

Two types of sheathed archaeella structures from *Haloarcula marismortui* differ in their outer layer architectures

Corresponding Author: Professor Matthias Wolf

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)

This paper shows cryo-EM structures of archaeella formed by two large archaeellins, ArlA2 and ArlB, from *Haloarcula marismortui*. Both proteins have extra D3 domain that makes outer sheath. Authors describe structural features and surface properties, and they suggest role in salt and temperature tolerance. The structures are first of this type, and the work is interesting and important. But some structural claims need more evidence, and cryo-EM methods should be described more fully. Also functional conclusions should be written more carefully as most of them are speculations/hypothesis for future work. Below are my specific comments.

Resolution is good for ArlB filament, but not for ArlA2 outer domain. Authors used AlphaFold3 model docking. Can authors explain if they tried focused refinement for ArlA2 D3 region?

If the outer domain of ArlA2 is flexible, please show extra evidence, for example 2D class averages or 3D variability analysis.

Since part of the model is predicted, it is better to say this clearly in the discussion as limitation.

Authors suggest that presence (or absence) of outer domain rotation (ArlB vs. ArlA2) can affect filament waveform and rigidity. Did authors try to look at filament waveform directly (for example micrographs at lower magnification or tomography), and is this any different than other standard size archaeella filament? Furthermore, I couldn't find evidence showing that these filaments are more rigid than other filaments that are made up of smaller proteins.

For ArlB, authors say ASU is tetramer, for ArlA2 it is monomer. Normally, such symmetry is visible in power spectrum. In case of ArlA2, where resolution is low, it would be good to show power spectra of raw filament segments to support the claim.

ArlB has four conformations in the ASU. Does this tell something about supercoiling, like what region of the ArlB protein contributes to the supercoiling? A bit more discussion will help.

For ArlA2 reconstruction, methods are not described. Please add this.

Was 3D classification or heterogeneous refinement tried to reduce heterogeneity and improve resolution?

Authors used SPA without trying the helical reconstruction? Or the helical reconstruction was attempted first?

Discussion connects structural features to adaptation in salt and temperature. These are interesting points, but they are speculation. At least this should be toned down and can be better represented as hypothesis for future work.

Fig. 1: Please label domains D1-D3 for both ArlA2 and ArlB.

Fig. 4: Show residues involved in contacts.

Fig. 5: Arrows for relative orientations of outer domains in four conformers will help clarity.

Abstract, line 23: typo 'of of'

Abstract, line 24: better to say "proteinaceous sheath" at first place to avoid confusion with lipid sheath.

What inter-box distances were used for filament tracing?

Was 3D variability in cryoSPARC tried to check flexibility?

At the end, I think the title can be modified. The current title puts focus on environmental adaptation, but the data shown in the paper are mainly structural. Better to change title to highlight the structural findings, and keep adaptation part only as discussion/hypothesis.

Reviewer #2

(Remarks to the Author)

In this paper, Meshcheryakov and co-authors report on new structures of two archaeella from the archaeal species *Haloarcula marismortui*. The archaeella described show so far unreported proteinaceous sheaths, formed by an insertion of an additional domain in the constituting archaeellin subunits. The two archaeella are ecoparalogs expressed at distinct environmental conditions. The orientations of the sheath-forming D3 domains differ in the two filaments, possibly giving them distinct mechanical properties. This paper should be published, as the data are of high quality and the findings have significant novelty. However, there are various points that should be addressed beforehand.

Major points:

1. When preparing the isolated archaeella for cryoEM, the authors diluted the sample with water. Can they comment on why this was necessary? Also, can they be sure that the structure was not impacted by the non-native buffer?
2. Line 127: The authors suggest that there is flexibility in the connection between the outer sheath and the core of the filament due to limited contacts between the D3 and core archaeellin domains. It would be interesting to show how the sheath and core move relative to each other, as this could provide insights into how the sheath modulates the mechanics of the archaeellum. For instance, the authors could use flexibility analysis to test this.
3. Line 139 – 140: To help the non-expert reader, I suggest that the authors elaborate a bit more on how the same site can coordinate monovalent, as well as divalent ions.
4. Line 145 following: It is unclear from the narrative and Fig. 4 how the D3 domains are rotated with respect to the core of the filament. Please can both be updated to make this clearer?
5. Line 161 following: As above, can you please clarify how the positions of the D3 domains of the ArlB filament relate to the core and how this is different in both filaments? This would best be done with an updated figure 4.
6. Line 176: Please can the authors clearly justify their interpretation that weak D3 dimer interactions would preserve the supercoiling properties of the archaeellum?
7. In line 187 the authors state that the dimer is “rigid”, which appears to contradict the statement above (line 176), as well as previous statements regarding the flexibility of the outer sheath (e.g. line 127). I suggest that the authors reconsider their interpretation and devise a clearer and more consistent discussion regarding these points.
8. Lines 191: The absence of structural data does not infer uniqueness; thus I suggest removing “adding to their uniqueness”.
9. What do the authors mean by “facilitation activities”?

Minor points:

1. Line 23: 2x “of”
2. Line 43: archaeal flagellum archaeellum
3. Line 87 – 91: This paragraph appears to be missing references.
4. Line 104: The authors may also want to reference Fig. 1 here. Please check the figure order; Fig. 2 is referenced before Fig. 1.
5. Line 95: never not
6. Line 113: electron Coulomb
7. Line 114: Also reference Fig 1 here.
8. Line 142: all any
9. Line 152: The authors are referring to Fig. 5, however, there is no such figure. Do they mean Fig. 4?
10. Line 159: electron coulomb
11. Line 170: Not all papers on archaeellum structures are cited, please could you complete the list?
12. Line 201: ArlB of....? Which species are the authors referring to?
13. Line 261: 20%
14. Line 414: Coulomb

Reviewer #3

(Remarks to the Author)

In this manuscript, Meshcheryakov et al. describe the structural basis of motility adaptation in the haloarchaeon *Haloarcula marismortui* by determining the cryo-EM structures of its two archaeellar filaments, ArlA2 and ArlB. Unlike typical archaeella, which are ~10 nm wide, both filaments were found to be nearly twice as thick due to an unprecedented outer sheath surrounding the conserved inner core. The sheath architectures differ: in ArlB, the outer domains rotate 180° to form stable dimers that enhance filament rigidity and create a highly negatively charged surface, possibly favoring function at high salinity. In contrast, ArlA2 filaments lack this rotation and show weaker interdomain contacts, which may lead to greater flexibility and adaptation to lower ionic strength and higher temperatures. These structural differences could help explain how the ecoparalogous archaeellins support survival across the fluctuating conditions of the Dead Sea.

Overall, the manuscript is well-written and easy to follow. However, I have several concerns regarding the cryo-EM analysis that should be addressed:

- (1) Major: The approximate helical symmetry (twist and rise) should be reported. It has been previously reported that archaeal flagella often exhibit a twist of approximately 108°, corresponding to ten protofilaments in a cross-section. This

appears to be the case in Fig. 2, and reporting these parameters would be beneficial to the community.

(2) Major: I am concerned that the symmetry of the ArlA2 filament (Fig. 1A) may not have been determined or applied correctly. The inner core appears to be resolved properly. However, the resolution of the outer domain drops dramatically, which is not common if the alignment were optimal. One possibility is that inner and outer domains have different symmetries, while the reconstruction was driven by the alignment of the inner domains, resulting in misalignment of the outer domains. A way to test this would be to first perform a helical refinement using a smaller box size to estimate the rise and twist. Then, using a slightly larger box, the authors could perform multiple helical refinements with multiples of the initial rise and twist (e.g., 2x, 3x, 4x and so on) to see if the outer domains become better resolved.

Minor points:

(3) Are the filaments supercoiled? As these are flagellar filaments, I would expect them to be. Therefore, it would be valuable to show micrographs, such as negative staining images, raw cryo-EM micrographs, or 2D class averages. This is required during the checklist stage at journals like Nature Communications, anyways.

(4) On line 287, "DeepEMhanser" should be corrected to "DeepEMhancer."

(5) For the benefit of a broader audience, please clarify in the introduction that the term "archaellum" is also called "archaeal flagellum" in some studies. So people outside the community will not be confused.

(6) The manuscript should clarify the rationale for including B-factors and the "proteins/ligands" column in the data table, as well as the map contour level in the legend for Fig. 1. These parameters are often not considered informative in cryo-EM studies, and I think they should be removed unless there is a specific justification.

(7) In lines 123-126, the authors begin numbering the archaellar domains with D1. A schematic diagram would be helpful for reader comprehension. Additionally, since bacterial flagellar domains start with D0, the authors might consider adopting this convention for consistency...

(8) What is the coordination distance between the putative Na⁺ ion and the adjacent oxygen atoms? Sodium and magnesium ions have distinct signature coordination distances (<https://journals.iucr.org/d/issues/2024/05/00/gm5103/index.html>). For example, the Na⁺-O distance is typically ~2.3 Å, whereas the Mg²⁺-O distance is closer to 2.0 Å. Reporting this distance could help confirm the ion's identity.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

Authors have addressed all my concerns. I have no further comments.

Reviewer #2

(Remarks to the Author)

The authors have addressed all my queries and I am now overall happy with the manuscript.

Reviewer #3

(Remarks to the Author)

The authors have addressed most of my points, and I have no further concerns. However, I noticed two items that need to be fixed or tested. I do not need to see the revision again.

(1) Table 2 appears to be blank. Was the wrong table attached?

(2) The outer domain symmetry was not addressed properly. For ArlA2, Figure 4a looks great and seems to exhibit canonical archaeal flagellum symmetry. For ArlB2, a dimer was formed along the right-handed 7-start (Fig 4c); this suggests the symmetry is either double the canonical archaeal flagellum symmetry (no seam) or 14 times the canonical flagellum symmetry (with a seam). If you draw a helical net, it should become obvious whether a seam exists. Either way, this is easy to test in helical refinement by first figuring out the helical symmetry for the inner core. Since you already have a model, you can even do this in Chimera/ChimeraX using mmaker to align one subunit to the adjacent one. Anyways, failing to identify the helical symmetry is embarrassing (I understand you already got the structure without knowing the exact symmetry), and I highly recommend having this addressed.

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REVIEWER COMMENTS

We thank all reviewers for their efforts to provide constructive feedback. This has significantly improved our manuscript.

In particular, we were able to improve all maps using additional refinement, helical reconstructions and local refinement. All atomic models are now derived from experimental data. We have added cryo-EM workflows and 3D variability analysis to better illustrate the structural flexibility of the filament.

An additional SI Figure 3 now shows negatively stained filaments at varying salt concentration, illustrating the stability of our samples at the chosen experimental conditions. This is further supported by salt-concentration-dependent trypsinolysis (SI Fig. 4).

Reviewer #1 (Remarks to the Author):

*This paper shows cryo-EM structures of archaella formed by two large archaellins, ArlA2 and ArlB, from Haloarcula marismortui. Both proteins have extra D3 domain that makes outer sheath. Authors describe structural features and surface properties, and they suggest role in salt and temperature tolerance. The structures are first of this type, and the work is interesting and important. But some structural claims need more evidence, and **cryo-EM methods should be described more fully**. Also functional conclusions should be written more carefully as most of them are speculations/hypothesis for future work. Below are my specific comments. Resolution is good for ArlB filament, but not for ArlA2 outer domain. Authors used AlphaFold3 model docking. Can authors explain if they tried focused refinement for ArlA2 D3 region? If the outer domain of ArlA2 is flexible, please show extra evidence, for example 2D class averages or 3D variability analysis.*

We thank the reviewer for their overall positive evaluation of our manuscript.

We have expanded the cryo-EM methods section (lines 336-355 in the revision).

Some of the claims about functional conclusions have been removed (e.g. about waveform and rigidity) or toned down.

We have performed additional refinements including helical averaging and tried masking and/or signal subtraction of the inner core with the goal to improve the outer domain density of ArlA2. These efforts led to an overall improvement of all maps, and the respective figures have been updated.

We have added 3D variability analysis (lines 356-363 in the revised, SI Fig.10/11, SI Movie 1/2)

Since part of the model is predicted, it is better to say this clearly in the discussion as limitation.

We were able improve the cryo-EM map of the ArlA2 filament. In the revised version both models are now based on experimental maps.

Authors suggest that presence (or absence) of outer domain rotation (ArlB vs. ArlA2) can affect filament waveform and rigidity. Did authors try to look at filament waveform directly

(for example micrographs at lower magnification or tomography), and is this any different than other standard size archaella filament?

The reviewer misunderstood our interpretation. We did not claim anywhere in the manuscript that the presence (or absence) of outer domain rotation (ArlB vs. ArlA2) can affect the filament waveform. The only occurrence in our original text mentioning waveform was related to bacterial flagella (line 262 in the revised MS, at the end of the discussion).

Nevertheless, based on the reviewer's concern, we performed TEM of negative stained samples at low magnification (SI Fig. 3). This analysis revealed no difference in the waveform between each filament type. However, this may be due to the effect of the stain, which does not represent native conditions.

To address the aspect of rigidity, we added a variability analysis of the single particle data in the present revised version (see added movies in FigShare). These movies provide further evidence that the filaments are flexible and can undergo a change in local curvature.

Furthermore, I couldn't find evidence showing that these filaments are more rigid than other filaments that are made up of smaller proteins.

We did not compare the rigidity of these archaella with others consisting of smaller archaellin subunits. We only make statements about the two filament types of *Har. marismortui*.

Figure 4, presented in the article by Pyatibratov et al. (2008), shows a more active movement of the ArlB strain compared to the ArlA2 strain on a semi-solid agar swarm plate. This could be an indirect indication of increased rigidity of the corresponding archaella (assuming that increased archaellum rigidity may lead to more efficient motility). Of course, it would be interesting to conduct such a study but the authors of the manuscript do not have the necessary tools. However, it seems to us that such a comparison will not provide much information, since the rigidity of the archaella of small archaellins can be influenced not only by their size, but also by other factors such as glycosylation, stronger interaction between domains in each specific case *etc.*, and can vary from case to case.

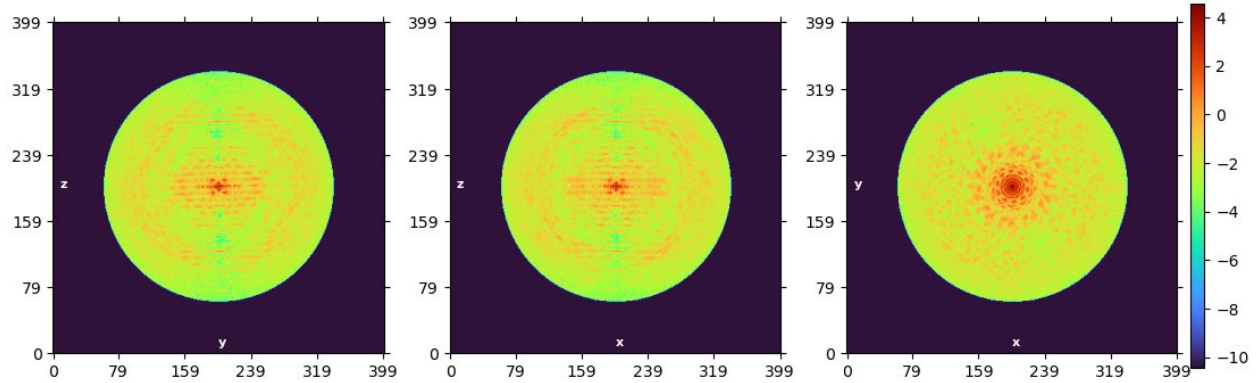
In summary, while we do discuss aspects of filament rigidity in our manuscript, this is a small part of the discussion and clearly designated as our interpretation. Evidence for a tighter interaction between the outer domains of ArlB (more rigid) is provided based on free energy calculations. Our revised manuscript adds movies of our 3D variability analysis, which suggest a larger magnitude of domain motion in ArlA2 (less rigid).

For ArlB, authors say ASU is tetramer, for ArlA2 it is monomer. Normally, such symmetry is visible in power spectrum. In case of ArlA2, where resolution is low, it would be good to show power spectra of raw filament segments to support the claim.

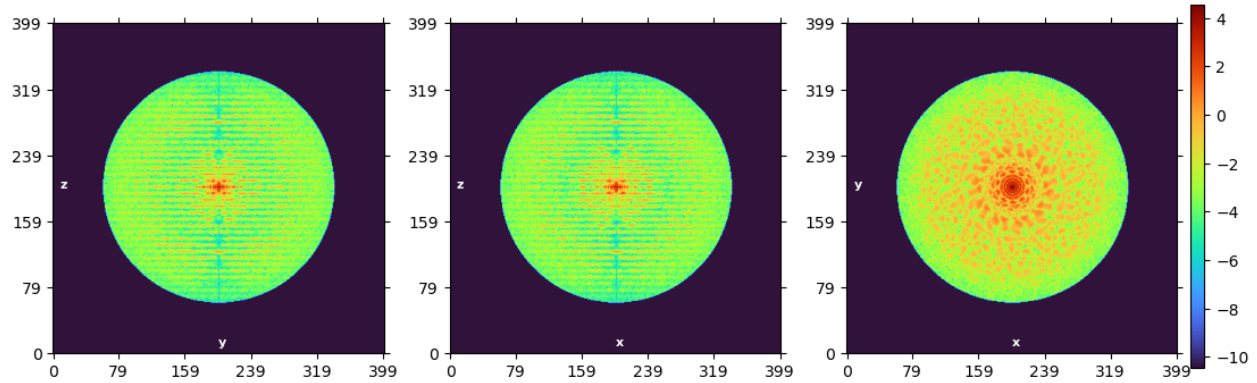
We were able to improve the resolution in ArlA2 (revised Figure 3a), which can now clearly resolve secondary structure.

A comparison of their respective power spectra does not show obvious differences (see below). However, small conformational changes in the outer domains are not expected to drastically change the helical lattice.

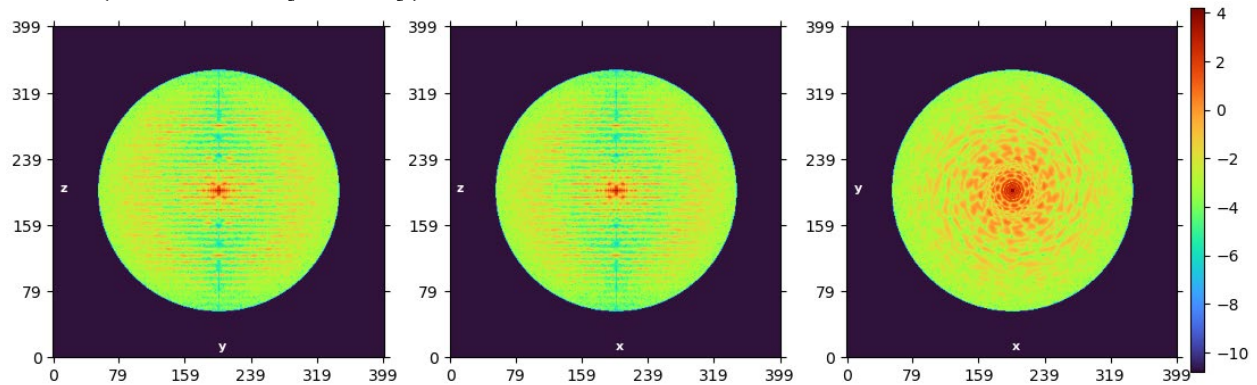
ArlB (no symmetry imposed)



ArlB (with helical symmetry)



ArlA2 (with helical symmetry):



ArlB has four conformations in the ASU. Does this tell something about supercoiling, like what region of the ArlB protein contributes to the supercoiling? A bit more discussion will help.

Using negative-stain TEM and analyzing cryoEM images at low magnification we could not obtain convincing evidence for a change in supercoiling (new SI Fig. 3).

There is not much information on archaeellar supercoiling. To our knowledge the filament wavelength has been reported only for two organisms: *Halobacterium salinarum* ($2.1 \pm 0.2 \mu\text{m}$) and *Saccharolobus islandicus* ($1.5 \mu\text{m}$). Since supercoiling is a complex topic that is influenced not only by the filament structure but also by the properties of the media as well as dynamic effects during swimming, and our structures represent only two among many, we do not feel confident enough to make general conclusions.

For ArlA2 reconstruction, methods are not described. Please add this.

Thank you. Added (line 341-346 in the revised).

Was 3D classification or heterogeneous refinement tried to reduce heterogeneity and improve resolution?

Both heterogeneous refinement and 3D classification were tried, but they did not improve the map.

Authors used SPA without trying the helical reconstruction? Or the helical reconstruction was attempted first?

In the revised manuscript we describe helical reconstruction for ArlA2 filament. We have also tried helical reconstruction for ArlB with all possible helical parameters that were calculated by CryoSPARC symmetry search job. Our analysis included integer multiples of the established rise and rotation parameters to account for an expanded asymmetric unit (as performed by Gambelli, L. et al. An archaeellum filament composed of two alternating subunits. *Nat. Commun.* 13, 710 (2022)). But none of these parameters resulted in reasonable density maps of the outer sheath of ArlB.

Discussion connects structural features to adaptation in salt and temperature. These are interesting points, but they are speculation. At least this should be toned down and can be better represented as hypothesis for future work.

We rephrased our interpretation about these points in the discussion (lines 213-221 in the revised).

The fact that ArlA2 and ArlB can adapt to different environmental conditions has been established. This was demonstrated by comparing the motility of strains that produce two types of archaeella in a semi-solid agar medium at different salinities and temperatures. Additionally, the stability of ArpA2 and ArlB archaeella was assessed using scanning calorimetry and limited

proteolysis (Pyatibratov et al., 2008, Figures 4-6; Syutkin et al., 2014, Fig. 2). Our work offers an interpretation of this observation at the structural level. Each filament type has different surface charges, which are known to be influenced by salt and pH, in other words, environmental conditions.

Fig. 1: Please label domains D1-D3 for both ArlA2 and ArlB.

Done (Figure 1).

Fig. 4: Show residues involved in contacts.

Done (Figure 4b, 4d).

Fig. 5: Arrows for relative orientations of outer domains in four conformers will help clarity.

Done (Figure 5).

Abstract, line 23: typo ‘of of’

Corrected. Thank you.

Abstract, line 24: better to say “proteinaceous sheath” at first place to avoid confusion with lipid sheath.

Done (line 29)

What inter-box distances were used for filament tracing?

Separation distance was 80 Å for both filaments.

Was 3D variability in cryoSPARC tried to check flexibility?

We have done the 3D variability analysis and added these results to the revised version (Supplementary Figures 10 and 11, Supplementary Movies 1 - 4).

At the end, I think the title can be modified. The current title puts focus on environmental adaptation, but the data shown in the paper are mainly structural. Better to change title to highlight the structural findings, and keep adaptation part only as discussion/hypothesis.

Thank you for this suggestion. We have changed the title accordingly to “Two types of sheathed archaellum structures from *Haloarcula marismortui* differ in their outer layer architectures”

Reviewer #2 (Remarks to the Author):

*In this paper, Meshcheryakov and co-authors report on new structures of two archaeella from the archaeal species *Haloarcula marismortui*. The archaeella described show so-far unreported proteinaceous sheaths, formed by an insertion of an additional domain in the constituting archaeellin subunits. The two archaeella are ecoparalogs expressed at distinct environmental conditions. The orientations of the sheath-forming D3 domains differ in the two filaments, possibly giving them distinct mechanical properties. This paper should be published, as the data are of high quality and the findings have significant novelty. However, there are various points that should be addressed beforehand.*

Major points:

1. When preparing the isolated archaeella for cryoEM, the authors diluted the sample with water. Can they comment on why this was necessary? Also, can they be sure that the structure was not impacted by the non-native buffer?

Both samples were diluted with salt-free Tris-HCl buffer not water. This omission was corrected in the methods (line 126 in the revised; line 320). Upon twofold dilution with sodium-free buffer, the filaments and the tertiary structure of their subunits remain intact. This is supported by data from EM (new SI Fig. 3) and limited proteolysis (new SI Fig. 4), which shows that the subunits within the archaeellum maintain stability, only losing it when the NaCl concentration falls below 1.4 M or after being heated to 90° Celsius in 3.4 M NaCl. To reduce background noise, samples must be diluted before plunging. For example, in the study of archaeella from haloarchaea *Halobacterium salinarum* by Sofer et al. (2024), the archaeellar solution was diluted four times with water, reducing the NaCl concentration from 4M to 1M.

2. Line 127: The authors suggest that there is flexibility in the connection between the outer sheath and the core of the filament due to limited contacts between the D3 and core archaeellin domains. It would be interesting to show how the sheath and core move relative to each other, as this could provide insights into how the sheath modulates the mechanics of the archaeellum. For instance, the authors could use flexibility analysis to test this.

Thank you for this suggestion. We have done 3D variability analysis and added the result to the main text (lines 182-192 in the revised; Supplementary Figures 10 and 11, Supplementary Movies 1-4).

3. Line 139 – 140: To help the non-expert reader, I suggest that the authors elaborate a bit more on how the same site can coordinate monovalent, as well as divalent ions.

This part of the text has been revised (lines 148-157 in the revision).

4. Line 145 following: It is unclear from the narrative and Fig. 4 how the D3 domains are

rotated with respect to the core of the filament. Please can both be updated to make this clearer?

Thank you for pointing this out. We have updated Fig 4 for better clarity and adjusted the referring narrative.

5. Line 161 following: As above, can you please clarify how the positions of the D3 domains of the ArlB filament relate to the core and how this is different in both filaments? This would best be done with an updated figure 4.

Done (Figure 4).

6. Line 176: Please can the authors clearly justify their interpretation that weak D3 dimer interactions would preserve the supercoiling properties of the archaellum?

Although supercoiling of archaellum is unexplored, one of the current ideas suggests that supercoiling of the archaellum is supported by continuous motion of individual archaellins. In this case, too rigid filament structure might prevent subunit motion and affect supercoiling. We have added a sentence to express this more clearly (lines 203-207 in the revision).

7. In line 187 the authors state that the dimer is “rigid”, which appears to contradict the statement above (line 176), as well as previous statements regarding the flexibility of the outer sheath (e.g. line 127). I suggest that the authors reconsider their interpretation and devise a clearer and more consistent discussion regarding these points.

Thank for pointing out this contradiction. We have imaged both filaments by negative stain TEM at low magnification (SI Fig. 3). However, we could not conclusively find any differences in rigidity. We have therefore removed or toned down claims about rigidity from our manuscript.

8. Lines 191: The absence of structural data does not infer uniqueness; thus I suggest removing “adding to their uniqueness”.

Thank you. Done

9. What do the authors mean by “facilitation activities”?

We replaced “facilitation activities” with “auxiliary functionality”.

Minor points:

1. Line 23: 2x “of”

Done

2. Line 43: archaeal flagellum $\diamond$ archaellum

Done

3. Line 87 – 91: This paragraph appears to be missing references.

Added

4. Line 104: The authors may also want to reference Fig. 1 here. Please check the figure order; Fig. 2 is referenced before Fig. 1.

Thank you for pointing this out. The order of Figures references was revised.

5. Line 95: never $\diamond$ not

Done

6. Line 113: electron $\diamond$ Coulomb

Done

7. Line 114: Also reference Fig 1 here.

8. Line 142: all $\diamond$ any

Done

9. Line 152: The authors are referring to Fig. 5, however, there is no such figure. Do they mean Fig. 4?

There are 5 figures.

10. Line 159: electron $\diamond$ coulomb

11. Line 170: Not all papers on archaellum structures are cited, please could you complete the list?

Thank you. Four additional references were added.

12. Line 201: ArlB of....? Which species are the authors referring to?

“Har. marismortui” was added after “ArlB”.

13. Line 261: 20%

Done

14. Line 414: Coulomb

Done

Reviewer #3 (Remarks to the Author):

In this manuscripts, Meshcheryakov et al. describe the structural basis of motility adaptation in the haloarchaeon *Haloarcula marismortui* by determining the cryo-EM structures of its two archaeellar filaments, ArlA2 and ArlB. Unlike typical archaeella, which are ~10 nm wide, both filaments were found to be nearly twice as thick due to an unprecedented outer sheath surrounding the conserved inner core. The sheath architectures differ: in ArlB, the outer domains rotate 180° to form stable dimers that enhance filament rigidity and create a highly negatively charged surface, possibly favoring function at high salinity. In contrast, ArlA2 filaments lack this rotation and show weaker interdomain contacts, which may lead to greater flexibility and adaptation to lower ionic strength and higher temperatures. These structural differences could help explain how the ecoparalogous archaeellins support survival across the fluctuating conditions of the Dead Sea.

Overall, the manuscript is well-written and easy to follow. However, I have several concerns regarding the cryo-EM analysis that should be addressed:

(1) Major: The approximate helical symmetry (twist and rise) should be reported. It has been previously reported that archaeal flagella often exhibit a twist of approximately 108°, corresponding to ten protofilaments in a cross-section. This appears to be the case in Fig. 2, and reporting these parameters would be beneficial to the community.

Thank you for this comment. We have added our helical analysis and listed the refined helical parameters for both ArlA2 and ArlB filaments in the main text and methods. Indeed, they are close to 108°.

(2) Major: I am concerned that the symmetry of the ArlA2 filament (Fig. 1A) may not have been determined or applied correctly. The inner core appears to be resolved properly. However, the resolution of the outer domain drops dramatically, which is not common if the alignment were optimal. One possibility is that inner and outer domains have different symmetries, while the reconstruction was driven by the alignment of the inner domains, resulting in misalignment of the outer domains. A way to test this would be to first perform a helical refinement using a smaller box size to estimate the rise and twist. Then, using a slightly larger box, the authors could perform multiple helical refinements with multiples of the initial rise and twist (e.g., 2x, 3x, 4x and so on) to see if the outer domains become better resolved.

Thank you for this practical suggestion.

We have tested different helical parameters for ArlA2. However, the best resolution was obtained with the “classical” archaellum parameters: 5.54 Å rise and 108.19° twist. These parameters were used for further symmetry expansion and local refinement of the outer sheath. Our analysis included integer multiples of the established rise and rotation parameters to account for an expanded asymmetric unit (as performed by Gambelli, L. et al. An archaellum filament composed of two alternating subunits. *Nat. Commun.* 13, 710 (2022)), but none of these parameters resulted in reasonable density maps of the outer sheath of ArlB.

Masking the inner core during the reconstruction with a cylindrical mask (and also alternatively signal-subtracting the inner domains) resulted in slight improvement. We conclude that the superhelical curvature of the filament causes sufficient variability in the local conformation of the outer domains of ArlA2 to blur their density upon helical averaging. This effect is less pronounced in ArlB, whose outer domains are pairwise linked, resulting in a better ordered filament assembly.

Thanks to these additional refinement efforts, the resolutions of all maps have improved overall, facilitating and adding confidence to our original interpretation. We present updated maps of the outer domains in Figure 3, which is now resolving secondary structural features of ArlA2 that match the AlphaFold model with high fidelity. We are therefore confident that our reconstruction reflects the structure correctly.

Minor points:

(3) Are the filaments supercoiled? As these are flagellar filaments, I would expect them to be. Therefore, it would be valuable to show micrographs, such as negative staining images, raw cryo-EM micrographs, or 2D class averages. This is required during the checklist stage at journals like Nature Communications, anyways.

Yes, they are. We added uncropped cryo-EM images showing long curved filaments (SI Fig 3a, SI Fig. 4a), and 2D class average images to Supplementary Figure 3.

(4) On line 287, "DeepEMhanser" should be corrected to "DeepEMhancer."

Done

(5) For the benefit of a broader audience, please clarify in the introduction that the term "archaellum" is also called "archaeal flagellum" in some studies. So people outside the community will not be confused.

Done. Thank you.

(6) The manuscript should clarify the rationale for including B-factors and the "proteins/ligands" column in the data table, as well as the map contour level in the legend for Fig. 1. These parameters are often not considered informative in cryo-EM studies, and I think they should be removed unless there is a specific justification.

We agree with the reviewer. These parameters have been removed.

(7) In lines 123-126, the authors begin numbering the archaellar domains with D1. A schematic diagram would be helpful for reader comprehension. Additionally, since bacterial flagellar domains start with D0, the authors might consider adopting this convention for consistency...

Thank you for the suggestion. We have revised the domain numbering starting from D0. There is a schematic diagram (Fig. 2a). We also added the domains positions to the ArlA2 and ArlB sequences in Supplementary Fig. 2.

(8) What is the coordination distance between the putative Na⁺ ion and the adjacent oxygen atoms? Sodium and magnesium ions have distinct signature coordination distances (<https://journals.iucr.org/d/issues/2024/05/00/gm5103/index.html>). For example, the Na⁺-O distance is typically ~2.3 Å, whereas the Mg²⁺-O distance is closer to 2.0 Å. Reporting this distance could help confirm the ion's identity.

This part of the text has been revised (lines 148-157). The average Me-O distance is about 2.25 Å.

Point by point response

We thank all the reviewers for their time and careful review.

Reviewer #1 (Remarks to the Author):

Authors have addressed all my concerns. I have no further comments.

Reviewer #2 (Remarks to the Author):

The authors have addressed all my queries and I am now overall happy with the manuscript.

Reviewer #3 (Remarks to the Author):

The authors have addressed most of my points, and I have no further concerns. However, I noticed two items that need to be fixed or tested. I do not need to see the revision again.

(1) Table 2 appears to be blank. Was the wrong table attached?

The table is now attached.

(2) The outer domain symmetry was not addressed properly. For ArlA2, Figure 4a looks great and seems to exhibit canonical archaeal flagellum symmetry. For ArlB2, a dimer was formed along the right-handed 7-start (Fig 4c); this suggests the symmetry is either double the canonical archaeal flagellum symmetry (no seam) or 14 times the canonical flagellum symmetry (with a seam). If you draw a helical net, it should become obvious whether a seam exists. Either way, this is easy to test in helical refinement by first figuring out the helical symmetry for the inner core. Since you already have a model, you can even do this in Chimera/ChimeraX using mmaker to align one subunit to the adjacent one. Anyways, failing to identify the helical symmetry is embarrassing (I understand you already got the structure without knowing the exact symmetry), and I highly recommend having this addressed.

We thank the reviewer for their detailed recommendation.

In this final revision, we added the identified helical parameters of ArlB to the manuscript (line 125). Comparison of the reconstruction with and without helical symmetry imposed revealed no noticeable differences.