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Structural Diversity of Bacterial Flagellar Motors

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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 19 January 2011

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has now been seen by three referees whose comments are shown below. You will be pleased to see that all three referees are positive about the manuscript and would support publication here after appropriate revision. Still, referee 1 feels strongly that some more experimental work should be included before we can ultimately publish the manuscript. We should therefore be happy to consider a revised manuscript in which the referees' criticisms and suggestions are addressed in an adequate manner. This will need to include some more genetic experimental work along the lines put forward by referee 1.

I should remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript. When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Peer Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html

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

Yours sincerely,

Editor

The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

The manuscript by Chen et al describes the structural differences of bacterial flagellar motors from 11 different species. It is a fascinating comprehensive structural study performed at the edge of current technology by combining high throughput data collection using electron tomography and bioinformatics. The research is well designed and performed and the structures are valuable in order to understand the complexity and variability of motor structures. The paper is overall well written and the figures well designed.

The authors further 'show' the localization of five proteins of the export machinery. Electron tomography in combination with the applied comparative genomics is probably the only and quite elegant method with which this could have been achieved since the export apparatus cannot be co-purified with the motor structure. However, this could (and should) be tested by genetic manipulation. It would thus be interesting to see the motor structure of a genetically manipulated bacterium that either lacks a gene coding for an export protein or expresses a fusion with a large reporter protein of one of these components. This could then provide the direct molecular insight required for publication in EMBO.

Specific comments:

- Whether the spherical density under the torus ring looks like a FliI ATPase hexamer is a testable hypothesis: would it be possible to align the structures of the motor with a mask on this spherical density allowing only small rotational and translational shifts to the particles that have contributed to the overall average. Such an alignment could improve the sharpness of the structure in the ATPase area to see more details.

- The authors and others previously showed different symmetries of separate motor components. Here they only state the 16-fold symmetries for the motors of spirochetes and 13-fold symmetry for H. gracilis. Clearly it is not possible to resolve all the symmetries of all the components. However the authors could indicate the symmetries that they must have reliably identified for some other components as stators.

- Sub-tomogram alignment: the structures seem to have different contrast in different parts of the structure suggesting that a tight mask was used around the components of interest (like C-rings of E. coli or S. enterica). Were all the masks customized or was a spherical mask always used? Please describe in more detail.

Figure 1: excellent figure. However, the quality of the V. cholerae motor could be improved (only 11 subtomograms were averaged for this particular motor).

Figure 2: why mixing up all 11 motors, including those from spirochetes that clearly have a different structure due to the filament not penetrating the outer membrane surface. Averages of sub-groups might be more informative.

Figure 3: stator assignments are not clear: there appears to be a randomly located blob placed in H. neptunium but, despite some densities being at around the same place in E. coli, none are assigned.

Figure 3: unconserved areas indicated in C. crescentus and H. neptunium. Are they noise? Collect more particles to make the point they are not. In contrast, some visible features are not marked as unconserved in H. hepaticus.

Figure 5: nothing new other then the 13-fold symmetry (see comments above), A and B should be at the same plane.

Point out the periplasmic layer(s) and comment on their number

Line 107: morphological and ecological diversity are obvious consequences of phylogenetic diversity. Please rephrase.

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Description of motor components. The authors should consequently cite previous work, where statements they made have been published previously. Currently this is an uneasy mix of statements that appear as if they were made for the first time by the authors but in fact have been reported by others. E.g. the FlgH absence and the FlgI mutant. Statements about the absence of FlgH have been made previously and the FlgI is mentioned almost just in passing as having been observed by Liu et al, who performed an excellent tomography analysis of the motor of this mutant.

General: the use of the term 'electron cryotomography' is irritating, as the term cryo-electron tomography was established first.

Referee #2 (Remarks to the Author):

The survey of flagellar motor structures here is a technically impressive and biologically informative piece of work. Major conclusions concern the nature of the conserved structural core (what it consists of and ways in which its size and shape vary), the probable locations of some proteins that function in flagellar export, and the presence of certain features, mainly in the outer regions of the basal body, that seem to distinguish the motors of phylogenetically diverse organisms. I believe the work represents an important contribution to the field, of quite great interest for the light it sheds on flagellar biology and also because it represents a nice example of what can be accomplished using state-of-the-art microscopy. My comments and concerns are for the most part minor:

1. The discussion of chemotaxis is not entirely accurate in that it represents the running and tumbling responses as basically symmetric, when in fact Berg's work showed that cells respond to increasing attractant or decreasing repellent by prolonging runs, and to the opposite sorts of stimuli--increasing repellent or decreasing repellent--by basically doing little or nothing different from before. Fairly strong repellent stimuli are needed to stimulate tumbling to any great degree.

2. The LPSMC rings are described early on but the T ring is only mentioned fairly late. It might be helpful to introduce this along with the others. It is not present in all species, but neither are the L and P rings.

3. (This is more in the nature of a comment/question than a request for additional information for this paper): All species studied here have an outer membrane (though one was a firmicute). The phylogenetic diversity represented here is certainly very considerable but why wasn't a 'typical" (and well studied) gram positive species such as Bacillus subtilis included?

4. I suspect that some aspects of the comparative sequence analysis/annotation might need to be re-visited. Present indications (work from the lab of Prof. Namba) are that FliJ is very important for flagellar export. An analysis of FliJ sequences shows that it is relatively poorly conserved, apparently functioning in some role that does not strongly constrain its sequence. In view of these considerations, the statement that FliJ is only present in five of the species seems surprising, and evoked a certain amount of suspicion in this reviewer. A closer look (at the supplemental table) shows that V. cholerae is indicated as not having a FliJ, but in fact it does. The same is true for E. coli. And regarding the others, I think it is important to look quite hard for FliJ homologs before stating that they are not present. It may be quite difficult to recognize by sequence similarity, and in cases where there is no synteny it may be hard to locate.

5. Is the low positioning of FliH -the asterisks--consistent with its known binding to FliN?

6. In the discussion of the relatively greater density in the lower part of the C ring in Campy and Helicobacter, it is indicated that whatever is going on, it is likely to involve FliG. What is the basis for this? Couldn't other C-ring proteins be involved, or is it known that FliG is the sole determinant of C-ring morphology?

7. The Y-shaped cross section of the C ring mentioned in the text does not seem to be reflected in the coloring used in figure 3.

8. Also concerning the informatics analysis: It is stated that FliY is found in S. enterica but that is not the case. It is true that Campylobacter has FliY, as stated in the paper, but importantly, it also has FliN. The same is true for helicobacter.
The result is that only one of the species here--T. primitia--has only FliY and no FliN (I am presuming this based on the assumption that T. primitia is like other Treponema species). When both are present, FliY might be a minority contributor, and we might not expect overt structural differences.

9. The assignment of some of the densities to 'stator' seems reasonable, but in the cases of Helicobacter and Campylobacter, I think the assignment should be treated with some caution, given how different they appear. If they really are stator densities, it would seem to indicate a surprisingly great variability in the number and/or organization of stator domains.

Referee #3 (Remarks to the Author):

The manuscript by Chen et al examines the structure of 11 motors from phylogenitically diverse species of bacteria by cryoelectron tomography. Once the structures of all eleven motors were determined, comparisons were made and discussed in detail with respect to motor sub-structure presence, organization, and location. Both similarities and differences were noted, and unassigned protein structures were proposed based on published information and DNA bioinformatic data. The results show that there is conservation of many motor flagellar substructure among the Bacteria. In addition, there was notable variation from one species to another. The work illustrates the power of using cryoelectron tomography in analyzing and comparing structures from diverse organisms.

In general, this is an excellent and exciting manuscript. It is very well written, insightful, and quite timely. Jensen and coworkers are among the leaders in this field, as they were the first to use cryoelctron tomography to study bacterial motors in situ. Based on the work presented by Chen et al, investigators can finally determine how well the flagellar motor structure is conserved in nature, and what type of differences can we expect when we examine specific species.

My specific comments are minor, and are listed below.

1. Page 4, line 73 and 74. Suggest that the word "never" be eliminated. All spirochetes examined to date do protrude their periplasmic flagella in a small fraction of cells (especially in stationary phase). These protruding periplasmic flagella do rotate, so protrusion is not associated with cell death. These protrusions are surrounded by the outer membrane sheath, but it is possible that there are regions on the protruding periplasmic flagella where there is no outer membrane (see Charon et al. J. Bacteriol. 1992. 174: 834-840-especially last paragraph of the results).

2. Page 4, line 73 and 74. The periplasmic flagella influences (or contorts as used here) primarily in domain of the cell where they reside, and in some cases this is not the entire cell as stated. Thus, spirochetes that have short periplasmic flagella influence the shape of the cell only at the cell ends where they reside. (See Charon et al. 1991. J. Bacteriol. 173:4820-4826, Bromley and Charon, J. Bacteriol. 1406-1412. 1979; Berg et al. 1978, Symp. Soc. For Gen Microbiol. 28:285-2294.; Kan and Wolgemuth, Biophys. J.93:54-61. 2007.

3. Page 4, line 91. Suggest adding the words "genetic analyses" after the "biochemical".

4. Page 10, line 218 and line 229. The C ring forming a Y shaped structure is not evident to this reviewer. Please clarify.

5. Page 10, lines 222 and 224 and elsewhere. Suggest adding the words " proposed" or "putative" before FliHIJ and FlhAB complexes. Although the authors propose that these sub-structures are composed of these proteins in the text of the manuscript, it has not proven that this is in fact the case.

6. Page 11, line 318. flbB has been previously identified as a putative motility gene in Borrelia burgdorferi (Ge et al. J. Bacteriol. 1997,197: 2289-2299.) Is the authors proposed flbB gene the same gene as proposed by Ge here?

1st Revision - authors' response 03 May 2011

We were pleased to see that all three referees were positive about the paper. In addition to the several textual suggestions, the main reviewer request was for experimental evidence that the spherical density below the torus is in fact the ATPase FliI, as we had argued. To achieve this we obtained a strain of *C. jejuni* lacking FliI, collected whole-cell tomograms, calculated an average, and showed that the density in question (and none others) was gone. Also as requested, we collected additional cryo-tomograms of *V. cholerae* to improve its average structure. As a result of this extra work, one more author has been added. Please find below detailed responses to all the referees' comments:

Referee #1 (Remarks to the Author):

The manuscript by Chen et al describes the structural differences of bacterial flagellar motors from 11 different species. It is a fascinating comprehensive structural study performed at the edge of current technology by combining high throughput data collection using electron tomography and bioinformatics. The research is well designed and performed and the structures are valuable in order to understand the complexity and variability of motor structures. The paper is overall well written and the figures well designed.

The authors further 'show' the localization of five proteins of the export machinery. Electron tomography in combination with the applied comparative genomics is probably the only and quite elegant method with which this could have been achieved since the export apparatus cannot be co-purified with the motor structure. However, this could (and should) be tested by genetic manipulation. It would thus be interesting to see the motor structure of a genetically manipulated bacterium that either lacks a gene coding for an export protein or expresses a fusion with a large reporter protein of one of these components. This could then provide the direct molecular insight required for publication in EMBO.

Among the organisms imaged, we felt *C. jejuni* was the best for this task since it is genetically tractable and its motor structure clearly exhibits the spherical density below the torus in question (which we propose to be the ATPase FliI). A mutant strain missing fliI was obtained, many cryotomograms of whole cells were recorded, and a new motor structure was produced. While the spherical density below the torus was completely gone, the rest of the motor was unperturbed, showing conclusively that the spherical density is FliI. This result is now presented in the revised Fig. 4D.

Specific comments:

- Whether the spherical density under the torus ring looks like a FliI ATPase hexamer is a testable hypothesis: would it be possible to align the structures of the motor with a mask on this spherical density allowing only small rotational and translational shifts to the particles that have contributed

to the overall average. Such an alignment could improve the sharpness of the structure in the ATPase area to see more details.

While we agree that higher resolution details would help test the hypothesis that the spherical density is FliI, fortunately we were able to prove this unambiguously by imaging a FliI deletion strain of *C. jejuni* (see above). Concerning the possibility that we could achieve a higher resolution structure of this element through masking and special alignment, we note that FliI occurs as both a monomer and a hexamer, it interacts with FliJ and FliH, and its contrast and shape in the various average motors is quite variable. The structure of the FliHIJ complex is therefore probably heterogeneous. Moreover, as a non-essential soluble protein without rigid attachment, the location it occupies is probably inconsistent. This all being said, it is true that if we aligned the tomograms locally about that density, more detail might emerge, but spherical densities are of course difficult to align. For all these reasons we focused instead on imaging the deletion.

- The authors and others previously showed different symmetries of separate motor components. Here they only state the 16-fold symmetries for the motors of spirochetes and 13-fold symmetry for H. gracilis. Clearly it is not possible to resolve all the symmetries of all the components. However the authors could indicate the symmetries that they must have reliably identified for some other components as stators.

We have looked carefully for symmetries in all the motor structures and the ones shown in Fig. 5 are the only ones we could detect. The reasons we can't detect the symmetry of other stators probably include (1) some stators may be smaller and therefore harder to detect, (2) some stators may be more dynamic, becoming blurred in the averages, (3) some stators may have higher symmetry than our resolution allows us to detect, or (4) in the cases of the cells that have single, polar flagella, the rod-shaped cells freeze lying flat on the grid, and so no direct “top” views of the motor were obtained, making stator symmetries harder to detect. The symmetries of other components of the motor (like the C or M rings) are likely too high to be resolved in our tomograms.

- Sub-tomogram alignment: the structures seem to have different contrast in different parts of the structure suggesting that a tight mask was used around the components of interest (like C-rings of E. coli or S. enterica). Were all the masks customized or was a spherical mask always used? Please describe in more detail.

A spherical mask large enough to include all the components of the motor structure was always used for the alignment, with an appropriate missing wedge to prevent orientational biases. One reason the contrast of the C-rings in *E. coli* and *S. enterica* is stronger than those of other bacteria may be that these cells were lysed before imaging, reducing molecular crowding.

Figure 1: excellent figure. However, the quality of the V. cholerae motor could be improved (only 11 subtomograms were averaged for this particular motor).

Thank you. We have now collected more cryo-tomograms of *V. cholerae* to obtain a motor structure of higher quality. The new average includes 16 subtomograms, has a pixel size of 1.9nm and a resolution of 8.3nm using the FSC criterion comparing half-data-sets with a threshold of 0.5. The structure is better defined but the features are unchanged, so the results and conclusions in the text are not changed.

Figure 2: why mixing up all 11 motors, including those from spirochetes that clearly have a different structure due to the filament not penetrating the outer membrane surface. Averages of sub-groups might be more informative.

In contrast to Fig. 1, which highlights the diversity of the motor structures, the purpose of Fig.2 is to show that they all share a conserved core. Thus while the spirochete motors don’t have the L-ring and have larger stators, their P ring (when present in *B. burgdorferi*), MS and C-rings and export apparati all appear in the same relative locations with regard to the rod and the inner

membrane as those of the other species. Readers should look to the averages of the individual motors for the details of their unique features.

Figure 3: stator assignments are not clear: there appears to be a randomly located blob placed in H. neptunium but, despite some densities being at around the same place in E. coli, none are assigned.

Fig.3 is a manual segmentation based on careful visual comparison of the various motors at the present resolution, but must be viewed as an interpretation rather than a set of certain conclusions. The stators in particular (the region we think is being referred to in this comment) range from clear to unclear for reasons such as those listed above (comment about symmetries). That being said, there is a density in the stator position in *H. neptunium* but none at the same position in *E. coli*, so we have left the markings as before. Nevertheless to address this reviewer concern we have modified the figure legend to clarify the interpretive nature of these segmentations.

Figure 3: unconserved areas indicated in C. crescentus and H. neptunium. Are they noise? Collect more particles to make the point they are not. In contrast, some visible features are not marked as unconserved in H. hepaticus.

Thank you for pointing out our inconsistent marking of *H. hepaticus* - this has now been corrected. Concerning adding more particles, in our experience, after a few tens of motors are included in an average, little is gained by adding more, likely because the limiting factors are the conformational heterogeneity of the motors and perhaps the contrast transfer function of the microscope. (We note, for instance, that our reconstruction of the *B. burgdorferi* motor looks remarkably similar to a recently published version that included an order of magnitude more particles (Liu et al., 2009)). Nevertheless to address these uncertainties and this reviewer's concern, we removed the marks in question on the unconserved areas in *C. crescentus* and *H. neptunium*.

Figure 5: nothing new other then the 13-fold symmetry (see comments above), A and B should be at the same plane. Point out the periplasmic layer(s) and comment on their number.

While the 16-fold symmetry of the spirochete motors has been published before (Murphy et al. 2005 and Liu. et al, 2009), we showed them again here alongside the 13-fold symmetry in *H. gracilis* to highlight the diversity seen, which is what this paper is about. Showing our reconstruction of the *B. burgdorferi* motor also allows readers to see how well it matches the previously published reconstructions from other groups. While we had originally deliberately chosen to show different planes through the two spirochete motors, we see the reviewer's point and now agree that it is better to show the same plane in all three motors. The figure has been modified accordingly. We're not sure what the reviewer meant by periplasmic layers, but he/she might have wanted to know how thick the slices shown are - if so, they are 1.9nm thick for *T. primitia*, 1.3nm thick for *B.burgdorferi* and *H. gracilis*. Alternatively, he/she might have wanted to know how many layers (slices) exhibit this symmetry. If so, in *T. primitia* and *B. burgdorferi*, the 16-fold symmetry was visible in several slices through the stator region. In *H. gracilis*, the plane shown is the only axial slice where the 13-fold symmetry is clearly seen.

Line 107: morphological and ecological diversity are obvious consequences of phylogenetic diversity. Please rephrase.

"Morphological","ecological" and "phylogenetic" were deleted and the new sentence is "To this end, we imaged the flagellar motors from 11 phylogenetically diverse bacteria chosen for their general interest as model organisms, involvement in animal host associations or free-living lifestyles, and suitability for electron cryotomography".

Line 121: what is an excellent correspondence? Delete the word 'excellent' or qualify.

Deleted. The new sentence is “Comparison of the *S. enterica* structure with a previous single-particle cryoEM reconstruction (Thomas et al, 2006) exhibited similar features at similar positions along the rod and C-ring (Fig. 2, left panel)”.

Line 177: 1. The hexameric structure of ATPase involved in T3SS export machinery from Pseudomonas was observed by single particle reconstruction and could also be cited here.

We believe the reviewer is referring to Natalie Strynadka's work on EscN (Nat. Struct. Mol. Biol. 2007 Feb;14(2):131-7), which is now referenced.

Line 212: I cannot find a C-ring in H. neptunium. Could the authors comment on this or collect more particles to the 27 that they already have to make the C-ring more visible.

We agree the contrast of the C-ring in *H. neptunium* is very weak. It must be conformationally heterogeneous, present in a small subset of motors, or just simply substantially smaller than the other C-rings. This is part of the diversity of motors we are reporting in this paper. The C-rings with the highest contrast are from *E. coli* and *S. enterica*, perhaps because the cells were gently lysed, reducing background. As discussed earlier, the number of particles beyond these 27 that would be needed to improve the structure substantially is beyond the scope of this work.

Description of motor components. The authors should consequently cite previous work, where statements they made have been published previously.

Currently this is an uneasy mix of statements that appear as if they were made for the first time by the authors but in fact have been reported by others. E.g. the FlgH absence and the FlgI mutant. Statements about the absence of FlgH have been made previously and the FlgI is mentioned almost just in passing as having been observed by Liu et al, who performed an excellent tomography analysis of the motor of this mutant.

More explicit references are now made to the pioneering work of Liu et al. and others, and we also moved this reference in particular up within the paragraph.

General: the use of the term 'electron cryotomography' is irritating, as the term cryo-electron tomography was established first.

While we have a slight preference for "electron cryotomography", we don't feel strongly about this, so if EMBO or the editor has a preference we're happy to change it. Unfortunately our field has not yet reached consensus on the best name. Other terms are also being used, including for instance "cryo-electron microscope tomography" and "cryogenic electron tomography". Years ago I (Jensen) was persuaded by certain leaders in the field to place the "cryo" prefix in front of "tomography" because in the related expression, "electron cryomicroscopy," it is the microscope and its sample that is cooled, not the electrons. This point seems to outweigh others in the minds of general structural biologists and crystallographers in particular, perhaps because in the names of techniques involving X-rays such as "X-ray crystallography", "small-angle X-ray scattering," and "soft X-ray microscopy" for instance, modifiers in front of "X-ray" describe characteristics of the X-rays. The other reason we like putting the "cryo" prefix in front of "tomography" is that it allows us to drop the word "electron" in many sentences and just write "cryotomography" without losing clarity.

Of course for the scientists who first developed cryo-EM (who were not doing what we now mean by "tomography"), it was important to distinguish their approach from traditional EM, and because the abbreviation "EM" was so well established, it seemed best to them to write "cryo-EM". We understand this, and have chosen to use cryo-electron tomography ourselves in other papers, for instance when we were discussing both traditional (room-temperature) EM and cryo-EM work, and the use of the abbreviations EM and cryo-EM conserved words and increased clarity.

Since receiving this reviewer comment, we've talked to others in the field about this and not heard any strong sentiments one way or the other. Most of our colleagues don't seem to care much.

Referee #2 (Remarks to the Author):

The survey of flagellar motor structures here is a technically impressive and biologically informative piece of work. Major conclusions concern the nature of the conserved structural core (what it consists of and ways in which its size and shape vary), the probable locations of some proteins that function in flagellar export, and the presence of certain features, mainly in the outer regions of the basal body, that seem to distinguish the motors of phylogenetically diverse organisms. I believe the work represents an important contribution to the field, of quite great interest for the light it sheds on flagellar biology and also because it represents a nice example of what can be accomplished using state-of-the-art microscopy. My comments and concerns are for the most part minor:

1. The discussion of chemotaxis is not entirely accurate in that it represents the running and tumbling responses as basically symmetric, when in fact Berg's work showed that cells respond to increasing attractant or decreasing repellent by prolonging runs, and to the opposite sorts of stimuli--increasing repellent or decreasing repellent--by basically doing little or nothing different from before. Fairly strong repellent stimuli are needed to stimulate tumbling to any great degree.

The discussion of chemotaxis has been modified so that it now reads "Signals from chemoreceptor arrays (Briegel et al, 2009) modulate the probability of the flagellar motor reversing direction (to spin clockwise) (Hazelbauer et al, 2008), which causes the bacterium to randomly re-orient. Thus, if a bacterium detects that it is swimming towards nutrients, the chemosensory system can prolong its movement in a specific direction."

2. The LPSMC rings are described early on but the T ring is only mentioned fairly late. It might be helpful to introduce this along with the others. It is not present in all species, but neither are the L and P rings.

Within the 11 species we studied, the L-ring is present in 9 species (only missing in the two spirochetes). The P-ring is present in 10 species (only missing in *T. primitia*). The T-ring is present in only one, *V. cholerae*. This is why we deferred the discussion of the T-ring until the "Unconserved densities" section.

3. (This is more in the nature of a comment/question than a request for additional information for this paper): All species studied here have an outer membrane (though one was a firmicute). The phylogenetic diversity represented here is certainly very considerable but why wasn't a 'typical"(and well studied) gram positive species such as Bacillus subtilis included?

Gram positive species tend to be larger and have dense cell walls, which makes obtaining motor structures difficult. Intact *B. subtilis* cells in particular are prohibitively large (1-2 microns thick) for cryotomography.

4. I suspect that some aspects of the comparative sequence analysis/annotation might need to be re-visited. Present indications (work from the lab of Prof. Namba) are that FliJ is very important for flagellar export. An analysis of FliJ sequences shows that it is relatively poorly conserved, apparently functioning in some role that does not strongly constrain its sequence. In view of these considerations, the statement that FliJ is only present in five of the species seems surprising, and evoked a certain amount of suspicion in this reviewer. A closer look (at the supplemental table) shows that V. cholerae is indicated as not having a FliJ, but in fact it does. The same is true for E.

coli. And regarding the others, I think it is important to look quite hard for FliJ homologs before stating that they are not present. It may be quite difficult to recognize by sequence similarity, and in cases where there is no synteny it may be hard to locate.

Thank you for your help on this enigmatic protein FliJ. Spurred on by the reviewer's comments, we have relaxed our criteria and re-annotated the entries in the table for *E. coli* and *V. cholerae*, but still could not detect FliJ-like sequences in *B. burgdorferi, H. neptunium, C. jejuni* or *H. hepaticus* (*H. hepaticus* has protein NP_860484.1, annotated as similar to *C. crescentus* FliJ, but using simple BLAST searches we did not detect similarity). We have also now noted these uncertainties in the text to be sure all readers are made aware.

5. Is the low positioning of FliH -the asterisks--consistent with its known binding to FliN?

The asterisks mark the known FliH-binding site on the FliI structure (Lane, O'Toole and Moore, J Biol Chem. 2006 Jan 6;281(1):508-17. Epub 2005 Oct 31), assuming (as explained in the text) that the C-terminus of FliI faces the membrane. We chose not to discuss the binding of FliH to components of the C-ring such as FliN because corresponding "bridge" densities were not seen in the reconstructions. FliH may shuttle dynamically between the export apparatus and C-ring.

6. In the discussion of the relatively greater density in the lower part of the C ring in Campy and Helicobacter, it is indicated that whatever is going on, it is likely to involve FliG. What is the basis for this? Couldn't other C-ring proteins be involved, or is it known that FliG is the sole determinant of C-ring morphology?

A number of studies have shown that the membrane-proximal part of the C-ring is composed of FliG. It is this area of the C-ring that we are attempting to describe here. We have altered the text to make this more clear and included additional references.

7. The Y-shaped cross section of the C ring mentioned in the text does not seem to be reflected in the coloring used in figure 3.

Yes, the shape of the C-ring near the membrane varies. In some cases it looks like a "Y" (see for instance *H. gracilis*), but in other cases it does not. In light of these variations and the overall uncertainties present, we deleted the reference to a “Y-shape” in the text.

8. Also concerning the informatics analysis: It is stated that FliY is found in S. enterica but that is not the case. It is true that Campylobacter has FliY, as stated in the paper, but importantly, it also has FliN. The same is true for helicobacter. The result is that only one of the species here--T. primitia--has only FliY and no FliN (I am presuming this based on the assumption that T. primitia is like other Treponema species). When both are present, FliY might be a minority contributor, and we might not expect overt structural differences.

We thank the reviewer for pointing out this mistake. Our naive mis-labelling of a Salmonella gene as fliY was due to the presence of another gene labelled fliY involed in cystine transport (e.g., Turner et al, J Bacteriol. 1999, 181(7):2192-8). This error has been fixed in the table. Consistent with the reviewer's and our expectations, no clear differences in C-ring morphology due to the presence of FliY were seen, as described in the text.

9. The assignment of some of the densities to 'stator' seems reasonable, but in the cases of Helicobacter and Campylobacter, I think the assignment should be treated with some caution, given how different they appear. If they really are stator densities, it would seem to indicate a surprisingly great variability in the number and/or organization of stator domains.

Yes - the variability present in the general stator region of the motors surprised us too, and definitely contributed to our decision to use as a title "Structural diversity of bacterial flagellar

motors". As described above, the segmentations shown in Fig. 3 are not certain, but are based on careful comparison of the relative shape and positions of all the densities observed in these motors. Clearly there are additional, as-yet-unidentified proteins located in the periplasms in *H. hepaticus* and *C. jejuni* that account for their unique structures.

Referee #3 (Remarks to the Author):

The manuscript by Chen et al examines the structure of 11 motors from phylogenitically diverse species of bacteria by cryoelectron tomography. Once the structures of all eleven motors were determined, comparisons were made and discussed in detail with respect to motor sub-structure presence, organization, and location. Both similarities and differences were noted, and unassigned protein structures were proposed based on published information and DNA bioinformatic data. The results show that there is conservation of many motor flagellar substructure among the Bacteria. In addition, there was notable variation from one species to another. The work illustrates the power of using cryoelectron tomography in analyzing and comparing structures from diverse organisms.

In general, this is an excellent and exciting manuscript. It is very well written, insightful, and quite timely. Jensen and coworkers are among the leaders in this field, as they were the first to use cryoelctron tomography to study bacterial motors in situ. Based on the work presented by Chen et al, investigators can finally determine how well the flagellar motor structure is conserved in nature, and what type of differences can we expect when we examine specific species. My specific comments are minor, and are listed below.

1. Page 4, line 73 and 74. Suggest that the word "never" be eliminated. All spirochetes examined to date do protrude their periplasmic flagella in a small fraction of cells (especially in stationary phase). These protruding periplasmic flagella do rotate, so protrusion is not associated with cell death. These protrusions are surrounded by the outer membrane sheath, but it is possible that there are regions on the protruding periplasmic flagella where there is no outer membrane (see Charon et al. J. Bacteriol. 1992. 174:834-840-especially last paragraph of the results).

Agreed. The word “never” has been deleted. The sentence is now “The flagella of spirochetes don’t typically pierce the outer membrane, but instead remain in the periplasm where they rotate and/or contort the cell (Kudryashev et al, 2010; Liu et al, 2009; Murphy et al, 2006).”

2. Page 4, line 73 and 74. The periplasmic flagella influences (or contorts as used here) primarily in domain of the cell where they reside, and in some cases this is not the entire cell as stated. Thus, spirochetes that have short periplasmic flagella influence the shape of the cell only at the cell ends where they reside. (See Charon et al. 1991. J. Bacteriol.173:4820-4826, Bromley and Charon, J. Bacteriol. 1406-1412. 1979; Berg et al. 1978, Symp. Soc. For Gen Microbiol. 28:285-2294.; Kan and Wolgemuth, Biophys. J.93:54-61. 2007.

Thank you again. The word “entire” has been deleted (see above).

3. Page 4, line 91. Suggest adding the words "genetic analyses" after the "biochemical".

The words are added and the sentence is now “X-ray crystallography has nevertheless revealed the structures of some of the isolated motor proteins, and complementary biochemical and genetic analyses have located many of them within the intact motor.”

4. Page 10, line 218 and line 229. The C ring forming a Y shaped structure is not evident to this reviewer. Please clarify.

See concern #7 from Reviewer #2 above.

5. Page 10, lines 222 and 224 and elsewhere. Suggest adding the words " proposed" or "putative" before FliHIJ and FlhAB complexes. Although the authors propose that these sub-structures are composed of these proteins in the text of the manuscript, it has not proven that this is in fact the case.

We have now definitively identified the spherical density as FliI by imaging a deletion strain (see above), so those sentences have been left unaltered, but instead of "FlhAB complex", we now use the ambiguous term "export platform" (new Page 11, line 235).

6. Page 11, line 318. flbB has been previously identified as a putative motility gene in Borrelia burgdorferi (Ge et al. J. Bacteriol. 1997,197:2289-2299.) Is the authors proposed flbB gene the same gene as proposed by Ge here?

Yes. Although flbB was previously implicated in the spirochaete flagellum, based on our bioinformatic studies we make the novel proposal here that FlbB is a structural component of the "P-collar". In the revised text we have added the citation for Ge et al., clarified what is novel about our prediction, and emphasized that it is still only speculation.

2nd Editorial Decision 16 May 2011

Thank you for sending us your revised manuscript. Our original referee 1 has now seen it again, and you will be pleased to learn that in his/her view you have addressed all criticisms in a satisfactory manner (see below). Please let me know whether you would like to follow the minor suggestion put forward by the referee. I will then formally accept the manuscript.

Thank you very much again for considering our journal for publication of your work.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

The authors have addressed all issues well by changing the text according to our and the other reviewers requests as appropriate and (most importantly) have conducted a concise experiment that has clarified our main concern. Congrats to a nice piece of work. As for the periplasmic layer: the one (or two) layers, sometimes made from peptidoglycan located in the periplasm - but this is minor. Just wondering if Figure 3 would look nicer if arranged in two rows of 6 panels.

Additional correspondence (author) 17 May 2011

Thanks for this wonderful news. We don't want to make any changes - we really can't say anything confidently about the periplasmic layers, and we proportioned Fig 3 the way we did because we imagined it would be a two-column figure at the top of one published page. Two rows of six panels would make the individual panels too small in our opinion, but we'll be happy to work with the production editor on this at the next stage.

