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

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 13 January 2017

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by three referees whose comments are shown below. As you can see from the comments, the referees express interest in the proposed mechanism of asymmetric FtsZ filament assembly during cell division. Given the referees' positive recommendations, I would like to invite you to submit a revised version of the manuscript, addressing the comments of all referees.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://emboj.embopress.org/about#Transparent_Process

We generally allow three months as standard revision time. Please contact us in advance if you need an extension of the revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed.

Please feel free to contact me if have any further questions regarding the revision. Thank you for the opportunity to consider your work for publication. I look forward to your revision.

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REFEREE REPORTS

Referee #1:

Review: "Short FtsZ filaments drive asymmetric cell envelope constriction at the onset of bacterial cytokinesis", Yao et al., EMBO Journal 2016

This is a very interesting and timely manuscript investigating FtsZ-based cell division. I enjoyed reading it very much. The authors first show by analysing new and existing cryoET volumes of unmodified cells that many show asymmetric cell constrictions. We have seen similar things (and will be scooped somewhat by this study). The authors then show that PG synthesis is asymmetric (not shown on the same cells, that would have been very difficult, although possible, using CLEM). Finally, they show that the constrictions correlate with the occurrence of FtsZ filaments by pointing at filaments at the expected distance from the membrane.

I found the manuscript to be well written and as convincing as these sorts of things can get without hypothesis-driven perturbations.

In summary, I recommend to work for publication in EMBO Journal as it provides important insights that constrain our view of how cells constrict during the FtsZ-based process. Much more mechanistic work will be required in future but knowing that somehow FtsZ filaments, and not complete rings, are able to produce indentations that correlate with PG synthesis guides us where to look next!

Some more specific comments in no particular order:

- There are several manuscript on biorxiv right now that are relevant to the work, references to those might need to be worked in?

- introduction: Szwedziak et al did not envisage that the force generated by overlap would constrict the cell - rather that it would allow the ring to constrict while the cell does around it

- two interlinked processes. But I am now convinced that the 'checkpointing' idea of ring formation is most likely not correct (constriction only after ring formation)!

- line 181-: possible that asymmetric incorporation is caused by the very short labelling times and only a few PG synthesis complexes going round?

- line 224: do like the argument of finding asymmetric PG synthesis mostly on convex sides of Caulobacter, which speaks against above point.

-line 237-: disappointing (for cryoET) that still not possible to see FtsZ in all early constricting cells. Method problem or related to mechanism? Treadmilling filaments?

- line 247-: what do the authors think happens to FtsZ in late cells? FtsZ gone? How narrow do they have to be for FtsZ to disappear? If something else took over abscission, is not likely we would know about mutants arrested in that stage?

- suggestion (not demanding this to be done): what happens in Caulobacter cells that are straight (no crescentin, for example)?

- line 265-: good to see that there might still be a role for a ring structure (making sure constriction works along a plane, so membranes can fuse efficiently)!

- why was P. mirabilis chosen and not, say, thin E. coli? Not clear and not really discussed. (line 307-) This was done because E. coli does not show asymmetric constriction, right?

- line 331-: how does PG synthesis know what to do? Template model needs mentioning (as in model at the end of Ethan's paper)?

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- what about FtsZ in cells without PG (Thermoplasma, for example and many other archaea)?

- line 351-: stating that in E. coli and B. subtilis always symmetric. Does this not mean that the title and abstract could be seen as somewhat misleading? Yes, the mechanism could be the same, but the morphology is quite different and the title implies that FtsZ always induces asymmetric constriction (to me).

- line 359-: argument about E. coli being straight (hence more likely to be symmetric) also applies to P. mirabilis, no? Similarly: can the authors comment on why the short FtsZ filaments would assemble only on one side of the C. crescentus cells? Are the two sides of P. mirabilis also different?

- line 372-: naturally, I like the discussion of problems with imaging non-functional GFP fusions of FtsZ. This is important to be reiterated. Maybe worth citing the latest papers on biorxiv using more functional fusions (Filho et al)?

- Figure 1A: Caulobacter, Legionella, Ralstonia, Thiomonas and possibly Salmonella cells could also be constricted on both sides as a dumbbell structure might be mechanically unstable and bend and kink on the grid?

- generally: it was not immediately clear to me what side 'convex' refers to when talking about Caulobacter cells (since FtsZ is on the inside of the cell, which inverts the curvature definition).

- Figure 3: cryoET will hopefully improve over the next few years through higher voltages, even better detectors, phase plates, FIB milling and much faster acquisition times. It must, looking at the pictures.

- the model in Figure 4 indicates that in the mid-constriction stage, the two sides of the invaginating cellular membrane catch up and are in-sync with each other. Would this not indicate that the cells are waiting for the Z-ring to form, and once the ring forms, actual cytokinesis can begin? Perhaps the constriction is only needed for Z-ring assembly?

- I see two constrictions in Figure 3C? What is the other one?

- Figure 3A and S4 - the FtsZ layer is very hard to see. Perhaps the authors could show additional panels with a bandpass filter applied? I do understand though the P. mirabilis cells are thick and this may not be possible.

- It is difficult to see the FtsZ layer in the panels of figure 1A. In how many cases is FtsZ seen? Could these be marked?

- Figure S8 could be ommitted and simply citing these studies maybe enough, although this is a question of taste and style.

- a difficult but important question: could the authors comment on whether asymmetric PG insertion or FtsZ polymerization occurs first?

- line 568 - the reference is duplicated in the bibliography.

Referee #2:

This manuscript shows some beautiful images of cells displaying asymmetric constrictions and tomograms of cells with FtsZ filaments. The main conclusion of the paper is that cell division starts asymmetrically in bacteria. This is supported by HADA labeling and finding FtsZ filaments associated with the asymmetric invagination. The results are all internally consistent. However, they are not well incorporated with the literature and I have problems when trying to relate the results presented here with what is known from other techiques. I give some examples below:

1) The number of filaments is low. Many labs have assessed the amount of FtsZ in the ring and find

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that it is in the range of 30% in E. coli (presumably Salmonella and Proteus) independent of the stage of constriction. This would be about 2000 molecules of FtsZ which would be about 8000 nm of filaments. Much more than observed here. This is also independent of the stage of constriction. I worry that the filaments are not preserved during analysis for whatever reason.
2) In most cells, certainly E. coli, B. subtilis and C. crescentus the Z ring is established well before constriction starts and is very dynamic whether or not constriction is occurring. In Cc it is known that the Z ring promotes cell elongation before there is a switch to constriction.
3) The pulses with HADA are quite long. In E. coli and other organisms one generally sees complete labelling of the septum in 1 minute.

Referee #3:

This thought-provoking study investigates the pattern of cytokinesis in a broad group of Gram-negative bacteria and provides evidence that many species, but not E. coli, initiate cell constriction asymmetrically on one side of the cell. By categorizing cells at different stages of cytokinesis, the authors find that many cells continue to divide asymmetrically, while others end up dividing symmetrically. To examine whether this phenomenon is caused by the one-sided localization of FtsZ and/or the peptidoglycan synthesis machinery, the authors use electron cryo-tomography (ECT) of intact cells and labeling of nascent peptidoglycan insertion using fluorescent D-amino acids (HADA). Focusing on the crescent-shaped Caulobacter crescentus and the rod-shaped Proteus mirabilis, the authors find that these species often briefly localize their division machinery to one side of the cell at the initial stage of cell division prior to encircling the cell with a circumferential ring that completes cytokinesis.

Although there is some repetition in the Discussion, this work is generally well presented and the conclusions about asymmetric constrictions are generally well supported by the data. The correlation between cell length, which is a proxy for the stage of cell division, with asymmetric localization of FtsZ or HADA (e.g. Fig. S3) is convincing, despite the relatively low number of cells showing asymmetric localization, presumably due to the transient nature of this stage. The authors use other reported examples of asymmetric constriction in bacteria, which helps put their data into context (albeit taking away some of the novelty).

The paper does have some weaknesses, however. One concerns the use of the word "short FtsZ filaments" in the title and elsewhere in the paper. I realize that the authors are making the case for filaments that are hundreds of nm in length in contrast to the several micron-long filaments claimed in other ETC reports. However, my initial impression from the paper's title was that really short (100 nm or less, perhaps) filaments are involved in asymmetric constrictions, which does not seem to be the case. In several model species including E. coli, B. subtilis and C. crescentus, the ability to observe discrete patches of FtsZ around the division site by super-resolution suggest that most FtsZ filaments cannot be very long, perhaps a maximum of several hundred nm within these patches. So the novelty in this work is more the asymmetry at division initiation than the shortness of the FtsZ filaments, which seems more like a logical consequence than a mechanism. I suggest this be clarified with some careful wording changes.

The other main concern is in the claim that the filaments observed by ETC in all the species are in fact FtsZ. Although there is strongly suggestive precedent from prior work, the species used in the present study are diverse and may harbor other filament-forming proteins. Although many of the filaments are beautifully imaged in the tomograms, additional filaments are also present that are not highlighted (see below). I realize that proving that these filaments are FtsZ would be onerous if not impossible, but I think more careful wording is needed indicating that while it is assume that they are FtsZ, it cannot yet be confirmed.

Other comments:

1) One puzzle is that among the Gram-negative bacteria examined, an initial asymmetric constriction cannot be detected in the most well-studied species, E. coli. The authors speculate that the nature of the Min system may impose symmetry at midcell, which is a reasonable guess. However, this would not explain the asymmetric constrictions in P. mirabilis, a close relative of E. coli with similar rod shape that presumably has a Min system that works similarly.

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2) In B. subtilis, fluorescence microscopy of ring proteins displayed a "two-dot" localization pattern prior to a more complete ring (e.g. PMID 11298280). Although this is not asymmetric, it is consistent with nucleation of a smaller assembly before extension into a larger ring structure.

3) A better explanation is needed for the HADA labeling on the convex side of C. crescentus cells. C. crescentus divides by constriction and not septation, so it is not clear what the HADA labeling on the convex side really shows other than there is more elongation on that side. This is not surprising given that the convex side, by definition, grows more than the concave side. Also, as shown by PMID 17501919, FtsZ has a role in elongation near midcell as well as constriction.

4) Fig. S4 bottom: the red line used to trace the intensity profile extends inward only to the first filament. However, additional filaments distal to the membrane are present in the image. Might these be FtsZ as well?

5) The segmentations are open to interpretation. While the evidence for filaments in cells such as #17 in Fig. S5 is strong, there are additional dark filaments more distal from the membrane on the same side of that cell as well as on the opposite side that are not highlighted. Could these be additional FtsZ filaments? In cell #24 and #30, the highlighted filament runs parallel with another filament, but the segmentation highlights only the outermost filament.

6) In Fig. S6, the cell at top left (unconstriction stage) appears to have a constriction on the left. This brings up the question of how often were outer membrane invaginations counted, whether real constrictions leading to division or not?

7) It is surprising that the S. enterica minicells overproducing FtsZ can constrict. Do they exhibit Z rings by fluorescence microscopy? It is not clear how these would form near the center of these minicells without their typical spatial guidance systems. Also, do the authors have any rationale for why the minicells show more asymmetric constrictions than symmetric constrictions?

8) Line 183: this should be modified to say "during division of rod-shaped cells that contain MreB and FtsZ"

9) Is there any way to distinguish between FtsZ protofilaments and FtsZ protofilament bundles from the ETC images? This distinction, if any, was not made clear in the paper.

10) Lines 294 and following, describing the rationale for using C. crescentus and P. mirabilis, are quite redundant with the first part of the Results.

11) Lines 329-333: An alternative to the idea that the initial short FtsZ polymers lengthen to encircle the cell is that additional FtsZ arcs nucleate elsewhere around the cell and initiate constriction at those additional sites. This fits with the patchy FtsZ rings seen by super-resolution. Moreover, these polymers or polymer rafts likely treadmill, as the authors cite Filho et al. (although it is not yet published in a peer reviewed journal). If FtsZ polymers indeed treadmill, it may not be necessary to invoke a sliding filament model at all.

12) Lines 341-342: A better answer to the observation of relatively few FtsZ filaments at the late constriction stage is that at least in E. coli, FtsZ leaves the deeply constricting ring before other division proteins (PMID 24506818). Thus, FtsZ filaments may be absent because they have migrated to new division sites.

13) Line 391: Would short (< 100 nm) FtsZ filaments at locations other than midcell even be detectable by ECT in native cells?

14) Line 417: It should be stated more explicitly how the HADA was obtained.

15) Fig. 2A: are the relative fluorescence values on the y-axes comparable? Same for Fig. 2B-what do the numbers signify?

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16) Fig. 3 legend: need to add that the scale bar in the second image in panel B is 50 nm, not 100 nm

17) Fig. 4: FtsZ also has been reported to form a sharp focus at the swarmer pole in C. crescentus prior to migrating to midcell. Have FtsZ polymers corresponding to these foci ever been detected by ECT?

18) Fig. EV5 is mislabeled as Fig. E5.

19) There is no mention of culture/media conditions for the various bacterial species obtained either in this work or from the paper that describes the Caltech Tomography Database. These would be important for anyone to reproduce the experiments.

1st Revision - authors' response 28 February 2017

We thank the reviewers for their very helpful questions and comments, which have guided us in improving the paper. Note that in addition to addressing the reviewers' concerns, to clarify the most important points we have extensively rearranged some of the figures.

Referee #1:

Review: "Short FtsZ filaments drive asymmetric cell envelope constriction at the onset of bacterial cytokinesis", Yao et al., EMBO Journal 2016

This is a very interesting and timely manuscript investigating FtsZ-based cell division. I enjoyed reading it very much. The authors first show by analysing new and existing cryoET volumes of unmodified cells that many show asymmetric cell constrictions. We have seen similar things (and will be scooped somewhat by this study). The authors then show that PG synthesis is asymmetric (not shown on the same cells, that would have been very difficult, although possible, using CLEM). Finally, they show that the constrictions correlate with the occurrence of FtsZ filaments by pointing at filaments at the expected distance from the membrane.

I found the manuscript to be well written and as convincing as these sorts of things can get without hypothesis-driven perturbations.

In summary, I recommend to work for publication in EMBO Journal as it provides important insights that constrain our view of how cells constrict during the FtsZ-based process. Much more mechanistic work will be required in future but knowing that somehow FtsZ filaments, and not complete rings, are able to produce indentations that correlate with PG synthesis guides us where to look next!

Some more specific comments in no particular order:

- There are several manuscript on biorxiv right now that are relevant to the work, references to those might need to be worked in?

Agreed. We have now referenced the following four bioRxiv submissions:

1. [Now published in Science] Treadmilling by FtsZ filaments drives peptidoglycan synthesis and bacterial cell division. AW Bisson-Filho, YP Hsu, GR Squyres, E Kuru, F Wu, C Jukes, Y Sun, C Dekker, S Holden, M VanNieuwenhze, YV Brun, EC Garner.

2. [Now published in Science] GTPase activity-coupled treadmilling of the bacterial tubulin FtsZ organizes septal cell-wall synthesis. X Yang, Z Lyu, A Miguel, R McQuillen, KC Huang, J Xiao.

3. Chiral vortex dynamics on membranes is an intrinsic property of FtsZ, driven by GTP hydrolysis. D Ramirez, DA Garcia-Soriano, A Raso, M Feingold, G Rivas, P Schwille.

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4. A polymerisation-associated conformational switch in FtsZ that enables treadmilling.
JM Wagstaff, M Tsim, MA Oliva, A García-Sanchez, D Kureisaite-Ciziene, JM Andreu, J Löwe.

- introduction: Szwedziak et al did not envisage that the force generated by overlap would constrict the cell - rather that it would allow the ring to constrict while the cell does around it

Szwedziak et al. proposed one model (among three) in which filament overlap generates constrictive force. Quoting from their text (Szwedziak et al., Architecture of the ring formed by the tubulin homologue FtsZ in bacterial cell division, eLife 2014, 3:e04601):

> **"Where is the energy coming from for sliding and constriction?**
> What drives constriction of the closed rings and filament sliding? We propose three possible mechanisms that may even act in concert: (a) maximising filament overlap via sliding, ...
>
> A. When the overlap between the filaments that are attracted to each other increases, more and more binding energy is produced. This has been proposed before to be theoretically sufficient for the constriction process ... It was suggested previously that instead of forming many intermolecular solid bonds, which would lead to avidity and a barrier to sliding, an attractive force over a longer distance would keep the filaments apart while interacting (Hörger et al., 2008). This is more akin to the liquid state of matter, where many transient homotypic interactions, counteracted by thermal motion, lead to a fluid situation without absolute order but still keeping the molecules together."

Just before this the authors emphasized their conclusion that constriction depended on a complete ring:

> "Since in this model force generation is dependent on a closed ring, the system becomes self-regulating since constriction will only commence after a complete ring has formed. If the cell is too large or not enough FtsZ is available, constriction will not begin. It is important to note that only a closed, continuous ring is required, but it may consist of a number of shorter, overlapping filaments ..."

In an attempt to be as accurate to the authors' own words as possible, we have revised the sentence to now say "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." We feel it is important to call attention to these points in the Introduction to prepare readers to appreciate how our new findings are different and why the differences matter mechanistically.

- two interlinked processes. But I am now convinced that the 'checkpointing' idea of ring formation is most likely not correct (constriction only after ring formation)!

Agreed.

- line 181-: possible that asymmetric incorporation is caused by the very short labelling times and only a few PG synthesis complexes going round?

Yes, though how many PG synthesis complexes were active during the labeling time is still unclear: it could be just one, which had not yet traversed to the other side of the cell, or it could be several, which all started and stopped on the same side, adding to the asymmetric incorporation.

- line 224: do like the argument of finding asymmetric PG synthesis mostly on convex sides of Caulobacter, which speaks against above point.

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Right - at least it shows that if there were multiple PG synthesis complexes formed during the labeling period, they all (or the strong majority) started and stopped on the convex side (outer curvature).

-line 237-: disappointing (for cryoET) that still not possible to see FtsZ in all early constricting cells. Method problem or related to mechanism? Treadmilling filaments?

What we know is that FtsZ filaments were much more commonly observed in dividing *C. crescentus* than dividing *P. mirabilis* cells. We think the most likely reason is the limitation of the method that the clarity of reconstructions goes down with cell thickness, but it is also possible that FtsZ filaments are shorter or more rare in *P. mirabilis* cells. Our guess is that the filaments treadmill similarly in both species, so that would not underlie the differences.

- line 247-: what do the authors think happens to FtsZ in late cells? FtsZ gone? How narrow do they have to be for FtsZ to disappear? If something else took over abscission, is not likely we would know about mutants arrested in that stage?

We don't know what happens to FtsZ in late cells, but our results are consistent with previous fluorescence studies (Den Blaauwen et al. *J Bacteriol*, 1999) that during the last stages of cell constriction, the central FtsZ ring disappears. This suggests FtsZ filaments might not be essential for the final stages of division. Late constriction must be comparably fast, since we don't have many snapshots of late stages. It is therefore difficult for us to say how narrow the cells are when FtsZ disappears. That being said, the most constricted *C. crescentus* cell in our data set which still had visible FtsZ filaments was 245 nm across (#46). The most constricted *P. mirabilis* cell in our data set which still had visible FtsZ filaments was 315 nm across (#33). We fully agree that if something else took over abscission, mutants arrested in that stage would have been found by now. One possibility is however that further new PG synthesis (in the absence of guiding FtsZ filaments) drives the final detachment. There is increasing evidence that new PG synthesis exerts constriction force on the cytoplasmic membrane and this facilitates cell constriction (Coltharp et al. *PNAS*, 2016; Egan and Vollmer *Front Microbiol*, 2015; Filho et al. *Science*, 2017). If so, the gene(s) responsible for abscission might be essential for cell growth, not just for division, so genetic screens may not identify them.

- suggestion (not demanding this to be done): what happens in Caulobacter cells that are straight (no crescentin, for example)?

We don’t know. We could guess that in the straight Caulobacter cells, the MipZ concentration would be pretty much the same all around the division plane. FtsZ might then begin to polymerize at random positions, resulting in a more symmetric constriction. It is a good idea to check in the future.

- line 265-: good to see that there might still be a role for a ring structure (making sure constriction works along a plane, so membranes can fuse efficiently)!

Good idea. Maybe so.

- why was P. mirabilis chosen and not, say, thin E. coli? Not clear and not really discussed. (line 307-) This was done because E. coli does not show asymmetric constriction, right?

P. mirabilis was chosen because while we were imaging it for another study, we saw several asymmetrically-constricted cells and realized it could complement the curved *C. crescentus* model system nicely. FYI we have seven examples of constricted *E. coli* cells in our database, and all appear symmetric. Our interpretation is that because the concentration of MinC is equal all around the division plane, FtsZ filaments polymerize in random locations on that plane, quickly resulting in

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a nearly symmetric constriction. But we suspect that even in *E. coli*, the very first FtsZ-filament/PG-synthetic-complex that assembles probably starts constriction, and so we could probably find rare examples of slightly asymmetrically-constricted cells at the very earliest stage if we imaged more.

- line 331-: how does PG synthesis know what to do? Template model needs mentioning (as in model at the end of Ethan's paper)?

By template model we think the reviewer means the idea that existing PG (the glycan strands) templates the deposition of new material (causes new glycan strands to be laid down parallel to the existing ones), but we don't see this idea explicitly in Ethan's Sept. 2016 biorxiv paper. Our reading of Ethan's paper finds the idea that circumferential treadmilling of FtsZ guides the deposition of new PG, without any comment on why FtsZ treadmills circumferentially. That all being said, we like the idea of existing circumferential glycan strands causing FtsZ filaments to treadmill circumferentially and lay down new circumferential strands very much, and have in fact published an extensive modeling study supporting similar notions in cell wall elongation (Nguyen et al., Coarse-grained simulations of bacterial cell wall growth reveal that local coordination alone can be sufficient to maintain rod shape. *PNAS*, 2015, 112: E3689-E3698).

- what about FtsZ in cells without PG (Thermoplasma, for example and many other archaea)?

This would be a very interesting follow-up project. Although don't know for sure, we can point out an interesting clue. As shown in Figure S2A, the asymmetric division of an algae plastid (Sato et al., The dynamic surface of dividing cyanelles and ultrastructure of the region directly below the surface in Cyanophora paradoxa. *Planta* 229: 781-91) suggests that incomplete FtsZ rings are capable of driving asymmetric constriction without a PG layer.

- line 351-: stating that in E. coli and B. subtilis always symmetric. Does this not mean that the title and abstract could be seen as somewhat misleading? Yes, the mechanism could be the same, but the morphology is quite different and the title implies that FtsZ always induces asymmetric constriction (to me).

Good point. We think we fixed this by adding the word "can" to the title and also revising the abstract.

- line 359-: argument about E. coli being straight (hence more likely to be symmetric) also applies to P. mirabilis, no? Similarly: can the authors comment on why the short FtsZ filaments would assemble only on one side of the C. crescentus cells? Are the two sides of P. mirabilis also different?

Yes, the same argument about *E. coli* being straight does also apply to *P. mirabilis*, so there is something importantly different about the two that should be studied further. Concerning the short filaments in *C. crescentus*, according to (Thanbichler and Shapiro, *Cell* 2006), MipZ, an FtsZ polymerization inhibitor in *C. crescentus*, forms a gradient where the lowest concentration is at the midplane. Given the fact that *C. crescentus* is a curved cell, the lowest concentration of MipZ should actually be on the convex (outer) side of the cell. This would explain why FtsZ filaments preferentially polymerize at the convex side. We don't know why the two sides of *P. mirabilis* are different. Maybe once FtsZ filaments nucleate in one (random) place, others follow in the same location. (Please also refer to our response to reviewer #3, question 1).

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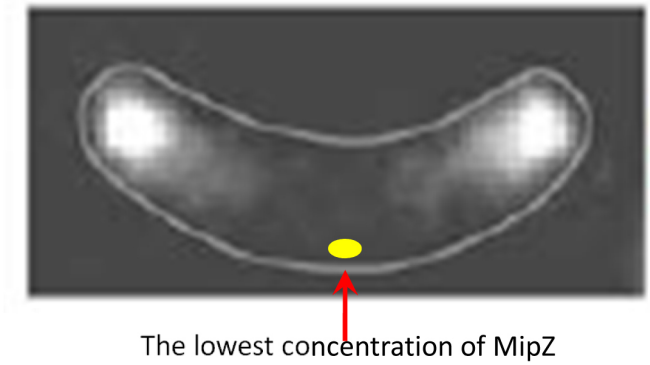
C. crescentus

MipZ-eYFP



The lowest concentration of MipZ

Adapted from Thanbichler and Shapiro with modification

Cell 2006 Jul 14;126(1):147-62.

Yellow dot and red arrow denote the lowest concentration of MipZ

- line 372-: naturally, I like the discussion of problems with imaging non-functional GFP fusions of FtsZ. This is important to be reiterated. Maybe worth citing the latest papers on biorxiv using more functional fusions (Filho et al)?

Added.

- Figure 1A: Caulobacter, Legionella, Ralstonia, Thiomonas and possibly Salmonella cells could also be constricted on both sides as a dumbbell structure might be mechanically unstable and bend and kink on the grid?

We're not sure we follow the Reviewer's thought process here, but we can say that we agree that deeply constricted cells could be flexible around the constriction and that plunge-freezing on grids could bend or kink cells. For instance, here are two rare cells that WERE NOT included in the analysis because they might have been squeezed by holes in the carbon film. These were easily identified by the proximity of the cell poles to a hole edge, and discarded.

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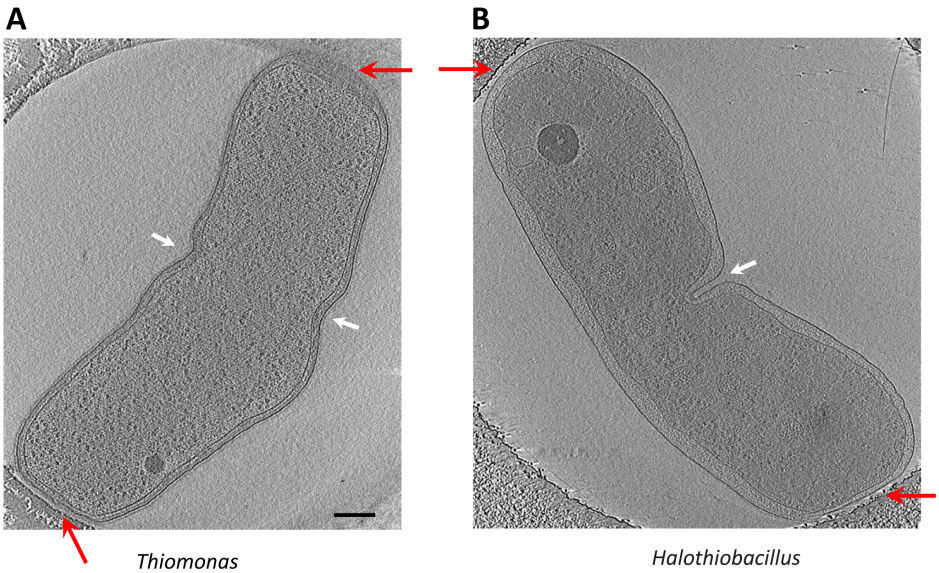

The plots of inverse radius of curvature (1/*r*) as a function of width ratio (W_{mid}/W) of *P. mirabilis* and *C. crescentus* cells (Figure EV2) show strong correlation (Pearson correlation coefficients of -0.85 in *P. mirabilis* and -0.82 in *C. crescentus*), however, indicating that the cells that were included in the analyses were not randomly kinked or distorted.

- generally: it was not immediately clear to me what side 'convex' refers to when talking about Caulobacter cells (since FtsZ is on the inside of the cell, which inverts the curvature definition).

Good point. We have replaced the terms “convex” and “concave” with “outer curvature” and “inner curvature” throughout the revised manuscript, respectively.

- Figure 3 *(Figure 5 in the revised manuscript)*: cryoET will hopefully improve over the next few years through higher voltages, even better detectors, phase plates, FIB milling and much faster acquisition times. It must, looking at the pictures.

Yes. It is a rapidly developing field.

- the model in Figure 4 *(Figure 7 in the revised manuscript)* indicates that in the mid-constriction stage, the two sides of the invaginating cellular membrane catch up and are in-sync with each other. Would this not indicate that the cells are waiting for the Z-ring to form, and once the ring forms, actual cytokinesis can begin? Perhaps the constriction is only needed for Z-ring assembly?

We think the Reviewer is distinguishing between constriction and cytokinesis, as if maybe there are two different stages ("constriction" needed for Z-ring assembly, then Z-ring drives "actual cytokinesis"), but that doesn't make much sense to us. We think constriction is cytokinesis. As the Reviewer has agreed above, constriction can initiate before a complete Z-ring forms. We don't know whether constriction is needed for a complete Z-ring to form, but we know individual short FtsZ filaments can polymerize before there is constriction, since we have examples of cells where a filament is visible but there is no constriction yet. We think individual short filaments polymerize and drive constriction, then more and longer filaments polymerize, eventually extending and treadmilling all around the ring. In some cells, they stay asymmetric for a long time (*Salmonella* minicells Figure 5E, *Belliella baltica* relative (Figure EV1), plastids from *C. paradoxa* (Figure S2A), *E. coli mreB*$^-$ mutant (Figure S2D), *H. mediterranei* (Figure S2F), and *R. hypermnestra*

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symbiont (Figure S2G) for example). In other cells, the ring develops so quickly it almost immediately looks symmetric (probably *E. coli*).

- I see two constrictions in Figure 3C *(Figure 5D in the revised manuscript)*? What is the other one?

We don't know, but it is not at mid-cell, and there are no FtsZ-like filaments there, so we did not include it in the analysis. The seven Legionella cells included in the analysis are all constricting at mid-cell.

- Figure 3A *(Figure 5A in the revised manuscript)* and S4 *(removed in the revised manuscript)* - the FtsZ layer is very hard to see. Perhaps the authors could show additional panels with a bandpass filter applied? I do understand though the P. mirabilis cells are thick and this may not be possible.

We have tried many different ways to do the reconstruction including using WBP and SIRT algorithms in combination with different bandpass filters. The results were similar.

- It is difficult to see the FtsZ layer in the panels of figure 1A. In how many cases is FtsZ seen? Could these be marked?

FtsZ is visible in *P. mirabilis*, *C. crescentus*, *H. neapolitanus c2*, *L. pneumophila*, *T. intermedia* and *S. enterica* minicells. We would rather not mark it in Figure 1A, however, since the organization of the paper is to first report the discovery of asymmetric division in these species, then show asymmetric deposition of new PG, then show these correlate with the asymmetric presence of FtsZ filaments.

- Figure S8 *(Figure S2 in the revised manuscript)* could be ommitted and simply citing these studies maybe enough, although this is a question of taste and style.

Agreed it is a matter of taste. We have chosen to include it to be sure readers see exactly the figures we are talking about, and how strongly they together reinforce the main and mechanistically very important conclusion that complete Z-rings are not required for constriction.

- a difficult but important question: could the authors comment on whether asymmetric PG insertion or FtsZ polymerization occurs first?

We don't know yet, but we think the answer could come from future Correlative Light and Electron Microscopy (CLEM) with a fluorescent D-amino acid derivative that doesn't fluoresce until it is incorporated into the PG layer. Cells dividing in the presence of this dye could be plunge-frozen and imaged first by cryo-fluorescence light microscopy (to see where new PG had been incorporated) and then by ECT (to see where FtsZ filaments were).

- line 568 - the reference is duplicated in the bibliography.

Thanks. Fixed.

Referee #2:

This manuscript shows some beautiful images of cells displaying asymmetric constrictions and tomograms of cells with FtsZ filaments. The main conclusion of the paper is that cell division starts asymmetrically in bacteria. This is supported by HADA labeling and finding FtsZ filaments associated with the asymmetric invagination. The results are all internally consistent. However, they are not well incorporated with the literature and I have problems when trying to relate the results presented here with what is known from other techiques. I give some examples below:

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1) The number of filaments is low. Many labs have assessed the amount of FtsZ in the ring and find that it is in the range of 30% in E. coli (presumably Salmonella and Proteus) independent of the stage of constriction. This would be about 2000 molecules of FtsZ which would be about 8000 nm of filaments. Much more than observed here. This is also independent of the stage of constriction. I worry that the filaments are not preserved during analysis for whatever reason.

The first paper to our knowledge reporting this kind of measurement is Stricker et al., *PNAS*, 2002. Here the authors expressed non-functional, GFP-tagged FtsZ from a plasmid and found that about 30% of the fluorescence was in the "ring" throughout division. Then, based on this finding, the authors calculated that "the Z-ring is on average six to seven protofilaments thick".
(i) We note that in what we called the "mid-constriction" stage (after which all cells were constricted on both sides and a complete FtsZ ring would have been seen in a fluorescence experiment, corresponding in *C. crescentus* to cell #s 33-46), there are on average 4.4 filaments per side, not so far from the 6-7 estimate given by Stricker.
(ii) Stricker et al.'s and many other fluorescence studies are suspect because they involve overexpression of non-functional FtsZ-GFP which disrupts native abundance, localization and function. As pointed out by Goley et al. *Molecular Microbiology*, 2011, wildtype FtsZ is strongly regulated at transcription and protein levels, and Goley et al. showed themselves that their artificially-induced FtsZ-GFP localized as foci at the swarmer cell pole or as a ring at the midplane *before* (in the cell cycle) western blots showed evidence of any wild-type FtsZ. (Please also see our response to Reviewer #3, question 17 below).
(iii) Seeing a fluorescent band at midcell does not necessarily mean there are filaments there, only that there are a lot of labeled proteins in that area. For instance, as we describe in the Discussion, super-resolution fluorescence light microscopy showed that FtsZ-Dendra2 in *C. crescentus* occupies a toroidal volume extending ~250 nm away from the membrane, and the authors made a special point that this was not just their resolution limit: they were confident the toroid really was that thick (Biteen et al., 2012). In ECT studies of native cells, however, clear FtsZ-like filaments (though see question 4 from Referee #3) have never been seen that far from the membrane - they are always ~16 nm away from the membrane (Li et al., 2007; Szwedziak et al., 2014b; plus the results of this paper). Thus the fluorescence is either coming from unnatural filaments (due to tags) or from unpolymerized labeled proteins concentrated at mid-cell. Both possibilities would cause Stricker's analysis to be an overestimate of the number of actual filaments expected in a wildtype cell.
(iv) Standard (room-temperature) fluorescence images are recorded of either live or chemically-fixed cells. If live, the signal from filaments is likely blurred by treadmilling during the exposure time. It is certain therefore that many monomers joining and leaving the filament will be found in the vicinity fluorescing, even though they are not yet or no longer part of the actual polymer. If the sample is chemically fixed, all kinds of unnatural aggregates are formed by random crosslinks - that is what "fixing" means.
(v) It is highly unlikely that filaments are not being preserved in our experiments. The samples are prepared by plunge-freezing, which cools the sample so fast that individual water molecules don't have time to crystallize before they stop rearranging. Whenever there is a problem (like a sticky plunger) and the sample does not freeze this quickly, we recognize it immediately because the water crystallizes (and this is easily seen in the cryo-EM). So we know our samples froze too fast for water molecules to crystallize. That's why we think it is so unlikely that protein filaments were not preserved. Some have questioned whether the blotting step that precedes plunge-freezing might cause some surface tension issue or some other problem that causes filaments to depolymerize just before they freeze. We think this is also very unlikely because it would not be expected to preferentially affect the side of the cell without invagination. As we summarize at the end of the Results section, in total we have tomograms of 25 cells or minicells with a constriction on just one side and visible FtsZ filaments. Among these, 19 had FtsZ filaments only on that same side, five had filaments on both sides, and only one had filaments on just the other (non-constricted) side, but this was likely a false-positive constriction (see text). If surface tension were causing filament depolymerization, one would expect a more even effect. That all being said. as we say in the text already we agree that we may not be resolving all the filaments present in the *P. mirabilis* cells because these cells are so thick.

2) In most cells, certainly E. coli, B. subtilis and C. crescentus the Z ring is established well before constriction starts and is very dynamic whether or not constriction is occurring. In Cc it is known that the Z ring promotes cell elongation before there is a switch to constriction.

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(i) As mentioned above, most of these papers relied on modified cells overexpressing non-functional fluorescently-tagged FtsZ and they interpreted all fluorescence near the midcell as FtsZ filaments. Labeled FtsZ does not share the same abundance, timing or function as native FtsZ, and likely also perturbs native FtsZ polymerization. Very importantly, as mentioned above, artificially-induced FtsZ-GFP localized as a ring at the midplane long before (in the cell cycle) western blots showed evidence of any wild-type FtsZ (Goley et al. *Molecular Microbiology*, 2011). So it is clear that previous work with artificially-induced non-functional FtsZ has given a false impression of how early the FtsZ ring appears.

(ii) In the papers we think the Reviewer is thinking of, light microscopy or traditional thin-section EM with fixed and stained cells was used to detect constriction, and as we have discovered here neither has been sensitive enough to recognize the early constriction asymmetry, so these methods are not detecting the earliest stages of constriction.

(iii) We can confirm that sometimes native FtsZ filaments are present before constriction starts, but these instances are rare, and there aren't very many filaments, so we doubt this condition lasts for long.

Here, we visualized native FtsZ filaments in unmodified WT cells and identified early constriction in cells that were unfixed and fully hydrated with high magnification EM. Our work differs from most previous studies both in materials and methods. Please also refer to response to Reviewer #3, question 3 for further comments on the Z ring promoting elongation before constriction.

3) The pulses with HADA are quite long. In E. coli and other organisms one generally sees complete labelling of the septum in 1 minute.

The extent of labeling depends on species, HADA concentration, culture density, temperature, culture medium and so on. We tried two pulse times under our conditions and found that 1.5 minutes revealed a population of long cells with evidence of new PG on only one side of the midcell. In 12/13 cases in *C. crescentus,* the new PG appeared on the outer curvature.

Referee #3:

This thought-provoking study investigates the pattern of cytokinesis in a broad group of Gram-negative bacteria and provides evidence that many species, but not E. coli, initiate cell constriction asymmetrically on one side of the cell. By categorizing cells at different stages of cytokinesis, the authors find that many cells continue to divide asymmetrically, while others end up dividing symmetrically. To examine whether this phenomenon is caused by the one-sided localization of FtsZ and/or the peptidoglycan synthesis machinery, the authors use electron cryo-tomography (ECT) of intact cells and labeling of nascent peptidoglycan insertion using fluorescent D-amino acids (HADA). Focusing on the crescent-shaped Caulobacter crescentus and the rod-shaped Proteus mirabilis, the authors find that these species often briefly localize their division machinery to one side of the cell at the initial stage of cell division prior to encircling the cell with a circumferential ring that completes cytokinesis.

Although there is some repetition in the Discussion, this work is generally well presented and the conclusions about asymmetric constrictions are generally well supported by the data. The correlation between cell length, which is a proxy for the stage of cell division, with asymmetric localization of FtsZ or HADA (e.g. Fig. S3) *(Figure EV3 in the revised manuscript)* is convincing, despite the relatively low number of cells showing asymmetric localization, presumably due to the transient nature of this stage. The authors use other reported examples of asymmetric constriction in bacteria, which helps put their data into context (albeit taking away some of the novelty).

By the way, we agree that the previously published images collected in Fig. S2 take away some of the novelty of our paper here because they already make the point that complete FtsZ rings are not required for constriction, but we felt this point had not been noticed sufficiently, perhaps because the images had not previously been gathered together.

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The paper does have some weaknesses, however. One concerns the use of the word "short FtsZ filaments" in the title and elsewhere in the paper. I realize that the authors are making the case for filaments that are hundreds of nm in length in contrast to the several micron-long filaments claimed in other ETC reports. However, my initial impression from the paper's title was that really short (100 nm or less, perhaps) filaments are involved in asymmetric constrictions, which does not seem to be the case. In several model species including E. coli, B. subtilis and C. crescentus, the ability to observe discrete patches of FtsZ around the division site by super-resolution suggest that most FtsZ filaments cannot be very long, perhaps a maximum of several hundred nm within these patches. So the novelty in this work is more the asymmetry at division initiation than the shortness of the FtsZ filaments, which seems more like a logical consequence than a mechanism. I suggest this be clarified with some careful wording changes.

Understood. We have tried to make the careful wording changes requested, but we think the following comments could be helpful:

(i) Because of the missing wedge, it is hard for us to estimate the lengths of the filaments. What is solidly shown here is that in wildtype cells, the presence of FtsZ filaments too short to extend all the way around the cell (not complete rings) correlates with early constriction.

(ii) Again, for all the reasons mentioned above, we consider fluorescence results based on non-functional GFP-fusions which are almost universally over-expressed from inducible promoters with perturbed abundance, timing, etc. to be interesting but inconclusive. So we still consider our findings here to have novelty and unique value, even though they are in many ways consistent with some fluorescence results.

(iii) We agree there is novelty in our finding that constriction begins asymmetrically, and that this is a major contribution of the paper.

(iv) We think our finding of short filaments correlating with constriction is not just a "logical consequence," but is mechanistically informative. We think this is proved by the fact that a previous ECT study (Szwedziak et al., eLife 2014, 3:e04601) concluded just the opposite (that complete rings were required), which led the authors to propose models that involve force-generation through filament sliding (see above). Our finding of incomplete rings (short filaments) driving initial constriction refutes this, and is therefore mechanistically informative.

The other main concern is in the claim that the filaments observed by ETC in all the species are in fact FtsZ. Although there is strongly suggestive precedent from prior work, the species used in the present study are diverse and may harbor other filament-forming proteins. Although many of the filaments are beautifully imaged in the tomograms, additional filaments are also present that are not highlighted (see below). I realize that proving that these filaments are FtsZ would be onerous if not impossible, but I think more careful wording is needed indicating that while it is assume that they are FtsZ, it cannot yet be confirmed.

We agree that there are other filaments in these cells, but our claim is that we have discovered that constriction in several species begins asymmetrically, and when one looks into those cells, there is a strong correlation between constriction site and filaments which match all the hallmark features of FtsZ (at the division site parallel to the division plane, ~4 nm thick, ~16 nm from the membrane). Now are all of these FtsZ, some of them, or none? To argue that none are FtsZ is completely unreasonable (since they possess all the hallmark features of the filaments that have been proven in previous work to be FtsZ). If even some are FtsZ, our point holds that FtsZ "rings" do not have to be complete to initiate constriction. That is our point. That being said, in deference to the reviewer's concern, we now refer to the filaments as "FtsZ-like."

Other comments:

1) One puzzle is that among the Gram-negative bacteria examined, an initial asymmetric constriction cannot be detected in the most well-studied species, E. coli. The authors speculate that the nature of the Min system may impose symmetry at midcell, which is a reasonable guess. However, this would not explain the asymmetric constrictions in P. mirabilis, a close relative of E.

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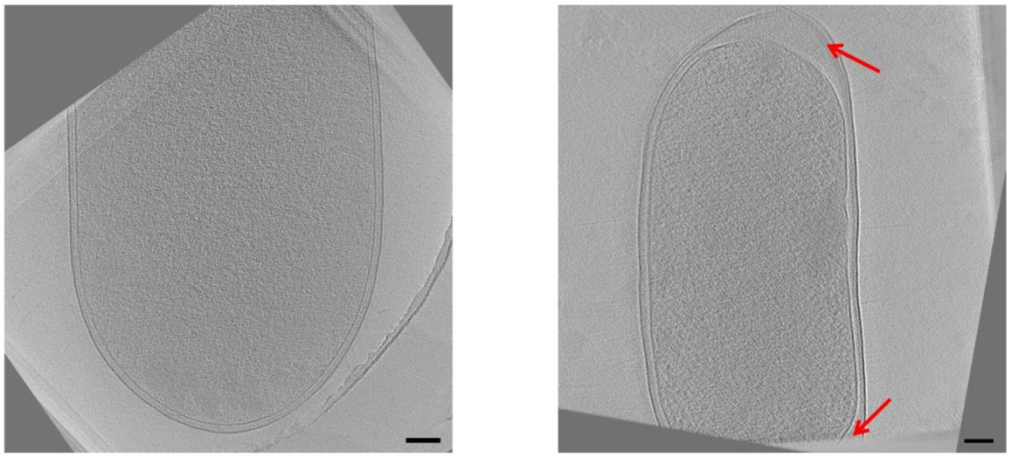
coli with similar rod shape that presumably has a Min system that works similarly.

We agree the differences between *E. coli* and *P. mirabilis* are interesting. *E. coli* may constrict asymmetrically very briefly - we don't know. As for *P. mirabilis*, we add for this Reviewer here that around 60% of *P. mirabilis* cells have a thick cell wall "cap" asymmetrically located on both poles. Furthermore, most of the cells are just slightly curved (see below and cells nos. 1-10 in Figure 2). Taken together, these observations suggest that the two sides of *P. mirabilis* may be different.



E. coli *P. mirabilis*

The red arrows indicate the thick cell wall cap localized asymmetrically at both poles
Scale bar, 100 nm

2) In B. subtilis, fluorescence microscopy of ring proteins displayed a "two-dot" localization pattern prior to a more complete ring (e.g. PMID 11298280). Although this is not asymmetric, it is consistent with nucleation of a smaller assembly before extension into a larger ring structure.

Good point. In the study referred to (PMID 11298280), the locations of ring proteins were imaged in fixed cells. Because the "two dots" were always on the left and right edges (not in-between), they suggest a complete, but thin ring (too thin to be seen over the top and bottom - nucleation at random locations would presumably result in dots in random locations, not always on the left and right sides). Perhaps FtsZ filaments nucleate at one or a few points and then very rapidly grow and treadmill all around the division plane, then the "ring" thickens.

3) A better explanation is needed for the HADA labeling on the convex side of C. crescentus cells. C. crescentus divides by constriction and not septation, so it is not clear what the HADA labeling on the convex side really shows other than there is more elongation on that side. This is not surprising given that the convex side, by definition, grows more than the concave side. Also, as shown by PMID 17501919, FtsZ has a role in elongation near midcell as well as constriction.

We're not sure what the Reviewer's concern is. We agree that all the HADA data shows is that there is new PG synthesis on one side. In *C. crescentus*, where the inner and outer curvatures are identifiable, this new PG consistently appears on the same side as constriction initiates and initial short FtsZ filaments form. Our conclusion is that short FtsZ filaments (rather than a complete ring) are sufficient to initiate constriction, constriction starts asymmetrically, and it is accompanied by new PG synthesis. Whether one wants to interpret this new PG synthesis as either "elongation" or "constriction" synthesis seems to us to assume that there are two distinct molecular modes of FtsZ-guided PG addition. Are there? Maybe not. That being said, the ECT data show that cells at that stage are constricting - the cell wall is being remodeled with a constriction on one side.

4) Fig. S4 *(removed in the revised manuscript)* bottom: the red line used to trace the intensity profile extends inward only to the first filament. However, additional filaments distal to the membrane are present in the image. Might these be FtsZ as well?

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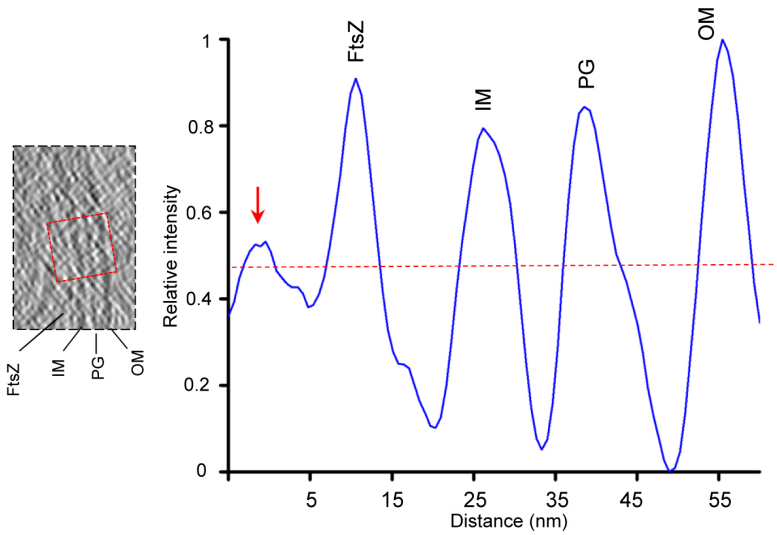
We have now extended the plot to explore this possibility. The average intensity profile across the red box area was calculated and plotted as a function of position. The "short filament" distal to the membrane (red arrow) is not nearly as significant as the first, FtsZ filament. Whether or not it is a filament of some kind we cannot say (please also see our response to the next question below). Note also that after our restructuring some of the figures, this no longer appears in the final paper.



5) The segmentations are open to interpretation. While the evidence for filaments in cells such as #17 in Fig. S5 *(Figure EV4 in the revised manuscript)* is strong, there are additional dark filaments more distal from the membrane on the same side of that cell as well as on the opposite side that are not highlighted. Could these be additional FtsZ filaments? In cell #24 and #30 *(mislabeled as #30 in the initial submission, fixed and labeled as #34 in the revised manuscript)*, the highlighted filament runs parallel with another filament, but the segmentation highlights only the outermost filament.

While there are many filamentous objects in cells (cytoskeletal filaments, nucleic acids, glycan strands), our focus here is on FtsZ filaments, which are recognized by being ~4 nm thick, ~16 nm from the membrane, smooth (not crooked), parallel to the division plane and near mid-cell. The only filaments we segmented fulfill all of these criteria, and we believe they are also the strongest (most clear) filaments in these images.

6) In Fig. S6 *(Figure EV1 in the revised manuscript)*, the cell at top left (unconstriction stage) appears to have a constriction on the left. This brings up the question of how often were outer membrane invaginations counted, whether real constrictions leading to division or not?

As mentioned in Figure S1 and the material and methods, we used the inner membrane to define constriction. So the answer to the question is outer membrane invaginations were never counted as constrictions. Such outer membrane undulations were actually common in unconstricted *H. neapolitanus* c2 cells and appeared all over the surface (not just at mid-cell).

7) It is surprising that the S. enterica minicells overproducing FtsZ can constrict. Do they exhibit Z rings by fluorescence microscopy? It is not clear how these would form near the center of these minicells without their typical spatial guidance systems. Also, do the authors have any rationale for why the minicells show more asymmetric constrictions than symmetric constrictions?

(i) We don't know whether they exhibit Z rings by fluorescence microscopy as overexpression of WT (not tagged) FtsZ was used to generate the minicells.

(ii) We agree with the reviewer that minicells are not expected to have spatial guidance systems, so

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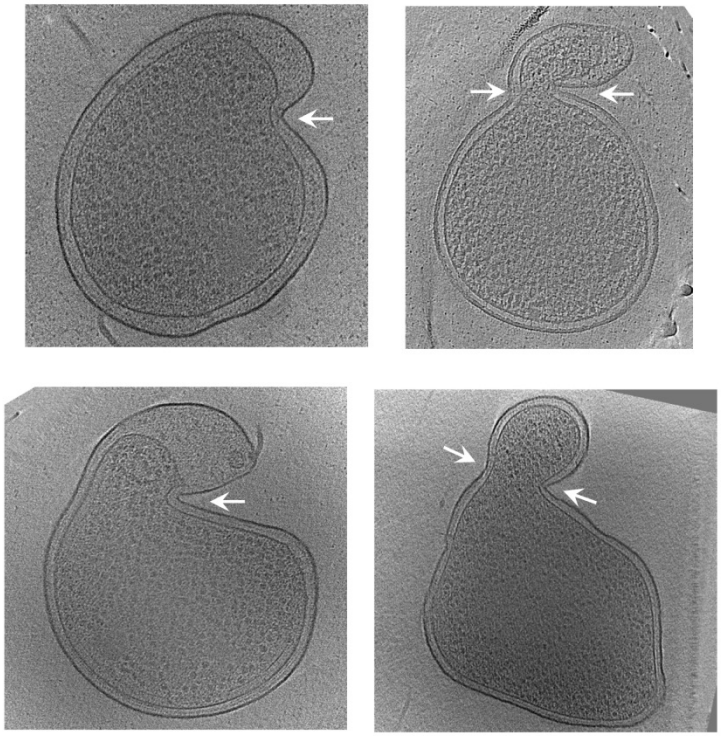
constrictions would likely appear essentially randomly, as shown in Figure 5E and in Figure 6D. Here are four more examples of uncentered, asymmetric constrictions:



White arrows indicate constriction

(iii) We would speculate that because the minicells are amorphous and without spatial guidance systems, FtsZ can nucleate stochastically anywhere. Once FtsZ has nucleated, filaments apparently extend from there. Note that *E. coli mreB* mutant spherical cells also exhibit non-centered asymmetric constrictions accompanied by incomplete FtsZ rings (Figure S2D).

8) Line 183: this should be modified to say "during division of rod-shaped cells that contain MreB and FtsZ"

Modified.

9) Is there any way to distinguish between FtsZ protofilaments and FtsZ protofilament bundles from the ETC images? This distinction, if any, was not made clear in the paper.

We are not sure what the reviewer means by "bundle." In all cases where multiple filaments are seen, the filaments lie next to each other roughly 6-7nm apart to form a flat band. Because the size of the filaments matches a single FtsZ protofilament (see below), we think these are individual protofilaments rather than any kind of bundle.

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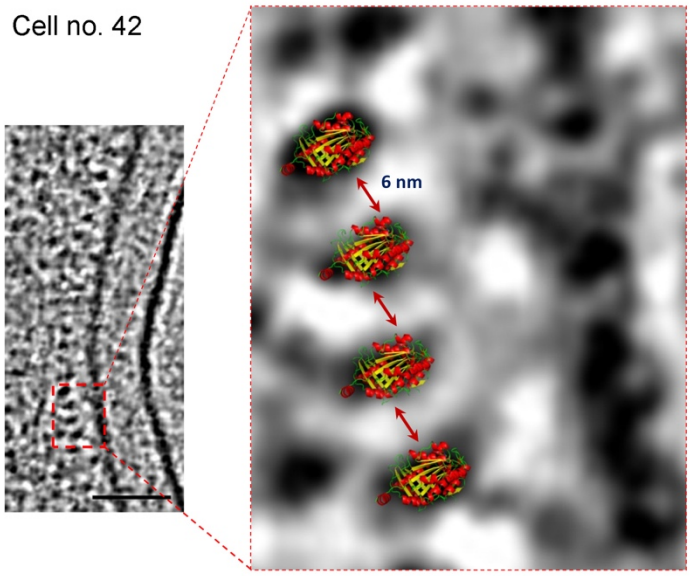

Enlarged views of the outer curvature side of *C. crescentus* cell no. 42. The band of filaments at the division site is seen on the left. Scale bar, 50 nm. Shown on the right is a close-up view of the boxed area on the left. Crystal structures of FtsZ (PBD ID: 1FSZ) shown as ribbon diagram were fitted into the FtsZ density. Based on prior biochemical data, the C-terminus of the molecule was oriented toward the inner membrane, although the exact orientation can only be guessed due to the resolution limit. Note that Szwedziak et al.'s cryotomograms of constricting liposomes show the same structure of FtsZ bands more clearly (Szwedziak et al., eLife 2014, 3:e04601).

10) Lines 294 and following, describing the rationale for using C. crescentus and P. mirabilis, are quite redundant with the first part of the Results.

Revised.

11) Lines 329-333: An alternative to the idea that the initial short FtsZ polymers lengthen to encircle the cell is that additional FtsZ arcs nucleate elsewhere around the cell and initiate constriction at those additional sites. This fits with the patchy FtsZ rings seen by super-resolution. Moreover, these polymers or polymer rafts likely treadmill, as the authors cite Filho et al. (although it is not yet published in a peer reviewed journal). If FtsZ polymers indeed treadmill, it may not be necessary to invoke a sliding filament model at all.

Agreed. It seems clear that in some species, one FtsZ filament "nucleus" predominates and treadmilling must not go around and around the cell, since constriction clearly proceeds deeply on one side of the cell before anything happens on the other (Figure S2 panel G is perhaps the most convincing example). In other cells (like perhaps *E. coli*?), FtsZ filaments might nucleate all around the division plane almost simultaneously and then rapidly treadmill away, resulting in essentially symmetric constriction.

12) Lines 341-342: A better answer to the observation of relatively few FtsZ filaments at the late constriction stage is that at least in E. coli, FtsZ leaves the deeply constricting ring before other division proteins (PMID 24506818). Thus, FtsZ filaments may be absent because they have migrated to new division sites.

Agreed. Revised accordingly with reference.

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13) Line 391: Would short (< 100 nm) FtsZ filaments at locations other than midcell even be detectable by ECT in native cells?

The Reviewer may be familiar with our ECT work on MreB, where we showed that at least in one influential *E. coli* strain, the extended helical filaments seen by fluorescence microscopy were artifacts of the YFP tag, and no helical filaments were visible in wildtype (untagged) cells (Swulius et al., *J. Bac*, 2012). In a preceding paper (Swulius et al., *BBRC*, 2011), we reported simulations where we computationally moved FtsZ filament densities in a tomogram to other places in the cell at different distances from and orientations with respect to the membrane and then used a variety of algorithms to search for them computationally. We found that for filaments approximately the width of FtsZ or MreB, noise prevented their routine identification until they were at least 80 nm long. So no, we would not likely have recognized short (<100 nm) FtsZ filaments away from midcell. That being said, new advances in ECT technology like better detectors, FIB-milling and phase plates are rapidly improving the situation, so we do not wish our colleagues to consider this a permanent limitation for future work.

14) Line 417: It should be stated more explicitly how the HADA was obtained.

Done.

15) Fig. 2A *(Figure 4 in revised manuscript)*: are the relative fluorescence values on the y-axes comparable? Same for Fig. 2B-what do the numbers signify?

No: because we used an automatic exposure mode to automatically determine the optimal exposure level, the exposure for individual cells varies. The numbers in Figure 4A and B are 1/10 values of the 8-bit gray values measured using the ImageJ software. We have added this explanation to the figure legend.

16) Fig. 3 legend *(Figure 5B in the revised manuscript):* need to add that the scale bar in the second image in panel B is 50 nm, not 100 nm

Thanks! (Note though that the panel has been removed in the revised manuscript for simplicity.)

17) Fig. 4 *(Figure 7 in the revised manuscript)*: FtsZ also has been reported to form a sharp focus at the swarmer pole in C. crescentus prior to migrating to midcell. Have FtsZ polymers corresponding to these foci ever been detected by ECT?

No. So far, our database contains about 2,300 tomograms of *C. crescentus* cells at various stages. These tomograms were collected by different lab members for diverse projects. Very occasionally, we have observed different kinds of filaments in and around the poles, but in most cases we don't know their identity, and none have looked like FtsZ (see Briegel et al., *Molecular Microbiology*, 2011 for examples).
Goley et al. *Molecular Microbiology*, 2011 showed that in cells overexpressing FtsZ-YFP, fluorescent foci appear in the swarmer cell pole 0-10 minutes post-synchrony, but in wildtype cells, as shown by western-blotting in Figure 8B, there is no detectable FtsZ present between 0-20 minutes after synchronization. These results suggest that there is no FtsZ protein in WT cells when overexpressed fluorescently-tagged FtsZ appears as foci on the swarmer pole, perhaps explaining why FtsZ-like filaments have not been seen there in cryotomograms.

18) Fig. EV5 is mislabeled as Fig. E5.

Thanks! Fixed.

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19) There is no mention of culture/media conditions for the various bacterial species obtained either in this work or from the paper that describes the Caltech Tomography Database. These would be important for anyone to reproduce the experiments.

Agreed. This has now been added and summarized in Table S3.

2nd Editorial Decision 15 March 2017

Thank you for submitting a revised version of your manuscript. The manuscript has now been seen by both referees, who find that their main concerns have now been addressed. There are just a few minor issues to be dealt with before formal acceptance here. Congratulations on a nice study!

1. Please add keywords and author contributions to the article.
2. Please update the references according to the EMBO Journal style (please see our author guidelines: http://emboj.msubmit.net/html/emboj_author_instructions.html)
3. Please update the callouts to Figures 7 and S1 - currently the full figure is referenced in the text instead of separate panels.
4. Figure 1A: images of Belliella, Legionella and Ralstonia appear to be identical to panels in Figure EV1. Please provide a different image in one of the figures.
5. Please add scale bars to Figures 2-3, Figure 5E, left panel, and Figure 6A-B, upper panels. If the scale is the same in these figures, please note in the legend.
6. In Figure 6, enlarged view also appears to be shown for other panels than 6B. Please correct the figure legend text.
7. There is a typo in Figure 7B (constrction), please correct. It might also be useful to increase the thickness of the lines indicating FtsZ filaments (especially in the early constriction stage panel) to ensure that they remain easily noticeable in the pdf version.

Please let me know if you have any further questions regarding this final revision step. Thank you again for giving us the chance to consider your manuscript for The EMBO Journal. I am looking forward to receiving the final version.

REFEREE COMMENTS

Referee #1:

The revisions made by Yao et al to their original manuscript are all reasonable and I recommend publication of the manuscript. They have referenced all the papers as requested and answered questions raised, if not in the manuscript, then in the response letter. I think that it is not possible with the current technology to have clearer pictures of bacterial cell division from thick bacterial cells. I do believe though that the Z-ring is complete in the mid-constriction stage (drawn as broken circles in new Figure 7), but there is no way to confirm this and future studies will tell.

Referee #3:

The authors have done a good job of responding to my comments/suggestions and those of the other reviewers, and the manuscript has improved as a result. The results in the revised paper will be useful for the field of bacterial cell biology and should help further our understanding of bacterial cell division mechanisms.

2nd Revision - authors' response 17 March 2017

Authors made requested editorial changes.

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YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

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Journal Submitted to: EMBO Journal
Manuscript Number: EMBOJ-2016-96235R

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ^2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Page 7 and page 8 for HADA labeling; page 5 for tomography.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	N/A
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	N/A
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	N/A
For animal studies, include a statement about randomization even if no randomization was used.	N/A
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	N/A
4.b. For animal studies, include a statement about blinding even if no blinding was done	N/A
5. For every figure, are statistical tests justified as appropriate?	Page 21 for correlation coefficient.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	N/A
Is there an estimate of variation within each group of data?	Page 21 for variation.
Is the variance similar between the groups that are being statistically compared?	Yes page 21.

C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo

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http://ClinicalTrials.gov
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http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun

http://datadryad.org

http://figshare.com

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http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	N/A
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	N/A

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	N/A
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	N/A
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	N/A

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	N/A
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	N/A
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	N/A
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	N/A
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	N/A
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	N/A
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	N/A

F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	N/A
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	N/A
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	N/A
21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section. Examples: **Primary Data** Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462 **Referenced Data** Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	N/A
22. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	PAGE 6 for software.

G- Dual use research of concern

23. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	N/A