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Architecture of the Flagellar Rotor

Koushik Paul, Gabriela Gonzalez Benet, Alexandrine M Bilwes, Brian R Crane and David Blair

Corresponding author: David Blair, University of Utah

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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.)

Correspondence 11 March 2011

Many thanks for submitting your manuscript EMBOJ-2010-77109. Please let me first apologise for the delay in getting back to you: as I told you, one of the referees was very late in returning his/her report. We have, however, now heard back from all three referees, whose comments are enclosed below.

As you will see, while referee 3 is enthusiastic about the paper, referees 2 and particularly 1 question the extent to which your model can be reconciled with various bits of published literature. Clearly this is a critical issue, and it would be very useful to us if you could prepare a point-by-point response to these reports, so that we can take your responses into consideration in order to make a well-informed decision on your study.

I look forward to hearing back from you.

Best wishes,

Editor
The EMBO Journal

REFeree REPORTS

Referee #1 (Remarks to the Author):

The authors group proposed an interesting structural model of the flagellar rotor a few years ago,

based on the effects of FliG mutations on the FliG-FliM interactions as well as on flagellar assembly and function. Now they solved the crystal structure of a complex of the middle domains of FliG and FliM, and together with the results of crosslinking studies on the interactions of the C-terminal domain of FliG and the middle domain of FliM, they show that the FliM middle domain, which forms the wall of the basal body C ring, binds to both the middle and C-terminal domains of FliG. The results not only confirm their previous model but also give them an opportunity to present an atomic model for the upper part of the C ring, which is thought to play active roles in torque generation and directional switching of motor rotation.

This is certainly an elaborate work, and all the experiments appear to be carefully done. The crystal structure and the crosslinking experiments are both sound, making the model proposal apparently persuasive. There are, however, a few critical points that appear to be unfavorable to the proposed model.

First, if the interactions between the stator and charged residues on the ridge of the FliG C-terminal domain are important for the process of torque generation, as the authors group has strongly put forward, the observations of the nearly equal 26 steps per revolution of the motor by optical measurements are not reconcilable with the model, in which each of the 26 FliG C-terminal domains is positioned on one of the FliM subunits of the C-ring with the 33-fold rotational symmetry. The authors made an excuse statement that the N-M domains of FliG that they assigned to the inner lobe of the C ring may also play an important role in the torque generation step because the inner lobes have the 26-fold symmetry, but this notion has no experimental support.

Second, since the structural model of the C-ring wall proposed previously by the authors group based on their FliM crystal structure is a 2.5 nm thick wall with a 4 nm repeat along the circumference, as depicted in Figure 6, it is rather difficult to believe that FliM forms the mechanically stable C-ring structure as observed by electron microscopy of either negatively-stained or frozen-hydrated specimen. The authors do not seem to have evidence for two distinct interactions of the FliM subunits, either.

Third, the inner lobe is gone in the basal body formed by a FliF-FliG fusion-deletion mutant with deletions of about C-terminal 60 residue of FliF and almost entire N-terminal domain of FliG. This put the FliG middle domain under the M ring, according to the model presented in this paper. Is this reconcilable with the involvement of the FliG middle domain in the torque generation process with 26 steps per revolution?

I understand that the authors are quite confident with the present model, but the crosslink studies are sometime very tricky because quite often non-physiological interactions are indicated. I would suggest the authors to include more clear statements that the present model still has some features that cannot explain some of the previous experimental results on the structure and functions of the flagellar motor. In that sense, the title may be too strong and should be rephrased to suggest that there are still some ambiguities.

Points of minor concern:

1. More labels of residues with their names and numbers should be put on the figures to guide readers in studying the structure. Not only the residue numbers but also the names should be stated in the text, desirably for both *T. maritima* and *E. coli*, to make it easier for readers to follow the descriptions of the model and structure. I suspect that Val-153 and Thr-149 on line 6 of page 10 may be Val-150 and Gln-149, respectively.
2. The distance between residues 117 and 166 in the FliGm-FliGm model in Figure 5B seems to be too far for crosslinking.
3. The cross-eye stereo view is used in the figures, but since the parallel view is more standard and people tend to try parallel view without reading the legend, it would be better to use the latter.
4. The last part of the legend for Supplemental Figure 4 contains a comment that appears to be from one of the authors to another.
5. Subunit clashes are not obvious in Supplemental Figure 6.
6. No explanations are given on the blue and red atoms in Supplemental Figure 9.

Referee #2 (Remarks to the Author):

This manuscript provides data supporting previous models of interaction between bacterial flagellar motor proteins, and incompatible with the models developed from the crystal structure published last year. Overall the manuscript is densely written and takes an effort to follow, but the data are relatively clear, and by having in vivo cross linking in addition to in vitro biophysical analyses are reasonably convincing. That said, it does not mean that the structure presented by Lee et al is wrong. Part of the Introduction and Discussion is directly aimed at the interpretation of their data. In fairness, while that data may have been over-extrapolated to other species, it may well be right for that species about which we know very little and which may not show "normal" swimming behaviour. Rather than suggest their model is not supported by the current data, it may be more accurate to say that there may be more than one correct model, depending on the species, and this model may well be more accurate for proteobacteria.

Specific points.

Page 6. I'm not sure it is strictly accurate to say FliG "functions directly in rotation"-it is not the "active" driving component, although it is the one that goes round, rather it is the passive component pushed by interaction with MotA (or at least that is how I see it)

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Page 8. I think some more detail of the Park et al 2004 paper may help understand what is meant by a surface with specific recognition, but not always associated. It can seem like a bit of a fudge to fit data, but may well be essential for transient interactions in complexes.

Page 10 Fig S3-what does swimming look like when examined microscopically, as I wouldn't say it was "largely" rescued looking at the swim plates

Page 10 Fig S4-there is an odd statement left at the bottom of the legend

Page 11. Bottom-I would suggest changing the last sentence to "subdomains of T.maritima are close to..... and are not in the orientation suggested by the A.aeolicus crystal...." They may both be right!

Page 15. I suggest moving Fig S6 into the main text as these data and fitting are important for the overall interpretation.

Page 15-16. The interpretation that we are looking at a flexible structure rather than a set of rigid rings is important for the overall interpretation of the complete data set and needs emphasising.

Page 16-I am not sure where the paragraph on NMR fits in or what is being interpreted from those data. I think it can be reduced

Referee #3 (Remarks to the Author):

The function of the bacterial flagellum as the motor device for bacterial motility largely depends on the C-ring structure present at the cytoplasmic face of the basal body. The C-ring is important for rotation and switching of the flagellum and consists of multiple copies of the multi-domain proteins FliG, FliM and FliY.

The authors present the crystal structure of the FliG(M)-FliM(M) domain heterodimer which was unknown before. The structure is nicely supported by the biochemical/genetic experiments and provides important insights into the structural arrangement of the full-length FliG and FliM proteins within the C-ring. The present data contradict a recently proposed model for rotor assembly (Lee et al., 2010) which was based on pure crystallographic evidences.

Overall, the referee believes that the manuscript and the data presented are of high quality and fulfill the standards of EMBO J.

Minor remarks:

- Please include a scheme with the domain topologies of FliG, FliM and FliY.
- Fig. 2A . Please label the N- and C-termini of FliG(M) and FliM(M)
- Fig. 2B. Please show the unbiased electron density for the 'EHPQR' and 'GGPG' motifs.

Correspondence

23 March 2011

Referee #1

The authors group proposed an interesting structural model of the flagellar rotor a few years ago, based on the effects of FliG mutations on the FliG-FliM interactions as well as on flagellar assembly and function. Now they solved the crystal structure of a complex of the middle domains of FliG and FliM, and together with the results of crosslinking studies on the interactions of the C-terminal domain of FliG and the middle domain of FliM, they show that the FliM middle domain, which forms the wall of the basal body C ring, binds to both the middle and C-terminal domains of FliG. The results not only confirm their previous model but also give them an opportunity to present an atomic model for the upper part of the C ring, which is thought to play active roles in torque generation and directional switching of motor rotation.

This is certainly an elaborate work, and all the experiments appear to be carefully done. The crystal structure and the crosslinking experiments are both sound, making the model proposal apparently persuasive. There are, however, a few critical points that appear to be unfavorable to the proposed model.

First, if the interactions between the stator and charged residues on the ridge of the FliG C-terminal domain are important for the process of torque generation, as the authors group has strongly put forward, the observations of the nearly equal 26 steps per revolution of the motor by optical measurements are not reconcilable with the model, in which each of the 26 FliG C-terminal domains is positioned on one of the FliM subunits of the C-ring with the 33-fold rotational symmetry.

The reviewer correctly notes that stator-rotor interactions involving the C-terminal domain of FliG have received the greatest emphasis in most previous discussions of the probable motor mechanism. We do not feel that the present model is irreconcilable with the observation of 26 steps per revolution. Indeed, our model specifically allows for 26 units of FliG to contact the stator (unlike the competing model of Stock and coworkers, which has ~34 copies of FliG). Thus, 26 steps are more readily realized in our model than in the competing alternative. The model suggests that the spacing between steps may not be precisely the same, but minor differences in the spacings between successive steps might have escaped detection by the experiments noted. As we suggest in the Discussion, the 26-fold steps can also be rationalized if the stator interacts not only with the FliG C-terminal domain but also with the more "inboard" parts of FliG (i.e., the domains at a more inward radius). Below, the reviewer will indicate concern that evidence for interactions of FliG with inboard FliM subunits have not previously been demonstrated. It is important to realize that mutational studies have not focused as intensively on the more-inboard domains of FliG, in part because interactions involving the C-terminal domain were demonstrated conclusively, focusing attention on that domain. Thus, the absence of evidence for such interactions in the present literature does not constitute evidence against them; it is only now, with the availability of both the full-length FliG structure and a clear working model for the organization of the protein, that we can begin to undertake a more systematic study of this possibility.

Further, we note that there is an observation in the literature that can be taken as support for an involvement of the more-inward parts of the rotor in producing the forces for rotation. The C-terminal parts of the MS-ring protein FliF are known to interact with these more-inward parts of FliG (specifically, with the FliG N-terminal domain). Jenal and co-workers reported that certain mutations in the C-terminal part of FliF give rise to an immotile (but normally flagellated) phenotype (Grunenfelder and others; J. Bacteriology, 2003). Given that the affected FliF segment is in close association with the FliG domains in the more-inward locations, this result fits with the suggestion that these domains (the FliG N-terminal and middle domains) make a contribution to torque generation. If only the C-terminal domain of FliG is involved then the result is harder to explain. We note this result in the revised manuscript, in connection with the proposal that the inner domains of FliG might contribute to rotation.

The authors made an excuse statement that the N-M domains of FliG that they assigned to the inner lobe of the C ring may also play an important role in the torque generation step because the inner lobes have the 26-fold symmetry, but this notion has no experimental support.

As just noted, the absence of evidence specifically in support of this idea does not constitute evidence against it, and the existence of FliF alleles that give paralyzed, flagellate phenotype can be taken as suggestive evidence in support of the proposal.

Second, since the structural model of the C-ring wall proposed previously by the authors group based on their FliM crystal structure is a 2.5 nm thick wall with a 4 nm repeat along the

circumference, as depicted in Figure 6, it is rather difficult to believe that FliM forms the mechanically stable C-ring structure as observed by electron microscopy of either negatively-stained or frozen-hydrated specimen.

If we understand this correctly, the reviewer's concern is that a structure of the kind proposed might not have the mechanical stability indicated by the durability of the EM specimens, or that would presumably be required for function. A close examination of the model indicates that its overall rigidity is likely to be at least as great as that implicit in the simpler, drum-shaped C-ring structures proposed previously. In the present model, simultaneous interaction of certain individual FliG subunits with two different FliM subunits introduces what are in effect triangle-shaped structural elements. The simpler models in which FliM occupies only a single position, in a roughly "picket-fence" kind of arrangement, would allow greater flexibility, especially in the FliG N-terminal and middle domains; in those models, all of the FliG N-terminal and middle domains are suspended between FliF (to which they are attached at an inner radius) and the FliM/FliGc assembly forming the outer part of the ring.

The authors do not seem to have evidence for two distinct interactions of the FliM subunits, either.

We believe that the paper actually does provide clear evidence of the two distinct interactions. One of the interactions of FliM with FliG is characterized in a fairly detailed way, by the co-crystal structure. The other interaction, involving the interaction between FliM and the FliG C-terminal domain, is also characterized fairly extensively, albeit not through full crystal-structure determination. Evidence for this second interaction, and constraints on the relative orientations of the proteins, was obtained by binding experiments, cross-linking experiments, and one instance of inter-genic suppression.

Third, the inner lobe is gone in the basal body formed by a FliF-FliG fusion-deletion mutant with deletions of about C-terminal 60 residue of FliF and almost entire N-terminal domain of FliG. This put the FliG middle domain under the M ring, according to the model presented in this paper. Is this reconcilable with the involvement of the FliG middle domain in the torque generation process with 26 steps per revolution?

We believe that the observations with the FliF-FliG fusion-deletion mutant are compatible with the idea that the more-inboard domains have a role in rotation of the normal motor. The fusion-deletion mutant showed a significant impairment in motility relative to wild-type; further, given the smaller overall size of the basal body in this mutant, we feel that it is almost certain to contain fewer copies of FliM, and thus might adhere to a simpler kind of architecture in which FliG and FliM are present in equal numbers, and interactions with the FliG inner domains (insofar as they are needed to move the stator past the "gaps" in the present architecture) would no longer be needed.

I understand that the authors are quite confident with the present model, but the crosslink studies are sometime very tricky because quite often non-physiological interactions are indicated.

The most-critical cross-linking experiments in the present study were those involving the FliM middle domain and the FliG C-terminal domain. Like the reviewer, we consider it quite important to establish the physiological relevance of the interactions. In the present case, an instance of inter-genic suppression was observed which involves the same pair of positions that gave the strongest cross-linking. Further, the interaction faces identified in the cross-linking experiments are the same as those identified in the protein-binding experiments, and mutations on these faces affect not only the binding interaction but also flagellar assembly.

I would suggest the authors to include more clear statements that the present model still has some features that cannot explain some of the previous experimental results on the structure and functions of the flagellar motor.

As noted in the detailed comments above, we feel that the present model can account for the well-established observations in the literature, and does not give rise to any substantive contradictions.

In that sense, the title may be too strong and should be rephrased to suggest that there are still some ambiguities.

It is true that we are not reporting a high-resolution crystal structure of the entire flagellar rotor, and that the present title might incorrectly give this impression. We have changed the title to "Architecture of the Flagellar Rotor," which we believe is a more accurate reflection of the content of the paper, and its emphasis on overall subunit organization.

Points of minor concern:

1. *More labels of residues with their names and numbers should be put on the figures to guide readers in studying the structure. Not only the residue numbers but also the names should be stated in the text, desirably for both T. maritime and E. coli, to make it easier for readers to follow the descriptions of the model and structure. I suspect that Val-153 and Thr-149 on line 6 of page 10 may be Val-150 and Gln-149, respectively.*

The involvement of residues names and numbers from two species does introduce some complexity. To clarify the matter, we have added a short description line to the Table that summarizes the cross-linking results, indicating explicitly that the amino acid names and numbers are for the *E. coli* protein (because the *E. coli* proteins were the ones used for the cross-linking experiments). A similar statement has been inserted at the point noted by the reviewer in the text, so the reader will know that Val-153 and Thr-149 refer to the residues in this part of the *E. coli* protein. Brief clarification have also been inserted at certain places in the text, to indicate that use of the *E. coli* proteins for the experiments and *E. coli* numbering in the descriptions of the results (pp. 7, 8, 10). *T. maritima* numbering is used in the discussion of the co-crystal structure, which used the *T. maritima* protein, and this is now noted explicitly.

2. *The distance between residues 117 and 166 in the FliGm-FliGm model in Figure 5B seems to be too far for crosslinking.*

Cross-linking between these positions is likely to require some motion of the domains. This was noted in the original study in which the cross-linking was observed (the conclusion was based at that time on considerations of the domain size and the inter-subunit distances). Considerable motion might be expected particularly in this domain of FliG, because it is the FliG domain that (in most positions within the ring) is not directly supported by interactions with underlying FliM subunits.

3. *The cross-eye stereo view is used in the figures, but since the parallel view is more standard and people tend to try parallel view without reading the legend, it would be better to use the latter.*

I have been of the same opinion myself, in the past; however, after using stereo-views more, and introducing them to others, I see as much merit in the cross-eyed representation as in the parallel view. (Students and colleagues tend to have better success with the cross-eye view if one is looking at a computer screen without use of optical aids). If there is a firm editorial policy on this specific point, however, we are happy to re-draw the figures accordingly.

4. *The last part of the legend for Supplemental Figure 4 contains a comment that appears to be from one of the authors to another.*

Yes. The author-to-author query has been removed.

5. *Subunit clashes are not obvious in Supplemental Figure 6.*

An additional panel has been added to Supplemental Figure 6 to make the clashes more apparent.

6. *No explanations are given on the blue and red atoms in Supplemental Figure 9.*

The red and blue coloring indicate the atoms at the termini produced by the (virtual) cleavage of the protein. This is noted in the revised manuscript.

Referee #2

This manuscript provides data supporting previous models of interaction between bacterial flagellar motor proteins, and incompatible with the models developed from the crystal structure published last year. Overall the manuscript is densely written and takes an effort to follow, but the data are relatively clear, and by having in vivo cross-linking in addition to in vitro biophysical analyses are reasonably convincing. That said, it does not mean that the structure presented by Lee et al is wrong. Part of the Introduction and Discussion is directly aimed at the interpretation of their data. In fairness, while that data may have been over-extrapolated to other species, it may well be right for that species about which we know very little and which may not show "normal" swimming behaviour. Rather than suggest their model is not supported by the current data, it may be more accurate to say that there may be more than one correct model, depending on the species, and this model may well be more accurate for proteobacteria.

We have tried to provide a fair and balanced view, and in particular do not wish to make the Lee et

al. model a major focus of the paper, but to put primary emphasis on the model that is proposed here, the data supporting it, and some of its implications. It is important, however, to note that the Lee model was not proposed as a model appropriate for *A. aeolicus*, but as a model for the motor of *E. coli* and *Salmonella*, and thus an appropriate framework for unifying the electron microscopic data, the extensive functional data, and previous structural and biochemical studies. To avoid confusion in the field, we believe that it is important to state clearly that the model of Lee et al. is incompatible with the findings of the present study, as well as previous results obtained in the study of this well-developed paradigm for the flagellum.

One cannot say definitively that an organization like that proposed by Lee et al. does not exist in any species. We do not feel that there are solid reasons for believing that this organization is probable in any species, however. It rests on the assumption that the inter-subunit contact observed in crystals is the same as that occurring in the assembled, functioning motor. Our view is that such an assumption should be tested by experiment, in any case---whether one is working with the actual motor of *A. aeolicus*, or *T. maritima*, or another species.

The similarity of crystal contacts involving the proteins of more than one species is such that one is understandably tempted to assume that it is relevant to the protein in cells. However, as we note in the paper, the surfaces involved are fairly well conserved--though for reasons unrelated to the crystal contact itself--and so having seen the contact in one species we might reasonably expect to see it in others. A further consideration is that the contact might occur in cells, but in the protein prior to its assembly into the motor, as a means of shielding these hydrophobic surfaces and preventing them from engaging in non-native interactions. We mentioned such a possibility (the occurrence of pre-assembly conformations different from the mature arrangement) in the paper, in connection with the NMR results on FliM and FliG. In the revised paper, we have added a brief additional statement, indicating that the FliGm-FliGc crystal contacts might similarly be accounted for in terms of a pre-assembly conformation.

Specific points.

Page 6. I'm not sure it is strictly accurate to say FliG "functions directly in rotation"- it is not the "active" driving component, although it is the one that goes round, rather it is the passive component pushed by interaction with MotA (or at least that is how I see it)

It is true that, at least according to a current model, the stator is the instigator of movement, executing the powerstroke that pushes on the rotor to drive it around. In this sense (and at least according to that model), the rotor is passive. Nevertheless, we suggest that FliG--in its role of directly contacting the stator and being directly pushed on by the stator--can be said to function directly in rotation. If we say that only the mechanically-actuating components are directly involved in rotation, then we would have to say that no part of the rotor is directly involved in rotation, which seems contrary to normal expectation, and somewhat confusing.

Fig2-This figure is really not very clear, even when closely applying the detail in the legend. It needs to be bigger, or have more of the structure present

We weren't sure what aspect of the figure is unclear; the figure was intended as a fairly simple rendering of the overall shape of the complex, with the region of particular interest (the interface) shown in the enlargements. As a space-saving alternative to enlarging the entire figure, we have included in the Supplemental Information (Figure 1) an additional panel, which is like Panel A of Figure 2, except showing the complex in stereo-view. We hope that by providing the interested reader with means of viewing the complex in stereo, the important information regarding the structure will now be more accessible.

Page 8. I think some more detail of the Park et al 2004 paper may help understand what is meant by a surface with specific recognition, but not always associated. It can seem like a bit of a fudge to fit data, but may well be essential for transient interactions in complexes.

Our intention was to point out that the properties of the GM interface (i.e. size, hydrophobic character, surface and charge complementarity) place it in a class of protein-protein interactions where the contact is known to be specific and have a distinct biological function (e.g. in proteins that associate during a signaling process), but that the participating partners are not always in contact as part of their function (contrasting for example an enzyme that is an obligate oligomer). In other words, this is a binary association of medium (μM or lower affinity). We have reworded this sentence to help clarify the comment.

p8. 1st paragraph, last line changed to:

"These parameters reflect an interface of medium affinity (ca $>\mu\text{M}$) often characteristic of binding partners that associate and dissociate as part of their function."

Page 10 Fig S3-what does swimming look like when examined microscopically, as I wouldn't say it was "largely" rescued looking at the swim plates.

Since "largely" could be taken to mean "mostly," and that is not the case, we have replaced "largely" with "substantially." In the microscope, most of the cells do swim, but their motility is less vigorous than the wild type. Their motility is much better than that of either single mutant.

Page 10 Fig S4-there is an odd statement left at the bottom of the legend

Yes; the odd statement was an author-to-author query that had escaped notice, and has been removed.

Page 11. Bottom-I would suggest changing the last sentence to "subdomains of T.maritima are close to.... and are not in the orientation suggested by the A.aeolicus crystal...." They may both be right!

The suggested language seems essentially the same as that in the manuscript; since the intention seems to be to indicate the possibility that there may be inter-species differences, allowing both possibilities to be correct, we have re-written this sentence to read:

"We suggest that the FliG_N and FliG_M subdomains are relatively close in the flagellar motor of *E. coli*, rather than being widely separated as suggested by the crystal-contact based model."

Page 15. I suggest moving Fig S6 into the main text as these data and fitting are important for the overall interpretation.

We feel that the information in Figure S6 is indeed fairly important, as confirmation of the consistency of the model with available information. We do not feel that these images are critical for the interpretation of the results or the overall formulation of the model, however. Given the constraints on space within the article, the complexity of the article as it stands, and the relative ease with which the interested reader can access the information in any case, we have elected to leave the figure in SI.

Page 15-16. The interpretation that we are looking at a flexible structure rather than a set of rigid rings is important for the overall interpretation of the complete data set and needs emphasising.

We feel that the results here can be interpreted in a fairly straightforward way, within the framework of the model proposed, with flexibility required only in certain parts to account for the cross-linking between certain positions in the FliG middle domain (the part that is in the "suspended" position in most cases, and supported directly by FliM in only a minority of the subunits). Global flexibility does not appear to be indicated, or required, and since we do not have means of knowing just how flexible the structure actually is, nor reasons for requiring that it be unusually flexible, we do not feel that further specific comment on these lines is necessary.

Page 16-I am not sure where the paragraph on NMR fits in or what is being interpreted from those data. I think it can be reduced

The NMR experiments give evidence of interactions that are quite different from those proposed here, and that (as noted in the paper) appear incompatible with the arrangement required by the rotor-stator interactions that have been demonstrated. They are, nevertheless, experimental findings, and so we did wish to overlook them but to provide an account of why we might expect to see them even if they do not reflect the final assembled state of the motor. The idea that conformations prior to assembly might differ from those in the motor seems a useful proposal, and since it might also account for the occurrence (and would predict evolutionary selection for) the FliG_M - FliG_C crystal contacts, we have added a phrase to that effect.

Referee #3

The function of the bacterial flagellum as the motor device for bacterial motility largely depends on the C-ring structure present at the cytoplasmic face of the basal body. The C-ring is important for rotation and switching of the flagellum and consists of multiple copies of the multi-domain proteins FliG, FliM and FliY.

The authors present the crystal structure of the FliG(M)-FliM(M) domain heterodimer which was

unknown before. The structure is nicely supported by the biochemical/genetic experiments and provides important insights into the structural arrangement of the full-length FliG and FliM proteins within the C-ring. The present data contradict a recently proposed model for rotor assembly (Lee et al., 2010) which was based on pure crystallographic evidences. Overall, the referee believes that the manuscript and the data presented are of high quality and fulfill the standards of EMBO J.

Minor remarks:

- Please include a scheme with the domain topologies of FliG, FliM and FliY.
- Fig. 2A . Please label the N- and C-termini of FliG(M) and FliM(M)
- Fig. 2B. Please show the unbiased electron density for the 'EHPQR' and 'GGPG' motifs.

The requested figure showing unbiased electron density for these key regions has been added, as an additional panel in Supplemental Figure 1.

1st Editorial Decision

04 April 2011

Please let me apologise again for the delay in the handling of your manuscript, but I have now heard back again from referee 1, to whom I sent your point-by-point response and revised manuscript. He/she finds your responses largely convincing, but still has some concerns as to whether your model is reconcilable with the known step size of the motor. Here are his/her comments:

"The responses by the authors are mostly satisfactory except for the point on the number of steps and the step size. It is not just 26 steps per revolution of the motor that has been detected but it is the angular step size that has been measured by optical nanophotometry. The observed step size is close to 13.8 degree (360/26), not 10.6 degree (360/34). Even if the accuracy of step angle measurement is not sufficiently high enough to distinguish the two cases, published data from several consecutive steps are clearly showing the difference. In order to reconcile the present model with the step size, the authors seem to have no choice other than giving up the role of the FliG C-terminal domain in torque generation and emphasizing the essential involvement of the FliG middle domain. This point should be discussed."

Despite this remaining concern, he/she is overall positive about publishing the manuscript in the EMBO Journal, and I would therefore like to invite you to submit a revised version of your study. The revision you sent earlier addresses most of the concerns of all three reviewers, but it would be important to ensure that the remaining concern of referee 1 is adequately discussed. Please can you therefore revise the manuscript accordingly and submit your new revision formally through our system?

I look forward to receiving the revised version of your manuscript.

Best wishes,

Editor
The EMBO Journal

1st Revision - authors' response

12 April 2011

Thanks again to you and to the reviewers. In response to the comment of reviewer 1, we have made an additional figure, for inclusion in the Supplemental Information, to illustrate that the major interactions can involve the FliG C-terminal domain and that including additional interactions involving the middle domain has the effect of both allowing movement past the gap positions and causing the step size to be close to 1/26th of a revolution, consistent with observation. The text has been rewritten slightly in the concluding paragraphs of the discussion, to indicate that the model is consistent with the step-size observations in the literature, and directing the reader to the new figure.

See also point-by-point response above.

2nd Editorial Decision

12 May 2011

Many thanks for submitting the revised version of your manuscript. I have now heard back from referee 2 (apologies for the delay with this), whose comments are enclosed below; as you know, Referee 1 has already commented on the revision. Referee 2 is satisfied with your responses to the concerns raised in the previous round of review, and so I am pleased to be able to tell you that we can accept your paper for publication. I do just have a few issues from the editorial side first.

Firstly, I saw that Keichi Namba's lab has just published a structural analysis of FliG: I haven't had the chance to read this paper in detail, so I don't know how it relates to your paper. I do, however, think it would be useful to the community if you could cite and briefly discuss this work in relation to yours.

Secondly, I would encourage you to include more of the materials and methods in the main body of the paper rather than in the supplementary information: you are currently well below our length limit (55,000 characters, while your manuscript is ~36,000 characters) and I think it would be useful to the reader if you could expand on the main materials and methods.

Thirdly, we require Conflict of Interest and Author Contributions statements for all published manuscripts; please can you include these?

If you could just make these changes to the text, and resubmit your manuscript, we will then be able to accept it without further delay.

Many thanks and best wishes,

REFEREE REPORT

Referee #2 (Remarks to the Author):

I think the paper is now fine, the experimental work is painstaking and well executed and the model well argued. My only real concerns the first time round were the rather evangelical approach to their model vs alternative models. That is still there, but they argue well in the rebuttal why they need to explain their data in the context of other models.

I think it is important that this paper is published as it provides a testable framework for future work.

2nd Revision - authors' response

17 May 2011

The uploaded version of the manuscript incorporates the changes suggested:

1. The recently published paper of Minamino et al. is discussed at several places in the paper.
2. More complete Experimental Procedures are not part of the body of the paper and have been deleted from Supplemental Information.
3. Author contributions are listed on the cover page.

We hope that with these recommended changes the paper will be suitable for publication. Thank you for your help with the manuscript.