Bacterial motility complexes require the actin-like protein, MreB and the Ras homologue, MglA

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1st Editorial Decision 22 April 2009

Thank you for submitting your manuscript for consideration by The EMBO Journal. I have received the final report from the three referees asked to evaluate your manuscript and I enclose their comments below.

As you will see from their reports the referees express potential interest in the findings, however, it is clear that they are also concerned that the proposed model for the localization of A- and S-motility factors by MglA and MreB may be turn out to be a significant over-simplification. This seems to be in part due to the different observed localization of proteins in cells without polymerized MreB and loss of functional MglA. To strengthen the model the referees suggest a number of important and helpful experiments. It needs to be determined if the localization of the motility proteins are directly linked to the binding of MreB and MglA (and the role of its GTPase activity) and if a direct interaction between these two proteins can be detected. The role of A22 also has to be shown to directly affect MreB and quantification of the data as requested by referee #2 should be provided. Overall, while there is interest in the study, there is also hesitation that other factors may be involved and potentially play a more important role, diminishing the impact of the study. I understand that the referees ask for a significant amount of new data but given the interest in the study and if you are able to provide the data required that in the end strengthens the study I would like to ask you to submit a revised version of the manuscript. The normal policy of the EMBO Journal is to allow three months for a period of revision, however, given the interest in the study, in this exceptional circumstance I would be willing to extend this deadline in order to give you the full opportunity to address the concerns raised by the referees.

I would like to remind you that it is 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 revisions included in the next, final version of the manuscript.

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

Yours sincerely,

Editor
The EMBO Journal

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REFEREE COMMENTS

Referee #1 (Remarks to the Author):

Localization of bacterial proteins to specific subcellular sites is commonly observed. However, little is known about how these proteins find their correct localization. In this manuscript the authors report on the roles of the actin-like protein MreB and the Ras-like protein MglA in localization of motility proteins in Myxococcus xanthus. The authors present evidence that MreB in M. xanthus forms helical filaments, which depolymerize in the presence of A22. Interestingly, depolymerization of MreB correlates with inhibition of the activity of the two motility systems in M. xanthus and results in the dispersal of clusters of the A-motility protein AglZ and in an alteration in the localization of the S-motility protein FrzS. MreB is only the second protein in M. xanthus shown to be important for both motility systems. The second protein important for both motility systems is the MglA protein. In an mglA deletion mutant AglZ and FrzS were also found to localize abnormally. MglA-YFP was observed to localize to the poles as well as to focal adhesion complexes. Finally, correct MglA localization depends on MreB.

This paper potentially adds significantly to our knowledge about protein localization by showing that MreB is required for the correct localization of AglZ, FrzS and MglA and that MglA, in turn, is required for AglZ and FrzS localization. MreB has been shown to form A22 sensitive filaments in several bacteria. Moreover, MglA was shown to be important for the localization of the A-motility protein RomR. Therefore, the novel and potentially very interesting finding in this paper is the connection between MreB, MglA and protein localization. However, as detailed below the work still appears preliminary.

The paper is very well-written and for a general readership. The logic is easy to follow.

Major comments

1. It is suggested that MreB may act through MglA to guide protein localization. However, A22 treated cells and mglA mutant cells show different localization of FrzS. How is that explained?

2. A22 results in abnormal localization of MglA, FrzS and AglZ. However, it is not clear that this effect depends on MreB. This question could be addressed by isolating A22 resistant MreB mutants (as was done in Caulobacter) and then check the effect of A22 on protein localization in these mutants.

3. MglA is referred to as a GTPase and a Ras homolog. To my knowledge it is not been shown that MglA has GTPase activity. Also, it is unclear whether MglA activity depends on GTPase activity. This could easily be tested in vivo as well as in vitro by analyzing constitutively active and inactive MglA mutants.

4. Quality of figures:
   Fig. 1B and Fig. 1C: The current versions are so small that the details described are very difficult/impossible to see.
   Fig. 2A: The current versions are so small that the details described are very difficult/impossible to see. Include in the legend a description of what happens at 6 min and 24 min.
   Fig. 3A: The current versions are so small that the details described are very difficult/impossible to see. 3B: Individual cells cannot be distinguished and, therefore, it is difficult to follow where the
AglZ clusters are localized before addition and after removal of A22.

Fig. 4A and 4B: The current versions are so small that the details described are very difficult/impossible to see. Would it be possible to include a schematic or phase contrast pictures to show the borders of the cells.

Fig. 6A and 6B: The current versions are so small that the details described are very difficult/impossible to see. Especially the numbering of the clusters in 6A is not evident.

Fig. 6C: The upper panel in this figure does not convincingly show that FrzS only co-precipitates in the MglA-YFP strain.

Referee #2 (Remarks to the Author):

This interesting study using A22 to depolymerise MreB in gliding Myxococcus indicates a role for the cytoskeleton in both A and S motility and also implicates a small Ras like GTPase. The data and the hypothesis are interesting and may well be right, but the authors need to be a little more circumspect in some of their interpretations as the data are still indirect. No direct interaction has been identified with MreB and, as other have suggested, some proteins may be localised or be confined to troughs between the helical protein polymer and therefore appear to co-localise. Some of the images are hard to be sure about as there is often not complete loss and recovery results in different patterns. Some of this might be helped if there was dual labelling and co-localisation of the proteins was measured.

Specific Comments
1. In Abstract - last sentence - MreB is actin-like, the last sentence is over interpreting.
2. The images in Fig 1A are interpreted as a helix, because of previous images. Without a z-stack it is hard to tell from the image that it is a helix and not spots. Is there a deconvoluted image.
3. The pitch seems very precise, as is based on a diffuse fluorescent image - I find it hard to believe you can be accurate to 3 decimal places. Remember the same pitch would be formed by proteins excluded from a MreB helix.
4. If MreB is forming a polymerised helical structure, why would there be bright foci - what do you think these might be?
5. Fig 3A does not seem to show complete dispersal as suggested on Page 9 - there are still clear areas of increased fluorescence. Figure 3B is much clearer and I'm not sure what 3A adds as the images appear to be treated differently - there seems to be much more fluorescence in AglZ/YFP +A22 than -A22 therefore without quantitation it is hard to interpret.
6. Again Fig 4 would benefit from quantitation. The spots are dimmer, but the difference between 9 min and 13 min doesn't look massively different between 17 and 25 (and why would it go down). These are very hard to interpret. Why would there only be partial loss.
7. Fig 5 - How was the relative protein localisation measured. Do you know what copy number is needed to see a focus?

Referee #3 (Remarks to the Author):

This manuscript by Mauriello and colleagues explores the role of the bacterial actin, MreB, in Myxococcus motility. The authors characterize MreB localization in Myxo, and demonstrate that a previously-described MreB inhibitor, A22, perturbs both A- and S-motility. The onset of motility inhibition correlates with A22's delocalization of two known motility factors, the A-motility protein AglZ, and the S-motility protein FrzS. In an effort to explore the link between MreB and the motility proteins, the authors characterize another previously-identified protein, MglA. MglA is found to localize to sites of both AglZ and FrzS action, MglA is delocalized by A22, and an mglA mutant affects the localization of both AglZ and FrzS. The authors therefore suggest a model in which MreB localizes MglA, which in turn localizes AglZ and FrzS, which in turn mediate A- and S-motility, respectively.

This is clearly a well-conceived paper on an interesting topic: MreB has been shown to play a number of important functions in a wide range of bacteria, but has not been previously characterized in Myxo and its roles in motility have been relatively underexplored. This work will therefore be of
broad interest to both the bacterial cell biology and motility fields. However, the authors aggressively promote an over-simplistic model for MreB/MglA-mediated FrzS and AglZ localization, which is not fully supported by the data. Moreover, important controls are missing in order to justify the conclusion that MreB is important for Myxo motility. Ultimately, this work may be appropriate for publication in EMBO, but a number of key issues need to be addressed.

Major issues:
1. The only way in which the authors probe the function of MreB is via A22 treatment at doses that are much (10-15 fold) higher than have been used in other systems. In order to demonstrate that this A22 treatment specifically perturbs MreB, the authors must provide controls, for example by isolating A22-resistant mreB alleles and showing that A22 no longer affects motility or protein localization in these mutants. Alternatively, a different genetic way of perturbing MreB, such as a depletion allele, could be used.
2. The authors propose a pathway in which MglA is localized by MreB and in turn recruits FrzS and AglZ. In that case, how do the authors explain the finding that unlike A22 treatment, the mglA deletion mutant still retains some FrzS and AglZ localization? If MreB does not affect AglZ/FrzS through other proteins, the expectation would be that A22 treatment would not further affect AglZ/FrzS localization in the mglA deletion. Is that so?
3. A second problem with the model stems from the fact that the FrzS and AglZ localizations are distinct from each other and overlap with different subdomains of the MglA localization. The fact that AglZ/FrzS do not simply go wherever MglA is demonstrates that MglA is both not fully necessary (see above), but also not sufficient to direct AglZ/FrzS localization; other factors must be involved. Identifying these additional factors is perhaps beyond the scope of this paper, but nevertheless, the authors' conclusions about the role of MglA is over-stated, diminishing the impact of the manuscript.
4. The authors argue that MglA may be directly interacting with MreB. An effort to demonstrate such an interaction, for example by biochemistry or a 2-hybrid assay would significantly strengthen the paper.

Minor issues:
A. The argument for the co-association of the various proteins studied here would be strengthened by co-localization experiments that examined their distributions in the same cells. Similarly, rather than having to rely on the similar kinetics of A22’s effects on motility and protein localization, the authors should correlate these phenotypes in the same cells.
B. The authors show that A22 inhibits the motility of individual A+S- and A-S+ cells (Fig. 2). They should also show the effect of A22 on wild-type cells.
C. In other bacterial systems, MreB forms a dynamic structure. Have the authors attempted to make a fluorescent protein fusion to MreB to examine its dynamics?
D. For all of the fluorescent micrographs, the authors should show both bright field and fluorescence images. For example, it would be nice to reassure the readers that the observed delocalization was not due to out-of-focus images.
E. In the discussion, the authors suggest that the dependence of AglZ localization on MreB supports their previously-published focal adhesion model for A-motility. However, in the simplest form of the focal adhesion model, the cytoskeleton binds and potentially anchors the focal adhesion, but is not actually required for its assembly (AglZ would still cluster but move more freely without anything to push against). The authors should either remove this point or discuss it more clearly and convincingly.

Referee #1 (Remarks to the Author):

Localization of bacterial proteins to specific subcellular sites is commonly observed. However, little is known about how these proteins find their correct localization. In this manuscript the authors report on the roles of the actin-like protein MreB and the Ras-like protein MglA in localization of motility proteins in Myxococcus xanthus. The authors present evidence that MreB in M. xanthus
forms helical filaments, which depolymerize in the presence of A22. Interestingly, depolymerization of MreB correlates with inhibition of the activity of the two motility systems in M. xanthus and results in the dispersal of clusters of the A-motility protein AglZ and in an alteration in the localization of the S-motility protein FrzS. MreB is only the second protein in M. xanthus shown to be important for both motility systems. The second protein important for both motility systems is the MglA protein. In an mglA deletion mutant AglZ and FrzS were also found to localize abnormally. MglA-YFP was observed to localize to the poles as well as to focal adhesion complexes. Finally, correct MglA localization depends on MreB.

This paper potentially adds significantly to our knowledge about protein localization by showing that MreB is required for the correct localization of AglZ, FrzS and MglA and that MglA, in turn, is required for AglZ and FrzS localization. MreB has been shown to form A22 sensitive filaments in several bacteria. Moreover, MglA was shown to be important for the localization of the A-motility protein RomR. Therefore, the novel and potentially very interesting finding in this paper is the connection between MreB, MglA and protein localization. However, as detailed below the work still appears preliminary.

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Major comments
1. It is suggested that MreB may act through MglA to guide protein localization. However, A22 treated cells and mglA mutant cells show different localization of FrzS. How is that explained?

Response to reviewer 1:

Fig. 5B shows that in non-motile ΔmglA cells, FrzS localizes at one cell pole rather than being asymmetrical bi-polar. Fig. 3C shows that after incubating with A22, the FrzS signal fades. We think that the reason why the localization of FrzS is more defective in the presence of A22 than in ΔmglA cells is that MreB drives FrzS localization not only via MglA, but also through other unidentified factors. In fact, MglA seems indispensable for the pole-to-pole trafficking of FrzS. We have therefore added a clarifying sentence to the abstract (page 2) and to the discussion on page 17.

See also response to comment 2 from reviewer 3.

2. A22 results in abnormal localization of MglA, FrzS and AglZ. However, it is not clear that this effect depends on MreB. This question could be addressed by isolating A22 resistant MreB mutants (as was done in Caulobacter) and then check the effect of A22 on protein localization in these mutants.

As described above (response to editors comments), to ensure that MreB was the direct target of A22, we constructed a M. xanthus strain carrying a V to A mutation in position 323 in the MreB protein. In the revised version of the manuscript, we show that this mutation, which confers resistance to A22 in C. crescentus (Gitai et al., 2005), also confers resistance to A22 in M. xanthus. Figure 1C now shows that the localization of MreBV323A, unlike MreB, is unperturbed in the presence of A22 (150 µg/ml). Additionally, Figure 2A and 2C now show that motility of mreBV323A cells is indistinguishable from wild type, but unperturbed in the presence of A22 (50µg/ml). Since motility of mreBV323A cells is unperturbed by A22 which we are confident that also protein localization is unaffected.

3. MglA is referred to as a GTPase and a Ras homolog. To my knowledge it is not been shown that MglA has GTPase activity. Also, it is unclear whether MglA activity depends on GTPase activity. This could easily be tested in vivo as well as in vitro by analyzing constitutively active and inactive MglA mutants.

The MglA GTPase activity has not yet been shown in vitro but sequence analysis (now included as Fig. S8), phylogenetics (Homologs of eukaryotic Ras superfamily proteins in prokaryotes and their novel phylogenetic correlation with their eukaryotic analogs. Dong JH, Wen JF, Tian HF. Gene. 2007 Jul 1;396(1):116-24) and genetic data (Complementation of sporulation and motility defects in a prokaryote by a eukaryotic GTPase. Hartzell PL. Proc Natl Acad Sci U S A.
1997 Sep 2;94(18):9881-6) strongly argue that MglA is a bona fide small GTPase. Purification of MglA, like for many other small GTases, has not proven to be an easy task. Also, we think that a deep biochemical analysis of the GTPase activity of MglA would be very interesting but we feel that it is beyond the scope of this work.

4. Quality of figures:
Fig. 1B and Fig. 1C: The current versions are so small that the details described are very difficult/impossible to see.

The resolution and size of Fig. 1B have been improved.
Old Fig. 1C is now Figure S2.

Fig. 2A: The current versions are so small that the details described are very difficult/impossible to see. Include in the legend a description of what happens at 6 min and 24 min.

We have removed the old Figure 2A from the revised manuscript. In the new version of the manuscript we added the construction of an A22 resistant mreBV323A strain that was used as control in all of the presented experiments. This rendered the old Figure 2A unnecessary.

Fig. 3A: The current versions are so small that the details described are very difficult/impossible to see. 3B: Individual cells cannot be distinguished and, therefore, it is difficult to follow where the AglZ clusters are localized before addition and after removal of A22.

Following the suggestion of reviewer 2 (comment 5) we eliminated the old Figure 3A. This figure has been replaced with a clearer new Figure 3A, which includes the original fluorescence micrographs and an overlay of fluorescence and bright field.

Fig. 4A and 4B: The current versions are so small that the details described are very difficult/impossible to see. Would it be possible to include a schematic or phase contrast pictures to show the borders of the cells.

The old Figures 4A and 4B have been replaced with a clearer new Figure 3B, which includes the original fluorescence micrographs and an overlay of fluorescence and bright field.

Fig. 6A and 6B: The current versions are so small that the details described are very difficult/impossible to see. Especially the numbering of the clusters in 6A is not evident.

The resolution and size of Figures 6A and 6B (now Figure 5A and 5B) have been improved.

Fig. 6C: The upper panel in this figure does not convincingly show that FrzS only coprecipitates in the MglA-YFP strain.

The old Figure 6C has been replaced by Figure 6A, which shows co-purification experiments using Tap-tagged FrzS or AglZ as baits. The eluate of the copurification experiments was analyzed by western immunoblotting. It was shown to contain AglZ or FrzS and MglA, indicating that the proteins directly interact consistent with the previously shown co-immuno precipitation experiment.

Referee #2 (Remarks to the Author):

This interesting study using A22 to depolymerise MreB in gliding Myxococcus indicates a role for the cytoskeleton in both A and S motility and also implicates a small Ras like GTPase. The data and the hypothesis are interesting and may well be right, but the authors need to be a little more circumspect in some of their interpretations as the data are still indirect. No direct interaction has been identified with MreB and, as other have suggested, some proteins may be localised or be confined to troughs between the helical protein polymer and therefore appear to co-localise. Some of the images are hard to be sure about as there is often not complete loss and recovery results in different patterns. Some of this might be helped if there was dual labelling and co-localisation of the proteins was measured.
Specific Comments
1. In Abstract-last sentence-MreB is actin-like, the last sentence is over interpreting.

We changed the sentence to "Interestingly, M. xanthus motility systems, like eukaryotic systems, utilize an actin-like protein and a small GTPase spatial regulator."

2. The images in Fig1A are interpreted as a helix, because of previous images. Without a z-stack it is hard to tell from the image that it is a helix and not spots. Is there a deconvoluted image.

Figure 1A shows Z sections of an already deconvolved image. While it is true that a 3D reconstruction would be necessary to make the definite conclusion that MreB forms a helix in M. xanthus, we believe that the quality of our images showing a continuous filament rather than only spots and dots, and the knowledge of MreB localization in other rod shaped bacteria allows us to suggest a helical localization pattern for M. xanthus MreB as well. We changed the text to "appearing to form helical filaments" on page 7 to tone down our conclusion.

3. The pitch seems very precise, as is based on a diffuse fluorescent image-I find it hard to believe you can be accurate to 3 decimal places. Remember the same pitch would be formed by proteins excluded from a MreB helix.

We agree with the reviewer. We have approximated the periodicity of the MreB helix to 0.47±0.1 nm.

4. If MreB is forming a polymerized helical structure, why would there be bright fociwhat do you think these might be?

We do not fully understand the role of the bright foci at the poles. This is the first time that such a structure is observed in a bacterium and we do not believe that it is an artifact as we observed it consistently throughout our experiments. We speculate that the brighter foci at the cells poles might be important for the efficient function of the A and S motors. However, we do not have any evidence for a biological role of this structure. In line 1-2 page 17, we added a sentence in the discussion section to state that we observe it but cannot yet assign a function to it.

5. Fig 3A does not seem to show complete dispersal as suggested on Page 9-there are still clear areas of increased fluorescence. Figure 3B is much clearer and I'm not sure what 3A adds as the images appear to be treated differently-there seems to be much more fluorescence in AglZ-YFP +A22 than -A22 therefore without quantitation it is hard to interpret.

In 3B after A22 is washed out the reformed clusters are in very different positions and there seem to be fewer larger clusters. If, post A22 MreB repolymerises and the proteins reassociate, why the very different pattern?

We have removed Figure 3A, as suggested by the reviewer. Figure 3B is now replaced by a similar figure showing the effect of A22 injection at 50 g/ml instead of 150 g/ml. In this new version of the manuscript, the injection experiments are done at 50 g/ml (unless specified) because Reviewer 3 objected that the doses might be too high.

We explain Figure 3B as follows: The AglZ clusters are very dynamic and their number and localization depend on velocity, which varies over the time (Mignot et al., 2007). Also, clusters assemble at the leading cell pole. When A22 is washed out, a single AglZ cluster is re-assembled at the leading cell pole. Then the initial recovery of motility coincides with the AglZ polar cluster remaining fixed while the cell moves forward leading to the appearance of multiple distributed clusters. Thus, there is no reason that the AglZ pattern after recovery be strictly identical to the pattern before treatment.

6. Again Fig 4 would benefit from quantitation. The spots are dimmer, but the difference
between 9 min and 13 min doesn’t look massively different between 17 and 25 (and why would it go down). These are very hard to interpret. Why would there only be partial loss.

We thank the reviewer for his/her comment and re-visited the experiments shown in Figure 4 extensively. Our new experiments show that there is no real significant difference between FrzS-GFP localization pattern in pilA+ and pilA- backgrounds after treatment with A22. We are now confident that FrzS-GFP fluorescence is in fact completely dispersed independently on the presence of the pili. Thus, we removed old Fig. 4 and replaced it with a new figure only showing the effect of A22 on FrzS-GFP localization in a wild type background.

7. Fig 5 - How was the relative protein localization measured. Do you know what copy number is needed to see a focus?

To quantitate subcellular localization of AglZ-YFP and FrzS-GFP in wild type or mglA backgrounds, cells were illuminated under similar conditions for each tested background and were scored for the presence of discrete detectable foci at the cell poles and along the cell body. It is unknown how many molecules are needed to detect a discrete focus, but under these conditions AglZ-YFP or FrzS-GFP foci could not be detected along the cell body or at both cell poles in an mglA mutant. Note that the micrographs are not shown with similar contrasts. For example, in panel A, the contrast of the micrograph showing AglZ-YFP in the mglA mutant is in fact significantly increased compared to the WT control to reveal the diffuse AglZ-YFP fluorescence resulting from mislocalization, which would otherwise be difficult to see if the WT and mglA mutant images had been treated identically.

Referee #3 (Remarks to the Author):

This manuscript by Mauriello and colleagues explores the role of the bacterial actin, MreB, in Myxococcus motility. The authors characterize MreB localization in Myxo, and demonstrate that a previously-described MreB inhibitor, A22, perturbs both A and S-motility. The onset of motility inhibition correlates with A22’s delocalization of two known motility factors, the A-motility protein AglZ, and the S-motility protein FrzS. In an effort to explore the link between MreB and the motility proteins, the authors characterize another previously-identified protein, MglA. MglA is found to localize to sites of both AglZ and FrzS action, MglA is delocalized by A22, and an mglA mutant affects the localization of both AglZ and FrzS. The authors therefore suggest a model in which MreB localizes MglA, which in turn localizes AglZ and FrzS, which in turn mediate A- and S-motility, respectively.

This is clearly a well-conceived paper on an interesting topic: MreB has been shown to play a number of important functions in a wide range of bacteria, but has not been previously characterized in Myxo and its roles in motility have been relatively underexplored. This work will therefore be of broad interest to both the bacterial cell biology and motility fields. However, the authors aggressively promote an oversimplified model for MreB/MglA-mediated FrzS and AglZ localization, which is not fully supported by the data. Moreover, important controls are missing in order to justify the conclusion that MreB is important for Myxo motility. Ultimately, this work may be appropriate for publication in EMBO, but a number of key issues need to be addressed.

Major issues:
1. The only way in which the authors probe the function of MreB is via A22 treatment at doses that are much (10-15 fold) higher than have been used in other systems. In order to demonstrate that this A22 treatment specifically perturbs MreB, the authors must provide controls, for example by isolating A22-resistant mreB alleles and showing that A22 no longer affects motility or protein localization in these mutants. Alternatively, a different genetic way of perturbing MreB, such as a depletion allele, could be used.

As discussed above, to ensure that MreB was the direct target of A22, we constructed a M. xanthus strain carrying a V to A mutation in position 323 in the
MreB protein. In the new version of the manuscript we show that such mutation, reported to confer resistance to A22 in C. crescentus (Gitai et al., 2005), confer resistance to A22 also in M. xanthus. The new results and figures that we provide are the following:

Figure 1C: The localization of MreBV323A, unlike MreB, is unperturbed in the presence of A22 (150 µg/ml).
Figure 2A and 2C: Motility of mreBV323A cells is indistinguishable from wild type, but unperturbed in the presence of A22 (50 µg/ml).

In addition to these new figures and experiments, we repeated all of the experiments with A22 using a concentration of 50 g/ml, much closer to the one using in other systems.

These experiments demonstrate that the A22 treatment specifically perturbs MreB in M. xanthus.

2. The authors propose a pathway in which MglA is localized by MreB and in turn recruits FrzS and AglZ. In that case, how do the authors explain the finding that unlike A22 treatment, the mglA deletion mutant still retains some FrzS and AglZ localization? If MreB does not affect AglZ/FrzS through other proteins, the expectation would be that A22 treatment would not further affect AglZ/FrzS localization in the mglA deletion. Is that so?

We agree with the reviewer that the fact that FrzS and AglZ (to a minor extent) retain some localization in mglA cells compared to the A22 treatment after which clusters are completely dispersed, suggests that MglA might not be the only factor recruited by the bacterial cytoskeleton for the positioning of motility proteins. We added a sentence in the abstract (page 2) and in the discussion on page 17. See also response to comment 1 from Reviewer 1.

3. A second problem with the model stems from the fact that the FrzS and AglZ localizations are distinct from each other and overlap with different subdomains of the MglA localization. The fact that AglZ/FrzS do not simply go wherever MglA is demonstrates that MglA is both not fully necessary (see above), but also not sufficient to direct AglZ/FrzS localization; other factors must be involved. Identifying these additional factors is perhaps beyond the scope of this paper, but nevertheless, the authors’ conclusions about the role of MglA is over-stated, diminishing the impact of the manuscript.

This question follows from the previous comment. Correct and modified accordingly in the result and discussion sections.

4. The authors argue that MglA may be directly interacting with MreB. An effort to demonstrate such an interaction, for example by biochemistry or a 2-hybrid assay would significantly strengthen the paper.

Testing a direct interaction between MglA and MreB has proven challenging because MglA is extremely difficult to purify. However, this revised version of the paper includes the following new interaction data and figures:

Figure 4A and B show in vitro cross-linking experiments supporting the direct interaction between MreB and the coiled-coil domain of AglZ. The result also suggests that this interaction can be favored by the presence of ATP.
Figure 4C shows the result of co-purification experiments using FrzS or AglZ TAPtagged as bait. The eluate of the co-purification experiments was analyzed by western immunoblotting. It was shown to contain FrzS or AglZ and MglA, confirming the interaction between these proteins.

Minor issues:
A. The argument for the co-association of the various proteins studied here would be strengthened by co-localization experiments that examined their distributions in the same
cells. Similarly, rather than having to rely on the similar kinetics of A22’s effects on motility and protein localization, the authors should correlate these phenotypes in the same cells.

The revised manuscript presents co-localization experiments in Figure 3C. We constructed a strain co-expressing AglZ-mCherry and FrzS-GFP. When we treated this strain with A22 and followed protein localization, we observed that the dispersal of AglZ and FrzS clusters occurred synchronously after the injection of A22. Similarly, clusters were reconstituted at the same time after the removal of A22. We explain this experiment on page 11 of the results section. These results show synergism between A- and S-motility and their co-dependence on the bacterial cytoskeleton and are now included as supplemental materials.

B. The authors show that A22 inhibits the motility of individual A+S- and A-S+ cells (Fig. 2). They should also show the effect of A22 on wild-type cells.

The effect of A22 on wild type cells, as well as on mreBV323A cells, is now shown in Figure 2A and 2C.

C. In other bacterial systems, MreB forms a dynamic structure. Have the authors attempted to make a fluorescent protein fusion to MreB to examine its dynamics?

We have repeatedly attempted to generate a functional MreB-GFP chimera using several strategies and none have so far been successful at generating a functional MreB-GFP fusion.

D. For all of the fluorescent micrographs, the authors should show both bright field and fluorescence images. For example, it would be nice to reassure the readers that the observed delocalization was not due to out-of-focus images.

Phase contrast and fluorescence images are now systematically shown in the figures. Importantly, all the time-lapse micrographs are captured with a Perfect Focusing System (Nikon) ensuring equivalent focus for all images at all times.

E. In the discussion, the authors suggest that the dependence of AglZ localization on MreB supports their previously-published focal adhesion model for A-motility. However, in the simplest form of the focal adhesion model, the cytoskeleton binds and potentially anchors the focal adhesion, but is not actually required for its assembly (AglZ would still cluster but move more freely without anything to push against). The authors should either remove this point or discuss it more clearly and convincingly.

We agree with the reviewer that the previously published focal adhesion model did not predict that the disassembly of the cytoskeleton would necessarily disperse the clusters. We now argue that the role of the MreB cytoskeleton is consistent with the previously observed periodicity of the clusters and that the new data suggests that the cytoskeleton is important both for positioning and stability of the adhesion sites.

2nd Editorial Decision 29 September 2009

You revised manuscript has now been re-evaluated by one of the original referees. As you will see from his/her comments, there are a number of outstanding clarifications and inconsistencies that need to be remedied prior to publication. In addition, while we agree that a detailed biochemical characterization of the putative GTPase activity of MglA is potentially beyond the scope of the manuscript, some evidence for the role of it GTPase activity would be beneficial for the study.

Please provide a response to the referees concerns when you provide your revised manuscript.

Thank you for the opportunity to consider your work for publication. I look forward to your
Yours sincerely,

Editor
The EMBO Journal

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REFEREE COMMENTS

Referee #1 (Remarks to the Author):

EMBOJ-2009-70853R

This is a revised version of a manuscript that I have previously reviewed.

Briefly, the authors report on the roles of the actin-like protein MreB and the Ras-like protein MglA in the localization of motility proteins in Myxococcus xanthus. The authors present evidence that MreB forms helical filaments, which depolymerize in the presence of A22. Depolymerization of MreB correlates with inhibition of the activity of the two motility systems (A and S) in M. xanthus and results in the dispersal of clusters of AglZ, a regulator of A-motility, and in an alteration in the localization of the S-motility protein FrzS. MreB is only the second protein in M. xanthus shown to be important for both motility systems. The second protein important for both motility systems is the MglA protein. In an mglA deletion mutant AglZ and FrzS were also found to localize abnormally. MglA-YFP was observed to localize to the poles as well as to focal adhesion complexes. Finally, correct MglA localization depends on MreB but not on AglZ or FrzS.

This paper potentially adds to our knowledge about protein localization by showing that MreB is required for the correct localization of AglZ, FrzS and MglA and that MglA, in turn, is also involved in AglZ and FrzS localization. MreB has been shown to form A22 sensitive filaments in several bacteria. Moreover, MglA was shown to be important for the localization of the A-motility protein RomR. Therefore, the most significant finding in the manuscript is the connection between MreB and MglA to protein localization.

In the revised version of the manuscript, the authors have now added additional experiments some of which address my initial concerns.

The paper is very well-written and for a general readership. The logic is easy to follow.

Major comments:
1. p.8-10: The experiment in Fig. S2A is a good control that A22 has an effect on cell shape in M. xanthus and E. coli. The experiments in Fig S2B show that the effect of A22 in E. coli on MreB is reversible. Experiments showing that 50 µg/ml A22 in M. xanthus results in MreB depolymerization should be included and it should be show that an effect of A22 at this concentration on MreB polymerization is reversible. These experiments can be done using immuno-fluorescence. These experiments are needed to strengthen the connection between MreB polymerization status and A22 on motility shown in Fig. 2. Moreover, in the experiments in Fig. 3, cells are treated with 150 µg/ml A22. Again here experiments to show that the effect os A22 on MreB polymerization is reversible should be included.

2. It is not clear what Fig. 2A is showing. The labeling of the Y-axis in the figure does not fit with the Figure legend.
In all experiments in Fig. 2: Include the A22 concentration.

3. The data in Fig. 2B reporting the effect of A22 on motility can be interpreted either way: There is some recovery of motility of M. xanthus cells after removal of A22 or there is no recovery. The only way to distinguish between these two possibilities is to perform statistical tests for significance.

4. Fig. S2B: In the text on p8/9 it is stated that A22 is added to a final concentration of 50 µg/ml but
in the legend it is 150 µg/ml. Please clarify.

5. What are the units on the X-axes in Fig. 2AB?
Include in the legend to Fig. S2A the concentration of A22 used.
Fig. S2B: Explain what happens at the marked time points.

6. In all experiments in Fig. 3: Include the A22 concentrations in the legend.

7. p. 10: Since the submission of the initial version of this manuscript and the revised version, the authors have published that AglZ is not part of the A-motility machinery but rather an inhibitor of the Frz system, which in turn acts as an inhibitor of the to be A-motility system. Nevertheless, throughout the manuscript the authors refer to AglZ as an A-motility protein and use AglZ as a marker protein to follow the effect of MreB on the localization of A-motility proteins. The text should be changed so that the authors' recent findings are correctly referred to. Moreover, according to these findings, MreB and MglA do not regulate the localization of an A-motility protein but of a protein (AglZ) that regulates the activity of the Frz system. Given that AglZ is not part of the A-motility system, it is not clear that AglZ is an appropriate marker protein for A-motility in the cytological studies.

8. The cell in Fig. 3B has a clear AglZ-YFP cluster at the leading pole but does not have focal adhesion complexes. The polar cluster clearly disappears after A22 addition and reappears upon removal of A22. However, it is not clear from this figure what happens to focal adhesion complexes.

8. Fig. 6C: Legend should be corrected to include correct lane numbering.

9. The experiment in Fig. 3S: The authors claim that localization of FrzS as well as AglZ recovers after removal of A22. However; the figure shows that AglZ localization does not recover.

10. Fig. 4:Panel A and B have been switched.

11. p. 14: It is stated that AglZ is not important for MglA localization. However, the opposite is claimed in Fig. S7.

12. Fig. 5B: Some of the light blue diamonds indicate the leading pole. What are the other light blue diamonds indicating?

13. p. 14, last para: The description of the effect on A22 on MglA localization is different on p. 14 and in the legend to Fig. 5C. Fig. 5C suggests that A22 has a dramatic effect on the clusters along the cell body as well as the polar clusters leading to a unipolar localization pattern.

14. The statements in the last para of the Introduction are for the most part not consistent with the reported observations: MglA has previously been identified as important for localization of a motility protein (RomR); MreB not only affects focal adhesion complexes but also the polar clusters; finally, the manuscript does not contain showing that focal adhesion clusters are essential for A-motility.

15. The data presented show that MglA is important for FrzS and AglZ localization and that the opposite is not true. The data presented also suggest that MglA interacts directly with FrzS and AglZ. Finally, the data show that MglA localizes to both poles as well as to clusters between the poles. In total, these observations seem to suggest that FrzS and AglZ should localize in the same pattern. Yet that is not the case. How is that explained? Along the same lines: Does MglA interact with MreB in vivo or in vitro

16. MglA is referred to as a GTPase and a Ras homolog. To my knowledge it is not been shown that MglA has GTPase activity. Also, it is unclear whether MglA activity depends on GTPase activity. This could easily be tested in vivo as well as in vitro by analyzing constitutively active and inactive MglA mutants.
I read the response of the authors but it still would be nice to have in order to make the comparison to eukaryotic systems.
We thank the reviewer for his careful examination of the manuscript. Below is a detailed response to the comments:

1. p.8-10: The experiment in Fig. S2A is a good control that A22 has an effect on cell shape in M. xanthus and E. coli. The experiments in Fig S2B show that the effect of A22 in E. coli on MreB is reversible. Experiments showing that 50 µg/ml A22 in M. xanthus results in MreB depolymerization should be included and it should be show that an effect of A22 at this concentration on MreB polymerization is reversible. These experiments can be done using immuno-fluorescence. These experiments are needed to strengthen the connection between MreB polymerization status and A22 on motility shown in Fig. 2. Moreover, in the experiments in Fig. 3, cells are treated with 150 µg/ml A22. Again here experiments to show that the effect of A22 on MreB polymerization is reversible should be included.

We think that the suggested experiments are not necessary since we have already provided many controls to show that A22 affects motility through its action on MreB. Specifically:

i) A22 disrupts MreB localization in M. xanthus.

ii) A22 affects cell shape in M. xanthus just like in E. coli.

iii) A22 affects motility reversibly, which shows that the A22 effect does not result from profound nonspecific lesions.

iv) Importantly, we show that the mreBV323A allele confers complete resistance to the A22-induced motility arrest.

v) We show that the mreBV323A allele confers resistance to the A22-induced dispersal of MreB.

Unfortunately, the experiments suggested by the reviewer are not possible in the absence of a functional MreB-GFP fusion because one must monitor depolymerization of MreB and re-polymerization on live cells and immunofluorescence (IF) can only be done with fixed cells. Technically, our incubation chamber cannot accommodate IF because antibodies do not diffuse through the agar layer.

2. It is not clear what Fig. 2A is showing. The labeling of the Y-axis in the figure does not fit with the Figure legend.

In all experiments in Fig. 2: Include the A22 concentration.

We now labeled the Y-axis in the figure "Relative cumulated distance" and explain in the figure legend that it is the distance travelled by a cell at a given time over the maximum distance travelled at the end of the time lapse (d/dmax) plotted over time.

3. The data in Fig. 2B reporting the effect of A22 on motility can be interpreted either way: There is some recovery of motility of M. xanthus cells after removal of A22 or there is no recovery. The only way to distinguish between these two possibilities is to perform statistical tests for significance.

We replaced the histogram representations of Figure 2B and 2C by more indicative box plot representations, moreover the statistical analysis is described in the supplemental materials, to show that there is indeed recovery. Recovery is also evident from the AglZ-YFP and FrzS-GFP fusions, which are rapidly re-localized once A22 is removed.
4. Fig. S2B: In the text on p8/9 it is stated that A22 is added to a final concentration of 50 µg/ml but in the legend it is 150 µg/ml. Please clarify.

We corrected the text and specified that we used 150 µg/ml. The experiment was performed to show that reversibility of A22 occurred even at the highest concentration.

5. What are the units on the X-axes in Fig. 2AB?
Include in the legend to Fig. S2A the concentration of A22 used.
Fig. S2B: Explain what happens at the marked time points.

Fig. 2AB. The units on the X-axes are minutes. We added the unit in the figure.
Fig. S2A. The concentration of A22 that we used was 150 µg/ml. We specified that in the figure legend.
Fig. S2B. We now explain the time points "A22 was added after 6 min (red arrow) and removed after 24 min (black arrow)."

6. In all experiments in Fig. 3: Include the A22 concentrations in the legend.
We have made the changes.

7. p. 10: Since the submission of the initial version of this manuscript and the revised version, the authors have published that AglZ is not part of the A-motility machinery but rather an inhibitor of the Frz system, which in turn acts as an inhibitor of the to be Amotility system. Nevertheless, throughout the manuscript the authors refer to AglZ as an A-motility protein and use AglZ as a marker protein to follow the effect of MreB on the localization of A-motility proteins. The text should be changed so that the authors' recent findings are correctly referred to. Moreover, according to these findings, MreB and MglA do not regulate the localization of an A-motility protein but of a protein (AglZ) that regulates the activity of the Frz system. Given that AglZ is not part of the A-motility system, it is not clear that AglZ is an appropriate marker protein for A-motility in the cytological studies.

The study by Mauriello et al., (2009) showed that AglZ is not a structural component of the A-motility machinery but does not argue that AglZ is not an Amotility protein. In fact, AglZ is a bona fide A-motility protein because its inactivation leads to a specific block of A-motility and it does not affect S-motility. All our results, published and unpublished are consistent with the notion that AglZ is a component of A-motility focal adhesion sites, which we show now also contain MglA, and it is thus the best available cytological marker to track these sites. The text now cites AglZ as a regulatory protein and we reference the paper by Mauriello et al. (2009). Page 10 line 17-19.

8. The cell in Fig. 3B has a clear AglZ-YFP cluster at the leading pole but does not have focal adhesion complexes. The polar cluster clearly disappears after A22 addition and reappears upon removal of A22. However, it is not clear from this figure what happens to focal adhesion complexes.

We agree that in the previous picture the selected frames only showed a few clusters. More representative frames are now included to show the fate of the internal clusters throughout the experiment.

8. Fig. 6C: Legend should be corrected to include correct lane numbering.

The correct lane numbering was included in the Fig. 6C Legend.

9. The experiment in Fig. 3S: The authors claim that localization of FrzS as well as AglZ recovers after removal of A22. However, the figure shows that AglZ localization does not recover.

The study by Mauriello et al., (2009) showed that AglZ is not a structural component of the A-motility machinery but does not argue that AglZ is not an Amotility protein. In fact, AglZ is a bona fide A-motility protein because its inactivation leads to a specific block of A-motility and it does not affect S-motility. All our results, published and unpublished are consistent with the notion that AglZ is a component of A-motility focal adhesion sites, which we show now also contain MglA, and it is thus the best available cytological marker to track these sites. The text now cites AglZ as a regulatory protein and we reference the paper by Mauriello et al. (2009). Page 10 line 17-19.
The reviewer is correct. This experiment is difficult to perform all the way to full recovery because mCherry bleaches significantly throughout the procedure making AglZ-recovery difficult to follow. We proved that AglZ-recovery occurs using the YFP tag. The point of the experiment is to show that AglZ and FrzS are dispersed synchronously when A22 is added, which is clearly shown. Thus, we removed the recovery panel of the figure when bleaching becomes a significant issue.

10. Fig. 4: Panel A and B have been switched.

We fixed it, Figure 4 is now Figure 5.

11. p. 14: It is stated that AglZ is not important for MglA localization. However, the opposite is claimed in Fig. S7.

The erroneous legend of figure S7 has been corrected and it is now consistent with the text.

12. Fig. 5B: Some of the light blue diamonds indicate the leading pole. What are the other light blue diamonds indicating?

All of the blue diamonds indicate the leading pole. The legend has been modified and it is now hopefully clear.

13. p. 14, last paragraph: The description of the effect of A22 on MglA localization is different on p. 14 and in the legend to Fig. 5C. Fig. 5C suggests that A22 has a dramatic effect on the clusters along the cell body as well as the polar clusters leading to a unipolar localization pattern.

Main text and figure legend are now consistent.

14. The statements in the last paragraph of the Introduction are for the most part not consistent with the reported observations: MglA has previously been identified as important for localization of a motility protein (RomR); MreB not only affects focal adhesion complexes but also the polar clusters; finally, the manuscript does not contain showing that focal adhesion clusters are essential for A-motility.

We corrected the last paragraph of the introduction and changed it to: "In this paper, we identified the actin-like protein MreB and the Ras-like protein MglA as critical components in the localization of the A- and S-motility proteins, FrzS and AglZ. We also found that MreB acts upstream to MglA in the positioning of polar motility proteins and the focal adhesion complexes. Finally, our data suggest that assembly of the focal adhesion clusters is an essential requirement for cells to achieve A-motility." We believe that our data strongly argue that the focal adhesion sites are critical for A-motility because their dispersal and re-appearance are always strictly correlated to arrest and restoration of motility. However, we replaced the word "show" with "suggest".

15. The data presented show that MglA is important for FrzS and AglZ localization and that the opposite is not true. The data presented also suggest that MglA interacts directly with FrzS and AglZ. Finally, the data show that MglA localizes to both poles as well as to clusters between the poles. In total, these observations seem to suggest that FrzS and AglZ should localize in the same pattern. Yet that is not the case. How is that explained? Along the same lines: Does MglA interacts with MreB in vivo or in vitro.

We have already addressed this comment in the previous rebuttal and hypothesized that additional specificity factors must exist to prevent for example, accumulation of FrzS along the clusters. This complexity was addressed in the text in the discussion section page 17 line 16- page 18 line 14 and further page 19 line 9-12.
16. MglA is referred to as a GTPase and a Ras homolog. To my knowledge it is not been shown that MglA has GTPase activity. Also, it is unclear whether MglA activity depends on GTPase activity. This could easily be tested in vivo as well as in vitro by analyzing constitutively active and inactive MglA mutants.

I read the response of the authors but it still would be nice to have in order to make the comparison to eukaryotic systems.

We have now added in vitro evidence that recombinant MglA can hydrolyze GTP. In vivo evidence that this activity is important for function is available because Hartzell and Kaiser (1991) characterized loss-of-function alleles (C23F and G81V) of MglA that mapped precisely to the Walker A and potential Walker B motifs in MglA, strongly suggesting that GTPase activity is essential for function. This observation is now reported in the discussion section.

Testing in vivo constitutive mutants would be designed to address the role of the MglA GTPase cycle and would require a large set of experiments that, while interesting, would fall beyond the scope of the present study. Indeed, the characterization of point mutants would require extensive additional experiments, including testing for protein stability, determining the kinetics of GTP hydrolysis, and characterizing motility behaviors. This study is a future goal of our research but we feel that it is beyond the scope of this study.