* spp. *enterica* minicells (D) division. Representative central x-y slices of 3D tomographic reconstructions of mid-constriction stage cells are shown on the left. Cell orientation is the same as in Fig 3. White arrows highlight the constriction sites and indicate the cross-sectional x-z slice plane. In (B), enlarged views of x-y slices of both the inner (left) and outer (right) curvature of the division site are shown, with yellow arrows indicating FtsZ-like filament cross sections. In all panels, the middle column shows an x-z slice of the division site. Yellow arrows indicate FtsZ-like filaments. Segmentations are shown at right, highlighting the inner (yellow) and outer (purple) membranes, and all FtsZ-like filaments observed (red). The two cells in (A) correspond to cells nos. 26 and 31 in Fig 2; the two cells in (B) correspond to nos. 38 and 42 in Fig 3. Scale bars, 50 nm in second column in (B); all other scale bars, 100 nm; scale is the same in upper and lower panels in (A) and (B); segmentations not to scale.

#26), with an average of 3.4 filaments/cell (noting, however, that because of the “missing wedge” of information in ECT, it is impossible to follow individual filaments all the way around a cell’s circumference (Iancu *et al*, 2005), so some of the filaments might have been multiply-counted if they continued all the way from the outer to the inner curvature). Nearly twice as many filaments were seen on the outer than inner curvature (50 to 29). In seven early-constriction stage cells (nos. 12, 16, 17, 20, 24, 25, and 32) and one mid-constriction stage cell (no. 34), FtsZ-like filaments were found only on the outer curvature of the cell (one example shown in Fig 5B and three more in Fig EV4); in only one cell (no. 19), a filament was found only on the inner curvature. Together these results show that during early-constriction, FtsZ filaments are more commonly found on the outer curvature of *C. crescentus*.

FtsZ-like filaments were observed in all 14 mid-constriction stage *C. crescentus* cells, ranging in number from only 1 (cell #34) to 19 (cell #38), with an average of 8.8 filaments/cell (again noting that some may have been multiply-counted; two example cells shown in Fig 6B). The ratio of filaments on the outer to inner curvature was still slightly biased toward the outer curvature (68 to 55), but much closer to parity than in the early-constriction stage. Many filaments were noticeably longer than those in the early-constriction stage cells, spanning the entire visible arc on one side of the cell, but shorter filaments (ending within the visible region) were also seen. Together the filaments formed a flat ribbon parallel to the membrane. No FtsZ-like filaments were observed in the three late-constriction stage cells (Fig 3), leading us at this point to define the boundary between “mid” and “late” stages as after the last cell with visible FtsZ-like filaments.

Following this analysis of the large *P. mirabilis* and *C. crescentus* data sets, we also looked for FtsZ-like filaments in the other 89 tomograms of dividing cells of other species. In all three *H. neapolitanus* c2 cells with unilateral constriction, a short FtsZ-like filament was detected on the constricting side and none was seen on the non-constricting side. The same was true for both asymmetrically constricting *L. pneumophila* cells. Finally, in *S. enterica* subsp. *enterica* minicells produced by an FtsZ-overexpressing strain, short FtsZ-like filaments were seen on the constricting side and not the other side of eight out of 13 unilaterally constricting cells (one example each of asymmetrically dividing *H. neapolitanus* c2, *L. pneumophila*, *S. enterica* subsp. *enterica* minicells with FtsZ-like filaments shown in Fig 5C–E). Unfortunately, the tomograms of the other three species (*Belliella baltica*-related strain, *T. intermedia*, and *R. eutropha*) were not high enough quality to detect filaments, in part because of cell thickness. Later in cell division in the three other species where FtsZ-like filaments were seen (*H. neapolitanus* c2, *L. pneumophila*, *S. enterica* subsp. *enterica* minicells), FtsZ-like filaments were seen on both sides of bilaterally constricting cells (two examples shown in Fig 6C and D), as in *P. mirabilis* and *C. crescentus*.

In summary, we have a total of 44 tomograms of cells or minicells constricting on just one side, and FtsZ-like filaments are visible in 25. Among these, in 19, the filaments appear only on the constricting side, in 5, the filaments are on both sides, and in just 1 (*C. crescentus* #12, the likely false positive), the filament is on the side opposite the constriction (the filament is on the outer curvature, and the putative constriction is on the inner). As mentioned above, while the effect of the “missing wedge” of information in

ECT makes it impossible to follow features all the way around a cell’s circumference (Iancu *et al*, 2005), in those cases where *no* filament portions were observed on one side of the cell, the data are sufficient to establish that the filaments did not form complete rings. These results reveal that the presence of short FtsZ filaments correlates in both time and location with asymmetric constriction, and complete rings are not necessary for constriction.

Discussion

Asymmetric early-constriction is a widespread property of bacterial cytokinesis

In multiple unmodified Gram-negative bacterial species, as well as non-replicating minicells containing FtsZ (Farley *et al*, 2016), we observed initial cell division constrictions on just one side of the division plane. We conclude that cell division initiates asymmetrically. Previous imaging studies missed asymmetric constriction likely because of limited resolution, specimen preparation, or number of samples: Light microscopy is limited to a few hundred nanometer resolution, and in many cases, the constrictions were only tens of nanometers deep. While higher resolution, traditional EM preparation methods rely on fixation and dehydration, which often fail to preserve membrane structure (Pilhofer *et al*, 2010). Previous ECT studies might have discovered this phenomenon, but they either imaged too few cells (Li *et al*, 2007) and/or focused on later stages of division ((Li *et al*, 2007; Szwedziak *et al*, 2014). Here, by imaging a large number of native cells by ECT throughout the process of division, the asymmetry of early cell division was recognized.

Accompanying asymmetric early-constriction in both time and place, we observed asymmetric PG synthesis and short FtsZ-like filaments. In *C. crescentus*, where the cell’s inherent curvature distinguishes the two sides, constriction, PG synthesis, and the presence of FtsZ-like filaments were all strongly biased toward the outer curvature (ratios of 9:1, 12:1, and 7:1, respectively, outer to inner curvatures, in cells where the feature was present on only one side). While it remains unclear whether the PG synthesis was MreB-, FtsZ-, or even crescentin-related (Cabeen *et al*, 2009), we believe it was part of the cell division process because the HADA labeling only appeared at midcell in cells whose lengths matched early-constriction.

These observations agree with previous studies that have observed asymmetric constriction and/or FtsZ localization in other systems (Appendix Fig S2). In the spherical cyanobacterial plastids of *Cyanophora paradoxa*, fluorescence microscopy showed FtsZ arcs underlying asymmetric indentation furrows (Sato *et al*, 2009; Appendix Fig S2A). In the spiral-shaped magnetotactic bacterium *Magnetospirillum gryphiswaldense*, asymmetric septation accompanied by FtsZ arcs was suggested to split the magnetosome chain (Katzmann *et al*, 2011; Appendix Fig S2B). Asymmetric FtsZ localization and/or constriction have been observed in several *E. coli* mutants including a temperature-sensitive mutant of *ftsZ* that induces spiral septa (Addinall & Lutkenhaus, 1996; Appendix Fig S2C), a spherical *mreB* mutant strain (Bendezu & de Boer, 2008; Appendix Fig S2D), and a strain overexpressing a MinC_C-MinD fusion (Rowlett & Margolin, 2014; Appendix Fig S2E). In the

pleomorphic archaeon *Haloferax mediterranei*, immunofluorescence of FtsZ revealed arcs that did not completely encircle the cells (Poplawski *et al*, 2000; Appendix Fig S2F). Recently, it was shown that a rod-shaped gammaproteobacterial symbiont living on nematodes constricts asymmetrically during division, initiating FtsZ assembly at the host-associated pole (Leisch *et al*, 2016; Appendix Fig S2G).

A model for constriction driven by dynamic FtsZ filaments

Taken together, all these results support a model for FtsZ-driven cytokinesis in which FtsZ polymerizes into short filaments, initially at one site on the division plane, and these short FtsZ arcs drive initial PG synthesis and constriction at that site (Fig 7A and B).

Complete FtsZ rings are not necessary for constriction to start. Our data also show that at least in *P. mirabilis* and *C. crescentus*, as cytokinesis proceeds, the number of filaments and their length both increase, until they often (but not always—see *C. crescentus* cell #34) overlap to form a continuous ring. Near the end of constriction, the number of FtsZ filaments decreases (e.g. cells 34, 36, and 41 in Fig 3), and we define here a “late-constriction” stage characterized by both the presence of deep furrows and the absence of FtsZ filaments, consistent with the previous observation in *E. coli* that FtsZ leaves the ring in late-constriction before other division proteins (Soderstrom *et al*, 2014).

This model reconciles the apparently conflicting results from the two previous ECT studies on *C. crescentus* (Li *et al*, 2007; Szwedziak *et al*, 2014), providing a more complete picture of the

Figure 7. Model of FtsZ-driven PG synthesis and constriction in *P. mirabilis* and *C. crescentus* cytokinesis.

A, B The diagrams show progression through cell division of *P. mirabilis* (A) and *C. crescentus* (B). The cell envelope profiles were drawn according to the experimental images. FtsZ filaments are shown in red, new PG incorporation in green, and outer and inner membranes in purple and yellow, respectively. In the early-constriction stage, short arc-like FtsZ filaments assemble on one side of the cell, recruiting PG synthesis and driving initial, asymmetric constriction of the cell envelope. Later, FtsZ filaments accumulate, forming a bundle of overlapping filaments that covers the entire division plane in the mid-constriction stage. Finally, these highly dynamic filaments drive PG synthesis and constriction symmetrically around the division plane until cytokinesis is complete.

highly dynamic FtsZ filaments that at various times throughout division take the form either of short isolated filaments or extended bundles. Our ECT “snapshots” agree with the model that FtsZ filaments are dynamic: While a clear pattern emerged (schematized in Fig 7), there were exceptions. In some cells, the location of filaments and membrane indentations did not match (e.g., cells 12, 19, and 29 in Fig 3), and there was not a smooth increase in numbers of filaments with deeper constriction (*C. crescentus* cells #33, 34, and 38 had 15, 1, and 19 filaments, respectively). This is consistent with constriction occurring on a timescale longer than the lifetime of individual filaments. In fact, fluorescence microscopy studies have shown that the half-life of FtsZ subunits in division assemblies is on the order of a few seconds in *E. coli* and *B. subtilis* (Anderson et al, 2004), and recent studies showed that FtsZ treadmills rapidly (Ramirez et al, 2016; Wagstaff et al, 2016; Bisson-Filho et al, 2017; Yang et al, 2017).

We did not observe asymmetric early-constriction in all bacterial species; for example, all of the dividing *E. coli* and *B. subtilis* cells in the Caltech Tomography Database appear symmetric. We doubt these phenotypes are the result of different mechanisms, but rather simply variations in the positioning of FtsZ. In *C. crescentus*, a gradient of MipZ guides positioning of FtsZ (Thanbichler & Shapiro, 2006). Since the cell is curved, the point of lowest MipZ concentration is probably at the center of the outer curvature; this may then serve as a nucleation site for FtsZ to assemble into short arcs and initiate constriction and PG synthesis. *E. coli*, by contrast, uses spatial oscillations of the Min proteins to inhibit FtsZ polymerization (Raskin & de Boer, 1999). Since *E. coli* cells are straight rods, this oscillation marks an entire midcell plane rather than a point, likely allowing FtsZ polymer nucleation anywhere around the cell with equal probability, quickly resulting in symmetric constriction. In spherical *E. coli* mutants (*pbpA*[−], *rodA*[−], and *mreB*[−]), a combination of Min oscillation and aberrant nucleoid position likely leads to asymmetric FtsZ nucleation and therefore asymmetric constriction (Iwaya et al, 1978; Wachi et al, 1987; Corbin et al, 2002). It remains unclear why division in the straight-rod-shaped *P. mirabilis* begins asymmetrically. In any case, these morphologically distinct division processes likely reflect differences in FtsZ localization, not function, and all are consistent with the model that complete FtsZ rings are not required for initial constriction, as suggested by Addinall and Lutkenhaus (1996).

Advantages of visualizing native FtsZ structures

Many previous fluorescence microscopy and super-resolution fluorescence microscopy results support our model that dynamic FtsZ filaments do not necessarily form closed rings in dividing cells (Thanbichler & Shapiro, 2006; Costa et al, 2008; Fu et al, 2010; Schofield et al, 2010; Goley et al, 2011; Jennings et al, 2011; Strauss et al, 2012; Buss et al, 2013, 2015). All of these studies, however, relied on artificially expressed fusion proteins that are not fully functional (Charbon et al, 2011) and, depending on the genetic constructs used, were often expressed too early and arrived at the division plane long before native FtsZ is even normally expressed in the cell cycle (Goley et al, 2011). Thus, these studies did not faithfully reveal the physiological spatial and temporal localization of native FtsZ. Also, unlike ECT, fluorescence microscopy cannot

distinguish between concentrated monomers and physically linked polymers (filaments). This is an important consideration when interpreting fluorescent images of FtsZ “rings” at the division plane (Ma et al, 1996; Sun & Margolin, 1998; Thanedar & Margolin, 2004). For example, super-resolution fluorescence light microscopy showed that FtsZ-Dendra2 in *C. crescentus* cells occupies a toroidal volume extending ~250 nm away from the membrane (Biteen et al, 2012). In ECT studies of native cells, however, FtsZ-like filaments have never been observed at such a distance from the membrane (Li et al, 2007; Szwedziak et al, 2014); it is therefore unlikely that these signals are being produced by filamentous FtsZ. Instead, it must reflect simply a concentration of FtsZ monomers at the midplane. In a recent report, ectopically expressed FtsZ-mEos2 in *E. coli* showed a punctate fluorescent volume at the midcell, as well as many non-midcell fluorescent clusters (Coltharp et al, 2016), but ECT of native cells has not revealed FtsZ-like filaments anywhere but at midcell. Thus, fluorescence microscopy and ECT are complementary: Fluorescence studies have the advantages of being able to label targets specifically and reveal dynamics (Ramirez et al, 2016; Wagstaff et al, 2016; Bisson-Filho et al, 2017; Yang et al, 2017), and ECT has the advantage of being able to visualize wild-type filaments directly in their native state.

Materials and Methods

ECT imaging

The cell strains and culture conditions used in this study are summarized in Appendix Table S3. For ECT imaging, cells growing in cultures were gently centrifuged and resuspended in 50 μ l fresh medium. This resuspension was mixed with fiducial markers (10 nm gold beads treated with bovine serum albumin (BSA) to prevent aggregation) and plunge-frozen on EM grids in a mixture of liquid ethane and propane (Tivol et al, 2008). Images were acquired using a 300 keV Polara transmission electron microscope (FEI) equipped with a GIF energy filter (Gatan) and a K2 Summit direct detector (Gatan). Tilt-series were collected from -60° to $+60^\circ$ in 1° increments using UCSF Tomography software (Zheng et al, 2007) with a defocus of -8μ m and total dosage of $160 \text{ e}^-/\text{\AA}^2$. Tomograms were calculated using IMOD software (Kremer et al, 1996). Segmentation and rendering were performed using the Amira package (FEI) and IMOD software.

Labeling nascent PG incorporation

For fluorescent labeling of PG, the procedure was adapted from (Kuru et al, 2012). HADA (7-hydroxycoumarin-3-carboxylic acid-amino-D-alanine) was provided by Dr. Yves Brun at Indiana University. Briefly, exponentially growing wild-type *C. crescentus* NA1000/CB15N cells in M2G medium ($\text{OD}_{600} = 0.3$) were incubated at room temperature for 1.5 min with 500 μ M HADA. Cells were then fixed on ice for 20 min by adding ice-cold anhydrous ethanol to a final concentration of 85%. Cells were washed three times with PBS buffer (pH 7.4), placed on agarose-coated slides and imaged with an Eclipse 90i light microscope (Nikon). The same protocol was used for labeling *P. mirabilis* cells except the cells were incubated at 30°C for 7.5 min. Fluorescence value profiles were

measured using ImageJ (Schneider *et al*, 2012). Length of a dividing cell was calculated by adding the two lengths from each terminus to the division plane. Cell lengths were measured using Photoshop CS6 (Adobe System Incorporated), and figures were generated using the software OriginPro 8.0 (OriginLab Corporation, <http://www.originlab.com>).

Measurements of cell width, radius of curvature, and indentation length

For data analysis, we wrote a software tool to measure the width and indentation near the constriction site on either side of the cell (Appendix Fig S1). The inner membrane was subsampled at 50-nm intervals and fitted to a centripetal natural cubic spline. The program uses either of two criteria to define constriction: (i) at the indentation site, if the program can find three curvature maxima, one concave (inner) curvature maximum and two convex (outer) maxima, the site is defined as a constriction; (ii) if the program can detect a site on the inner membrane with $r < 300$ nm, the program also defines this site as a constriction.

Expanded View for this article is available online.

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Author contributions

QY and GJJ conceived and designed the experiments. QY, Y-WC, MB, CVI, AB, and DG performed the experiments. AJJ wrote the software and made measurements. QY, AJJ, CMO, and GJJ analyzed the data. QY, AJJ, Y-WC, CMO, and GJJ wrote the paper.

Conflict of interest

The authors declare that they have no conflict of interest.

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