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FtsA forms actin-like protofilaments

Piotr Szwedziak, Qing Wang, Stefan MV Freund and Jan Löwe

Corresponding author: Jan Löwe, MRC-Laboratory of Molecular Biology

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1st Editorial Decision 02 December 2011

Thank you for the submission of your manuscript to The EMBO Journal. We have now received the reports from the two referees that were asked to evaluate it, which I copy below.

As you will see, while both referees' comments are quite positive and referee #1 only ask for certain clarifications, referee #2 is more concerned with some of your conclusions. Given these encouraging reports, I would like to invite you to submit a revised version of your manuscript

Please be aware that your revised manuscript must address the referees' concerns and their suggestions should be taken on board. Acceptance of the manuscript will depend on a positive outcome of a second round of review and I should also remind you that it is 'The EMBO Journal' policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript.

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We generally allow three months as standard 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. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

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Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1

FtsA is an actin like protein (with a unique and quite different domain structure; it lacks domain 1B but has an additional domain 1C located elsewhere in the structure) required for bacterial cell division. Since it binds both FtsZ and the membrane it can link FtsZ polymers to the membrane to promote Z ring formation. Two lines of evidence (certain mutants form rod-like structures in vivo and FtsA from streptococcus forms polymers in vitro) indicate FtsA can polymerize although the structure of such polymers is unknown. There has been one suggestion for FtsA self interaction published (Proteins. 2003, 50(2):192-206.), but it is a very speculative model (and based upon the work here it is wrong). This paper tackles 1) the structure of FtsA polymers and 2) the interaction between FtsA and FtsZ, which has been fairly well examined. The paper does not flow well (due to writing style and leaves out certain references, e.g. the one above). The introduction could be rewritten to better highlight the function of ftsA (tehter FtsZ filaments to the membrane) by combining the 2nd and 4th paragraphs of the introduction. The role of ftsA should also be pointed out in the second sentence of the abstract

This paper sets out to examine the interaction between FtsA and FtsZ. Although this interaction is well characterized in vivo it has not been confirmed in vitro. The first results confirm the in vivo findings of Pichoff and Lutkenhaus: 1) the tail of FtsA is required for membrane binding and 2) the tail of FtsZ interacts with domain 2B of FtsA. The results demonstrate how FtsA can form actin like filaments (the novel domain 1C of FtsA occupies the space of domain 1A (on the next molecule). This, along with the genetic evidence that self assembly is important, is a nice advance for the field as it documents the structure of FtsA filaments.

Comments/questions:

1) Fig 1. It is clear that ftsA does not pellet with GTP (part 1B) or with ATP (part 1C), however, the paper goes on to argue that FtsA polymerizes. Why dosen't FtsA polymerize in this experiment? Are the conditions different?
2) How does the peptide conformation bound to FtsA compare to it bound to ZipA?
3) How does the structure of the peptide-FtsA complex compare with the genetic analysis of important residues found by Haney et al. J Biol Chem. 2001 276(15):11980-7; a reference missing from the reference list. (it should be cited in the introduction).
4) The Kd is reported at 51 μM. This seems very weak. Any comment?
5) To what extent is this ftsA structure different from the previous reported structure from this lab. Why was this different structure obtained?
6) Why is the Z peptide present in the FtsA structure when ATP was present but not ATPγS?
7) What is to be made of the observation that FtsA forms filaments of vesicle surfaces but not in solution?
8) Page 9 near the bottom; Pichoff and Lutkenhaus used GFP-FtsA with C-terminal truncations to observe FtsA polymers as done here Mol Microbiol. 2007 64(4):1129-38)
9) Page 10, FtsA that can't bind the membrane forms filaments in vivo but not in vitro. Shouldn't polymers form in vitro?
10) Page 13, last part of first complete paragraph starting with "Certainly, the ..." It is not clear what the authors are saying here. This relates to point 2 above. Is the structure of the peptide different in the ZipA-peptide versus the FtsA-peptide complexes? Is the structure consistent with the mutations by Haney et al. ?

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Referee #2

FtsA is a conserved bacterial homolog of actin involved in cytokinesis. In E. coli and B. subtilis, FtsA is known to bind to the conserved carboxy-terminal tail of the bacterial tubulin FtsZ via its 2B domain. In addition, the carboxy-terminal tail of FtsA serves as a membrane targeting helix (MTS). Here, using an FtsZ carboxy-terminal peptide and FtsA lacking the MTS, both from Thermotoga maritima to facilitate crystal formation, Szwedziak et al. provide the first atomic structure of this FtsA-FtsZ interaction. They then go on to characterize an FtsA dimer that could be crystallized under certain conditions. It is known that FtsA lacking its MTS can form polymer bundles or sheets in vivo, and their FtsA dimer structure fits nicely into EM density maps of these FtsA polymers. This dimer structure packs similarly to MreB, another bacterial actin homolog, with some changes at the dimer interface due to the presence of FtsA's unique and relatively bulky 1c subdomain. This similar subunit orientation to MreB allays some concerns that the FtsA polymers are artifacts of concentrations much higher than physiological. To show further that FtsA polymers may be physiologically relevant, the authors make mutations at the dimer interface of B. subtilis FtsA that abolish polymer formation and claim that these mutant FtsAs are defective for FtsA function in vivo (unfortunately, in vivo experiments cannot be done easily in T. maritima).

The clear strengths of this manuscript are the first FtsZ-FtsA interaction at the atomic level and the demonstration that FtsA can form actin-like filaments with subunit interactions that are similar to those of actin and MreB. These seem to be two separate interactions, and the FtsZ-FtsA interaction is not emphasized relative to the FtsA-FtsA interaction (see title). The models shown in the final figure attempt to connect the relevance of these two interactions, although there is no evidence that FtsA normally forms extensive polymers in vivo (dimerization of FtsA has been shown, but not credited properly by the authors). The use of FtsAs from different species in different in vitro and in vivo assays is sometimes confounding and does not enhance the focus of the paper.

Thus, the story comes across as a very convincing FtsA-FtsZ molecular interaction that meshes with previous genetic data, and an FtsA-FtsA interaction with unclear physiological relevance. This is troublesome as the FtsA polymers are mostly lacking the MTS and are apparent only after being massively overproduced, while levels of FtsA, at least in E. coli cells, are relatively low (~1000 molecules per cell more or less). This means that if FtsA polymers are present in normal cells, they cannot be very long or very numerous. The in vivo studies are not too convincing (see comments below). As a result, the manuscript is somewhat unfocused, and the data become less convincing as it goes on, with the emphasis on the more problematic findings. Also, as suggested above, prior work on FtsA self-interaction has not been adequately referenced, giving the false impression of novelty for some of the conclusions. Most of these issues can probably be dealt with by judicious revision of the text, higher quality micrographs in Fig. 4 and carefully chosen caveats about physiological relevance.

Major comments:

1) Page 3: the authors state that "known" sources of energy in the divisome include nucleotide hydrolysis by FtsA. This has not been proven, and the authors actually state later (page 14) that ATPase activity has not been shown for any organism. The wording should be changed.

2) Page 5, 4th line from bottom: "lost" is too strong a word; replace with "reduced".

3) Page 6, top paragraph: Optimal membrane binding by FtsA is supposed to need an active membrane potential (Strahl and Hamoen, PNAS, 2010). Do these data contradict that idea?

4) It was mentioned in the legend for Fig. 2 that the TmFtsZ "does not directly interact with the nucleotide binding site containing ATP". However, van den Ent and Lowe 2000 show that H6 of TmFtsA helps to close the adenosine binding site and H7 comprises part of the adenosine hydrophobic binding site. Because the TmFtsZ makes contact with these same regions, it could be affecting how TmFtsA is interacting with nucleotide, although in the discussion they do say they tested for ATPase activity in the presence of the peptide and membranes and saw no changes. Another possibility would be to look in the presence of full-length TmFtsZ. Specific contacting residues of contact are only mentioned in the legend for Figure 2, but this should be mentioned in the text. It is significant that these residues (except Lys293) were also identified by Pichoff and

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Lutkenhaus 2007 for E. coli.

5) TmFtsZ binding to TmFtsA between helices H6 and H8 was only mentioned in the legend of Figure 2, but it would be useful in the text of the paper as well.

6) Page 7, bottom: Can the authors explain why they did not detect the TmFtsZ peptide in the TmFtsA dimer crystals? Might the FtsA dimerization inhibit binding to TmFtsZ? ? It doesn't in the dimer form of FtsA with the TmFtsZ peptide, so why the stabilized filament? Can the authors model how the full length TmFtsZ would fit alongside, given the peptide contacts they show in the crystal?

7) Why in one case is ATP-gamma-S required to see FtsA polymers, while in another case no nucleotide was needed for polymers to form on the lipid monolayer?

8) Page 10: No reason is given for the significantly larger spacing of EcFtsA subunits in the polymers vs. the others. Might the E. coli polymers be aberrant?

9) Page 10: using residue numbers for the different FtsAs is confusing. It would be better to use residue numbers for the truncations and simply use EcFtsA, BsFtsA or TmFtsA for the full length proteins.

10) Bottom of page 10 and Fig. 4: these mcherry fusions are massively overproduced compared to native FtsA in the T7 system. While this is stated in the figure legend, it should also be stated explicity in the text. Therefore, the FtsA polymers observed, while interesting and informative in terms of structure, are most likely not physiologically relevant. Only a few cells in the population seem to harbor them (panel a).

11) The poor quality of the images makes it hard to distinguish between the polymers in the top 3 panels and the supposed uniform fluorescence in the bottom 3 panels. I would argue that if the mCherry-FtsA polymers in the bottom 3 panels were just very thick, they would appear as shown. The mCherry channel and the green channel seem to be out of register.

12) Self-interaction of an MTS-deleted FtsA may require the presence of native WT FtsA to anchor the polymers to the membrane. This seems to be the case, as the polymers are aligned with the cytoplasmic membrane and not free-floating in the cytoplasm.

13) Proper interpretation of the "uniform" fluorescence of the three mutants in the bottom 3 panels of 4a requires that we know that the fusion proteins are stably made at the same levels as the mutants in the top 3 panels, and not proteolyzed between the mCherry and FtsA (which would yield uniform fluorescence). There is no information about amounts or sizes of the fusions.

14) It is not clear what the lower right panel in 4b is trying to show.

15) The in vivo section (pages 11-12) is weak. The 'polymerization-deficient' mutants were not shown to actually be deficient for in vitro assembly, but relying solely on the ability of the mCherry fusions to form visible polymers in cells. This evidence itself was weak (see comment above).

16) Also, the use of the ftsAts mutant is problematic. It is possible that the mutant FtsA's being overproduced interact with the resident ts FtsA differently than with no FtsA or with WT FtsA (as was done in the mCherry experiments in Fig. 4a, see comment 13 about this likely possibility). As a result, the phenotypes might not be solely caused by the mutants, but by an interaction between mutant and native ts FtsA, even at the restrictive temperature. As FtsA is not essential in B. subtilis, it is puzzling why the authors did not use an ftsA null mutant or at least a depletion strain for these experiments, as mixed FtsA interactions would confound their phenotypes.

17) The cell lengths and cell division efficiencies are not that different between WT and mutant. Moreover, phase contrast was used to measure cell lengths. As B. subtilis readily forms chains of cells, sometimes division septa are hard to see in phase contrast and need membrane staining to see more clearly. As a result, the authors have no way of knowing if some of those longer cells are septating chains or true filaments. Indeed, many of the mutants with 'filaments' have many smaller relatively normal-length cells around them. If the mutant FtsAs are defective, then why are there so

many short cells? A histogram of binned cell lengths might be a better way to display the data once the data are more rigorously collected.

18) FtsA overproduction has been shown to strongly inhibit cell division. How do we know that some of the results for the non-polymerizing FtsA are not in part due to toxic effects of overproduction? Furthermore, it is possible that the untagged mutant FtsAs K145A, M147E, and I278K were not stable in vivo. This would be a trivial reason why their complementation activities were lower and could therefore not complement the ts FtsA strain.

19) Page 12, lines 7-8: "absence of FtsA filaments (monomeric FtsA is still present)"; the absence of visible FtsA polymers after massive overproduction does not mean that monomeric FtsA is still present in cells. It is possible that most or all FtsA is present as dimers or oligomers (see also comment 21).

20) Page 12, line 9-10: Assuming that cell filamentation is worse with the FtsA mutants and assuming that these mutants really are worse at polymerizing than WT FtsA, increased cell length alone does not "strongly imply a role for FtsA in... Z-ring formation" because Z rings were never examined. At best, the authors can claim only that it implies a role in cell division, and even this is suspect (see comments above).

21) FtsA self-interaction and its potential role in cell division have been studied in depth by several groups (Yim et al. J. Bacteriol., 2000, Shiomi & Margolin, Mol. Microbiol., 2007, 2008) as well as a very recent paper in press Mol. Micro. from the Lutkenhaus lab. The present study is therefore not the first to address this issue. Indeed, the previous studies all made predictions about how FtsA oligomerization state might affect cell division and the Z-ring, and while the issues are by no means settled, they should be cited and discussed.

22) Page 12, line 21: the word "severe" is inaccurate. The effect is modest at best.

23) Page 14, line 5: What is the reason to think that the results in Feucht et al. were due to contamination?

24) Page 14, lines 15-17: Apart from the S. pneumoniae FtsA, a full length hypermorphic FtsA (FtsA*) was purified from E. coli and shown to interact with FtsZ and decrease overall FtsZ assembly (Beuria et al., J. Biol. Chem., 2009). Recently, wild-type E. coli FtsA was reconstituted with FtsZ in liposomes (Jimenez et al., J. Biol. Chem., 2010).

25) Page 15, top: A recent PALM study in E. coli showed that FtsZ forms multiple filaments in a ~110 nm zone around midcell (Fu et al., PLos One, 2010).

26) Fig. 1c: where is the supernatant only control for FtsA alone?

27) Why were membranes needed for the EM in Fig. 3? Why were membranes needed here but no membranes (and instead ATP gamma S) in previous data? No explanation is given.

28) A close up view of the FtsA-FtsA dimer interface(s) would be helpful and parallel the similar view of the FtsA-FtsZ interface. This is especially true if the emphasis of the paper is on the FtsA-FtsA interaction (as the title implies).

29) Carettoni et al (Proteins, 2003) proposed a fairly detailed FtsA-FtsA interaction model based on co-variation and phage display data. This model had two FtsA monomers interacting head to tail with a 180-degree twist. Based on the crystal structure here, this model appears to be incorrect. A mention of this would be useful, as the Carettoni model was to date the only molecular model for FtsA-FtsA interaction.

30) The schematic in Fig. 6D is misleading, as the FtsZ:FtsA ratio is significantly higher than 1:1 in B. subtilis and E. coli. The alternative idea, mentioned in the Discussion, is that FtsA forms short filaments or oligomers on the membrane, to which FtsZ binds, but which are dependent on FtsZ to get there. This is similar to what has been already depicted in several reviews on bacterial cell division.

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31) The schematic in Fig. 6C is not particularly illuminating, as this was already known from previous work. Also, in B. subtilis, Z rings still form in the absence of FtsA, so how do the authors imagine that they tethered to the membrane?

Minor comments:
1) Page 8, line 11: Fig. 6a is cited out of order.
2) Panel d in Fig. 1 should have lane numbers at the top, as the text (p. 6) refers to them.
3) It would have been helpful if the corresponding residues in T. maritima and/or E. coli were given for the FtsA mutants.

1st Revision - authors' response 10 February 2012

Reviewer #1

1. *Fig 1. It is clear that ftsA does not pellet with GTP (part 1B) or with ATP (part 1C), however, the paper goes on to argue that FtsA polymerizes. Why dosen't FtsA polymerize in this experiment? Are the conditions different?*

   No conditions have been found during this work that allow FtsA polymerisation in solution, *in vitro, with the important exception of the monolayer assay*. The reason for this is probably that the C-terminal amphipathic helix needs to be in contact with the membrane for polymerisation to occur at test-tube concentrations and in the absence of significant crowding. Our observation that FtsA polymerises is based on the crystal structure, the monolayer assay, in-cell tomography and fluorescence microscopy of labelled proteins in cells (where crowding is very high). As mentioned in the manuscript, conventional EM trials where we attempted to polymerise FtsA in solution and then apply on a carbon-coated grid failed and this is in line with the reviewer's observation that it does not pellet when nucleotides are added.

2. *How does the peptide conformation bound to FtsA compare to it bound to ZipA?*

   Please see Supplementary Figure 9 and Page 13 second top paragraph. Apologies if this was not made clear.

3. *How does the structure of the peptide-FtsA complex compare with the genetic analysis of important residues found by Haney et al. J Biol Chem. 2001 276(15):11980-7; a reference missing from the reference list. (it should be cited in the introduction).*

   This study is confirmed by our data showing the 8-16 last residues of FtsZ being important for FtsA binding and of course the crystal structure of FtsA in complex with the last residues of FtsZ, all done with *Thermotoga* proteins, showing that this interaction seems pretty much universal. The authors identified several residues important for FtsA binding within the C-terminal FtsZ region. Our biochemical assays support these data and the crystal structure provides atomic details of the interaction. It is now cited, apologies, this was an omission.

4. *The Kd is reported at 51 ± 9.9 µM. This seems very weak. Any comment?*

   True, although peptide interactions of this sort tend to be in the micromolar range. To us it seems that the FtsZ system is designed in a way that enables easy binding and dissociation of interacting partners. Particularly, one has to take into account that the FtsZ C-terminus is a busy region for protein interactions, including FtsA, ZipA, MinC, as determined by others previously. In a situation like this it may be unwise to design very high affinities. Another reason to believe the Kd is as high as indicated by ITC comes from our NMR studies, where we could observe not only shifted resonances, but disappeared peaks as well, which is the case when proteins do not interact tightly (medium micromolar regime).

5. *To what extent is this ftsA structure different from the previous reported structure from this lab. Why was this different structure obtained?*

   Structures of FtsA's monomers presented here and in the previous paper from our lab are very similar, with some differences concerning the 1C subdomain, which is slightly tilted. However, the crystal packings are entirely different. One of the structures presented here contains an FtsA dimer in the asymmetric unit. The other unit cell consists of an FtsA monomer, but they are packed head-to-tail and form a filament. Previous FtsA structures did not pack as polymers. After obtaining the FtsA dimer structure with the FtsZ peptide bound we decide to crystallise the complex in the presence if ATPgS, and this is how we obtained polymer-containing crystals.
   Why are they now different? Possibly one simple reason. Fusinita's structure from 2001 was obtained with His_6-tagged protein (C-terminal).

6. *Why is the Z peptide present in the FtsA structure when ATP was present but not ATPgS?*

   As mentioned in comment 5, these two crystal structures exhibit different crystals packings. It might be the case, that there is no enough space for the peptide to fit in between FtsA's polymers, but frankly, we do not know why this is the case. This discrepancy does not affect the logic of our story so this was not investigated, although there might be a story of behind this – for another paper.

7. *What is to be made of the observation that FtsA forms filaments of vesicle surfaces but not in solution?*

   As mentioned in the manuscript (page 14) it might be the case that FtsA polymers form more readily on vesicle surfaces since lipids provide charge compensation and thus lower activation energy. Please note that binding of the amphipathic helices to the surface provides additional binding energy to the system that is not available in the in-solution case and this probably makes the difference between getting filaments and not. Note that for the crystallisation MUCH higher protein concentrations are used, shifting towards polymerisation. Another case of this (admittedly from us) is documented here for MreB: Salje *et al.*, Mol Cell 2011.

8. *Page 9 near the bottom; Pichoff and Lutkenhaus used GFP-FtsA with C-terminal truncations to observe FtsA polymers, as done here Mol Microbiol. 2007 64(4): 1129-38)*

   We are aware of this study and it is now mentioned and cited (in introduction as prior evidence for polymerisation), thanks for this.

9. *Page 10, FtsA that can't bind the membrane forms filaments in vivo but not in vitro. Shouldn't polymers form in vitro?*

   In vitro assays with the purified, truncated FtsA protein did not yield any polymers, even in the presence of crowding agents or different divalent metal ions. See comment 1, reviewer #1. The difference to conditions in cells is huge, mostly crowding and protein concentration, since the proteins were overexpressed. Please note that this is the reason why we employ cellular tomography here (and SIM fluorescence microscopy) since polymerisation in cells is known to be so much easier.

10. *Page 13, last part of first complete paragraph starting with "Certainly, the ..." It is not clear what the authors are saying here. This relates to point 2 above. Is the structure of the peptide different in the ZipA-peptide versus the FtsA-peptide complexes? Is the structure consistent with the mutations by Haney et al. ?*

    We meant that the binding surface of the FtsA consists of alpha helices, whereas in case of ZipA the interacting site is mainly beta sheets. The peptide structures

are compared on Supplementary Figure 9. This paragraph has been rephrased and the suppl. Figure showing the superposition is now referenced.

Reviewer #2

Some general comments:

FtsA used for crystallisation trials was full-length, as determined by electrospray masspec. This is important, since we found that the tails of both FtsA and FtsZ become cleaved very easily during purification and the difference in molecular weight is not apparent on gels. Importantly, we suspect many published studies are ignorant of this problem.

There are no EM density maps shown in this paper, we are not sure what the referee meant.

In this work we presented two different crystal forms: one is an FtsA dimer with the FtsZ peptide bound, and the other is an FtsA monomer, which is packed in the crystal as a polymer (head to tail). Also the protein's 'dimerisation' and polymerisation abilities are not necessarily the same feature. We believe that what is sometimes called dimerisation in the literature to be polymerisation, in fact.

In our opinion using proteins from different species along with a variety of techniques applied only strengthens the paper as it shows that the protein's features are not confined to one particular species and we have made considerable efforts to show that *E. coli*, *B. subtilis* and *T. maritima* form filaments when overexpressed, in order to link all different organisms and findings together. Obviously, there are limitations as to what is possible, determined by some protein's behaviour in the test tube, especially *E. coli* FtsA.

This work shows that FtsA polymers can be formed under relatively low concentrations (1-2 μM) as demonstrated by the lipid monolayer assay and obviously here the protein contains the MTS. Rueda et al. proposed (Rueda et al., 2003, J Bacteriol, 185, 3344-51) that FtsA's intracellular concentration is about 0.5 μM, plus crowding.

We would also like to thank the reviewer for giving clear advice at the end of the general section. This is very much appreciated and a welcome departure from current practice in peer review.

1. *Page 3: the authors state that "known" sources of energy in the divisome include nucleotide hydrolysis by FtsA. This has not been proven, and the authors actually state later (page 14) that ATPase activity has not been shown for any organism. The wording should be changed.*

   We agree. Changed to "but potential or theoretical sources of energy in the system (apart from binding energies) are nucleotides associated with FtsA and FtsZ (inner divisome), as well as cell wall synthesis from precursors in the periplasm (outer divisome)"

2. *Page 5, 4th line from bottom: "lost" is too strong a word; replace with "reduced".*

   Replaced with "[…] is significantly reduced and the trace amount of FtsA in the pellet fraction is probably due to some unspecific interactions."

3. *Page 6, top paragraph: Optimal membrane binding by FtsA is supposed to need an active membrane potential (Strahl and Hamoen, PNAS, 2010). Do these data contradict that idea?*

   In their study, Strahl and Hamoen observed that some proteins that are membrane-associated, including FtsA, loose their cellular localisation upon addition of a specific proton-ionophore. Our data indicate that FtsA's amphipathic C-terminal helix is critical for membrane binding so it is conceivable that also in this case the membrane potential stimulates the interaction with the lipid bilayer since part of the interaction with the membrane is electrostatic (positive charge on peptide, negative charge on membrane). One should note that the amphipathic helix can be

replaced with the helix from MinD (Pichoff and Lutkenhaus, 2005, Mol Microbiol, 55, 1722-34) or even with the transmembrane domain of MalF (Shiomi and Margolin, 2008, Mol Microbiol, 67, 558-69). In conclusion, our new data does not provide any insight into this phenomenon.

4. *It was mentioned in the legend for Fig. 2 that the TmFtsZ "does not directly interact with the nucleotide binding site containing ATP". However, van den Ent and Lowe 2000 show that H6 of TmFtsA helps to close the adenosine binding site and H7 comprises part of the adenosine hydrophobic binding site. Because the TmFtsZ makes contact with these same regions, it could be affecting how TmFtsA is interacting with nucleotide, although in the discussion they do say they tested for ATPase activity in the presence of the peptide and membranes and saw no changes. Another possibility would be to look in the presence of full-length TmFtsZ. Specific contacting residues of contact are only mentioned in the legend for Figure 2, but this should be mentioned in the text. It is significant that these residues (except Lys293) were also identified by Pichoff and Lutkenhaus 2007 for E. coli.*

    By "does not *directly* interact with the nucleotide binding site containing ATP" we meant that the peptide is bound on the FtsA surface, not in the proximity of the cleft between subdomains 1A, 2A and 2B where the nucleotide sits. However, one cannot exclude that it might regulate FtsA's ATP metabolism through H6. Since the FtsZ C-terminal peptide is the only interacting site (and is attached to the core domain by a long and flexible linker) it is unlikely that full-length TmFtsZ would provide new insights. Specific contacts have now been mentioned in the text. Note that, again, we could not detect any ATPase hydrolysis by FtsA, with or without peptide.

5. *TmFtsZ binding to TmFtsA between helices H6 and H8 was only mentioned in the legend of Figure 2, but it would be useful in the text of the paper as well.*

    We agree. Added.

6. *Page 7, bottom: Can the authors explain why they did not detect the TmFtsZ peptide in the TmFtsA dimer crystals? Might the FtsA dimerization inhibit binding to TmFtsZ? ? It doesn't in the dimer form of FtsA with the TmFtsZ peptide, so why the stabilized filament? Can the authors model how the full length TmFtsZ would fit alongside, given the peptide contacts they show in the crystal?*

    We are aware that showing two similar structures might be confusing. To clarify, we obtained two FtsA crystal structures: a) the first one contained an FtsA dimer (each FtsA molecule had the FtsZ peptide bound and had an endogenous ATP molecule at 0.65 occupancy) per asymmetric unit. This structure is shown in Fig. 2. b) The second structure contained an FtsA monomer (with ATPgS added to the crystallisation trials and in the active site of the structure) per asymmetric unit. Although we added the FtsZ peptide, it did not show up in the electron density map. There may be a million reasons for this – but it was repeatable. Moreover, in case b), the protein crystallised as a continuous, true filament and the crystallographic a axis is aligned with the protofilament longitudinal axis. This structure is shown in Fig. 3.

    So yes, we did not find the peptide in the FtsA polymer structure and it might be triggered by any of many factors, for instance: different crystal packing or different buffer conditions that interfere with peptide binding. It is difficult to explain whether it is physiologically relevant, most likely not. Since it does not affect our story, we did not investigate this further.

    Given the flexible linker between FtsZ's interacting and core domains it is difficult to unequivocally model the FtsA:FtsZ complex. However, by means of electron cryo-tomography one could find out what the distance is between the two proteins and guess the linker conformation. We have that data available and it beautifully matches the numbers shown in figure 6c. This is for another paper.

7. *Why in one case is ATP-gamma-S required to see FtsA polymers, while in another case no nucleotide was needed for polymers to form on the lipid monolayer?*

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ATPgS was only used to obtain crystals where FtsA crystallised as a protofilament. We did not provide any ATP in the lipid monolayer assay but it turned out that a fraction of the protein contained the nucleotide carried over from the purification.
At the moment it is not clear what ATP is needed for. FtsA certainly binds the nucleotide but ATPase activity has not been demonstrated. We think that lipid binding in the monolayer assay reduces the energy required for polymerization and thus bypasses the need for ATP, and the same may be true for the crystals.

8. *Page 10: No reason is given for the significantly larger spacing of EcFtsA subunits in the polymers vs. the others. Might the E. coli polymers be aberrant?*

Yes, we assume that *E. coli* polymers might adopt a different conformation and our tomography results indicate so. It has been mentioned in the text that *E. coli* polymers appear to be helical, in contrast to straight *T. maritima* filaments and we consider that clear enough. The filaments in figure 4b top left (*E. coli*) do not look like simple straight protofilaments to us. The resolution in the tomograms is not sufficient to figure this out, unfortunately. But to us this does not matter: all we demonstrate here is that the protein forms filaments, clearly.

9. *Page 10: using residue numbers for the different FtsAs is confusing. It would be better to use residue numbers for the truncations and simply use EcFtsA, BsFtsA or TmFtsA for the full-length proteins.*

Done.

10. *Bottom of page 10 and Fig. 4: these mcherry fusions are massively overproduced compared to native FtsA in the T7 system. While this is stated in the figure legend, it should also be stated explicity in the text. Therefore, the FtsA polymers observed, while interesting and informative in terms of structure, are most likely not physiologically relevant. Only a few cells in the population seem to harbor them (panel a).*

During the preparation of this revision we significantly improved methodology for this experiment and completely repeated it since it is key to the logic (inducing at a lower OD, expressing for an hour and using a new super-resolution Nikon N-SIM microscope at Newcastle, UK, which Jeff Errington and Richard Daniel allowed us to use for a few days, many thanks to them!) The new pictures are shown in Figure 4a. Now the fusions are less overproduced, however, it is still far more than a normal intracellular concentration of FtsA. Also, under the new conditions, all cells displayed fluorescence so the sentence "The T7 system was used so not all cells showed strong fluorescence" has been removed.

11. *The poor quality of the images makes it hard to distinguish between the polymers in the top 3 panels and the supposed uniform fluorescence in the bottom 3 panels. I would argue that if the mCherry-FtsA polymers in the bottom 3 panels were just very thick, they would appear as shown. The mCherry channel and the green channel seem to be out of register.*

Done and improved by means of super-resolution microscopy using the Nikon N-SIM at Newcastle, UK (with traditional epi images presented in the Supplement in case that is an issue). We really hope this is clear now – we are certainly very happy with the new data.

12. *Self-interaction of an MTS-deleted FtsA may require the presence of native WT FtsA to anchor the polymers to the membrane. This seems to be the case, as the polymers are aligned with the cytoplasmic membrane and not free-floating in the cytoplasm.*

This is an attractive idea. Acquiring more fluorescence microscopy images (especially those done with the N-SIM technology) allows us to speculate on FtsA polymer localisation. They tend to form twisted sheets that are distributed along

cells. They clearly seem to stick to the membrane. This may be through native FtsA, although this is unlikely for protein from different species. Alternatively, the polymers may only appear in the space between the membrane and the nucleoid, the only space available to them in the crowded cell.

13. *interpretation of the "uniform" fluorescence of the three mutants in the bottom 3 panels of 4a requires that we know that the fusion proteins are stably made at the same levels as the mutants in the top 3 panels, and not proteolyzed between the mCherry and FtsA (which would yield uniform fluorescence). There is no information about amounts or sizes of the fusions.*

    We are confident that the polymerisation-deficient mutants (K145A, M147E, I278K) are made at a similar level compared to the other mutant (S46F) that still clearly polymerises. Certainly, the images do not look like that and care was taken so to be able to compare image brightness levels as a rough estimate of protein levels. They are all heavily over-expressed. Also, a new Western blot with anti-FtsA antibodies (Supplementary Figure 7b) shows that the all fusion proteins are made in comparable quantities, eliminating the concerns of the reviewer.

14. *It is not clear what the lower right panel in 4b is trying to show.*

    It shows that BsFtsA forms membrane invaginations that are similar to those that are caused by TmFtsA (the upper right panel), although they are not that prominent. This is difficult to show in 2 dimensions.

15. *The in vivo section (pages 11-12) is weak. The 'polymerization-deficient' mutants were not shown to actually be deficient for in vitro assembly, but relying solely on the ability of the mCherry fusions to form visible polymers in cells. This evidence itself was weak (see comment above).*

    We politely disagree with the reviewer that this section is weak and we hope that the new data alleviates at least some of these concerns (see reply to point #13, reviewer 2).

16. *Also, the use of the ftsAts mutant is problematic. It is possible that the mutant FtsA's being overproduced interact with the resident ts FtsA differently than with no FtsA or with WT FtsA (as was done in the mCherry experiments in Fig. 4a, see comment 13 about this likely possibility). As a result, the phenotypes might not be solely caused by the mutants, but by an interaction between mutant and native ts FtsA, even at the restrictive temperature. As FtsA is not essential in B. subtilis, it is puzzling why the authors did not use an ftsA null mutant or at least a depletion strain for these experiments, as mixed FtsA interactions would confound their phenotypes.*

    We are aware of the existing B. subtilis FtsA null mutants (Beall and Lutkenhaus, 1992, J Bacteriol, 174, 2398-403; Jensen et al., 2005, J Bacteriol, 187, 6536-44). However, it is striking that in both cases FtsZ had to be placed under the Pspac promoter in order to knock-out FtsA. We wanted to avoid additional side effects linked to FtsZ not being after the native promoter. In our complementation experiment we used negative (truncated product) as well as positive (wild-type, S46F) controls. Even if there are some residual amounts of ts FtsA they apparently do not interfere with both wild-type and S46F, so we assume this is also the case for K145A, M147E and I278K.

17. *The cell lengths and cell division efficiencies are not that different between WT and mutant. Moreover, phase contrast was used to measure cell lengths. As B. subtilis readily forms chains of cells, sometimes division septa are hard to see in phase contrast and need membrane staining to see more clearly. As a result, the authors have no way of knowing if some of those longer cells are septating chains or true filaments. Indeed, many of the mutants with 'filaments' have many smaller relatively normal-length cells around them. If the mutant FtsAs are defective, then why are there so many short cells? A histogram of*

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binned cell lengths might be a better way to display the data once the data are more rigorously collected.

It is possible that some of filamentous cells might be septating slower than normal cells but it still indicates that cell division is impaired to a certain degree. Also, we could not observe any of these (filamentous cells) in case of wild-type and S46F (as clearly indicated by much smaller SDs of these populations). Moreover, the acquired images were inspected manually. We do agree that the presence of normal–length cells around filamentous cells is somewhat worrying and most likely means that some sub-populations of the cells undergo unaffected cell division but to our surprise these cells were also present when the truncated Q87Stop protein was analysed. Also, previous work (Jensen et al., 2005, J Bacteriol, 187, 6536-44) revealed that the mean length of ftsa null mutant cells was 22.6 ± 11.9 μm – this is a fairly large standard deviation associated with a wide dispersion of cell length. In our opinion showing a histogram does not provide any additional information and may not be as clear for a reader as comparing mean lengths. But we do agree that this data is not as conclusive as one may anticipate. It probably indicates that there maybe functional overlap in the system, as has been reported before by others.

18. *FtsA overproduction has been shown to strongly inhibit cell division. How do we know that some of the results for the non-polymerizing FtsA are not in part due to toxic effects of overproduction? Furthermore, it is possible that the untagged mutant FtsAs K145A, M147E, and I278K were not stable in vivo. This would be a trivial reason why their complementation activities were lower and could therefore not complement the ts FtsA strain.*

It has been shown (Beall and Lutkenhaus, 1992, J Bacteriol, 174, 2398-403) that FtsA expressed from the Pspac promoter showed apparent complementation of the ts FtsA filamentation phenotype and our work confirms these findings (wild-type as well as S46F). This means that the expression level is right and fulfils an in vivo requirement for a proper FtsZ/FtsA ratio.

19. *Page 12, lines 7-8: "absence of FtsA filaments (monomeric FtsA is still present)"; the absence of visible FtsA polymers after massive overproduction does not mean that monomeric FtsA is still present in cells. It is possible that most or all FtsA is present as dimers or oligomers (see also comment 21).*

We strongly believe that our crystal structure of the FtsA protofilament allowed us to rationally design polymerisation-deficient mutants as shown by the fluorescence assay. Moreover, our findings have been very recently confirmed by an independent study from the Lutkenhaus lab (Pichoff et al., 2012, Mol Microbiol, 83, 151-167) – we performed mutagenesis for B. subtilis FtsA, the Lutkenhaus lab for E. coli FtsA, we used a fluorescence assay, they employed yeast-two-hybrid to detect FtsA-FtsA interaction. Satisfactory, both results agree to a very large extend when mapped onto the crystal structure of the T. maritima FtsA protofilament presented in this paper (added as Suppl. Figure 8, *please consult, it is worth seeing*). The fact that the Y2H data shows the same interaction sites as indicated by our protofilament indicates to us that probably all interactions between FtsA and FtsA are in the form of polymers, short or long. Another interesting outcome of the comparison of our data with Pichoff et al 2012 is that there seem to be more interactions on the flat side of FtsA, which, when compared with other actin-binding proteins is the inside of the double filament. This may indicate that FtsA also forms double filaments. Together with membrane binding this creates more parallels between FtsA and MreB double filaments, bound to membranes through an amphipathic helix, as reported recently by us (Salje, Mol Cell 2011).

20. *Page 12, line 9-10: Assuming that cell filamentation is worse with the FtsA mutants and assuming that these mutants really are worse at polymerizing than WT FtsA, increased cell length alone does not "strongly imply a role for FtsA in... Z-ring formation" because Z*

rings were never examined. At best, the authors can claim only that it implies a role in cell division, and even this is suspect (see comments above).

The role of FtsA in Z-ring formation has been extensively studied (Addinall and Lutkenhaus, 1996, J Bacteriol, 178, 7167-72; Pichoff and Lutkenhaus, 2002, EMBO J, 21, 685-93; Shiomi and Margolin, 2008, Mol Microbiol, 67, 558-69). This and previous (Pichoff and Lutkenhaus, 2005, Mol Microbiol, 55, 1722-34) work strongly suggest that the main role of FtsA is to attach the Z-ring to the membrane. Indeed, in this study we did not analyse the Z-ring formation, however, the phenotype of the B. subtilis strains indicates that it has a role in cell division. This has been reworded.

21. *FtsA self-interaction and its potential role in cell division have been studied in depth by several groups (Yim et al. J. Bacteriol., 2000, Shiomi& Margolin, Mol. Microbiol., 2007, 2008) as well as a very {Pichoff et al., 2012, Mol Microbiol, 83, 151-167} in press Mol. Micro. from the Lutkenhaus lab. The present study is therefore not the first to address this issue. Indeed, the previous studies all made predictions about how FtsA oligomerization state might affect cell division and the Z-ring, and while the issues are by no means settled, they should be cited and discussed.*

Unfortunately, previous reports are based on a model that now turns out to be incorrect (Carettoni, Proteins 2003). Also some make, in our opinion, some false assumptions. For instance (Yim et al., 2000, J Bacteriol, 182, 6366-73) claims that the C-terminus of FtsA is important for self-interaction by Y2H since each truncated protein species was able to interact with itself and with other truncated FtsAs but not with full length FtsA. We think it maybe caused by the fact that the full-length protein is membrane associated and not present in the yeast nucleus. An extra paragraph to discuss these unfortunate issues has now been put in the Discussion section.

22. *Page 12, line 21: the word "severe" is inaccurate. The effect is modest at best.*

We agree, wording was too strong and it has been changed. We have taken great care to be sure the assay employed produces consistent results.

23. *Page 14, line 5: What is the reason to think that the results in Feucht et al. were due to contamination?*

We might think of several reasons: first, Feucht et al. did not provide any catalytic mutants (missing negative control). Really, one should alsways perform the assay with both wild-type and mutant proteins purified in exactly the same manner and then ideally see a large difference. We have found over the years that it is just too easy to co-purify impurities that are not visible on a gel but significantly contribute to the obtained signal, especially with slow NPTases like the ones discussed here (when compared to *real* enzymes).

24. *Page 14, lines 15-17: Apart from the S. pneumoniae FtsA, a full length hypermorphic FtsA (FtsA*) was purified from E. coli and shown to interact with FtsZ and decrease overall FtsZ assembly (Beuria et al., J. Biol. Chem., 2009). Recently, wild-type E. coli FtsA was reconstituted with FtsZ in liposomes (Jimenez et al., J. Biol. Chem., 2010).*

Since *T. maritima* proteins are very well behaving and can be purified using relatively mild conditions (no denaturing agents, detergents or non-physiological salt concentrations) we used these for our in vitro analyses. Anyway, ATPase activity for both *S. pneumoniae* and *E. coli* proteins has not been demonstrated and a convincing proof for *B. subtilis* is absent (see reply to point #23).

25. *Page 15, top: A recent PALM study in E. coli showed that FtsZ forms multiple filaments in a ~110 nm zone around midcell (Fu et al., PLos One, 2010).*

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This work has now been cited, although we are not great fans of this study. It is very descriptive and hence not well supported. PALM is in its infancy and is quite intrusive as a method.

26. *Fig. 1c: where is the supernatant only control for FtsA alone?*

The supernatant only control for FtsA alone in Fig.1c looked exactly the same as the supernatant only control for Fig.1b and was hence excluded. The pellet is included to show there is nothing wrong with the protein in this particular experiment.

27. *Why were membranes needed for the EM in Fig. 3? Why were membranes needed here but no membranes (and instead ATP gamma S) in previous data? No explanation is given.*

Our initial trials were mainly focused on conventional EM in order to visualise FtsA polymers in a similar way to Lara et al., 2005, Mol Microbiol, 55, 699-711. However, no satisfactory results were obtained. Next we had the idea that membrane binding (through the amphipathic helix) might facilitate polymerisation, which turned out to be correct. As explained in the text, membranes might lower the energy needed for polymerisation, although there was some ATP bound to the protein (co-purified). ATPgS was used only in crystallisation trials and this is a very different situation, where the protein is roughly 100 times more concentrated and in very different (and strange) conditions (pH, precipitant etc.)

28. *A close up view of the FtsA-FtsA dimer interface(s) would be helpful and parallel the similar view of the FtsA-FtsZ interface. This is especially true if the emphasis of the paper is on the FtsA-FtsA interaction (as the title implies).*

This is going to be difficult since so many residues are involved. We have given up on the encyclopaedic figures a long time ago since interested readers will always download the structure(s) and have a look themselves, rather than trusting the authors. We think this is a better approach since mapping of 'interacting amino acids' always includes some form of guessing.

29. *Carettoni et al (Proteins, 2003) proposed a fairly detailed FtsA-FtsA interaction model based on co-variation and phage display data. This model had two FtsA monomers interacting head to tail with a 180-degree twist. Based on the crystal structure here, this model appears to be incorrect. A mention of this would be useful, as the Carettoni model was to date the only molecular model for FtsA-FtsA interaction.*

We do agree that the Carettoni model was until now the only available. It does appear to be wrong based on our data and on the recent data published by Pichoff et al, Mol Micro 2012. We would like to try to avoid citing work that is wrong, this seems counterproductive, given the dubious uses naïve citiation counts have obtained in recent years.

30. *The schematic in Fig. 6D is misleading, as the FtsZ:FtsA ratio is significantly higher than 1:1 in B. subtilis and E. coli. The alternative idea, mentioned in the Discussion, is that FtsA forms short filaments or oligomers on the membrane, to which FtsZ binds, but which are dependent on FtsZ to get there. This is similar to what has been already depicted in several reviews on bacterial cell division.*

Yes, Fig. 6D meant to show only a small section of the Z-ring, particularly the area where FtsZ filaments are attached to the membrane by a short stretch of FtsA. One should not assume that the A-ring covers the circumference of the cell. We have changed the figure legend to make this clearer and also the text. We would like to keep A-ring, since FtsA in fluorescence images does appear as a ring.

It might be that FtsA has been speculated to form filaments before, but here we show solid evidence how that is achieved and that is the purpose of this report.

31. *The schematic in Fig. 6C is not particularly illuminating, as this was already known from previous work. Also, in B. subtilis, Z rings still form in the absence of FtsA, so how do the authors imagine that they tethered to the membrane?*

This figure aims at summarising our current knowledge about the FtsZ:FtsA complex, emerging from both previous and this work. To our knowledge, the only study on the Z-ring formation in the absence of FtsA is (Jensen et al., 2005, J Bacteriol, 187, 6536-44) where the authors claim that FtsZ mainly localised as regular diffuse bands and occasionally they could come across sharp Z-rings (frequency 10%). It is difficult to speculate how these could be tethered to the membrane but they are certainly not functional (the cells are massively elongated). We are not convinced this needs discussing in the text since there is no real problem for our study coming from this data. There clearly is functional redundancy in the system, as shown by the fact that FtsA is not essential for *Bacillus*.

Minor comments:

1) Page 8, line 11: Fig. 6a is cited out of order.

Removed since the same thing can be inferred from figure 3 at this point in the text.

2) Panel d in Fig. 1 should have lane numbers at the top, as the text (p. 6) refers to them.

Done, apologies.

3) It would have been helpful if the corresponding residues in T. m15aritima and/or E. coli were given for the FtsA mutants.

Provided in Supplementary Fig. 6.

2nd Editorial Decision 28 February 2012

Thank you for the submission of your revised manuscript to The EMBO Journal. It has been sent to one of the original reviewers, who now considers that his/her concerns have been properly addressed and your manuscript is almost ready for publication. As you will see below referee #2 still points out to some minor details that need your attention before your manuscript can be officially accepted.

Browsing through the manuscript myself I have noticed that the statistical analysis of the results depicted in figure 5 is not optimally described. Statistical analyses must be described either in the Materials and Methods section or in the legend of the figure to which they apply. Although you have included a definition of the error bars used, we also require explicitly stating the number of independent experiments performed for the analysis. If this number is less than three, use of error bars is not appropriate and one representative experiment should be provided, clearly indicating this fact. Along these lines, albeit not absolutely necessary, we recommend the use of statistical significance analysis tools to further strengthen the interpretation of the results.

Also, we now encourage the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. Although optional at the moment, would you be willing to provide a PDF file per figure that contains the original, uncropped and unprocessed scans of all or key gels used in the figures? The PDF files should be labeled with the appropriate figure/panel number, and should have molecular weight markers; further annotation could be useful but is not essential. The files will be published online with the article as supplementary "Source Data" files. If you have any questions regarding this initiative do not hesitate to contact me.

Thank you very much again for your patience. I am looking forward to seeing the revised, final version of your manuscript.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #2

The authors have done a good job to address most of my concerns and those of the other referee. While the structural data are superb, I still think that the in vivo complementation data, while suggestive, are not that conclusive about FtsA polymerization being important for function without definitive proof that division septa are absent in the filamentous B. subtilis cells. The authors have soft-pedaled this fairly well, although it might be good to modify the wording in the abstract.

The Pichoff and Lutkenhaus genetic results (2012) do indeed corroborate nicely with the structural studies presented here. However, the Pichoff and Lutkenhaus mutants that decrease FtsA self-interaction (polymerization?) based on their assays are functional--in fact, they have gain of function properties (bypass of ZipA). This would seem to contradict the idea that polymerization is needed for FtsA function. Readers of the present paper will notice this, so the authors need to figure out how to deal with this apparent contradiction.

In the Discussion, which overall is very nicely written, it would be relevant to mention the Beuria et al. paper in JBC (284:14079, 2009) as to my knowledge it was the first example of in vitro interactions between FtsZ and FtsA (albeit a mutant).

2nd Revision - authors' response 02 March 2012

Point-by-point reply Manuscript EMBOJ-2011-80096R2

Editor:

Browsing through the manuscript myself I have noticed that the statistical analysis of the results depicted in figure 5 is not optimally described. Statistical analyses must be described either in the Materials and Methods section or in the legend of the figure to which they apply.

An additional paragraph on this has been added clarifying what error bars represent (page 28):

Cell measurements and statistical analysis

“About 500 cells for each FtsA variant were manually inspected and measured. Then average cell lengths and standard deviations over the given number of cells were calculated for each FtsA variant and plotted as bars and error bars, respectively, in Fig. 5b. “

If this number [of independent experiments] is less than three, use of error bars is not appropriate and one representative experiment should be provided, clearly indicating this fact.

Page 6, ITC analysis – instead of showing a mean value and standard deviation of two experiments we presented just two values, in order to conform with the guideline:

“Binding of the FtsZ peptide to FtsA is in the micromolar range with a K_d of 45 - 58 μM, as determined by two independent isothermal titration calorimetry experiments.” ….

Reviewer #2

The authors have soft-pedalled this fairly well, although it might be good to modify the wording in the abstract.

We modified the wording in the abstract, changing 'significant' to 'elongated':

"Mutants that disrupt polymerization also show an elongated cell division phenotype in a temperature sensitive FtsA background, demonstrating the importance of filament formation for FtsA's function in the Z-ring."

The Pichoff and Lutkenhaus genetic results (2012) do indeed corroborate nicely with the structural studies presented here. However, the Pichoff and Lutkenhaus mutants that decrease FtsA self-interaction (polymerization?) based on their assays are functional--in fact, they have gain of function properties (bypass of ZipA). This would seem to contradict the idea that polymerization is needed for FtsA function. Readers of the present paper will notice this, so the authors need to figure out how to deal with this apparent contradiction.

Page 15 - this has been now discussed:

"Interestingly, the mutants impaired in self-interaction identified in by Pichoff et al. can bypass the requirement for ZipA (another membrane anchor) and still support cell division in *E. coli*. This is most likely possible because recruitment of downstream proteins by FtsA is unaffected and the protein is still functional in the absence of ZipA. In contrast, FtsA in *B. subtilis* is presumably the only membrane tethering agent for FtsZ and as shown in this work, polymerization-deficient mutants trigger elongated cells. "

In the Discussion, which overall is very nicely written, it would be relevant to mention the Beuria et al. paper in JBC (284:14079, 2009) as to my knowledge it was the first example of in vitro interactions between FtsZ and FtsA (albeit a mutant).

Page 13 – the Beuria et al. (2009) paper has now been referenced:

"It has previously been shown that the last 15-20 residues of FtsZ are highly conserved and form a region for interactions with other proteins, such as ZipA, MinC and FtsA (Shen and Lutkenhaus, 2009) and mutated *E. coli* FtsA R286W could be co-pelleted with FtsZ (Beuria et al., 2009)."