nature portfolio

Peer Review File

Structural insights into the assembly mechanism of the molecular bushing in the bacterial flagellar motor

Corresponding Author: Professor Keiichi Namba

This manuscript has been previously submitted at another journal. This document only contains information relating to versions considered at Communications Biology.

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
In this paper, Yamaguchi and colleagues present a model of how the flagellar P- and L-rings assemble. To achieve this, they solve the structure of the P-ring in a polyrod mutant using single particle cryo-EM analysis and then compare this structure to known structures of the LP-rings associated with flagellar motors. They show that the P-ring in the polyrod mutant, where no L-ring is present, adapts an elliptical shape that is tilted by ~ 2.7 degrees resulting in strong interactions between the P-ring and the rod. The subsequent assembly of the L-ring induces a conformational change in the P-ring to adapt a circular structure that is detached from the rod so that these two rings can act as a bushing during motor rotation.
The paper is a beautiful work that is well written, nicely illustrated with solid data and I am supportive of its publication.
I only have the following questions to the authors:
1- Can the authors give a reason of why they decided to use the polyrod mutant instead of an L-ring mutant with a single P-ring? Is that to obtain more particles? Can the authors elaborate a little bit on the polyrod in the introduction (preferably with a nice example from the cryo-EM micrographs)? How many P-rings on average on a polyrod? Are they equidistant? Clarifying that in text will be helpful.
2- A minor detail regarding the model, it is believed that there is always a pool of P- and L-rings proteins in the periplasm that assemble once the rod is built (see https://pmc.ncbi.nlm.nih.gov/articles/PMC208602/). In other words, the model can be revised by having a pool of these two proteins prior to flagellar assembly.
3- I am confused of how the authors obtained figure 4f. Have the authors used the published L-ring structure (from LP-rings assembled with the motor) that they manually docked on their polyrod structure? Or are both of the PL-rings from a published structure that the authors manually tilted around the rod? Can the authors elaborate on how they made this figure?
4- There is a recent preprint that reports the structure of the P- and L- rings alone without the rod when they are present as a relic (after flagellar assembly, see https://www.biorxiv.org/content/10.64898/2025.12.02.691832v1). I am curious how the structure of the P-ring in the polyrod compares to the P-ring in these PL-subcomplexes?
5- The sentence in lines 207-208 does not read well “Negatively charged residues in the L-ring its distal part”
Good luck to the authors in their revision.

Reviewer #2

(Remarks to the Author)
The paper presents cryoEM structure of the LP-ring complex in the bacterial flagellar motor, focusing on the interactions and dynamics of its components during assembly. The results provide novel insights into the assembly mechanism of the molecular bushing in the bacterial flagellar motor. The structural results are state of the art, typical of the Namba group ever since the first flagellum structure paper by Namba et al. I don’t have main concerns of the paper and only could suggest the following areas where improvements could be made for publication:
1. Clarity in Figures: Some figures, such as Fig. 1 and Fig. 3, could benefit from clearer labeling and annotations to make the visual data more accessible to readers unfamiliar with the subject matter. Scale bars, colour keys should be included within the figure displays (not necessary in legends).
2. Discussion: The paper could be more impactful by discussing the potential impact of the findings on understanding

bacterial motility and its relevance to bioengineering applications, similar to those discussed by Lou et al. (PMID: 40627653).
3. Comparative Analysis: The paper could provide a more in-depth comparison of the PaPR structure with other similar structures in different bacterial species to highlight evolutionary differences or similarities.
By addressing these areas, the paper could enhance its readability, accessibility, and impact on the scientific community.

Reviewer #3

(Remarks to the Author)
This manuscript from Yamaguchi et al. reports a cryo-EM structural analysis of the P-ring attached to polyrod (PaPR), revealing the assembly mechanism of the LP-ring complex in the Salmonella flagellar motor. The work provides strong structural insights into how the P ring attaches to the rod and how the L ring assembly triggers P ring detachment to form a functional bushing. The experimental design, data quality, and interpretation are excellent. Below are minor points for clarification that could further strengthen the manuscript.

Minor points

1. Physiological relevance of the polyrod-based PaPR structure
The use of the flgG (G65V) polyrod mutant is an effective strategy to capture the intermediate P–ring–rod interaction state. However, because the polyrod differs from the native distal rod in length and local environment, the authors may wish to clarify further which structural features of the PaPR are most likely conserved in the native assembly intermediate. In particular, could the authors comment on whether the observed 2.7° tilt and ellipticity are expected to occur transiently during normal LP ring assembly in vivo?

2. Quantitative assessment of rod–P ring interactions
The manuscript describes increased hydrogen bonding between FlgI and FlgG in the PaPR compared to the LP ring. While the qualitative analysis is convincing, a brief quantitative summary, such as approximate numbers or interaction distribution per subunit, would help readers better appreciate the energetic contribution of these interactions to P-ring stabilization.

3. Flexibility of the GSS region and symmetry mismatch
The discussion of the FlgG specific sequence (GSS) as a flexible element accommodating symmetry mismatch between the rod and P ring is particularly insightful. It would be helpful if the authors could further discuss whether the observed GSS flexibility might also contribute to mechanical buffering during high-speed rotation after LP ring completion, in addition to its role in assembly.

4. Potential role of the peptidoglycan layer and outer membrane constraints
The proposed model emphasizes steric clashes and electrostatic repulsion during L-ring assembly as the trigger for P-ring detachment. Since the PaPR structure lacks the native peptidoglycan layer and outer membrane context, a short discussion on how these cellular constraints might modulate or reinforce the proposed mechanism would strengthen the physiological interpretation.

5. Generality of the proposed assembly mechanism
In the discussion part, the authors may consider briefly discussing whether similar tilted or elliptical intermediate states could exist in other bacterial species or related rotary systems, and whether this mechanism might represent a general strategy for assembling free-standing bushings around rotating shafts.

6. Helical Refinement informations
The helical symmetry parameters (64.9°rotation, 4.31 A rise, lines 122-123) determined by the Symmetry Search Utility in cryoSPARC are reported in the text; however, the authors do not present this information in Table 2 or Supplementary Fig. 3. It would be helpful to include the symmetry search results (e.g., a mean-squared-error plot from cryoSPARC) in Supplementary Figure 3 to demonstrate how these parameters were validated.
These additions would help readers better understand the helical reconstruction process and facilitate reproduction of the analysis.

Overall, these points are intended as minor clarifications rather than criticisms. The manuscript is well written, technically rigorous, and makes a substantial contribution to our understanding of bacterial flagellar assembly.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have satisfactorily answered all my questions. Congratulations on this nice work!

Reviewer #3

(Remarks to the Author)
The authors have addressed all my comments in their revised manuscript.

Response to the comments by the reviewers

To Reviewer #1

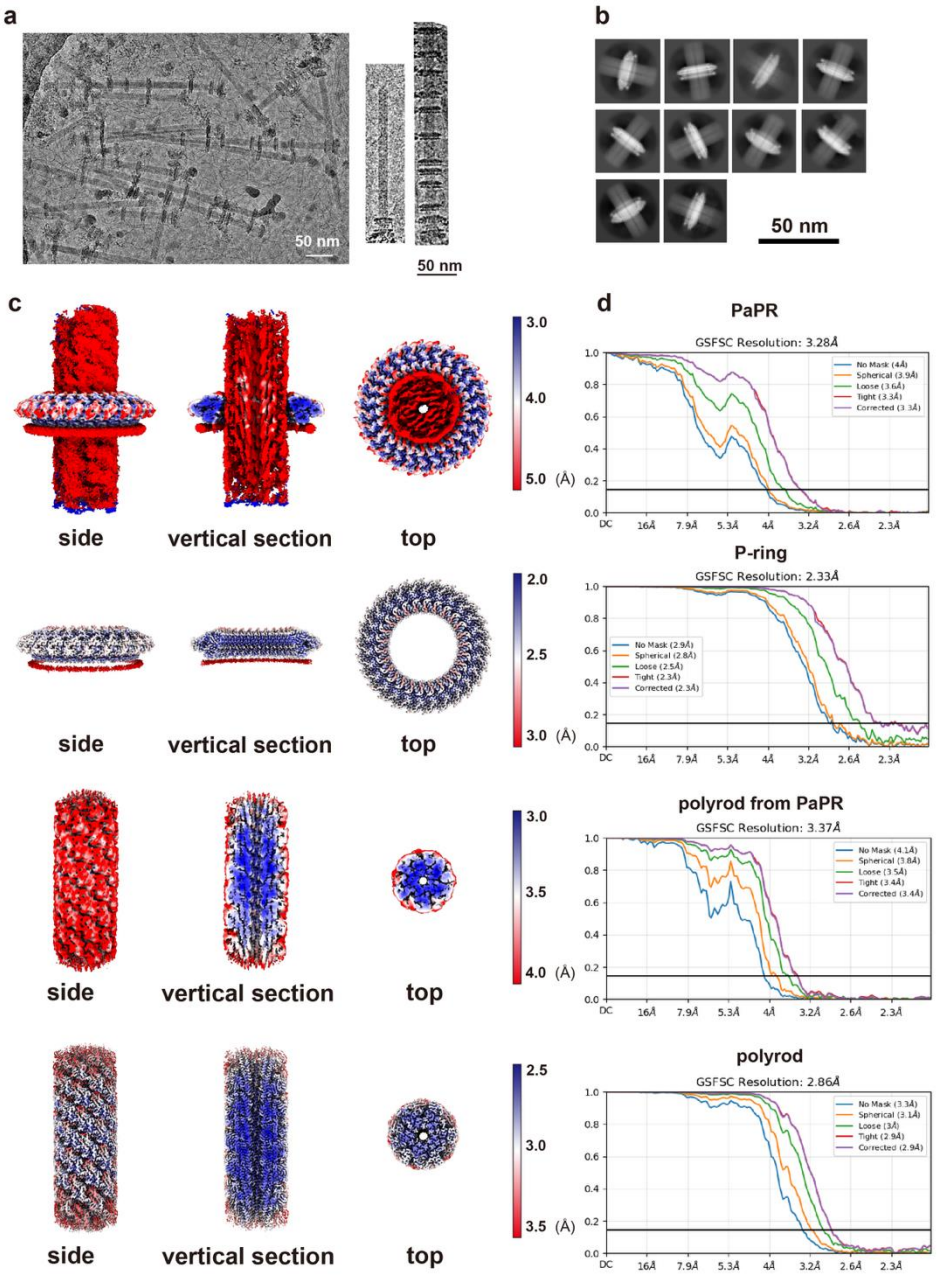
> In this paper, Yamaguchi and colleagues present a model of how the flagellar P- and L-rings assemble. To achieve this, they solve the structure of the P-ring in a polyrod mutant using single particle cryo-EM analysis and then compare this structure to known structures of the LP-rings associated with flagellar motors. They show that the P-ring in the polyrod mutant, where no L-ring is present, adapts an elliptical shape that is tilted by ~ 2.7 degrees resulting in strong interactions between the P-ring and the rod. The subsequent assembly of the L-ring induces a conformational change in the P-ring to adapt a circular structure that is detached from the rod so that these two rings can act as a bushing during motor rotation.

> The paper is a beautiful work that is well written, nicely illustrated with solid data and I am supportive of its publication.

Thank you very much for your recognition and support.

> I only have the following questions to the authors:
> 1- Can the authors give a reason of why they decided to use the polyrod mutant instead of an L-ring mutant with a single P-ring? Is that to obtain more particles? Can the authors elaborate a little bit on the polyrod in the introduction (preferably with a nice example from the cryo-EM micrographs)?

Yes, we used the polyrod mutant to obtain more particles per micrograph than using the candlestick basal body. We added a phrase and a sentence in Introduction (lines 88 - 90) to guide readers to a typical cryoEM image of the P-ring on the polyrod (Supplementary Fig. 2a) and to explain the reason why we used this mutant strain. We also added a similar sentence in the beginning of Results (lines 98-100).



Supplementary Fig.2

We estimated the average numbers of P-rings per micrograph observed for PaPR in this study and for HBB in the previous study. By considering the difference in magnification used in these two studies, 59,000× for PaPR and 40,000× for HBB, the particle number per physical area was increased by 6.8 times by using PaPR. We described it in the first section of Methods.

>How many P-rings on average on a polyrod? Are they equidistant? Clarifying that in text will be helpful.

Thank you for your advice. We added the following paragraph in the beginning of Results (lines 101 – 108). We also added two representative views of PaPR in Supplementary Fig. 2a, one with few and the other with many P-rings.

We purified PaPR by sucrose density gradient ultracentrifugation for cryoEM data collection. After observation of each fraction by negative stain EM, we selected a fraction that disperses well and contains polyrods with as many P-rings attached as possible. Since longer polyrods with higher-number of P-rings tend to aggregate, those fractions were excluded. The P-rings were attached on polyrods in a non-equidistant manner, as observed in the previous study[24]. Some PaPR contains more than 10 P-rings, whereas others contain only few (Supplementary Fig. 2a). On average, 4.2 P-rings were attached per polyrod.

> 2- A minor detail regarding the model, it is believed that there is always a pool of P- and L-rings proteins in the periplasm that assemble once the rod is built (see https://pmc.ncbi.nlm.nih.gov/articles/PMC208602/). In other words, the model can be revised by having a pool of these two proteins prior to flagellar assembly.

Thank you for your suggestion. We added the following phrase in Introduction (lines 68 – 69).

… and FlgH and FlgI are pooled in the periplasm until the rod is assembled[18].

We also modified Fig. 5 and supplementary Fig.1 accordingly and revised the discussion (lines 289 – 292) as follows.

FlgA, FlgH and FlgI are secreted to the periplasm via Sec translocon independently of the other flagellar proteins[18,20], and FlgH is delivered to the outer membrane by a sorting system such as Lol. The rod component proteins FliE, FlgB, FlgC, FlgF and FlgG are secreted via the flagellum-specific type III export apparatus.

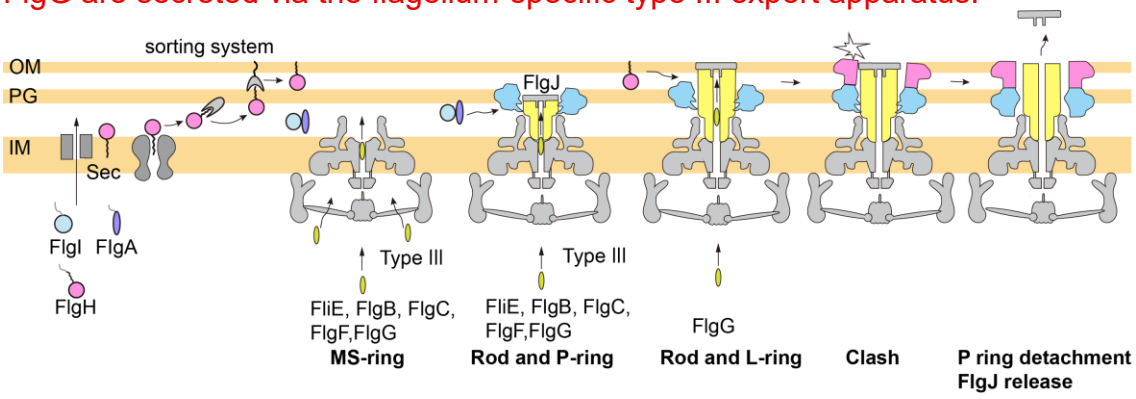
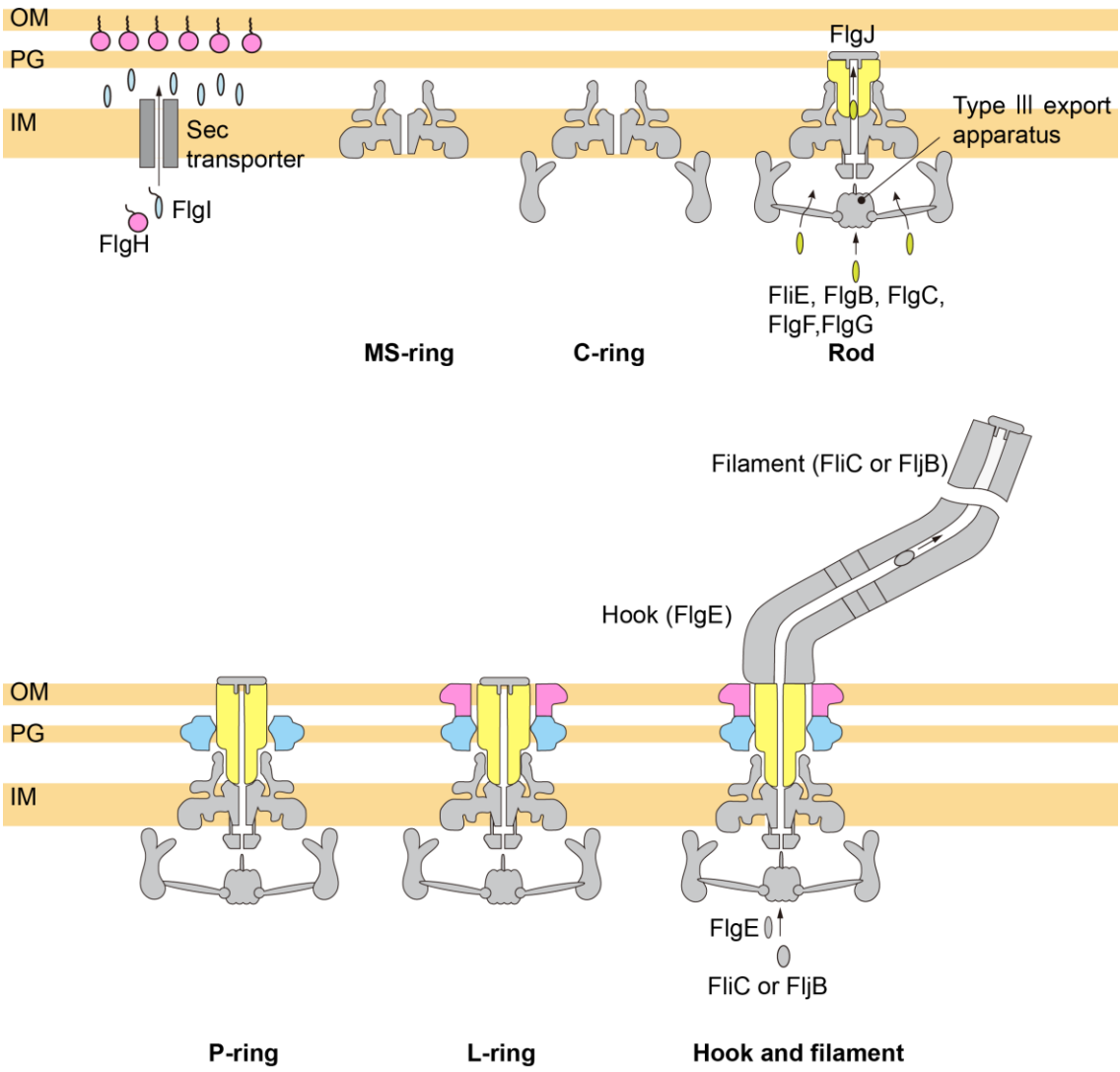


Fig. 5



Supplementary Fig.1

> 3- I am confused of how the authors obtained figure 4f. Have the authors used the published L-ring structure (from LP-rings assembled with the motor) that they manually docked on their polyrod structure? Or are both of the PL-rings from a published structure that the authors manually tilted around the rod? Can the authors elaborate on how they made this figure?

We took the L-ring structure from the published LP-ring structure of the hook-basal body and aligned it on the P-ring in PaPR by using UCSF Chimera. Therefore, the P-ring is from PaPR, and the L-ring is from the LP-ring of HBB.

> 4- There is a recent preprint that reports the structure of the P- and L- rings alone without the rod when they are present as a relic (after flagellar assembly, see https://www.biorxiv.org/content/10.64898/2025.12.02.691832v1). I am curious how

the structure of the P-ring in the polyrod compares to the P-ring in these PL-subcomplexes?

We could not directly compare them because the reference manuscript is only found in bioxiv and the PDB file are not yet made public.

It is most likely that the P-ring structure in the orphan LP-rings Wangbiao *et al.* found is circular as it is in the HBB, because they are free from the rod.

> 5- The sentence in lines 207-208 does not read well “Negatively charged residues in the L-ring its distal part”

Thank you. We modified it as follows (lines 219 – 223).

Negatively charged residues of FlgH in the distal part of the L-ring ...

> Good luck to the authors in their revision.

Thank you.

To Reviewer #2

> The paper presents cryoEM structure of the LP-ring complex in the bacterial flagellar motor, focusing on the interactions and dynamics of its components during assembly. The results provide novel insights into the assembly mechanism of the molecular bushing in the bacterial flagellar motor. The structural results are state of the art, typical of the Namba group ever since the first flagellum structure paper by Namba et al. I don’t have main concerns of the paper and only could suggest the following areas where improvements could be made for publication:

Thank you very much.

> 1. Clarity in Figures: Some figures, such as Fig. 1 and Fig. 3, could benefit from clearer labeling and annotations to make the visual data more accessible to readers unfamiliar with the subject matter. Scale bars, color keys should be included within the figure displays (not necessary in legends).

Thank you for your advice. We added scales and color kyes in Figs. 1 and 3.

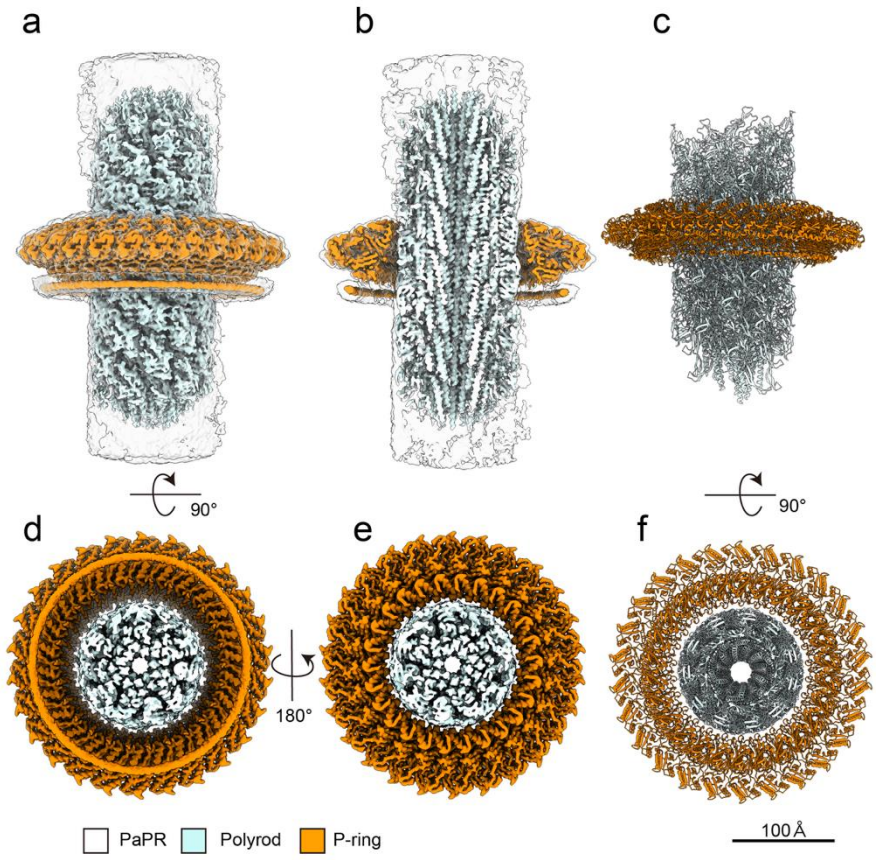


Fig.1

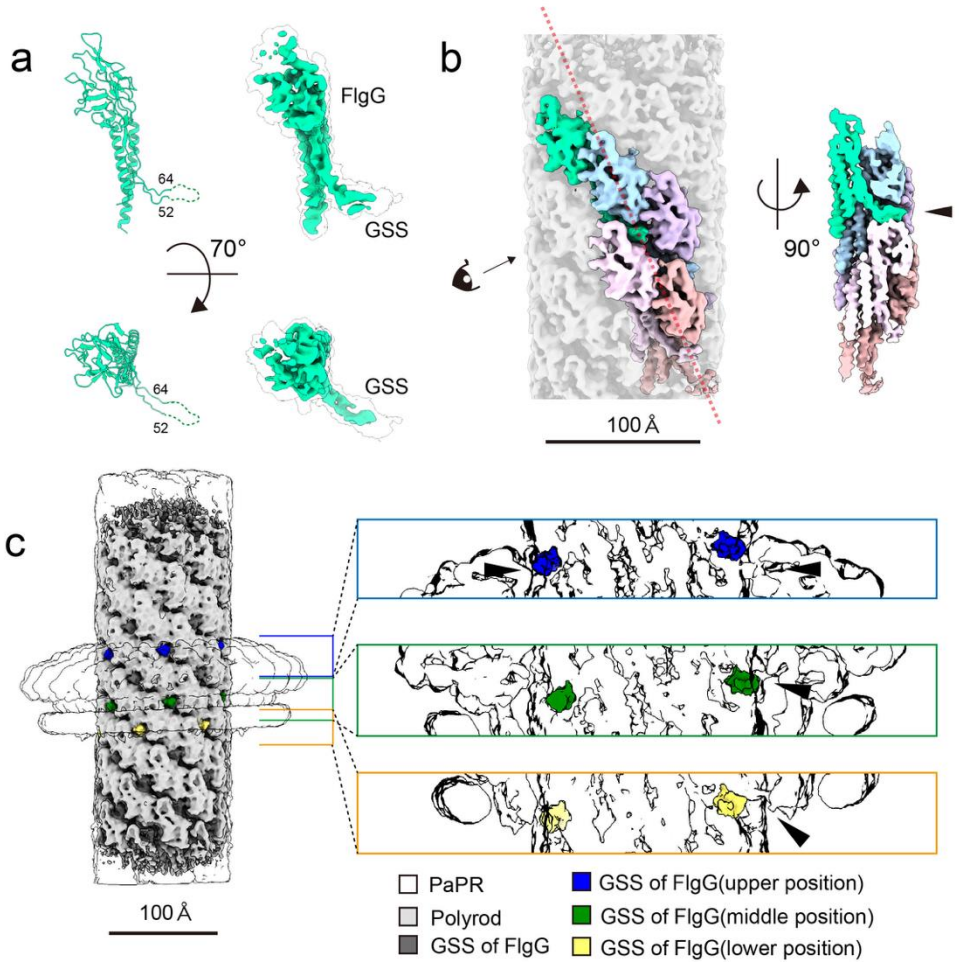


Fig.3

> 2. Discussion: The paper could be more impactful by discussing the potential impact of the findings on understanding bacterial motility and its relevance to bioengineering applications, similar to those discussed by Lou et al. (PMID: 40627653).

Thank you for your suggestion. We added a paragraph copied below as the last paragraph of Discussion (lines 328 – 338), discussing on application examples that utilize the self-assembly mechanisms of the flagellar motor.

Bacterial flagella are widely used in bacterial motility and is directly involved in pathogenicity. Therefore, understanding the assembly mechanisms of the flagellar motor provides valuable insights for developing strategies to combat infectious diseases. Furthermore, the mechanisms underlying the autonomous assembly of the flagellar motor offer important design principles for nanodevices and nanotechnology. The engineering of autonomously self-assembling nanomachine is still challenging. It is especially tricky to design and generate a free-standing part, like the LP-ring as a bushing, in a complex mechanical nanodevice by self-assembly. Therefore, biologically derived self-assembly mechanisms underlying the flagellar motor indeed serve as a

valuable model for engineering applications. Thus, our finding provides a good foundation for future advances in both medical and industrial applications.

> 3. Comparative Analysis: The paper could provide a more in-depth comparison of the PaPR structure with other similar structures in different bacterial species to highlight evolutionary differences or similarities.

Thank you for your advice. We agree that comparing the PaPR structure with those of other bacterial species could be important for evaluating the evolutionary difference, but unfortunately there is no similar structures to compare with at the moment.

> By addressing these areas, the paper could enhance its readability, accessibility, and impact on the scientific community.

Thank you for your kind comments.

To Reviewer #3

> This manuscript from Yamaguchi et al. reports a cryo-EM structural analysis of the P-ring attached to polyrod (PaPR), revealing the assembly mechanism of the LP-ring complex in the Salmonella flagellar motor. The work provides strong structural insights into how the P ring attaches to the rod and how the L ring assembly triggers P ring detachment to form a functional bushing. The experimental design, data quality, and interpretation are excellent. Below are minor points for clarification that could further strengthen the manuscript.

Thank you for your nice comments.

> Minor points
> 1. Physiological relevance of the polyrod-based PaPR structure
The use of the flgG (G65V) polyrod mutant is an effective strategy to capture the intermediate P–ring–rod interaction state. However, because the polyrod differs from the native distal rod in length and local environment, the authors may wish to clarify further which structural features of the PaPR are most likely conserved in the native assembly intermediate. In particular, could the authors comment on whether the observed 2.7° tilt and ellipticity are expected to occur transiently during normal LP ring assembly *in vivo*?

Thank you for your comment on the important point. The position of the P-ring as part of the LP-ring in the HBB is nearly at the bottom end of the distal rod formed by FlgF and FlgG, but it is known from the high-resolution structure of the LP-ring around the rod in the HBB that the rod protein that FlgI forming the P-ring directly faces is FlgG, not FlgF.

The fact that many P-rings are attached on the polyrod formed by FlgG also indicate that the intermolecular interaction between the P-ring and distal rod is formed by FlgI and FlgG. These observations assure that the PaPR structure represents the physiological one as an intermediate of the LP-ring assembly process. It is therefore highly likely that the interaction between the rod and P-ring is the same in both PaPR and normal flagellar motor, and the 2.7° tilt and elliptic shape are expected to occur transiently during LP-ring assembly *in vivo*. We added the following sentence at line 232 in the first paragraph of Discussion (lines 232 – 239).

This allows the P-ring in the PaPR structure to form more hydrogen bonds with the rod surface to firmly associate with the rod. This represents a physiological intermediate of the LP-ring assembly process, known as the candlestick structure[5,23] produced by deletion of L-ring protein FlgH. The P-ring is tightly associated with the distal rod formed by FlgG in the candlestick structure. It is therefore highly likely that the interaction between the rod and P-ring is the same in both PaPR and normal flagellar motor, and the 2.7° tilt and elliptic shape are expected to occur transiently during LP-ring assembly in vivo.

> 2. Quantitative assessment of rod–P ring interactions
The manuscript describes increased hydrogen bonding between FlgI and FlgG in the PaPR compared to the LP ring. While the qualitative analysis is convincing, a brief quantitative summary, such as approximate numbers or interaction distribution per subunit, would help readers better appreciate the energetic contribution of these interactions to P-ring stabilization.

Because the P-ring has C26 symmetry and the rod has a helical symmetry, each subunit interacts with different residues, and their interactions are not uniformly distributed. Therefore, we counted the total number of hydrogen bonds and added the following sentence (lines 153 – 155).

They potentially form nearly three time as many hydrogen bonds as those in the LP-ring (in total, 31 hydrogen bonds in PaPR and 13 in the LP-ring).

> 3. Flexibility of the GSS region and symmetry mismatch
The discussion of the FlgG specific sequence (GSS) as a flexible element accommodating symmetry mismatch between the rod and P ring is particularly insightful. It would be helpful if the authors could further discuss whether the observed GSS flexibility might also contribute to mechanical buffering during high-speed rotation after LP ring completion, in addition to its role in assembly.

We agree that the flexibility of GSS might also contribute to mechanical buffering for high-speed rotation, but since we do not have any experimental clue, we would refrain from discussing it. We consider that the water molecules at the rod and LP-ring interface play a major role as the mechanical buffer for high-speed rotation.

> 4. Potential role of the peptidoglycan layer and outer membrane constraints
The proposed model emphasizes steric clashes and electrostatic repulsion during L-ring assembly as the trigger for P-ring detachment. Since the PaPR structure lacks the native peptidoglycan layer and outer membrane context, a short discussion on how these cellular constraints might modulate or reinforce the proposed mechanism would strengthen the physiological interpretation.

Thank you for raising a good point. We added the following paragraph in Discussion to discuss the potential role of the peptidoglycan layer and outer membrane (lines 311 – 327).

It is curious that the L-ring rarely assembles on the P-ring of PaPR even though FlgH is present in this mutant. Spatial arrangement of the rod with the peptidoglycan layer and the outer membrane might have an important role for LP-ring assembly. The P-ring in the normal basal body is formed within the peptidoglycan layer, and the axial positioning of the P-ring on the distal rod is determined by the interactions of FlgI with peptidoglycan. In the polyrod mutant, however, many P-rings associate with the abnormally elongated rod that remains in the periplasm[24], indicating that the peptidoglycan layer is not essential for P-ring assembly. Given that FlgI remains in the periplasm while FlgH is transported to the outer membrane via a sorting system, L-ring assembly on the P-ring appears to require the outer membrane to be located rather closely to the P-ring for FlgH to associate with FlgI in the P-ring. These observations indicate that an appropriate positioning of the peptidoglycan layer and the outer membrane is crucial for coordinating the proper assembly of the P-ring and L-ring with rod elongation. Because it is quite tricky to design and generate a free-standing bushing around the rotating drive shaft by self-assembly, it is highly likely that the mechanism of LP-ring assembly we found in this study represents a general strategy used in other bacterial species and related rotary systems.

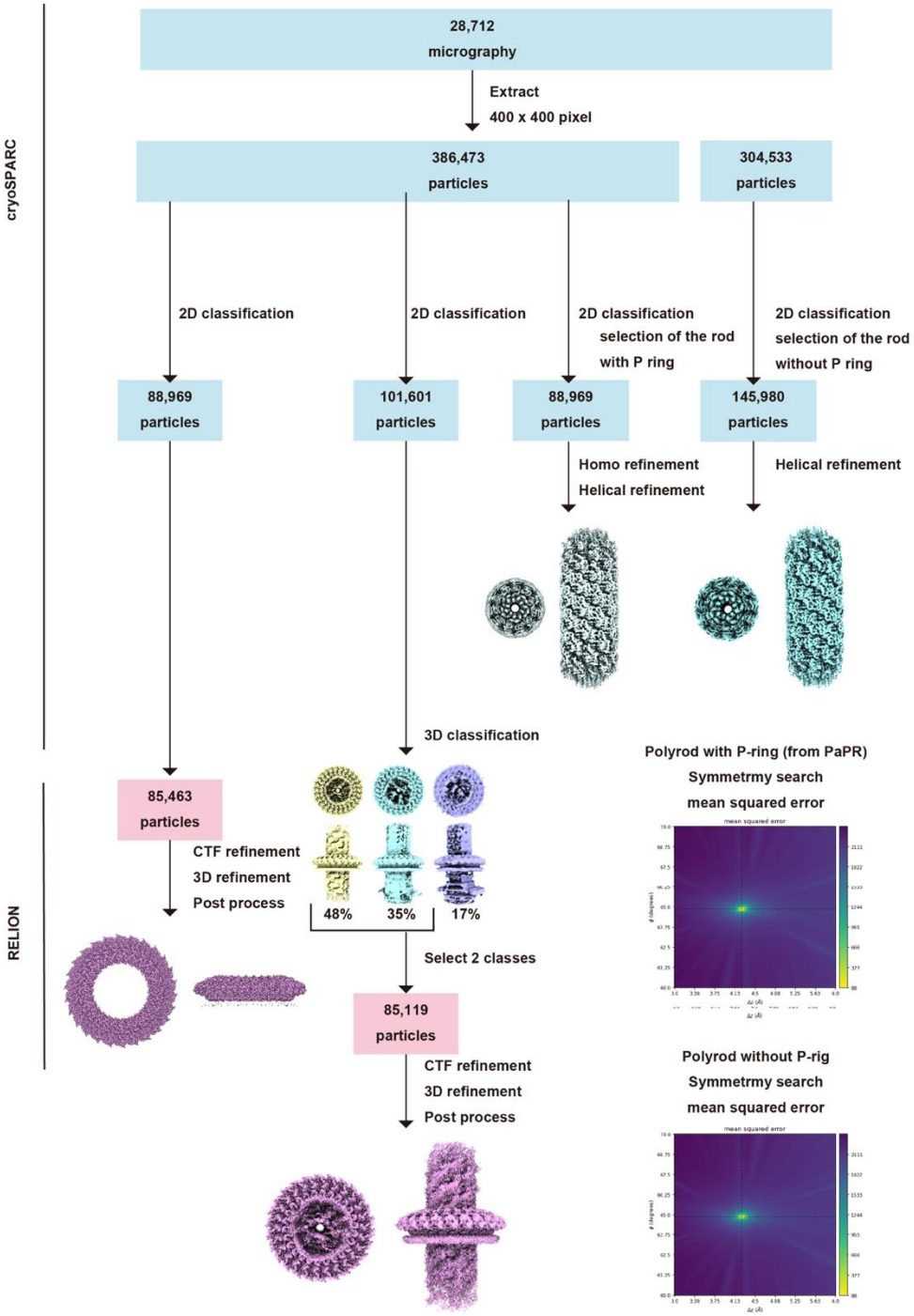
> 5. Generality of the proposed assembly mechanism
In the discussion part, the authors may consider briefly discussing whether similar tilted or elliptical intermediate states could exist in other bacterial species or related rotary systems, and whether this mechanism might represent a general strategy for assembling free-standing bushings around rotating shafts.

We added the following sentence at the bottom of the second last paragraph of Discussion (lines 324 – 327).

Because it is quite tricky to design and generate a free-standing bushing around the rotating drive shaft by self-assembly, it is highly likely that the mechanism of LP-ring assembly we found in this study represents a general strategy used in other bacterial species and related rotary systems.

> 6. Helical Refinement informations
The helical symmetry parameters (64.9°rotation, 4.31 A rise, lines 122-123) determined by the Symmetry Search Utility in cryoSPARC are reported in the text; however, the authors do not present this information in Table 2 or Supplementary Fig. 3. It would be helpful to include the symmetry search results (e.g., a mean-squared-error plot from cryoSPARC) in Supplementary Figure 3 to demonstrate how these parameters were validated. These additions would help readers better understand the helical reconstruction process and facilitate reproduction of the analysis.

Thank you for your advice. We added the mean-squared-error plots from cryoSPARC in Supplementary Fig. 3 and helical parameters in Table 2.



Supplementary Fig.3

> Overall, these points are intended as minor clarifications rather than criticisms. The manuscript is well written, technically rigorous, and makes a substantial contribution to our understanding of bacterial flagellar assembly.

Thank you for your kind comments.