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Novel transient cytoplasmic rings stabilize assembling bacterial flagellar motors

Grant Jensen, Mohammed Kaplan, Catherine Oikonomou, Cecily Wood, Georges Chreifi, Poorna Subramanian, Davi Ortega, Yi-Wei Chang, Morgan Beeby, and Carrie Shaffer
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Transaction Report:

(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. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

Dear Prof. Jensen,

Thank you again for the submission of your manuscript entitled "Novel transient cytoplasmic rings stabilize assembling bacterial flagellar motors" and for your patience during the review process. We have now received the reports from the referees, which I copy below.

As you can see from their comments, all four referees are very positive towards your work and although they point out to some concerns that will require your attention before your manuscript can be published, most of them are relatively minor.

Based on the overall interest expressed in the reports, I would like to invite you to address the comments of all referees in a revised version of the manuscript for The EMBO Journal. I should add that it is our policy to allow only a single major round of revision and that it is therefore important to resolve the main concerns at this stage. As already mentioned, I believe the concerns of the referees are reasonable and addressable, but we are aware that many laboratories cannot function at full efficiency during the current COVID-19/SARS-CoV-2 pandemic, so contact me if you have any questions, need further input on the referee comments or if you anticipate any problems in addressing any of their points. Please, follow the instructions below when preparing your manuscript for resubmission.

I would also like to point out that as a matter of policy, competing manuscripts published during this period will not be taken into consideration in our assessment of the novelty presented by your study ("scooping" protection). We have extended this 'scooping protection policy' beyond the usual 3 month revision timeline to cover the period required for a full revision to address the essential experimental issues. Please contact me if you see a paper with related content published elsewhere to discuss the appropriate course of action.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: https://www.embopress.org/page/journal/14602075/authorguide#transparentprocess

Again, please contact me at any time during revision if you need any help or have further questions.

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

Best regards,

David

David del Alamo, PhD.
Editor
The EMBO Journal

When submitting your revised manuscript, please carefully review the instructions below and include the following items:

1) a .docx formatted version of the manuscript text (including legends for main figures, EV figures and tables). Please make sure that the changes are highlighted to be clearly visible.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure).

3) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point response to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

4) a complete author checklist, which you can download from our author guidelines (https://wol-prod-cdn.literatumonline.com/pb-assets/embo-site/Author Checklist%20-%20EMBO%20J-1561436015657.xlsx). Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the RPF.

5) Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript.

6) We require a 'Data Availability' section after the Materials and Methods. Before submitting your revision, primary datasets produced in this study need to be deposited in an appropriate public database, and the accession numbers and database listed under 'Data Availability'. Please remember to provide a reviewer password if the datasets are not yet public (see

https://www.embopress.org/page/journal/14602075/authorguide#datadeposition). If no data deposition in external databases is needed for this paper, please then state in this section: This study includes no data deposited in external repositories. Note that the Data Availability Section is restricted to new primary data that are part of this study.

Note - All links should resolve to a page where the data can be accessed.

7) When assembling figures, please refer to our figure preparation guideline in order to ensure proper formatting and readability in print as well as on screen:
http://bit.ly/EMBOPressFigurePreparationGuideline

Please remember: Digital image enhancement is acceptable practice, as long as it accurately represents the original data and conforms to community standards. If a figure has been subjected to significant electronic manipulation, this must be noted in the figure legend or in the 'Materials and Methods' section. The editors reserve the right to request original versions of figures and the original images that were used to assemble the figure.

8) For data quantification: please specify the name of the statistical test used to generate error bars and P values, the number (n) of independent experiments (specify technical or biological replicates) underlying each data point and the test used to calculate p-values in each figure legend. The figure legends should contain a basic description of n, P and the test applied. Graphs must include a description of the bars and the error bars (s.d., s.e.m.).

9) We would also encourage you to include the source data for figure panels that show essential data. Numerical data can be provided as individual .xls or .csv files (including a tab describing the data). For 'blots' or microscopy, uncropped images should be submitted (using a zip archive or a single pdf per main figure if multiple images need to be supplied for one panel). Additional information on source data and instruction on how to label the files are available at .

10) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online (see examples in https://www.embopress.org/doi/10.15252/embj.201695874). A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc. in the text and their respective legends should be included in the main text after the legends of regular figures.

- For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends in a single PDF file called *Appendix*, which should start with a short Table of Content. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc. See detailed instructions regarding expanded view here: .

- Additional Tables/Datasets should be labelled and referred to as Table EV1, Dataset EV1, etc. Legends have to be provided in a separate tab in case of .xls files. Alternatively, the legend can be supplied as a separate text file (README) and zipped together with the Table/Dataset file.

11) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Detailed instructions are available at .

Further information is available in our Guide For Authors: https://www.embopress.org/page/journal/14602075/authorguide

Revision to The EMBO Journal should be submitted online within 90 days, unless an extension has been requested and approved by the editor; please click on the link below to submit the revision online before 26th Dec 2021:

Link Not Available

Referee #1:

In this study, Kaplan and colleagues analyze different assembly states of flagellar motors of diverse species. They show that assembly intermediates of a subset of these motors contain additional cytoplasmic rings that are required for stator ring assembly, and that FliO and FliQ are required for the assembly of the cytoplasmic rings.

Overall, this is an interesting and well worked out study, and the results will be of interest to microbiologists and molecular

biologists interested in flagellar biology and biomolecular machines.

My main remarks are the following:
There are no resolution assessments for the tomography and subtomogram averaging work, which makes it difficult to evaluate the level of detail of the reconstructions.

A main drawback of the (inherent) lack of resolution is that the extra components cannot be identified. Did the authors try to perform any bioinformatics searches for candidate genes encoding the proteins making up the extra rings?

Minor point:
L56 "inside-fashion": I guess this should be "inside-out fashion"?

Referee #2:

This study employs powerful tomographic methods together with a comparative (species-comparison) approach to very good effect. The images demonstrate the occurrence of features that occur only in motors of species that encounter high loads, at only certain stages of assembly. I believe the results convincingly establish the appearance then disappearance (or movement/motional blurring) of novel ring features that contact the top of the C-ring. Appearance of the ring features correlate with features in the periplasm that are believed to stabilize the stators (structures that are presumably necessitated by operation at high torque). The presentation is very clear, especially considering the number of different complexes, proteins, and species involved and the attendant possibilities for confusion. Together, the results provide significant advance in our understanding of flagellar assembly, specifically establishing a role for transient feature in orchestrating certain events. The likely involvement of FliO and FliQ (components of the centrally-located type-III secretion apparatus) in forming the new ring features is quite unexpected and raises interesting questions for further study.
Minor questions/concerns:
1. line 56. Was inside-out intended?
2. If just the MS-ring protein FliF is overproduced (in Salmonella), lots of MS-rings are formed. How does this fit with the idea that MS ring forms only once the T3SS apparatus is formed?
3. In the section comparing FliP* with FliP* FliQ, I wondered about delta-fliQ cells. Do the 67-nm ring, stators, and lower cage reappear when FliQ is absent but FliP is still present?
4. Fig. 5F. Are there some angled densities near where the cytoplasmic ring previously was?
5. Investigators who have been doing subtomogram averaging for many years have presumably developed an instinct for which features are real and which are not, but for the naive reader, there are some mysteries. I wondered what criteria, or cutoffs, are used to determine which features are to be highlighted in the schematics. I see additional features in the STA in 2F, e.g., which are lighter than the highlighted feature and at larger radius. Are features such as this considered to be down in the noise? Other examples are in 4A (angled densities between FlhA density and the cytoplasmic ring) and 4C (densities at smaller radius than the highlighted cytoplasmic ring). How do we know what to ignore? A related point is that in panels where an STA is not shown, it is harder to evaluate how accurately the schematic reflects the features present in an STA. Can STAs be shown for those cases, or is there a basis for saying they don't need to be shown?
6. line 330. does CheY lock these motors in the CCW state or CW state? (Or one way in some species and the other way in other species?) I think it's actually CW in Vibrio; not sure about the others.
7. that FliO and FliQ themselves form the rings is suggested to be doubtful on the grounds that they are membrane proteins. But there is an association between the rings and the formation of periplasmic structures, as if a membrane protein or proteins might be involved; if parts of the proteins are in extended conformation, density might appear some distance from the membrane.
8. I was left wanting some specific proposals for how the new features might benefit flagellar assembly. Is it too soon to speculate a bit about what's going on in terms of some specific mechanism for coordinating events in the cytoplasm (presence of the upper C-ring) with events in the periplasm (beginning to assemble the stator-stabilizing structures)?

Referee #3:

The authors studied in situ structures of the bacterial flagellar basal bodies of diverse Proteobacteria that produce high-torque motors by electron cryo-tomography and discovered novel cytoplasmic rings that interact with torque generating ring formed by FliG and are present only transiently during flagellar assembly. These cytoplasmic rings assemble in the very early stage and disappear after the stator units are assembled with their stabilizing rings in the periplasm. They also imaged many mutants in Helicobacter pylori and identified that two of the core complex proteins of the flagellar type III protein export apparatus, FliO and FliQ, are required for the assembly of these novel cytoplasmic rings.

It is well known that transient structures are present and required for bacterial flagellar assembly, such as the FlgJ cap for rod

assembly and the FlgD cap for hook assembly. However, this is the first discovery of a transient structure in the cytoplasm, and this ring structure plays an essential role in the completion of high-torque flagellar motor assembly to make the motors functional. This discovery was also made possible only by careful and elaborate in situ structural studies by electron cryo-tomography.

Although images of subtomogram averages showing structural details of intermediate structures are not available in all cases unfortunately due to small numbers of tomograms, and this makes it rather difficult for readers to make confident judgements on the reliability of structural interpretations given by the authors in such cases where subtomogram averages are not available, the main conclusion is well supported by the data presented in the manuscript. Therefore, this study certainly advances our understanding of the complex and dynamic features of the flagellar assembly process.

Minor points:
1. Line 56: The authors probably meant "inside-out fashion".
2. Line 276: Fig. S2D is probably Fig. S4D.
3. Line 325: "the stators and their scaffolds" are mainly in the membrane and periplasm and not in the cytoplasm "to assemble around the torque ring" formed by FliG.

Referee #4:

In this paper, the authors use cry-electron tomography to characterise assembly intermediates of the bacterial flagellum in-situ. Specifically, they identify a previously-unknown ring structure on the cytosolic side, present early during the assembly process but not at later stages. Importantly, this structure is observed in a range of bacteria across the kingdom, suggesting that it represents a fundamental element in flagellum assembly. While the exact nature of this ring remains unclear, this is nevertheless a significant contribution in our understanding of flagellum biogenesis.
The main limitation of this work, is that in multiple instances, the authors did not identify enough of the studied sub-complexes to perform sub-tomogram averaging; in those instances, it is often difficult to be certain that the structures described by the authors are really there. For this reason, the authors should be a bit more cautious in their interpretation of the data in those cases.

Some major comments:

- In each species/mutants, it would be really helpful to describe how many of each assembly intermediates were observed, in proportion to fully assembled flagella. A table recapitulating this would be very helpful.

- The authors should also specify if the various bacteria are Monotrichous, amphitrichous or peritrichous.

- The attribution of density is done by comparison with Salmonella/e.coli, where mutants or high-resolution structures have been reported. This is a reasonable assumption, but in the absence of mutants in the species described here, this remains speculative. I do not suggest that the authors do all the mutants to validate the attribution of the corresponding denisties, but a more cautious tone should be adopted throughout.

- For the fliP* H. Pylori strain, the authors report a smaller diameter for the complex. Based on the dimensions, and considering the existing high-resolution structure of the basal body (MS ring in particular), could the authors speculate on what the stoichiometry might be for FliF and Flig, for WT Vs flip*?

- I did not find any information about any of the sub-tomogram averaging being deposited in the EMDB. One could argue that some of them only include few particles in the average, but at least the fliP* mutant, where Flip and FliC are docked, should be deposited.

- Why does the MS complex looks different in the dFlhBc Vs dFlhAc C. Jejuni (figure 4)?

- As mentioned above, for the mutants where sub-tomogram averaging could not be obtained, densities are very difficult to see; in particular for figures 3B, 5B and 5E, I cannot confidently see the C-ring, unless other evidence is provided I would argue that these could also be the flT3SScc sub-complex. For those cases, the confidence of the conclusions should be toned down.

Minor comments:
Line 56: I think the authors mean "inside-out fashion"?
Lines 112-113: For clarity, the authors should specify which bacterial species/phyla are used.
Line 276: Should be S4D, not S2D

Point-by-point response to the referees' comments

Referee 1:

In this study, Kaplan and colleagues analyze different assembly states of flagellar motors of diverse species. They show that assembly intermediates of a subset of these motors contain additional cytoplasmic rings that are required for stator ring assembly, and that FliO and FliQ are required for the assembly of the cytoplasmic rings.

Overall, this is an interesting and well worked out study, and the results will be of interest to microbiologists and molecular biologists interested in flagellar biology and biomolecular machines.

We thank the reviewer for her/his insightful and constructive comments that allowed us to improve our manuscript.

My main remarks are the following:
There are no resolution assessments for the tomography and subtomogram averaging work, which makes it difficult to evaluate the level of detail of the reconstructions.

We have added FSC curves for all subtomogram averages with more than 50 particles as new Figures EV2, EV6, EV9 and EV10. Based on these curves, these averages have resolutions of ~6-7 nm.

A main drawback of the (inherent) lack of resolution is that the extra components cannot be identified. Did the authors try to perform any bioinformatics searches for candidate genes encoding the proteins making up the extra rings?

We indeed tried to use bioinformatics to identify candidates for the extra rings, but there are challenges to this approach. First, flagellar genes are not always clustered and the organization of clusters varies between species (Liu & Ochman, 2007). This makes it difficult to identify potential candidates. For example, when we examined the gene context of *fliM* using *S. oneidensis* as a reference, we found that it varied widely among species in which we identified the rings (see figure below). Second, we lack a clear negative control (a species where the rings are definitely not associated with motor assembly). For these reasons, we cannot speculate at this stage what protein(s) form these extra rings, but we hope that this will be the subject of future study.

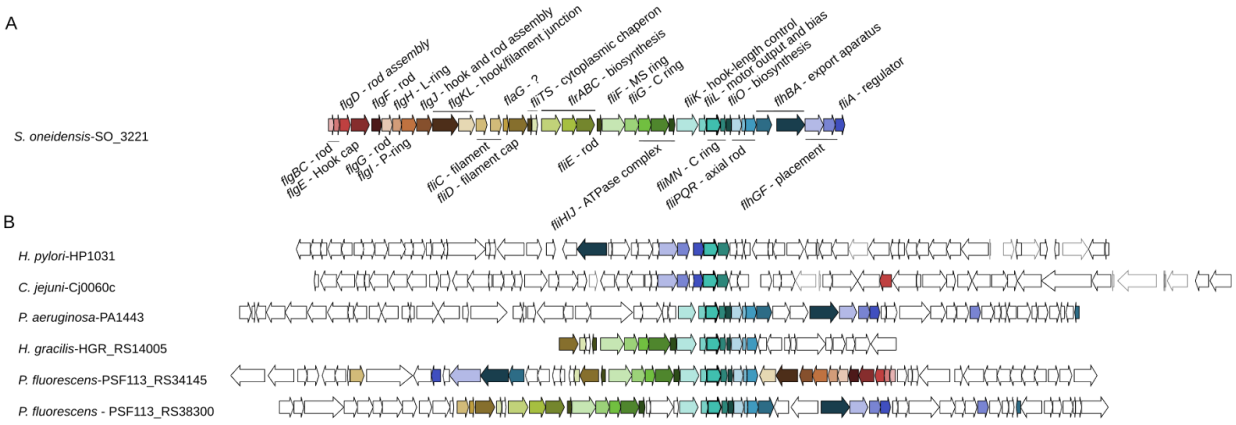

Figure: Gene context of *fliM* for *S. oneidensis* (A) and other organisms in this study (B). Genes encoding known components of the flagellar motor are color coded.

Minor point:
L56 "inside-fashion": I guess this should be "inside-out fashion"?

Corrected.

Referee 2:

This study employs powerful tomographic methods together with a comparative (species-comparison) approach to very good effect. The images demonstrate the occurrence of features that occur only in motors of species that encounter high loads, at only certain stages of assembly. I believe the results convincingly establish the appearance then disappearance (or movement/motional blurring) of novel ring features that contact the top of the C-ring. Appearance of the ring features correlate with features in the periplasm that are believed to stabilize the stators (structures that are presumably necessitated by operation at high torque). The presentation is very clear, especially considering the number of different complexes, proteins, and species involved and the attendant possibilities for confusion. Together, the results provide significant advance in our understanding of flagellar assembly, specifically establishing a role for transient feature in orchestrating certain events. The likely involvement of FliO and FliQ (components of the centrally-located type-III secretion apparatus) in forming the new ring features is quite unexpected and raises interesting questions for further study.

We thank the reviewer for her/his insightful and constructive comments that allowed us to improve our manuscript.

Minor questions/concerns:
1. line 56. Was inside-out intended?

Yes, we have corrected this typo.

2. If just the MS-ring protein FliF is overproduced (in Salmonella), lots of MS-rings are formed. How does this fit with the idea that MS ring forms only once the T3SS apparatus is formed?

In our cryo-ET data, we also see that the MS- and C-rings (along with some periplasmic components) can still assemble in the absence of a fully-assembled T3SS apparatus. This is evident in *H. pylori* and *C. jejuni* mutants that we imaged in this study (for example, see the MS-complex of *H. pylori fliP** in Fig. 3E). As we discuss in the manuscript, this might reflect different evolutionary histories of these subcomplexes (see lines 408-412 in the manuscript). However, it raises an intriguing question: if the MS-ring can assemble in the absence of a fully-assembled T3SS apparatus (as in *H. pylori fliP**), then how does it (the MS-ring) normally assemble around the T3SS in wild-type cells? To highlight this question, we have added the following sentence to the manuscript:

"*This ability of the MS-ring to assemble independently of a fully-assembled fT3SScc in these mutants raises the question of how FliF finds and assembles around the fT3SScc in wild-type cells*." (lines 395-396)

In addition, we investigated a new mutant, *H. pylori ΔfliF fliP**, which lacks the MS-ring protein FliF. We collected 455 new cryo-tomograms and failed to identify any flagellar-related complexes in these cells. We added these new results to the revised manuscript.

3. In the section comparing FliP* with FliP* FliQ, I wondered about delta-fliQ cells. Do the 67-nm ring, stators, and lower cage reappear when FliQ is absent but FliP is still present?

This is an excellent question. We do not have a ΔfliQ mutant in a wild-type FliP background (all the mutants in this study were constructed in the *fliP** background in order to capture early flagellar intermediates), but in future work we hope to study the effect of ΔfliQ, and other mutants, in cells with functional FliP.

4. Fig. 5F. Are there some angled densities near where the cytoplasmic ring previously was?

Yes, there are some rather washed-out densities there. One possible explanation is that some of the particles have (parts of) the C-ring assembled, but we cannot be sure. We have now added this point to the text:

"Nonetheless, some faint, angled densities were present in the MS-complex in this mutant, in the location where the novel rings would otherwise appear (Fig. 5F, middle panel)." (lines 276-277).

5. Investigators who have been doing subtomogram averaging for many years have presumably developed an instinct for which features are real and which are not, but for the naive reader, there are some mysteries. I wondered what criteria, or cutoffs, are used to determine which features are to be highlighted in the schematics. I see additional features in the STA in 2F, e.g., which are lighter than the highlighted feature and at larger radius. Are features such as this considered to be down in the noise?
Other examples are in 4A (angled densities between FlhA density and the cytoplasmic ring) and 4C (densities at smaller radius than the highlighted cytoplasmic ring). How do we know what to ignore? A related point is that in panels where an STA is not shown, it is harder to evaluate how accurately the schematic reflects the features present in an STA. Can STAs be shown for those cases, or is there a basis for saying they don't need to be shown?

We thank the reviewer for raising this important point. The three examples mentioned all average a low number of particles. In such cases, we interpret only the parts of the average that are unambiguously above the noise (for example the novel cytoplasmic ring in Fig. 2F, which is also unambiguously present at other stages like Fig. 2D). For fainter densities, like the ones at a larger radius in Fig 2F, we can't exclude that they originate from noise due to the low number of particles in this average (the same applies for the averages shown in 4A and 4C). Unfortunately, this is a limitation of imaging transient intermediates in wild-type cells (the 7 particles in Fig. 2F came from 76 tomograms). In addition, some intermediates are less abundant than others, which might be related to the dynamics of flagellar assembly (where some stages are short-lived compared to others). When only one or a few examples are

available, we cannot calculate an STA. In schematics of these complexes, to the best of our ability, we highlight only the unambiguous features that we can identify. In response to reviewer feedback (see our response to Referee 4 below), we now explicitly point out that conclusions based on individual examples are tentative.

We have also added this statement to the beginning of the Results section:
"*Note that schematics and interpretation of complexes where not enough examples were found to produce a subtomogram average are tentative.*" (lines 131-133).

6. line 330. does CheY lock these motors in the CCW state or CW state? (Or one way in some species and the other way in other species?) I think it's actually CW in Vibrio; not sure about the others.

Thank you, it should be a clockwise state. We have corrected this sentence in the revised manuscript.

7. that FliO and FliQ themselves form the rings is suggested to be doubtful on the grounds that they are membrane proteins. But there is an association between the rings and the formation of periplasmic structures, as if a membrane protein or proteins might be involved; if parts of the proteins are in extended conformation, density might appear some distance from the membrane.

Previous biochemical studies suggest that FliQ is present in a monomeric form in the fT3SScc and FliO is required for fT3SScc assembly but disassociates as soon as it is formed (see (Fabiani *et al*, 2017; Fukumura *et al*, 2017)). Together with previous studies indicating a regulatory role for FliO in the expression of other flagellar genes in *H. pylori* (see (Tsang & Hoover, 2014)) this leads us to hypothesize that these proteins do not form the rings themselves. However, as the reviewer points out, we cannot exclude the alternative possibility, so we have added the following to the manuscript:

"*As both FliO and FliQ are transmembrane proteins, we postulate that these fT3SScc proteins are involved in regulating other proteins that form (or are required to form) the rings, rather than forming the rings directly. Previous biochemical studies have indicated a regulatory role for FliO in H. pylori, where it is required for the optimal expression of other flagellar genes (Tsang & Hoover, 2014). However, direct physical involvement of these proteins in the formation of the novel cytoplasmic rings cannot be totally excluded*." (lines 337-343).

8. I was left wanting some specific proposals for how the new features might benefit flagellar assembly. Is it too soon to speculate a bit about what's going on in terms of some specific mechanism for coordinating events in the cytoplasm (presence of the upper C-ring) with events in the periplasm (beginning to assemble the stator-stabilizing structures)?

Our current hypothesis is that these rings might be required in high-torque motors to induce a conformational change in FliG that facilitates its interaction with the stators (MotA), stabilizing them until their periplasmic scaffolds assemble. It is interesting that these rings have not been observed in *E. coli* and *Salmonella* (which have variable number of stators depending on the viscosity of the external environment, see (Lele *et al*, 2013)). This might be because: 1) the rings are only present in motors with a stable stator ring, or 2) the rings are also present in *E. coli* and *Salmonella* but were lost during purification of assembly intermediates in previous studies (Kubori *et al*, 1992). Future studies, including *in situ* cryo-ET imaging of *E. coli* and *Salmonella*, should shed light on the rings' mechanism. We have added the following discussion to the revised manuscript:

"It is possible that these rings lock FliG in a certain conformation that stabilizes the stators before other periplasmic scaffolds are built in high-torque motors. Previous studies on the motor of S. enterica*, which does not have a fixed stator ring but rather a variable number of stators depending on the external environment, did not report these rings* (Kubori *et al*, 1992)*. This could be either because the novel rings are found only in high-torque motors with a stable stator ring and absent in* E. coli *and* S. enterica*, or because they were lost in purification of assembly intermediates in* S. enterica*."* lines (358-364).

Referee 3:

The authors studied in situ structures of the bacterial flagellar basal bodies of diverse Proteobacteria that produce high-torque motors by electron cryo-tomography and discovered novel cytoplasmic rings that interact with torque generating ring formed by FliG and are present only transiently during flagellar assembly. These cytoplasmic rings assemble in the very early stage and disappear after the stator units are assembled with their stabilizing rings in the periplasm. They also imaged many mutants in Helicobacter pylori and identified that two of the core complex proteins of the flagellar type III protein export apparatus, FliO and FliQ, are required for the assembly of these novel cytoplasmic rings.

It is well known that transient structures are present and required for bacterial flagellar assembly, such as the FlgJ cap for rod assembly and the FlgD cap for hook assembly. However, this is the first discovery of a transient structure in the cytoplasm, and this ring structure plays an essential role in the completion of high-torque flagellar motor assembly to make the motors functional. This discovery was also made possible only by careful and elaborate in situ structural studies by electron cryo-tomography.

Although images of subtomogram averages showing structural details of intermediate structures are not available in all cases unfortunately due to small numbers of tomograms, and this makes it rather difficult for readers to make confident judgements on the reliability of structural interpretations given by the authors in such cases where subtomogram averages are not available, the main conclusion is well supported by the data presented in the manuscript. Therefore, this study certainly advances our understanding of the complex and dynamic features of the flagellar assembly process.

We thank the reviewer for her/his insightful and constructive positive comments that helped us to improve our manuscript. Regarding the interpretation of intermediates with too few examples to average, please see our response to Referee 2 above.

Minor points:
1. Line 56: The authors probably meant "inside-out fashion".

Corrected.

2. Line 276: Fig. S2D is probably Fig. S4D.

Corrected.

3. Line 325: "the stators and their scaffolds" are mainly in the membrane and periplasm and not in the cytoplasm "to assemble around the torque ring" formed by FliG.

Thank you, this sentence was inaccurate. We have corrected this section to read:

"It is possible that these rings lock FliG in a certain conformation that stabilizes the stators before other periplasmic scaffolds are built in high-torque motors. Previous studies on the motor of S. enterica*, which does not have a fixed stator ring but rather a variable number of stators depending on the external environment, did not report these rings* (Kubori *et al*, 1992)*. This could be either because the novel rings are found only in high-torque motors with a stable stator ring and absent in* E. coli *and* S. enterica*, or because they were lost in purification of assembly intermediates in* S. enterica*."* lines (358-364).

Referee 4:

In this paper, the authors use cry-electron tomography to characterise assembly intermediates of the bacterial flagellum in-situ. Specifically, they identify a previously-unknown ring structure on the cytosolic side, present early during the assembly process but not at later stages. Importantly, this structure is observed in a range of bacteria across the kingdom, suggesting that it represents a fundamental element in flagellum assembly. While the exact nature of this ring remains unclear, this is nevertheless a significant contribution in our understanding of flagellum biogenesis.

The main limitation of this work, is that in multiple instances, the authors did not identify enough of the studied sub-complexes to perform sub-tomogram averaging; in those instances, it is often difficult to be certain that the structures described by the authors are really there. For this reason, the authors should be a bit more cautious in their interpretation of the data in those cases.

We thank the reviewer for insightful comments that helped us to improve our manuscript. As discussed in response to Referee 2 above, we have now modified the manuscript to use more tentative language in the cases where no sub-tomogram averages are available.

Some major comments:

- In each species/mutants, it would be really helpful to describe how many of each assembly intermediates were observed, in proportion to fully assembled flagella. A table recapitulating this would be very helpful.

Done. We added Table 1 to the revised manuscript, detailing how many tomograms were collected and how many examples of each assembly stage were identified in each species.

- The authors should also specify if the various bacteria are Monotrichous, amphitrichous or peritrichous.

Done. This information now appears in Expanded View Table 1 in the revised manuscript.

- The attribution of density is done by comparison with Salmonella/e.coli, where mutants or high-resolution structures have been reported. This is a reasonable assumption, but in the absence of mutants in the species described here, this remains speculative. I do not suggest that the authors do all the mutants to validate the attribution of the corresponding denisties, but a more cautious tone should be adopted throughout.

Agreed. We have modified our comparison of our MS-complex subtomogram average to the high-resolution structure of the *Salmonella* MS-ring to read:

" The improved quality of the subtomogram average of the MS-complex in the fliP strain allowed us to tentatively assign densities to FliF and FliG (Fig. EV7), and compare our structure to available high-resolution structures of the purified MS-ring* (Johnson *et al*, 2020; Kawamoto *et al*, 2021). *Manually fitting the high-resolution structure of the purified MS-ring from S. enterica (PDB 6SCN)* (Johnson *et al*, 2020) *into the average allowed us to provisionally assign densities to different periplasmic domains including the drive-shaft-housing ring (the Beta collar), the C-ring template (the ring building motif 3 (RBM3) ring) and the RBM2+RBM1 rings. Additionally, fitting the crystal structure of FliG (which is known to interact with FliF) from Aquifex aeolicus (PDB 3HJL)* (Lee *et al*, 2010) *suggested that the dense ring directly surrounding the (here absent) $FlhA_C$ densities probably contains the C-terminus of FliF ($FliF_C$) and the N- and middle domains of FliG ($FliG_{MN}$), while the peripheral ring contains the C-terminus of FliG ($FliG_C$) (Fig. EV7)." (Lines 196-206).*

- For the fliP* H. Pylori strain, the authors report a smaller diameter for the complex. Based on the dimensions, and considering the existing high-resolution structure of the basal body (MS ring in particular), could the authors speculate on what the stoichiometry might be for FliF and Flig, for WT Vs flip*?

Recent high-resolution work has shown that FliF is capable of building rings with different stoichiometries with only very minor structural changes required to build the various symmetries (Johnson *et al*, 2020). For example, there is an increase of only 2.3 Å in the diameter of the B-collar of the MS-ring between the 34- and the 33-fold symmetries. Given that the resolution of our average of the *fliP** MS-ring (which is better than the corresponding WT MS-ring) is ~6 nm (following the suggestion of Referee 1 above, Figures EV2, EV6, EV9 and EV10 now show the resolutions of our sub-tomogram averages), unfortunately we cannot speculate on what symmetries these rings have.

- I did not find any information about any of the sub-tomogram averaging being deposited in the EMDB. One could argue that some of them only include few particles in the average, but at least the fliP* mutant, where Flip and FliC are docked, should be deposited.

We deposited all structures containing more than fifty particles to the EMDB and they will be released upon publication of this work:
the MS-complex of *H. pylori fliP**(EMD-25704), MS-complex of *H. pylori ΔfliQ fliP**(EMD-25705), MS-complex of *H. pylori ΔfliM fliP** (EMD-25703), and fully assembled motor of *H. gracilis* (EMD-25702). The reviewers can access this data by logging in to EMDB deposit system using the following deposition ID's:
D_1000261739 (EMD-25705)
D_1000261738 (EMD-25704)
D_1000261737 (EMD-25703)
D_1000261736 (EMD-25702)

The password is: rnapolymerase.

- Why does the MS complex looks different in the dFlhBc Vs dFlhAc C. Jejuni (figure 4)?

This is a good point and we do not know why. It could be because different components of the fT3SScc are being assembled inside the MS-ring in these different mutants.

- As mentioned above, for the mutants where sub-tomogram averaging could not be obtained, densities are very difficult to see; in particular for figures 3B, 5B and 5E, I cannot confidently see the C-ring, unless other evidence is provided I would argue that these could also be the flT3SScc sub-complex. For those cases, the confidence of the conclusions should be toned down.

We agree and now use tentative language when discussing these stages in the revised manuscript. In addition, we added movies EV1-EV5, which scroll through the 3-D volumes of the stages shown in Figures 2B, 2D, 3B, 5B and 5E, to help the reader see these complexes better than in single 2-D projection figures.

The relevant sections of the revised manuscript now read:
"Note that schematics and interpretation of complexes where not enough examples were found to produce a subtomogram average are tentative." (lines 131-133).

"They were also not readily apparent in cryotomograms of the C-complex, although we did not find enough examples of the complex for subtomogram averaging to definitively confirm this. In fully-assembled H. pylori flagella, a periplasmic structure called the cage surrounds the rod and the MS-ring and stabilizes the stator ring (Qin et al., 2017). In individual particles, we observed density that may correspond to the lower part of this cage in the C-complex, suggesting that it is assembled before the rest of the basal body (Fig. 3B and Movie EV3)." (lines 172-178).

"However, we saw no difference in the MS-complexes present in the ΔflgS fliP strain compared to fliP* (Fig. 5 A), nor in two individual examples of the C-complex (Fig. 5B and Movie EV4)."* (lines 250-252).

"Putative C- complexes in ΔfliQ fliP cells additionally lacked the lower part of the cage and the stators normally present at this stage (Fig. 5 E and Movie EV5), although not enough examples were identified to produce a subtomogram average (see Table 1)."* (lines 269-272)

Minor comments:
Line 56: I think the authors mean "inside-out fashion"?

Yes, corrected.

Lines 112-113: For clarity, the authors should specify which bacterial species/phyla are used.

Done (lines 122-124 of the revised manuscript).

Line 276: Should be S4D, not S2D

Corrected, thank you.

References

Fabiani FD, Renault TT, Peters B, Dietsche T, Gálvez EJC, Guse A, Freier K, Charpentier E, Strowig T, Franz-Wachtel M, *et al* (2017) A flagellum-specific chaperone facilitates assembly of the core type III export apparatus of the bacterial flagellum. *PLOS Biology* 15: e2002267

Fukumura T, Makino F, Dietsche T, Kinoshita M, Kato T, Wagner S, Namba K, Imada K & Minamino T (2017) Assembly and stoichiometry of the core structure of the bacterial flagellar type III export gate complex. *PLOS Biology* 15: e2002281

Johnson S, Fong YH, Deme JC, Furlong EJ, Kuhlen L & Lea SM (2020) Symmetry mismatch in the MS-ring of the bacterial flagellar rotor explains the structural coordination of secretion and rotation. *Nature Microbiology*

Kubori T, Shimamoto N, Yamaguchi S, Namba K & Aizawa S-I (1992) Morphological pathway of flagellar assembly in Salmonella typhimurium. *Journal of Molecular Biology* 226: 433–446

Lele PP, Hosu BG & Berg HC (2013) Dynamics of mechanosensing in the bacterial flagellar motor. *Proceedings of the National Academy of Sciences* 110: 11839–11844

Liu R & Ochman H (2007) Origins of Flagellar Gene Operons and Secondary Flagellar Systems. *J Bacteriol* 189: 7098–7104

Tsang J & Hoover TR (2014) Requirement of the Flagellar Protein Export Apparatus Component FliO for Optimal Expression of Flagellar Genes in Helicobacter pylori. *Journal of Bacteriology* 196: 2709–2717

Dear Prof. Jensen,

Thank you for the submission of your revised manuscript to The EMBO Journal. The paper has been seen by two of the original referees who now consider that you have properly dealt with all of their major concerns. However, before we can proceed with the acceptance of your study, there are a couple of editorial issues that need your attention:

- Two of the datasets you provide (i.e., EMD-25702 and EMD-25705) do not seem to be accessible. Please check.

Please provide the paper's synopsis, composed of:
- a short 'blurb' text summarizing in two sentences the study (max. 250 characters) and three to four 'bullet points' highlighting the main findings. Bullet points and standfirst text should be submitted as a separate manuscript file in LaTeX, RTF or MS Word format. This text has not been provided.
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I am pleased to inform you that your manuscript has been accepted for publication in the EMBO Journal.

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