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Large ring polymers align FtsZ polymers for normal septum formation

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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 30 August 2010

Thank you for submitting your manuscript to the EMBO Journal. This is a resubmission of manuscript # 73239 that was rejected before review in 2009 as further data in support of that the ability of SepF to form rings is important for its function was needed for consideration here. As the resubmission added such additional insight it was sent out for full review. We have now received the comments back from the three referees who we asked to review the paper and their remarks to the authors are provided below.

As you can see below, the referees find the analysis interesting and suitable for publication here. They also raise a number of technical concerns that would have to be addressed in order to consider publication here in particular to further substantiate the finding that the SepF rings are physiological relevant. Also the organization of the text and the discussion needs some work. Therefore, should you be able to address the raised concerns we would consider a revised manuscript. I should point out that it is EMBO Journal policy to allow a single major round of revision only and it is therefore important to resolve the raised issues at this stage. 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 initiative, please visit our website: http://www.nature.com/emboj/about/process.html

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

Yours sincerely,

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Editor
The EMBO Journal

REFEREE REPORTS

Referee #1 (Remarks to the Author):

This study suggests a potential role for a ring-forming protein (SepF) in bundling the cytoskeletal protein FtsZ during Bacillus subtilis septation. This idea is attractive and novel, and the topic (cell division and regulation of the cytoskeleton) is of general interest. The data are convincing and importantly the mutant analysis gives credence to the hypothesis that the SepF rings are physiologically relevant. I have a few suggestions for improvement.

The presentation and especially the organization of the paper could be improved. The results do not have to be told in the chronological order they were obtained. The flow of the story would be much better if the results were reordered, for example, by starting with the characterization of the wild-type protein and finishing with all the mutants.

To obtain reliable estimations of SepF and FtsZ concentrations, they need to plot quantitative Western blot data from multiple experiments.

Fig. 4C lacks error bars.

It is always troubling to see polymerization buffer that contain 10 mM MgCl2. I understand that others have used it for FtsZ polymerization, but at this concentration of divalent ions, a lot of proteins polymerize in vitro (but not in vivo). It would be reassuring to see that the authors can recapitulate their findings in lower Mg2+ concentrations (preferentially under 5 mM), which would be more physiological (unless there is evidence that the concentration of Mg2+ is this high in B. subtilis?). For instance, does SepF still form rings and does it still stimulate FtsZ bundling in lower Mg2+ concentrations?

Minor:
p.4: "This phenotype can be restored by overexpression of ftsA." is confusing. Does this mean that the phenotype is suppressed by overexpression or that overexpression recapitulates the phenotype. Please clarify.

p.5: Based on the first sentence, it appears that the SepF mutants were purified, yet the section is dedicated to ring structures of the wild-type protein. The text needs to be revised. I would suggest a careful proofreading and reorganization of the entire manuscript.

p.6, pH7.4 is catalogued as "physiological". Is the intracellular pH in B. subtilis known? Has it be determined that pH 6.5 is not physiological whereas pH 7.4 is?

It would be nice to show that it is the pH and not the buffer (Tris-HCl vs MES-NaOH) that makes the difference by using different buffers.

They should show the EM data for the SepF mutants (A98V and F124S) and FtsZ that are currently presented as data not shown. Instead of tubular structures, shouldn't we see a mixture of FtsZ protoflaments and SepF mutant ring structures?

Referee #2 (Remarks to the Author):

SepF interacts with FtsZ and plays an important role in cell division of Gram positives and Cyanobacteria. In Bacillus subtilis, it is not essential for division per se, but cells divide slowly and septa show morphological abnormalities in its absence. In addition, sepF mutations are synthetically lethal with mutations in ftsA or ezrA, encoding two other FtsZ-interacting proteins, suggesting SepF plays a partially redundant role in assembly of the Z ring.

Here, the authors find that B.subtilis SepF is about as abundant as FtsZ itself and that the purified protein spontaneously assembles in large closed rings of about 50 nanometer diameter. When SepF is included in FtsZ polymerization assays, these rings organize FtsZ polymers into long tubules that look spectacular. The authors selected for two SepF mutants that interfere with Z-ring assembly and cause trans-dominant lethality, when overexpressed in ftsA- mutants. Evidence suggests that both are defective in interaction with FtsZ, and one is also less prone to form SepF rings in vitro.
In addition, the authors introduced mutations near the C-terminus of SepF, and studied one (G135N) that still interacts with FtsZ but fails to form SepF rings in vitro. This mutant, furthermore, fails to correct the phenotypes of SepF- cells, indicating that the abilty of SepF to assemble into the 50nm rings is physiologically relevant. The authors propose that SepF rings help to keep FtsZ protofilaments properly organized in the Z ring.

This is an exciting report. The finding that purified SepF can self-assemble in such regular and large closed-ring assemblies is most unexpected. The fact that these rings organize FtsZ polymers into tubules in vitro is also remarkable, and demonstrates that ringed SepF is fully capable of engaging FtsZ. The EM images are beautiful, and the properties of the various SepF mutants provides some confidence that SepF rings are a physiologically relevant form of the protein.

I find concluding language too strong at places, genetic characterizations of the mutants seem incomplete, and more experimental detail would be helpful. What readers will desire most, however, is more guidance in how these surprising results could be fitted with current models of the Z ring, and with the know phenotypes of sepF- cells. I recommend reorganizing the text to include a proper discussion section.

Specific comments

1) Abstract

a) Line 4, This is inaccurate without qualification. In B.subtilis, SepF is only required for cell division when FtsA or EzrA are missing.
b) Second to last sentence. The conclusion that 'SepF rings are required...' is too strong without direct evidence that SepF indeed forms rings in vivo. It may not. We 'propose'.. is a suitable term.

Results

2) The results show that when the A98V or F124S mutants are overexpressed in a delta-ftsA strain, they interfere with Z ring assembly. Later evidence indicates that these mutants are defective in binding FtsZ. This predicts that, as is shown for the G135N mutant, these mutants should not be able to correct sepF- cells at any level of expression. I suggest this be confirmed.

3) I assume that the N-terminal ends of the SepF proteins were completely native after protease cleavage of the MBP affinity tag, but this should be made explicitly clear.

4) On page 8, please also provide estimated average copynumbers of SepF and FtsZ per cell.

5) Figure 2 also shows a V131E mutation and an arrow indicating a deletion at E141 or so. These are not described in the text or legend, but do raise my curiosity. What were their phenotypes?

6) Please clarify what buffer was used for the experiments in figures 3 and 7B. Does SepF ring formation require Mg++?

7) It is unclear how the values in fig 4a were derived. The legend says 'relative' increase in pelleting, but relative to what in each case?

8) Did the buffer in figure S2b also include GTP and Mg++? If not, it is not comparible to panel A.

9) It is not clear what the light scattering panel in 4B is supposed to show.
a) Is this pH 7.4 buffer at 50 mM or 300 mM KCL?
b) The one experiment in panel 4B doesn't tell us much. Is the rise in scatter at b due to FtsZ-SepF

complex formation or just to SepF itself?
c) One might expect formation of FtsZ-SepF tubules to yield a massive increase in scatter. Why is this not observed? Perhaps a higher wavelength would detect these large structures better?

10) It is interesting that SepF does not affect the GTPase activity of FtsZ as lateral contacts in the tubules would tend to stabilize the FtsZ polymers. One possibility is that FtsZ filaments on the tubule are spaced too far apart to make such contacts. Figure 5D suggests that there may be some gaps between the longitudinal fibres. I wonder if the authors noticed any variability in the density of FtsZ filaments in tubules, or if this is just too hard to tell from the EM images.

11) Does tubule formation require Mg++? If not, leaving it out should result in very stable tubules without splayed ends.

12) Legend to figure 7. A figure S6 is mentioned, but I can't find it.

13) Figure 8 panel B begs the question if the A89V and F124S mutants then failed to localize to Z rings in sepF cells, as predicted by their poor interaction with FtsZ in panel A.

14) Another prediction is that unlike the A98V and F124S mutants (Figure 1B), the G135N mutant should not be transdominant when overexpressed in a ftsA- strain, specifically. Was this confirmed?

15) page 11, line 4. 'shows' is too stong. 'indicates' is more suitable.

16) Some points that could be further adressed briefly in a discussion section.
a) Synthetic lethality suggests some overlapping function of FtsA and SepF. One important function of FtsA is to tether FtsZ polymers to the membrane. How do the authors think SepF rings help accomplish this?
b) In the introduction (page 4) the authors describe difficulties in understanding the fact that sepF- cells are synthetic lethal with either ftsA or ezrA. Can this now be understood any better?
c) If I understand the model in figure 9 correctly, SepF rings might keep FtsZ polymers confined to a narrow area at the site of division, and it is 'spreading out' of Z polymers in sepF- cells that causes abnormally thick murein deposition? The model raises many questions, however. Confinement would only work if there is a mechanism to prevent multiple 'tubule' structure from forming adjacent to each other, correct?. And, how do the authors envision the tubules engaging the membrane, for example? Either just a few FtsZ polymers on one side of the tubule contact the membrane at any one time, or the membrane wraps around rhe tubule?
d) The deliberations on page 8 (lines 2-18) would be significantly more effective when integrated with the proposed model in a proper discussion section. Especially, the likely low density of FtsZ polymers in the Z-ring before and during initial constriction renders it hard to visualize how SepF rings (also limited in number, perhaps around 150 rings maximally?) would have much impact during those stages.
e) Has the localization of SepF been confirmed by immunofluorescence? As the used SepF-GFP fusions likely don't associate with SepF rings directly, it is conceivable that some interesting localization pattern has been missed.

17) Other points
a) Page 4, line 4. Replace ftsA with sepF.
a) Table S1; CRK600 or CRK6000?
b) Figure 8A, the A98V mutation is mislabeled A89V
h) The arrows mentioned in the legend to fig.S3 are not shown.

Referee #3 (Remarks to the Author):

In previous work, the authors identified the cell division protein SepF as a positive regulator of division in the Gram-positive bacterium Bacillus subtilis. Although not essential for viability, SepF is required for both efficient septation and normal septal morphology. Work from another laboratory (Singh, et al 2008) indicated that SepF likely functions by promoting lateral interaction between FtsZ protofilaments. Using both genetic and biochemical approaches this paper seeks to extend this earlier work.

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Using in vitro assembly assays, the authors find that SepF forms what appear to be ring-like structures, which, in the presence of FtsZ, encircle groups of FtsZ protofilaments and promote the formation of large tubular structures. (These images are quite striking!) Although unlikely to be physiologically relevant, these tubules are consistent with a bundling function for SepF as previously reported by Singh et al (2008).

Next, taking advantage of the lethality associated with mutations in both SepF and the highly conserved cell division protein FtsA, the authors conduct a screen in which they identify two independent dominant negative mutations in SepF. These mutants, together with analysis of a small C terminal deletion mutant, and a SepF GFP fusion, suggest a model in which geometrically precise interactions between SepF monomers are required to coordinate SepF's ability to promote the bundling of FtsZ protofilaments both in vivo and in vitro.

While the authors' data indicate that precisely coordinated interactions between SepF monomers are required to promote FtsZ assembly, it does not necessarily prove that SepF circles are physiologically relevant structures as the authors propose. Specifically, although the C terminal deletion mutant data support the idea curved SepF multimers are essential for SepF driven bundling of FtsZ protofilaments, these data do not prove the circular form of SepF is the biologically active form of the protein. In fact, these data also support a model in which short, curved multimers of SepF serve to bundle the small number of FtsZ protofilaments currently thought to constitute the FtsZ ring.

Comments:
1. It is intriguing that SepF only functions at pH conditions under which FtsZ tends to exist as single stranded protofilaments rather than bundles (lower pH promotes bundling between FtsZ protofilaments in the absence of any effector proteins).
2. Although the authors examine the effect of SepF on assembly of FtsZ polymers at varying concentrations of FtsZ, I do not see any data regarding the critical concentration of SepF required for oligomerization and/or circle formation in vitro. This type of data would go a long way towards clarifying the ability of SepF to form open as well as closed circles in vitro.
3. Similarly, what is the nature of FtsZ polymers assembled in the presence of lower levels of SepF? Are tubules still observed?
4. How many SepF monomers are predicted to present in a complete SepF circle? How does this information translate to the number of complete circles potentially available to promote lateral interactions beween FtsZ protofilaments in a living cell?
5. The addition of sucrose gradient or similar analysis of cell lysates would be one means of determining the oligomerization state of SepF in vivo. Such data is vital to making the case that is the ring form of SepF is indeed physiologically relevant.
6. The actions of inhibitors of FtsZ assembly provide a potential explanation for the lack of large SepF induced FtsZ tubules observed in vivo.

1st Revision - authors' response 22 October 2010

Response to referees

Referee #1

The presentation and especially the organization of the paper could be improved. The results do not have to be told in the chronological order they were obtained. The flow of the story would be much better if the results were reordered, for example, by starting with the characterization of the wild-type protein and finishing with all the mutants.
> The organization of the results has been changed as suggested.

To obtain reliable estimations of SepF and FtsZ concentrations, they need to plot quantitative Western blot data from multiple experiments.
> The value for SepF is based on four separate experiments (two at 30 ∞C, and two at 37 ∞C), and the FtsZ concentration was measured at both 30 ∞C and 37 ∞C. Since the FtsZ values were close to

previously published concentrations this was not repeated. We have now added the data from the different experiments to Fig. S1.

Fig. 4C lacks error bars.
> A new graph (Fig. 2C) including error bars has been included (based on four independent experiments).

It is always troubling to see polymerization buffer that contain 10 mM MgCl2. I understand that others have used it for FtsZ polymerization, but at this concentration of divalent ions, a lot of proteins polymerize in vitro (but not in vivo). It would be reassuring to see that the authors can recapitulate their findings in lower Mg2+ concentrations (preferentially under 5 mM), which would be more physiological (unless there is evidence that the concentration of Mg2+ is this high in B. subtilis?). For instance, does SepF still form rings and does it still stimulate FtsZ bundling in lower Mg2+ concentrations?
> We have repeated the experiments with different Mg2+ concentrations. SepF rings are readily formed even in the absence of Mg2+, however the formation of tubules with FtsZ requires some Mg2+, but we could go as low as 1 mM Mg2+. This is close to physiological concentrations (e.g. Rivas et al., 2000, JBC 275(16)). We have now added this information to the main text (page 5, lines 14-15, and page 7, lines 15-17).

Minor:
p.4: "This phenotype can be restored by overexpression of ftsA." is confusing. Does this mean that the phenotype is suppressed by overexpression or that overexpression recapitulates the phenotype. Please clarify.
> This is a mistake and it should have been ‘overexpression of sepF’. The sentence has been corrected (page 4, lines 3-4).

p.5: Based on the first sentence, it appears that the SepF mutants were purified, yet the section is dedicated to ring structures of the wild-type protein. The text needs to be revised. I would suggest a careful proofreading and reorganization of the entire manuscript.
> The text has been reorganized and now the analysis of the mutants follows that of the description of the wild type protein (as suggested by the referee).

p.6, pH7.4 is catalogued as "physiological". Is the intracellular pH in B. subtilis known? Has it be determined that pH 6.5 is not physiological whereas pH 7.4 is?
> Neutrophiles maintain an internal pH of about pH 7.5 to pH 8.0, and when the internal pH of E. coli is lower than pH 7, growth ceases (Booth, Mic Rev, 1985). Breeuwer et al. (Appl. & Env. Mic. 1996) have shown that the internal pH in B. subtilis varies between pH 7 to pH 9 when the medium pH increases from pH 5 to pH 9. It is therefore reasonable to assume that an internal pH of 6.5 is not physiological relevant for B. subtilis (and E. coli). We have added ‘more’ to the text (‘more physiological pH’), and we have added the two references (page 6, lines 16-17).

It would be nice to show that it is the pH and not the buffer (Tris-HCl vs MES-NaOH) that makes the difference by using different buffers.
> We have tested also HEPES and MES buffers with the same results. We have added this to the text (page 6, lines 17-19).

They should show the EM data for the SepF mutants (A98V and F124S) and FtsZ that are currently presented as data not shown. Instead of tubular structures, shouldn't we see a mixture of FtsZ protoflaments and SepF mutant ring structures?
> With mutant A98V no clear rings were seen when mixed with FtsZ, which is as expected since this mutant is disturbed in ring formation. Mutant F124S makes normal rings and we could discern rings in the presence of FtsZ. EM images have now been included as supplementary figure (Fig. S5).

Referee #2:

I find concluding language too strong at places, genetic characterizations of the mutants seem incomplete, and more experimental detail would be helpful. What readers will desire most, however, is more guidance in how these surprising results could be fitted with current models of the Z ring,

and with the know phenotypes of sepF- cells. I recommend reorganizing the text to include a proper discussion section.
> See details below, and we have now included a proper discussion section.

Specific comments

1) Abstract

a) Line 4, This is inaccurate without qualification. In B.subtilis, SepF is only required for cell division when FtsA or EzrA are missing.
> This is too detailed for the abstract, but we have changed the sentence to; SepF is required for normal cell division (page 2, line 6).

b) Second to last sentence. The conclusion that 'SepF rings are required...' is too strong without direct evidence that SepF indeed forms rings in vivo. It may not. We 'propose'.. is a suitable term.
> Changed to 'We propose' (page 13, line 5).

2) The results show that when the A98V or F124S mutants are overexpressed in a delta-ftsA strain, they interfere with Z ring assembly. Later evidence indicates that these mutants are defective in binding FtsZ. This predicts that, as is shown for the G135N mutant, these mutants should not be able to correct sepF- cells at any level of expression. I suggest this be confirmed.
> This is indeed the case, and we have now added to the text that expression of these mutants does not compensate for the absence of wild type SepF (page 8, lines 12-14).

3) I assume that the N-terminal ends of the SepF proteins were completely native after protease cleavage of the MBP affinity tag, but this should be made explicitly clear.
> Yes this is indeed the case; the Xa-cleavage site is positioned immediately upstream of SepF. We have now mentioned this in the Experimental Procedures (page 14, lines 13-14).

4) On page 8, please also provide estimated average copynumbers of SepF and FtsZ per cell.
> Has been added to the text (page 5, line 28 ñ page 6, line 1).

5) Figure 2 also shows a V131E mutation and an arrow indicating a deletion at E141 or so. These are not described in the text or legend, but do raise my curiosity. What were their phenotypes?
> There seems some confusion because we do not see a V131E mutation in the picture, and the arrow is positioned between amino acids 133 and 134. To make the picture clearer we have placed the amino acid positions below the alignment and removed the arrow (new Fig. 6, was previously Fig. 2).

6) Please clarify what buffer was used for the experiments in figures 3 and 7B. Does SepF ring formation require Mg++?
> The buffers used have now been described in the legends (Fig 3 is now Fig 1). SepF does not require Mg2+ to make rings. We have added this to the text (page 5, lines 14-15).

7) It is unclear how the values in fig 4a were derived. The legend says 'relative' increase in pelleting, but relative to what in each case?
> This is indeed confusing and we have removed 'relative'. A better description of the method is now added to the Experimental Procedures (page 16, lines 3-5).

8) Did the buffer in figure S2b also include GTP and Mg++? If not, it is not comparible to panel A.
> The same buffer was used, except for the presence of GTP. We now mention this in the legend of Fig. S2.

9) It is not clear what the light scattering panel in 4B is supposed to show.
a) Is this pH 7.4 buffer at 50 mM or 300 mM KCL?
b) The one experiment in panel 4B doesn't tell us much. Is the rise in scatter at b due to FtsZ-SepF complex formation or just to SepF itself?
c) One might expect formation of FtsZ-SepF tubules to yield a massive increase in scatter. Why is this not observed? Perhaps a higher wavelength would detect these large structures better?
> Indeed we expected to observe a strong light scattering signal from the tubules, but this was not

the case. We have tried different conditions, including changing the wavelength, without success. We agree that Fig. 4B (Fig. 2B in the revision) does not reveal much information. However, since light scatter is often used in FtsZ polymerization studies we felt it important to show that under more physiological polymerization conditions (pH 7.4 and 300 mM KCl) no clear dynamic light scatter response is detectable. We have described this aspect more extensively in Fig. S3.

10) It is interesting that SepF does not affect the GTPase activity of FtsZ as lateral contacts in the tubules would tend to stabilize the FtsZ polymers. One possibility is that FtsZ filaments on the tubule are spaced too far apart to make such contacts. Figure 5D suggests that there may be some gaps between the longitudinal fibres. I wonder if the authors noticed any variability in the density of FtsZ filaments in tubules, or if this is just too hard to tell from the EM images.
> Unfortunately, the resolution of the images is insufficient to say much about this.

11) Does tubule formation require Mg++? If not, leaving it out should result in very stable tubules without splayed ends.
> Polymerization of FtsZ is essential for tubule formation and this process is stimulated by Mg2+. Tubule formation required at least 1 mM Mg2+, and this is now added to the text (page 7,lines 15-17)

12) Legend to figure 7. A figure S6 is mentioned, but I can't find it.
> This has been a mistake and the remark has been removed.

13) Figure 8 panel B begs the question if the A89V and F124S mutants then failed to localize to Z rings in sepF cells, as predicted by their poor interaction with FtsZ in panel A.
> Yes these mutants no longer accumulate at midcell and the GFP signal is diffuse. We have now added this to the text (page 9, lines 16-17).

14) Another prediction is that unlike the A98V and F124S mutants (Figure 1B), the G135N mutant should not be transdominant when overexpressed in a ftsA- strain, specifically. Was this confirmed?
> Yes this was confirmed, and we have added this now to the text (page 10, lines 20-21).

15) page 11, line 4. 'shows' is too stong. 'indicates' is more suitable.
> Changed as suggested (page 10, line 22).

16) Some points that could be further adressed briefly in a discussion section.
a) Synthetic lethality suggests some overlapping function of FtsA and SepF. One important function of FtsA is to tether FtsZ polymers to the membrane. How do the authors think SepF rings help accomplish this?
> At the moment we do not have an answer to this question. SepF has no membrane binding sequence. An alternative explanation is that both proteins stimulate the assembly of FtsZ protofilaments.

b) In the introduction (page 4) the authors describe difficulties in understanding the fact that sepF- cells are synthetic lethal with either ftsA or ezrA. Can this now be understood any better?
> The confusion originated from the fact that FtsA is considered a positive regulator and EzrA a negative regulator of Z-ring assembly (as mentioned in the introduction). Now we know that SepF is not simply a stimulator of FtsZ protofilament assembly but that is seems to be required for the proper organization of this assembly. The protein can therefore not be simply regarded as a positive regulator of FtsZ polymerization. We have added this to the text (page 4, lines 20-22).

c) If I understand the model in figure 9 correctly, SepF rings might keep FtsZ polymers confined to a narrow area at the site of division, and it is 'spreading out' of Z polymers in sepF- cells that causes abnormally thick murein deposition? The model raises many questions, however. Confinement would only work if there is a mechanism to prevent multiple 'tubule' structure from forming adjacent to each other, correct?. And, how do the authors envision the tubules engaging the membrane, for example? Either just a few FtsZ polymers on one side of the tubule contact the membrane at any one time, or the membrane wraps around rhe tubule?
> Presumably, the limited number of FtsZ molecules hampers the formation of multiple tubules. We have moved the discussion on this matter from the result section to the new Discussion (page 12, lines 1-8). At the moment it goes too far to speculate how the tubules might interact with the cell

membrane.

d) The deliberations on page 8 (lines 2-18) would be significantly more effective when integrated with the proposed model in a proper discussion section. Especially, the likely low density of FtsZ polymers in the Z-ring before and during initial constriction renders it hard to visualize how SepF rings (also limited in number, perhaps around 150 rings maximally?) would have much impact during those stages.
> We have now combined these deliberations and the Conclusion section into a proper Discussion section (page 11, line 10 ñ page 12, line 9). Currently, we do not know how many SepF molecules constitute a SepF ring, and a discussion on the number of SepF rings in the cell is highly speculative.

e) Has the localization of SepF been confirmed by immunofluorescence? As the used SepF-GFP fusions likely don't associate with SepF rings directly, it is conceivable that some interesting localization pattern has been missed.
> No, we have not confirmed the localization by immunofluorescence. The spatial resolution of immunofluorescence with bacterial cells is rather low compared to GFP fusions, and it seems unlikely that we gain more information with this technique.

17) Other points
a) Page 4, line 4. Replace ftsA with sepF.
a) Table S1; CRK600 or CRK6000?
b) Figure 8A, the A98V mutation is mislabeled A89V
h) The arrows mentioned in the legend to fig.S3 are not shown.
> All the corrections have been made. In case of Fig. S3, the legend now indicate that GTP was added after 1 minute of equilibration (instead of arrows).

Referee #3:

While the authors' data indicate that precisely coordinated interactions between SepF monomers are required to promote FtsZ assembly, it does not necessarily prove that SepF circles are physiologically relevant structures as the authors propose. Specifically, although the C terminal deletion mutant data support the idea curved SepF multimers are essential for SepF driven bundling of FtsZ protofilaments, these data do not prove the circular form of SepF is the biologically active form of the protein. In fact, these data also support a model in which short, curved multimers of SepF serve to bundle the small number of FtsZ protofilaments currently thought to constitute the FtsZ ring.
> The referee raises an important point, and we agree that short curved multimers of SepF will fit the model as well, and will also result in an ordered assembly. We have added this conclusion to the Discussion (page 12, lines 20-22).

Comments:
1. It is intriguing that SepF only functions at pH conditions under which FtsZ tends to exist as single stranded protofilaments rather than bundles (lower pH promotes bundling between FtsZ protofilaments in the absence of any effector proteins).
> It is now increasingly recognized that it is important to study FtsZ polymerization under physiological conditions, especially if it comes to investigating the effect of other proteins on FtsZ polymerization (e.g. Scheffers, 2008, FEBS letters 582(17), Mohammadi et al., 2009, Biochemistry 48(46), Gonzalez et al., 2003, JBC 278(39)).

2. Although the authors examine the effect of SepF on assembly of FtsZ polymers at varying concentrations of FtsZ, I do not see any data regarding the critical concentration of SepF required for oligomerization and/or circle formation in vitro. This type of data would go a long way towards clarifying the ability of SepF to form open as well as closed circles in vitro.
> We have tried to address this question but ran into practical difficulties. The only reliable way to test for ring formation is EM. However, reducing the concentration of the protein makes it increasingly difficult to find rings on the grid, but this is not necessary related to a presumed critical concentration required for ring formation. We were able to reduce the SepF concentration 60x (0.1 μM) and, with some effort, still detected some rings. This information has now been added to the

text (page 5, lines 15-17).

3. Similarly, what is the nature of FtsZ polymers assembled in the presence of lower levels of SepF? Are tubules still observed?
> We have tested different concentrations and with 3 µM SepF tubules were still detected. With 1 µM SepF no tubules were seen. We have added this information to the text (page 7, lines 11-13).

4. How many SepF monomers are predicted to present in a complete SepF circle? How does this information translate to the number of complete circles potentially available to promote lateral interactions beween FtsZ protofilaments in a living cell?
> We have seriously considered this, and were tempted. However, it is highly speculative to deduct the diameter of a protein from its molecular weight and to assume that the protein functions as a monomer. Therefore, we have refrained from this, albeit persuasive, exercise.

5. The addition of sucrose gradient or similar analysis of cell lysates would be one means of determining the oligomerization state of SepF in vivo. Such data is vital to making the case that is the ring form of SepF is indeed physiologically relevant.
> Previous crosslinking and pulldown experiments have shown that SepF forms part of a large protein complex (including FtsZ: Ishikawa et al., 2006, Mol. Mic. 60(6)). Determining the real oligomerization state of SepF in vivo is therefore very difficult, and maybe impossible, and goes beyond the scope of this study.

6. The actions of inhibitors of FtsZ assembly provide a potential explanation for the lack of large SepF induced FtsZ tubules observed in vivo.
> So far different EM techniques, including CryoEM tomography, have never detected a clear Z-ring or a 'divisome complex' in Gram-positive bacteria (e.g. Zuber et al., 2006, J. Bacteriol. 188(18)). This does not mean that the Z-ring or the divisome does not exist. The same can be argued for SepF induced FtsZ tubules.

2nd Editorial Decision 08 November 2010

Thank you for submitting your revised manuscript to the EMBO Journal. I asked the original referees #2 and 3 to look at the revised version and I have now received their comments.

Both referees find the revised manuscript improved and support its publication in the EMBO Journal. However, they also request a number of text changes, before acceptance here. Referee #3 would like to see a better discussion about how the in vitro activities of SepF contribute to its in vivo function. I would like to ask you to respond to these remaining points in a final revision. Once we receive the revised version, we will proceed with its acceptance for publication here. When you send us your revision, please include a cover letter with an itemised list of all changes made, or your rebuttal, in response to comments from review.

I look forward to seeing the final version.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #2 (Remarks to the Author):

I'm satisfied with the responses to my previous comments. Only two small points for correction:

1) Abstract
Lines 12-13. For accuracy: add "in vitro' after 'align FtsZ filaments" and 'normal' before 'cell division'.

2) Discussion
Page 11, line 7. The reference to Raychaudhuri is incorrect (should be EMBOJ, 1999).

Referee #3 (Remarks to the Author):

Overall this is a much improved manuscript. However, I am still at a loss as to precisely how SepF's in vitro activity translates to in vivo function, one of my primary concerns in my original review.

Although the authors say in their response to Reviewer 3, "different EM techniques...have never detected a clear Z-ring" this is not entirely accurate. While traditional EM has not provided any information about the structure of the FtsZ ring, recent work using three state-of-the art techniques (electron cryotomography, PALM and STED microscopy) all suggest that the ring is a discontinuous helix composed of short FtsZ polymers linked together at only a few points rather than large, cable like bundles. See Jennings PC, Cox GC, Monahan LG, Harry EJ. 2010 Micron PMID 20933427 and Fu G, Huang T, Buss J, Coltharp C, Hensel Z, Xiao J. 2010 PLoS One 5, (2010) PMID 20856929 and Li Z, Trimble MJ, Brun YV, Jensen GJ.2007 EMBO J. 26. Notably, all three papers failed to detect any large tubular structures of FtsZ. Although the authors suggest a model in which SepF might coordinate interactions between only a few FtsZ polymers, a model consistent with these three papers, none of these manuscripts are cited in the current version of the manuscript.

I would therefore request that the authors add a section to the discussion in which they specifically discuss SepF activity in light of these three papers. In particular, the fairly of any state of the art technique to observe large tubular structures of FtsZ similar to those observed by G¸ndo du et al needs to be addressed. For example, SepF really limits FtsZ assembly to a defined area, as the authors suggest, this may explain the observation of Jennings et al that the "ring" is more or less a fixed width.

2nd Revision - authors' response 26 November 2010

Rebuttal Referee #3

Referee #3 cites three papers that use novel techniques (cryoEM, PALM and STED) to reveal details of FtsZ polymerization in bacterial cells (Li et al. 2007 EMBO J., Fu et al. 2010 Plos One, Jennings et al. 2010 Micron, respectively), and the referee would like to see these papers discussed in relation to our findings.

We feel that the data in these three papers do not contribute to a better understanding of our results for the following reasons:

- The CryoEM tomography paper of Li et al., uses Caulobacter crescentus, and the paper of Fu et al. uses the new PALM technique with Escherichia coli. Both species are Gram-negative bacteria and do not contain a SepF homologue. It is therefore difficult to see how the results of Li et al., and Fu et al., can help in the interpretation of our findings. Gram-negative bacteria divide in a different manner (constriction) compared to Gram-positive bacteria (cross-wall), and in the discussion we describe this difference in relation to the presence of SepF.

- The CryoEM data of Fu et al. seem to underestimate the number of FtsZprotofilaments, and both Li et al. and Jennings et al. criticize this study in the introduction of their own papers.

- Li et al. use an FtsZ-FP fusion that is not active (in cell division) and that seems to be impaired in the interaction with other proteins, as they suggest on page 2 of their paper. In addition, for most of the PALM images the cells were chemically fixed, which can distort cellular structures (as the

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authors acknowledge).

- Jennings et al. uses the novel STED technique to study the localization of FtsZ in Bacillus subtilis. However, in this paper almost nothing is said about the Z-ring, and the paper focuses primarily on the helical arrangement of FtsZ throughout the cell. Possibly this is due to the fact that, although STED increase the resolution a bit, it is still insufficient to draw clear conclusions on the arrangement of FtsZ protofilaments in the Zring. In addition, the authors were forced to fix the cells with methanol to use STED, and such treatment can easily disrupt sensitive structures.

Finally, referee #3 mentioned that our findings 'may explain the observation of Jennings et al that the "ring" is more or less a fixed width'. I have carefully read the Jennings paper, but there is no description or quantification of the width of the Z-ring. The authors give only values for the pitch of the helical FtsZ structures that are found throughout the whole cell (Jennings et al., Table 1), and that seem unrelated to the Z-ring.

For these reasons, and since these papers do not support or disprove our conclusion that SepF helps to align FtsZ protofilaments, we have decided not to discuss these papers in the discussion.

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