

***In situ* imaging of the bacterial flagellar motor reveals stable inner- and outer-membrane-associated sub-complexes during motor assembly and disassembly**

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

1st Editorial Decision

5th Dec 2018

Thank you for submitting your manuscript on bacterial flagellar motor assembly and disassembly to The EMBO Journal. Your study has been sent to three referees for evaluation and we have now received their reports, which are enclosed below for your information.

As you can see, the referees find your study potentially interesting. However, they also raise several critical points that need to be solved before they can support publication in The EMBO Journal. In particular, referee #1 requests you to provide direct evidence that the observed outer membrane complexes are made of flagellar P/L-rings and to analyze the formation of these rings in a *fliF* mutant of *Pseudomonas aeruginosa*. In addition, s/he suggests that the identification of a plug component that seals the outer membrane of the P-L ring disassembly would strengthen your model. Referee #2 and referee #3 are concerned that the study fails to address the physiological roles of motor disassembly and the molecular mechanism of assembly/disassembly, respectively.

We agree with the referees that these are important points and addressing all of them would be essential to pursue publication of your findings in The EMBO Journal. In addition, we will need strong support from the referees for publication here. Given the overall interest of your study, I would like to invite you to submit a revised version of the manuscript according to the referees' requests. I should add that it is The EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version.

I realize that addressing all the referees' criticisms will require a lot of additional time and effort and be technically challenging. I would therefore understand if you were to choose not to undergo an extensive revision here and rather pursue a submission at an alternative venue, in which case please

inform us at your earliest convenience.

REFeree REPORTS:

Referee #1:

The flagellum is a remarkably complex macromolecular machine. How bacteria control and regulate the assembly of such complex structures remains poorly understood. In the present manuscript, Kaplan et al. used cryo-electron tomography to analyze flagella assembly and disassembly products of *Legionella pneumophila*, *Pseudomonas aeruginosa* and *Shewanella oneidensis*. The main discovery of the present manuscript is the observation of apparently stable outer membrane complexes (presumably made of the P-L rings). Kaplan et al. speculate that these outer membrane complexes constitute disassembly products of

The present manuscript comes at a similar time as a very similar study by the Beeby lab (bioRxiv preprint doi: 10.1101/367458), which also observed apparent 'relic' structures (i.e. disassembly products) in various species (*Plesiomonas shigelloides*, *Vibrio cholerae*, *Vibrio fischeri*, *Shewanella putrefaciens* and *Pseudomonas aeruginosa*). Evidently, this is an interesting and timely investigation made possible by the recent advances in cryo-electron tomography.

While quite similar in their overall conclusions, the Beeby study, however, goes somewhat beyond the observations reported in the present manuscripts by Kaplan et al. In particular, an important experiment of Ferreira et al is their demonstration that the bacteria eject their flagella at the base of the flagellar hook when nutrients are depleted, thus providing a biological explanation for the observed phenomenon.

Further, I have the following comments that might help to clarify the author's conclusions:

- 1) Direct evidence that the observed outer membrane complexes are made of the flagellar P/L-rings would be useful. Fluorescent protein fusions can be used to unequivocally identify proteins in cryo-electron tomography experiments. GFP might not fold properly in the periplasm, however functional periplasmic mCherry fusions have been reported (PMID 24688673).
- 2) The current 'inside-out' model for flagella assembly proposes that the P/L-rings form around a completed rod. Thus, the most important experiment in the manuscript is the analysis of the formation of P-L rings in a *fliF* mutant of *Pseudomonas aeruginosa*. The flagellar basal body (and rod) does not form in the absence of the MS-ring (made of *FliF*) and consistent with the 'inside-out' model, the authors did not find any P-L rings in this mutant. However, this being a negative result, the authors should exclude any potential negative feedback on flagellar gene expression from the lack of the MS-ring. Alternatively, analysis the presence of P-L rings in a mutant of the proximal rod or ATPase of the flagellar export apparatus might be useful. Such mutants would allow to demonstrate the formation of flagellar inner membrane complexes, thus providing an internal control for unaltered flagellar gene expression.
- 3) Finally, the identification of a plug component that seals the outer membrane of the P-L ring disassembly complexes would dramatically strengthen the authors model.

Minor comments:

- 1) Lines 57-59: The core components of the flagellar export apparatus is thought to assemble first (PMID 28771466, 28771474). This is similar to the assembly of the injectisome inner membrane complex (PMID 20876096).
- 2) Lines 65-66: The recent literature strongly supports the assembly of the P-L rings only around a completed rod (PMID 24748615).
- 3) Line 77: Citation of ref. 35 is incomplete?

4) Lines 99-100: It is unclear what the authors mean with the distance between the P-L ring complexes and the inner membrane complex. How can the distance be only 5 nm from the inner to the outer membrane? Are the inner membrane complexes still connected to the peptidoglycan or what would cause the close localization next to the outer membrane complexes?

5) Line 146: Revise sentence 'the P- and L-rings, together with their associated rings...'

6) Lines 166-167: I have a very hard time understanding how the rod would find already assembled P/L-rings in the outer membrane and then precisely hit the P/L-ring pore in order to continue with hook assembly in this 'outside-in' model. While the T3SS (the actual protein export system) of the injectisome and the flagellum are closely related, this is not true for the outer-membrane components. The injectisome Secretin does not have homologs in the flagellum and therefore the assembly process might be very different. Evidently, this should be discussed in more details.

7) Lines 171-172: Revise sentence: Only the filaments that were broken mechanically were observed to re-grow, but not the filaments, which were denatured using the laser pulse.

Referee #2:

The authors present interesting data showing the in situ structures of the bacterial flagellar motors of three different bacterial species in their assembly and disassembly processes revealed by electron cryotomography. Although the assembly and disassembly processes of the flagellar motors have been reported before by electron microscopy of the flagellar basal body structures isolated from the cells of many different mutant strains of *E. coli* and *Salmonella* that form intermediate motor structures due to deficiency in component proteins, this is the first report on these processes visualized and studied in situ with different bacterial species. Many types of interesting intermediate structures possibly in the assembly and disassembly processes are presented in 3D in fine details by electron cryotomography and subtomogram averaging. The data are of high quality and well documented in a rather compact form, giving new insights into the motor assembly and disassembly processes as well as nicely confirming previous findings from in vitro studies.

My only concern is that the physiological roles of motor disassembly in the three bacterial species studied in this work are not described at all unlike for *Caulobacter crescentus*, which ejects the flagellum as part of a specific programmed developmental process. It would be more comprehensible if something about this point is mentioned in either introduction or discussion.

Referee #3:

This well structured and nicely illustrated paper investigates assembly intermediates of different bacterial flagellar motors using electron cryo tomography. It focuses on the questions how these molecular machines assemble and disassemble and reports data from many different strains. The authors use subtomogram averaging to reveal different intermediates from the different species. They also generate (FlgH) and image (FlgH, FliF, FlaA/B) strains with specific genes deleted to examine the events leading to motor disassembly. Although the paper is well written and the figures well illustrated, I am not completely convinced that the study is at par with others published in EMBO Journal as it fails to reveal a clear molecular mechanisms of assembly/disassembly. This could be addressed by expressing mutated motor proteins in a way that they stall motor assembly or disassembly at predicted points. The latter probably necessitating conditional expression of the mutants after motor formation. Electron cryo tomography should then reveal the expected structures. In doing so the study could give deeper molecular mechanistic insight. One minor issue is the overuse of adjectives like dramatically, striking etc

1st Revision - authors' response

20th Feb 2019

See next page

Editor's comments:

As you can see, the referees find your study potentially interesting. However, they also raise several critical points that need to be solved before they can support publication in The EMBO Journal. In particular, referee #1 requests you to provide direct evidence that the observed outer membrane complexes are made of flagellar P/L-rings and to analyze the formation of these rings in a fliF mutant of Pseudomonas aeruginosa. In addition, s/he suggests that the identification of a plug component that seals the outer membrane of the P-L ring disassembly would strengthen your model.

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We agree with the referees that these are important points and addressing all of them would be essential to pursue publication of your findings in The EMBO Journal. In addition, we will need strong support from the referees for publication here. Given the overall interest of your study, I would like to invite you to submit a revised version of the manuscript according to the referees' requests.

We thank the editor and reviewers for their helpful comments. We have now performed several additional experiments. We have now imaged three new flagellar mutants in *Pseudomonas aeruginosa*, one new flagellar mutant in *Shewanella oneidensis* and collected and reconstructed ~250 new tomograms. Moreover, we have applied mass spectrometry analysis and negative-stain EM imaging. These new experiments resulted in several new panels in Figures 3 and 5, a new supplementary figure S3, two supplementary tables, three supplementary lists and 1 major dataset in the mass spectrometry PRIDE database. We have revised the manuscript accordingly. See detailed responses to specific reviewers' comments below. We now feel that we have addressed all concerns and request reconsideration of our manuscript for publication in the EMBO Journal.

Point-by-point response to the referees' comments:

The flagellum is a remarkably complex macromolecular machine. How bacteria control and regulate the assembly of such complex structures remains poorly understood. In the present manuscript, Kaplan et al. used cryo-electron tomography to analyze flagella assembly and disassembly products of Legionella pneumophila, Pseudomonas aeruginosa and Shewanella oneidensis. The main discovery of the present manuscript is the observation of apparently stable outer membrane complexes (presumably made of the P-L rings). Kaplan et al. speculate that these outer membrane complexes constitute disassembly products of

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While quite similar in their overall conclusions, the Beeby study, however, goes somewhat beyond the observations reported in the present manuscripts by Kaplan et al. In particular, an important experiment of Ferreira et al is their demonstration that the bacteria eject their flagella at the base of the flagellar hook when nutrients are depleted, thus providing a biological explanation for the observed phenomenon.

We appreciate the reviewer's comparison of our manuscript with the complementary study from the Beeby lab that confirms and boosts confidence in our conclusions.

To address the biological explanation, since we observed these sub-complexes in many tomograms of cells grown to high OD₆₀₀, we speculated that the process of flagellar disassembly was related to entering stationary phase. We therefore counted flagella in negatively stained micrographs of cells in exponential and stationary phases (see Materials and Methods for details). From 100 cells in each condition, we counted 48 unambiguously cell-attached flagella in the exponential-phase sample, but only 20 in the stationary-phase. We conclude that flagellar disassembly is triggered in high OD₆₀₀ conditions and have added this experiment and discussion to the manuscript.

Further, I have the following comments that might help to clarify the author's conclusions:

1) Direct evidence that the observed outer membrane complexes are made of the flagellar P/L-rings would be useful. Fluorescent protein fusions can be used to unequivocally identify proteins in cryo-electron tomography experiments. GFP might not fold properly in the periplasm, however functional periplasmic mCherry fusions have been reported (PMID 24688673).

The experiment the reviewer suggests is an elegant one. However, we think it is unlikely to succeed in the case of PL sub-complexes as it has been previously shown that FlgI and FlgH (the P- and L-ring proteins) are present in a stable disassociated form in the periplasm even before the other flagellar proteins [1]. Therefore, fluorescence signal in the periplasm could not be unambiguously assigned to sub-complexes, particularly given the decreased signal-relative-to-noise observed in cryogenic fluorescence microscopy.

Therefore, to address the reviewer's point, we performed an alternative experiment, imaging *Pseudomonas aeruginosa* cells lacking the P-ring protein FlgI. We did not find any PL sub-complexes in 50 tomograms of $\Delta flgI$ cells. We did, however, observe structures consisting of the inner membrane complex and the rod, similar to structures previously purified from the same mutant of *Salmonella* [2]. The absence of outer membrane sub-complexes in *P. aeruginosa* cells lacking FlgI complements our similar finding in *Shewanella oneidensis* MR-1 cells without FlgH (the L-ring protein). We believe this dependence, along with the fact that the sub-complexes have the same shape and dimensions as embellished PL rings in fully-assembled motors strongly supports our claim that the observed complexes are PL sub-complexes. We have added these new results to Figures 3 and 5 of the manuscript and modified the text accordingly.

2) *The current inside-out' model for flagella assembly proposes that the P/L-rings form around a completed rod. Thus, the most important experiment in the manuscript is the analysis of the formation of P-L rings in a fliF mutant of Pseudomonas aeruginosa. The flagellar basal body (and rod) does not form in the absence of the MS-ring (made of FliF) and consistent with the 'inside-out' model, the authors did not find any P-L rings in this mutant. However, this being a negative result, the authors should exclude any potential negative feedback on flagellar gene expression from the lack of the MS-ring. Alternatively, analysis the presence of P-L rings in a mutant of the proximal rod or ATPase of the flagellar export apparatus might be useful. Such mutants would allow to demonstrate the formation of flagellar inner membrane complexes, thus providing an internal control for unaltered flagellar gene expression.*

To address this point, we performed the following experiments:

- 1- As the reviewer suggested, we imaged two additional mutants of *Pseudomonas aeruginosa*: $\Delta flgG$ (rod mutant) and $\Delta flhA$ (ATPase mutant). In 36 tomograms of $\Delta flgG$ and 22 tomograms of $\Delta flhA$ cells, no PL sub-complexes were observed. As the reviewer anticipated, in $\Delta flgG$ cells we did observe the inner membrane complex containing the MS ring, C-ring and a density likely corresponding to the ATPase. This is consistent with the classical work of Kubori et al. [2] who imaged purified complexes from *Salmonella* $\Delta flgG$ cells and saw only the MS-ring (early protocols for flagellar motor purification did not retain the switch complex [3]). We added this new data to Figures 3 and 5 and modified the main text.
- 2- We generated and imaged a *Shewanella oneidensis* MR-1 mutant lacking the MS-ring protein FliF (see updated Materials and Methods). Similar to what we observed in *P. aeruginosa* $\Delta fliF$ cells, we did not see any PL sub-complexes in 45 tomograms of *S. oneidensis* $\Delta fliF$ cells. We did, however, observe the presence of chemoreceptor arrays in these cells. Flagellar genes are expressed in a hierarchical way with the chemoreceptor genes belonging to the “late genes” (see Ref.[4] for a discussion of *E. coli* and *Salmonella* and Ref.[5] for a discussion of *S. oneidensis*). Therefore, the presence of chemoreceptor arrays in $\Delta fliF$ cells strongly indicates that (at least some of) the flagellar genes are still being expressed. We added this figure to the manuscript as Figure S3 and added a description of the result to the main text.

3) *Finally, the identification of a plug component that seals the outer membrane of the P-L ring disassembly complexes would dramatically strengthen the authors model.*

We agree with the reviewer that identifying the plug would be very interesting. Towards that end we purified membrane-associated fractions and used mass spectrometry to identify candidate proteins. As discussed above, we observed a relationship between the presence of PL sub-complexes and the OD₆₀₀ of the sample. We therefore compared membrane fractions from high OD₆₀₀ (HOD) and low OD₆₀₀ (LOD) samples (see Materials and Methods for details). This yielded a list of ~1,500 proteins that showed significant fold-changes between the two samples.

To identify candidate proteins that might be involved in the membrane sealing mechanism we performed the following analysis:

First, as a control we examined flagellar proteins that are not part of the PL sub-complex but are part of the inner-membrane sub-complex (FliF and FliG). These proteins showed a fold-change of ~1 between the HOD and LOD samples:

Table 1: Mass spectrometry results of the fold-change in flagellar motor proteins related to the inner-membrane sub-complex in the HOD and LOD samples. LFQ = label-free quantitation.

Protein ID	Protein name	Gene name	Signal peptide	Peptides	Molecular weight	LFQ intensity HOD/LOD
Q8ECB5	Flagellar M-ring protein	fliF SO_3228		9	61.901	1.1
Q8ECB6	Flagellar motor switch protein FliG	fliG SO_3227		7	38.248	0.8

As an interesting note, as described above, by negative staining we observed ~2X as many assembled flagella in LOD as in HOD samples. Since the HOD/LOD fold-change of FliF and FliG is close to 1, we speculate that the inner membrane sub-complex is less stable than the outer membrane sub-complex which is in agreement with our ECT data (where we detected many more PL sub-complexes than inner-membrane sub-complexes in all three species).

As a second control we examined proteins that form the T- and H-rings (FlgO, FlgP, FlgT, MotX and MotY) that decorate the PL sub-complex. The HOD/LOD fold-change was between 1.6-2.8 for these proteins:

Table 2: Mass spectrometry results of the fold-change in H- and T-ring proteins in the HOD and LOD samples. LFQ = label-free quantitation.

Protein ID	Protein name	Gene name	Signal peptide	Peptides	Molecular weight	LFQ intensity HOD/LOD
K4PU01	Smf-dependent flagellar motor protein MotY	motY SO_2754	SIGNAL 1 19 {ECO:0000256 SAM:SignalP}.	6	32.971	2.8
Q8EC86	Flagella assembly protein FlgT	flgT SO_3258	SIGNAL 1 26 {ECO:0000256 SAM:SignalP}.	7	43.89	2.3
Q8EC88	Outer membrane lipoprotein required for	flgP SO_3256		8	16.822	1.6

	flagellar motility FlgP					
Q8EC87	Outer membrane lipoprotein required for flagellar motility FlgO	flgO SO_3257	SIGNAL 1 23 {ECO:0000256 SAM:SignalP}.	7	22.713	2.3
Q8EAG6	Flagellar rotation associated protein MotX	motX SO_3936	SIGNAL 1 31 {ECO:0000256 SAM:SignalP}.	5	24.609	2.2

These tables are now added to the manuscript as Tables S1 & S2.

We then searched for candidate proteins(s) that might be involved in the sealing mechanism using the following strategy:

- I- We made a list of all periplasmic proteins present in the *Shewanella oneidensis* MR-1 genome (520 proteins; see List S1 in the revised manuscript).
- II- As PL sub-complexes were observed in all three species investigated here (*L. pneumophila*, *P. aeruginosa* and *S. oneidensis*), we reasoned that a protein(s) involved in sealing the outer membrane after flagellar disassembly would most likely be present in these three species. Therefore, we identified the subset of proteins in List S1 with homologues in the three species (50 proteins, see List S2).
- III- As the T- and H- ring proteins showed an HOD/LOD fold-change between 1.5-3, we expected the candidate protein(s) to have a comparable ratio. Hence, we looked for proteins in List S2 with HOD/LOD fold-changes between 1-10 in the MS data. This yielded a list of 11 candidate proteins (List S3).

We have now modified the manuscript to include these results. Further narrowing down these candidates will require extensive molecular biology and imaging and is beyond the scope of the current study. We foresee significant challenges to such work. If the plug is formed by a dedicated protein, disruption of the protein is likely to lead to make cells inviable. Alternatively, the plug may be formed by a conformational change in an integral component(s) of the flagellar motor. These possibilities will be explored in future work. In the meantime, we expect our MS data to be beneficial to a broad spectrum of scientists and have therefore submitted the raw data to ProteomeXchange via the PRIDE database. Once our manuscript is accepted we will remove the password protection. In the meantime, reviewers can access the data by logging into:

<http://www.ebi.ac.uk/pride>

with the following credentials:

Username: reviewer23899@ebi.ac.uk

Password: 3RK3Nvag

1) Lines 57-59: *The core components of the flagellar export apparatus is thought to assemble first (PMID 28771466, 28771474). This is similar to the assembly of the injectisome inner membrane complex (PMID 20876096).*

We have added these references and modified the text accordingly.

2) Lines 65-66: *The recent literature strongly supports the assembly of the P-L rings only around a completed rod (PMID 24748615).*

Thank you. We have added this reference to the text.

3) Line 77: *Citation of ref. 35 is incomplete?*

Corrected.

4) Lines 99-100: *It is unclear what the authors mean with the distance between the P-L ring complexes and the inner membrane complex. How can the distance be only 5 nm from the inner to the outer membrane? Are the inner membrane complexes still connected to the peptidoglycan or what would cause the close localization next to the outer membrane complexes?*

We apologize for our misleading wording. We measured the lateral distance between the center of the inner membrane sub-complex and the center of the PL sub-complex. This is the distance between the two centers in the direction parallel to the plane of the membranes. In other words, it is a measure of how well the inner-membrane sub-complex is aligned directly underneath the outer-membrane sub-complex. It is not the distance between the membranes (which ranged between 30-60 nm in these cases).

We have clarified this in the text:

“In these cases, the lateral distance (in the direction of the plane parallel to the membranes) between the PL sub-complex and the IM sub-complex ranged from 60 nm to 5 nm.”

5) Line 146: *Revise sentence 'the P- and L-rings, together with their associated rings...'*

Done. We now write *“the embellished P- and L-rings”*

6) Lines 166-167: *I have a very hard time understanding how the rod would find already assembled P/L-rings in the outer membrane and then precisely hit the P/L-ring pore in order to continue with hook assembly in this 'outside-in' model. While the T3SS (the actual protein export system) of the injectisome and the flagellum are closely related, this is not true for the outer-membrane components. The injectisome Secretin does not have homologs in the flagellum and therefore the assembly process might be very different. Evidently, this should be discussed in more details.*

We agree with the reviewer on this point. Our incentive to write that speculative sentence was the observation of an inner-membrane sub-complex next to a PL sub-complex (but not completely underneath it, please see for example Figure 2D). As these inner-membrane sub-complexes have an intact MS-ring we don't think they are the result of a disassembly process as the MS-ring is digested during flagellar disassembly. The complexes are sufficiently close to each other that if the inner membrane sub-complex were assembling and continued to grow a steric clash would occur with the pre-existing PL sub-complex. This observation obviously needs further exploration in the future, though. Therefore, in consideration of the reviewer's comment, we decided to remove this speculation from text.

7) Lines 171-172: Revise sentence: Only the filaments that were broken mechanically were observed to re-grow, but not the filaments, which were denatured using the laser pulse.

Done.

Reviewer 2:

The authors present interesting data showing the in situ structures of the bacterial flagellar motors of three different bacterial species in their assembly and disassembly processes revealed by electron cryotomography. Although the assembly and disassembly processes of the flagellar motors have been reported before by electron microscopy of the flagellar basal body structures isolated from the cells of many different mutant strains of E. coli and Salmonella that form intermediate motor structures due to deficiency in component proteins, this is the first report on these processes visualized and studied in situ with different bacterial species. Many types of interesting intermediate structures possibly in the assembly and disassembly processes are presented in 3D in fine details by electron cryotomography and subtomogram averaging. The data are of high quality and well documented in a rather compact form, giving new insights into the motor assembly and disassembly processes as well as nicely confirming previous findings from in vitro studies.

My only concern is that the physiological roles of motor disassembly in the three bacterial species studied in this work are not described at all unlike for Caulobacter crescentus, which ejects the flagellum as part of a specific programmed developmental process. It would be more comprehensible if something about this point is mentioned in either introduction or discussion.

We thank the reviewer for her/his comments. As described above in the first response to Reviewer 1, we speculated that the process of flagellar disassembly was related to entering stationary phase, since many of the tomograms where we observed these sub-complexes were from cells grown to high OD₆₀₀. We therefore counted flagella on cells in exponential and stationary phase (detailed in the updated Materials and Methods) and found that flagellar disassembly is triggered at high OD₆₀₀ values, likely as a result of nutrient depletion. We have added the description and discussion of this experiment to the revised manuscript.

Reviewer 3:

This well structured and nicely illustrated paper investigates assembly intermediates of different bacterial flagellar motors using electron cryo tomography. It focuses on the questions how these molecular machines assemble and disassemble and reports data from many different strains. The authors use subtomogram averaging to reveal different intermediates from the different species. They also generate (FlgH) and image (FlgH, FliF, FlaA/B) strains with specific genes deleted to examine the events leading to motor disassembly. Although the paper is well written and the figures well illustrated, I am not completely convinced that the study is at par with others published in EMBO Journal as it fails to reveal a clear molecular mechanisms of assembly/disassembly. This could be addressed by expressing mutated motor proteins in a way that they stall motor assembly or disassembly at predicted points. The latter probably necessitating conditional expression of the mutants after motor formation. Electron cryo tomography should then reveal the expected structures. In doing so the study could give deeper molecular mechanistic insight.

We thank the reviewer for her/his comments. As described above in the response to Reviewer 1, we have now prepared and imaged two additional *P. aeruginosa* mutants and one new *S. oneidensis* MR-1 mutant.

The additional mutants are:

- 1- *P. aeruginosa* lacking the rod protein (FlgG)
- 2- *P. aeruginosa* lacking the P-ring protein (FlgI)
- 3- *S. oneidensis* MR-1 lacking the MS-ring protein (FliF). Please note that we had already imaged this mutant in *P. aeruginosa*.

In each case, our results were consistent with the classical work of Kubori et al. in *Salmonella* [2]. From 36 tomograms of $\Delta flgG$ cells, we found (in two cases) the inner membrane complex (C-ring, MS-ring and the ATPase). From 50 tomograms of $\Delta flgI$ cells we observed the expected “extended rivet” structure, but with the switch complex also present as in the $\Delta flgG$ strain (the switch complex was not preserved in classical purification protocols [3]). Interestingly, we observed the “extended rivet” structure lacking the switch complex in lysed cells. This is in accordance with previous results showing that the motor disassembly process in *Caulobacter crescentus* begins with the cytoplasmic part of FliF which leads to the disintegration of the C-ring [6–10]. This is also in agreement with our observation of a disassembled motor in a lysed *L. pneumophila* cell (Figure 2 O-Q). In 45 tomograms of $\Delta fliF$ *S. oneidensis* cells, no structure related to the flagellar motor could be detected (other than chemoreceptor arrays, see response to Reviewer 1), again similar to what Kubori et al described. We have added these results and their discussion to the revised manuscript.

One minor issue is the overuse of adjectives like dramatically, striking etc.

We have removed these adjectives from the text.

References:

1. Jones CJ, Macnab RM (1990) Flagellar assembly in *Salmonella typhimurium*: analysis with temperature-sensitive mutants. *J Bacteriol* **172**: 1327–1339.
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Thank you for submitting a revised version of your manuscript. Your study has been seen by the original referees and their comments are enclosed below for your information.

As you will see, while referee #2 and #3 find that all criticisms have been sufficiently addressed and recommend the manuscript for publication, referee #1 is concerned that the physiological role of flagellum disassembly have to be strengthened (point 1). In addition, s/he stresses that the chemoreceptor arrays in Δ fliF cells do not allow to drawing any conclusions about the normal expression of flagellar genes (point 2). Please note that while his/her concern about flagellar gene expression is well taken, we do not request you to address point 2 from this referee. We instead agree with point 1 by referee #1 and request you to further investigate the physiological role of flagellum disassembly by either performing the experiment suggested by this reviewer or other suitable experimental approaches. This view was also shared by referee #2 and #3 during cross-commenting.

I thank you again for giving us the chance to consider your manuscript for The EMBO Journal and look forward to your revision.

REFeree REPORTS:

Referee #1:

Kaplan et al. have performed substantial additional experiments and extensively revised their manuscript. Despite great efforts, the authors were unable to identify the plug component that seals the outer membrane of the P-L ring disassembly complexes. However, the provided mass spectrometry data will be very useful to identify the potential plug component in future studies. In addition, the authors provide additional evidence for the 'inside-out' model of flagella assembly. While the manuscript is substantially improved, I have the following main concerns:

1) Page 7, line 177ff: The provided evidence concerning the physiological role of flagella disassembly requires further substantiation. The authors claim that *Shewanella* disassembles their flagella when they enter stationary phase at high OD based on counting of cell-attached flagella. While this is a plausible mechanism, the authors do not control for alternative explanations e.g. many bacteria downregulate flagella gene expression upon entering stationary phase. One possible experiment to demonstrate that nutrient depletion triggers flagella disassembly is the following: Grow *Shewanella* to log phase (low OD), divide the culture and resuspend in either 'spent' media of another high OD culture, fresh medium, minimal medium or minimal medium supplemented with rich medium (LB) and then determine the number of cell-attached flagella.

2) Fig. S3. I am not sure that I can follow the argument that the presence of chemoreceptor arrays in Δ fliF cells 'strongly' indicates that flagellar genes are expressed normally. As noted by the authors, expression of the chemoreceptor genes (= 'late genes') is dependent on the activity of the flagella-specific sigma factor FliA (at least in *E. coli* or *Salmonella*). FliA, however, is inactive in a Δ fliF mutant because its cognate anti-sigma factor FlgM is not secreted outside the cell? It is thus unclear, what the presence of chemoreceptor arrays in a Δ fliF mutant means, but it does not allow to draw conclusions concerning normal flagellar gene expression.

Minor comment:

3) The authors misunderstood my previous suggestion concerning an experiment to validate that the observed outer membrane complexes are made of flagellar P/L-rings. I was proposing to use cryo-EM visualizations of the outer membrane structures in the WT and strains expressing translational fluorescent protein fusions to P/L-ring components in order to unequivocally determine if the observed structures are in fact flagellar P/L-rings or not. However, the experiment performed by the authors is also an elegant one to resolve this issue.

Referee #2:

The authors sufficiently addressed the points raised by the three reviewers including mine by carrying out additional experiments and revising the manuscript by incorporating the new results into it. The revised manuscript is much improved, and I have no further concern with it.

Referee #3:

The authors added a number of mutant bacteria to their study and generated a large set of tomograms, which in my view improved the conclusions of their study and answered what I asked. I hence endorse publication although not with the highest level of enthusiasm.

2nd Revision - authors' response

28th Mar 2019

Editor's comments:

Thank you for submitting a revised version of your manuscript. Your study has been seen by the original referees and their comments are enclosed below for your information.

As you will see, while referee #2 and #3 find that all criticisms have been sufficiently addressed and recommend the manuscript for publication, referee #1 is concerned that the physiological role of flagellum disassembly have to be strengthened (point 1). In addition, s/he stresses that the chemoreceptor arrays in Δ fliF cells do not allow to drawing any conclusions about the normal expression of flagellar genes (point 2). Please note that while his/her concern about flagellar gene expression is well taken, we do not request you to address point 2 from this referee. We instead agree with point 1 by referee #1 and request you to further investigate the physiological role of flagellum disassembly by either performing the experiment suggested by this reviewer or other suitable experimental approaches. This view was also shared by referee #2 and #3 during cross-commenting.

We thank the editor and the reviewers for the helpful comments. We performed the experiment which reviewer 1 suggested to further investigate the physiological role of flagellum disassembly. Furthermore, we modified our statement about the expression of the flagellar genes in Δ fliF *S. oneidensis* cells in consideration to the comment of reviewer 1. We now feel that we have addressed all concerns and request reconsideration of our manuscript for publication in the EMBO journal.

Point-by-point response to the referees' comments:

Reviewer 1:

Kaplan et al. have performed substantial additional experiments and extensively revised their manuscript. Despite great efforts, the authors were unable to identify the plug component that seals the outer membrane of the P-L ring disassembly complexes. However, the provided mass spectrometry data will be very useful to identify the potential plug component in future studies. In addition, the authors provide additional evidence for the 'inside-out' model of flagella assembly. While the manuscript is substantially improved, I have the following main concerns:

1) Page 7, line 177ff: The provided evidence concerning the physiological role of flagella disassembly requires further substantiation. The authors claim that *Shewanella* disassembles their flagella when they enter stationary phase at high OD based on counting of cell-attached flagella. While this is a plausible mechanism, the authors do not control for alternative explanations e.g. many bacteria downregulate flagella gene expression upon entering stationary phase. One possible experiment to demonstrate that nutrient depletion triggers flagella disassembly is the following: Grow *Shewanella* to log phase (low OD), divide the culture and resuspend in either 'spent' media of another high OD culture, fresh medium, minimal medium or minimal medium supplemented with rich medium (LB) and then determine the number of cell-attached flagella.

We thank the reviewer for her/his comment. Based on the reviewer's suggestion, we performed the following experiment:

We grew *S. oneidensis* cells overnight in LB medium at 30° C with shaking at 175 rpm. In addition, we prepared another culture of *S. oneidensis* in LB and grew it (under the same above-mentioned conditions) for 6 hours. Subsequently, both cultures were spun down at 3000x g for 10 minutes at room temperature. The pellet of the 6-hour culture was resuspended in 100 µl of its supernatant and 25 µl was added to each of the following:

- 1- 1 ml of the supernatant of the overnight culture ("spent" medium).
- 2- 1 ml of minimal medium
- 3- 1 ml of fresh LB culture
- 4- 1 ml of a medium that contains 0.5 ml fresh LB and 0.5 ml minimal medium.

These four cultures were incubated at 30° C with shaking at 175 rpm for 3 hours. Then, we counted the number of cell-attached flagella in each of these samples using negative-stain electron microscopy. The result is:

- 1- 9 cell attached-flagella were counted in 60 cells of the spent LB medium.
- 2- 8 cell attached-flagella were counted in 60 cells of the minimal medium.
- 3- 44 cell-attached flagella were counted in 60 cells of the fresh LB medium.
- 4- 19 cell-attached flagella were counted in 60 cells of the medium consisting of 0.5 ml fresh LB and 0.5 ml minimal medium.

This result is in accordance to what we reported in our previously-submitted manuscript. We added this result to the main text and modified the Materials and Methods section accordingly.

2) Fig. S3. I am not sure that I can follow the argument that the presence of chemoreceptor arrays in Δ fliF cells 'strongly' indicates that flagellar genes are expressed normally. As noted by the authors, expression of the chemoreceptor genes (= 'late genes') is dependent on the activity of the flagella-specific sigma factor FliA (at least in *E. coli* or *Salmonella*). FliA, however, is inactive in a Δ fliF mutant because its cognate anti-sigma factor FlgM is not secreted outside the cell?

It is thus unclear, what the presence of chemoreceptor arrays in a Δ fliF mutant means, but it does not allow to draw conclusions concerning normal flagellar gene expression.

In consideration of the reviewer comment, we modified the main text as the following:

"therefore the presence of chemoreceptor arrays indicates that (at least some of the) flagellar/chemotaxis genes are being expressed in this mutant."

3) The authors misunderstood my previous suggestion concerning an experiment to validate that the observed outer membrane complexes are made of flagellar P/L-rings. I was proposing to use cryo-EM visualizations of the outer membrane structures in the WT and strains expressing translational fluorescent protein fusions to P/L-ring components in order to unequivocally determine if the observed structures are in fact flagellar P/L-rings or not. However, the experiment performed by the authors is also an elegant one to resolve this issue.

We apologize to the reviewer for this misunderstanding.

3rd Editorial Decision

8th Apr 2019

Thank you for submitting a revised version of your manuscript. It has now been seen by two of the original referees whose comments are shown below.

As you will see, referee #1 finds that his/her remaining concerns are sufficiently addressed and recommends the manuscript for publication. Also, s/he suggests that discussing the recent study by Ferreira, J. L. et al. (2019) would further strengthen your conclusions.

In addition, there are a few editorial issues concerning text and figures that I need you to address before we can officially accept the manuscript.

REFEREE REPORTS:

Referee #1:

Kaplan et al. have appropriately addressed all my remaining concerns and I recommend publication of the manuscript.

In addition, the recently published study by Ferreira et al. nicely supports the author's conclusions concerning the existence of stable outer-membrane-embedded sub-complexes and flagella disassembly under low nutrient conditions (Ferreira, J. L. et al. γ -proteobacteria eject their polar flagella under nutrient depletion, retaining flagellar motor relic structures. PLoS Biol 17, e3000165 (2019)).

It might be useful for the reader if the authors would briefly discuss the supporting findings of Ferreira et al. in their discussion.

3rd Revision - authors' response

11th Apr 2019

The authors performed the requested editorial changes.

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Grant J Jensen

Journal Submitted to: The EMBO Journal

Manuscript Number: EMBOJ-2018-100957R

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

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

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

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

2. Captions

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

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

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

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

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

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

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Is the variance similar between the groups that are being statistically compared?	NA
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C- Reagents

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

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

D- Animal Models

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

E- Human Subjects

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

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'.	We submitted our Mass spectrometry results of isolated membrane fractions from <i>Shewanella oneidensis</i> at different OD600 values to the ProteomeXchange via the PRIDE database. The project accession number is PXD012554. The data is still until password protection (the login information is available to the reviewers in the point-to-point file). If the paper is published we will remove the password protection to make the data available for public and provide the accession number in the paper.
Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
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