

Ultrastructural and atomic characterization of biofilm-associated extracellular filaments in *Shewanella oneidensis* MR-1

Corresponding Author: Professor Cong Liu

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)

The Authors use cryo-EM and cryo-ET to characterize the nano-scale filamentous structures that are formed by *Shewanella oneidensis* biofilms. Overall, I think the study is well done and the results presented would be of interest to different fields. The Authors characterize different sized filamentous structures and identify three of them. They then apply known protein subunits and model how those units would fit together to form the shape of the structures they observe under cryo-ET with relatively good fits. The conclusions laid out in the manuscript are logical based on previous reports and are supported by the data. I think there needs to be some additional discussion to contextualize the results and more clearly articulate why knowing this structural information is important to achieve better strains of *Shewanella* for bioenergy and environmental technologies, which is the stated goal of the Authors. My specific comments are below.

Major Comments:

There have been other studies that have used cryoEM and cryoET to characterize different filaments, both with intact cells and purified filament preparations (doi.org/10.1038/s41467-020-15650-w; doi.org/10.1038/s41467-022-35164-x). The Authors state that this is the first study that uses these tools to identify an MshA and PilA from *Shewanella*, but I do not see the importance of gaining this structural information for these particular filaments laid out in the discussion. Please expand on this to better contextualize the results. We know from previous work that these filaments are important for biofilm formation and electron transfer (in coordination with cytochromes) and we know that oxygen depletion increases the presence of these filaments (doi.org/10.1128/AEM.01615-16) in a biofilm. How will the results here in deriving a structure for these filaments advance the knowledge of the field? Is there something about the difference of the filament types under the different conditions that would guide us toward harnessing these cells more effectively?

What controls were run to confirm that the filaments were what they are hypothesized to be? e.g., were any pilA or mshA mutants tested to see if these structures disappeared as expected? Or could planktonic cells drop cast onto a microgrid and frozen to assess non-biofilm cells?

Regarding the discussion of the conductive properties of the filaments elucidated via AFM (around L265), is it possible that the conductivity of the filaments is affected by the absence of an electrochemical driving force on the growth substrate (i.e. the cells are not grown on a poised electrode)?

Regarding the putative eDNA filament conductivity results/discussion, there is precedence for eDNA enabling electron transfer in other organisms that produce redox shuttles (see: doi.org/10.1016/j.cell.2020.07.006). *S. oneidensis* also produces redox active compounds, could a similar mechanism be at play here?

L367: Throughout the materials and methods, there needs to be some description of how many microgrids were used for each analysis and how many separate images were used for the analysis. I am currently left wondering if this work was all done on one image from one grid per condition. Table 1 says 40 frames, but are all frames from 1 grid? My question stems from biological variability. If you inoculated another serum bottle with the same conditions, would you see the same thing?

Minor Comments:

Were the grids dehydrated before freezing? I think not based on how I am reading the methods, but please be explicit here

as that can cause structural changes in samples.

L124: "data collection was focused on cell-free regions of the grids enriched in extracellular material". I understand the reason for doing this. How far from the cells were these measurements taken?

L311: Small typo: "we the first atomic-resolution structure...".

L367: Worded confusingly. Makes it sound like the cells were added to the serum bottle and then autoclaved.

L369: I am not understanding the experimental setup here. Are the serum bottles the reactors? Are they open to the atmosphere in the InvivoO2 workstation? Was there any agitation or were the bottles stagnant?

Figure 1: I would like to see the raw image without all of the colored traces, especially for B and C. I understand why the Authors chose to include the colored lines to point out the filament, but seeing both the raw and processed images side by side would be valuable.

I understand why the Authors are labelling the unidentified structure as eDNA based on the modelling work, but I would be more conservative and say "putative eDNA" or something to that effect since I did not see other evidence presented that it was eDNA.

Reviewer #2

(Remarks to the Author)

Dear authors,

Your study provides valuable structural insights into *Shewanella oneidensis* MR-1 biofilms, notably delivering the first high-resolution structure of an MSHA-type pilus and native-context models of PiliA pili and flagella. The combination of cryo-EM, cryo-ET, and functional assays is impressive and advances the field significantly.

However, several points could strengthen your manuscript:

- The identity and function of the ~2 nm flexible filaments and the newly observed non-periodic filaments under anaerobic conditions remain unclear. Additional biochemical or genetic validation is needed to better characterize these components.
- The multimeric protein aggregates in the biofilm matrix were resolved only at limited resolution, preventing definitive identification. Higher-resolution structural analysis or complementary biochemical studies would improve understanding of their.
- While your structural data and conductivity assays argue against pili-mediated electron conduction, clarifying these findings and discussing alternative electron transfer mechanisms in more depth would benefit readers and avoid confusion given prior literature.
- Functional correlations between structure and biofilm physiology are currently limited. Consider integrating or discussing potential genetic or mutational experiments to link filament structure with biofilm formation and activity.
- The biofilms were grown under controlled lab conditions on EM grids. A brief discussion addressing how these conditions might differ from natural environments and the possible impact on filament expression or biofilm architecture would add valuable context.
- Figure 1's heavy overlay of segmentation colors obscures underlying structural details; alternative visualization strategies could improve clarity.
- Emphasizing current limitations in functional assignment of unidentified structures will help prevent overinterpretation of preliminary data.

Overall, the methodology and data analysis are sound, and your findings merit publication after minor revisions addressing these points. These enhancements would increase the clarity, biological relevance, and impact of your work.

Thank you for your important contribution.

Reviewer #3

(Remarks to the Author)

Li et al., Ultrastructural and atomic characterization of biofilm-associated extracellular filaments in *Shewanella oneidensis* MR-1

The authors present a structural study by cryo-ET into the extracellular matrix of biofilm-producing bacteria (*Shewanella*

oneidensis). They find several types of filamentous structures which they analysed in detail on high-resolution 3-D structures obtained by cryo-ET rendering (on whole cells) and cryo-EM 3D-averaging. However, for obvious reasons, the cryo-EM studies were conducted on cell-free areas with enriched filamentous material. They claim that the surface structures and electrostatic patterns of these extracellular filaments play a crucial role in stabilizing biofilms, and in metal reduction, which may have biomedical implications. Their study on oxygen limitation is remarkable, though, figure 5 is hard to interpret for an outside reader, esp. the micrographs.

Overall, this is a well-executed study on extracellular matrix of bacteria in biofilms. I cannot see any major flaws with the methods applied here. These biofilms are indeed a current topic of many research labs (including labs dealing with space flight!), and their implications in our life are not well studied yet. The novelty aspect of this work is a bit overrated. E.g., bacterial flagella and pili have been studied since quite some time, but typically in isolation and not within the context of a bacterial biofilm. Hence, I believe this paper deserves publication after some revision.

Some comments:

One of the big issues always with cellular cryo-ET is the correct identification of densities in the micrographs. Hence, I am not always fully convinced that the structures indicated (e.g., Fig. 5) are indeed what the authors want to claim, or if these are simply unidentified impurities (e.g., from lysed cells). This is much less of an issue with isolated material, or the detached filaments discussed in figure 2.

The authors conducted AFM studies, but they are not presented in the main text, only in the supplementary data. The point of supplementary data is to further explain what has been shown in the main text, NOT to add additional data of a completely different kind since they base a good portion of their conclusion on these measurements, discussed in the results. This may reflect my own antiquated view, but, nevertheless, I would like to see an AFM figure in the main text.

Figures:

The figures are well made and informative. I would add a side-by-side image of areas with and without rendering (esp. figure 1). Currently, the rendering hides the actual structures underneath.

As already mentioned above, figure 5E is essentially asking the reader to believe what the authors claim. In my opinion, there is no way to distinguish octamers from tetramers or even other particles. This panel has to be presented at a much larger scale.

Figure 6 is mostly imagination and guess-work, at best representing a single momentary shot. It should be accompanied by an overview micrograph where these biofilm arrangement can be seen unobstructed.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

Overall, the Authors addressed all of the comments I had and added significant depth to the discussion in order to contextualize the results. I only have one minor comment outstanding and would recommend publication of this work.

In Figure 1B and C, my impression from the response to comments is that the overlay would be removed and put next to the image so that the raw structure in the images could be observed. That does not seem to have happened here.

Reviewer #2

(Remarks to the Author)

Dear Authors,

Thank you for your thorough and careful revisions. I have reviewed the revised manuscript and detailed responses to my comments. The additional analyses and new contextual discussion significantly enhance the clarity, depth, and balance of the work.

Regarding the ~2 nm and non-periodic filaments (Q1): I appreciate the inclusion of comparative LC-MS/MS analysis and the clear explanation of methodological limits. Your cautious treatment of these filaments as unidentified structures pending biochemical confirmation is appropriate. The explicit plan for DNase/protease assays and genetic tagging provides confidence that future studies will resolve their nature.

On the multimeric aggregates (Q2): The expanded subtomogram averaging workflow, added figures, and integration with proteomics present a well-substantiated, transparent analysis. The tentative identification of the E2 component of the 2-oxoglutarate dehydrogenase complex is reasonable and clearly labeled as a working hypothesis.

On pili conductivity and alternative EET routes (Q3): The clarified scope of conclusions and expanded discussion now align well with current understanding and prevent confusion with earlier nanowire literature. The inclusion of possible eDNA-mediated shuttle retention enriches the discussion of extracellular electron transfer.

On functional correlations (Q4): The new comparative data from filament-deletion mutants convincingly connect structural

attributes with physiological behavior. These results add an important dimension to the study.

On environmental context (Q5): The newly added commentary thoughtfully situates the grid-based biofilm model relative to natural settings and outlines pertinent next steps.

On visualization (Q6) and interpretative caution (Q7): The new supplementary figures and explicit statements about current limitations greatly improve readability and interpretive balance.

Overall, the manuscript is now substantially strengthened and achieves an excellent balance between technical rigor and biological relevance. I commend the authors for their comprehensive revisions and careful phrasing, which make the study both accessible and scientifically robust.

I recommend acceptance pending final editorial checks.

With best regards,

Open Access This Peer Review File is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made.

In cases where reviewers are anonymous, credit should be given to 'Anonymous Referee' and the source.

The images or other third party material in this Peer Review File are included in the article's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder.

To view a copy of this license, visit <https://creativecommons.org/licenses/by/4.0/>

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

* The referees' comments are in *Italic*. Author's responses are in blue. All revisions are highlighted in red in the revised manuscripts.

Reviewer(s)' Comments to Author:

Reviewer #1:

Remarks to the Author

The Authors use cryo-EM and cryo-ET to characterize the nano-scale filamentous structures that are formed by *Shewanella oneidensis* biofilms. Overall, I think the study is well done and the results presented would be of interest to different fields. The Authors characterize different sized filamentous structures and identify three of them. They then apply known protein subunits and model how those units would fit together to form the shape of the structures they observe under cryo-ET with relatively good fits. The conclusions laid out in the manuscript are logical based on previous reports and are supported by the data. I think there needs to be some additional discussion to contextualize the results and more clearly articulate why knowing this structural information is important to achieve better strains of *Shewanella* for bioenergy and environmental technologies, which is the stated goal of the Authors. My specific comments are below.

REPLY: We thank the reviewer for the careful evaluation and for highlighting the need to more clearly articulate the broader significance of our native-context structural results. We address these points in the revised manuscript, and we respond to the reviewer's specific questions in detail below.

Major Comments:

Q1: There have been other studies that have used cryoEM and cryoET to characterize different filaments, both with intact cells and purified filament

preparations (doi.org/10.1038/s41467-020-15650-w; doi.org/10.1038/s41467-022-35164-x). The Authors state that this is the first study that uses these tools to identify an MshA and PilA from *Shewanella*, but I do not see the importance of gaining this structural information for these particular filaments laid out in the discussion. Please expand on this to better contextualize the results. We know from previous work that these filaments are important for biofilm formation and electron transfer (in coordination with cytochromes) and we know that oxygen depletion increases the presence of these filaments (doi.org/10.1128/AEM.01615-16) in a biofilm. How will the results here in deriving a structure for these filaments advance the knowledge of the field? Is there something about the difference of the filament types under the different conditions that would guide us toward harnessing these cells more effectively?

REPLY: We thank the reviewer for this thoughtful comment and for pointing out the need to better articulate the significance of resolving PilA and MshA structures in *Shewanella* biofilms. Following this suggestion, we now include matched phenotypic and electrochemical readouts for Δ MshA, Δ PilA, and Δ Fla strains, and we expand the Discussion to interpret these functional differences in the context of the native-context structural features resolved here and to clarify how the atomic models can inform structure-guided strategies for engineering biofilm architecture for bioenergy and environmental applications. In addition, we have now cited the studies suggested by the reviewer in the revised manuscript to properly contextualize prior cryo-EM and cryo-ET filament work and oxygen-dependent filament enrichment

To strengthen the biological relevance of the structural assignments, we examined Δ MshA, Δ PilA, and Δ Fla strains in parallel with wild-type MR-1 using the same biofilm conditions (Fig. R1-1). Δ MshA showed markedly reduced biofilm coverage and a pronounced decrease in ferrihydrite reduction and microbial fuel cell output, whereas Δ PilA remained comparable to wild type in

these readouts. Δ Fla exhibited a modest reduction, consistent with a more indirect contribution through surface colonization. Together, these phenotypes align with the native-context structural observations, particularly the extensive MshA-rich filament networks, and provide a functional framework for interpreting why PilA and MshA filaments play distinct roles in MR-1 biofilms. More broadly, the atomic models define concrete, structure-informed targets that can be tested in future mutagenesis or engineering studies to tune pilus interactions and biofilm architecture.

Accordingly, we added corresponding content in the Results section “Structural analysis of flagella and pili”, the last paragraph, as follows:

*“To connect the filament structures to biofilm physiology, we compared wild-type MR-1 with deletion mutants lacking each filament (Δ MshA, Δ PilA, and Δ Fla). Fluorescence imaging showed that biofilm coverage was markedly reduced in Δ MshA relative to wild type, whereas Δ PilA displayed no significant difference and Δ Fla exhibited a modest decrease (Fig. S18A-E). Consistent with these trends, ferrihydrite reduction activity, quantified as Fe(II)/total Fe (%), was strongly impaired in Δ MshA, but remained comparable to wild type in Δ PilA and Δ Fla (Fig. S18F). In microbial fuel cell measurements, Δ MshA produced a significantly lower output voltage and Δ Fla showed a modest decrease, while Δ PilA again was not significantly different from wild type (Fig. S18G). These phenotypes are in line with prior genetic studies showing that MshA plays a dominant role in *S. oneidensis* biofilm formation and can impact electrochemical activity, whereas PilA typically has a more limited contribution, and flagella mainly influence surface colonization and motility-related biofilm development¹⁻⁴. And the strong Δ MshA defects also align with our structural analysis, in which MshA pili exhibit a more balanced, patchy surface charge distribution and larger solvent-accessible surface area than PilA, features that are well suited to promote filament–filament and filament–matrix interactions that stabilize biofilm architecture.”*

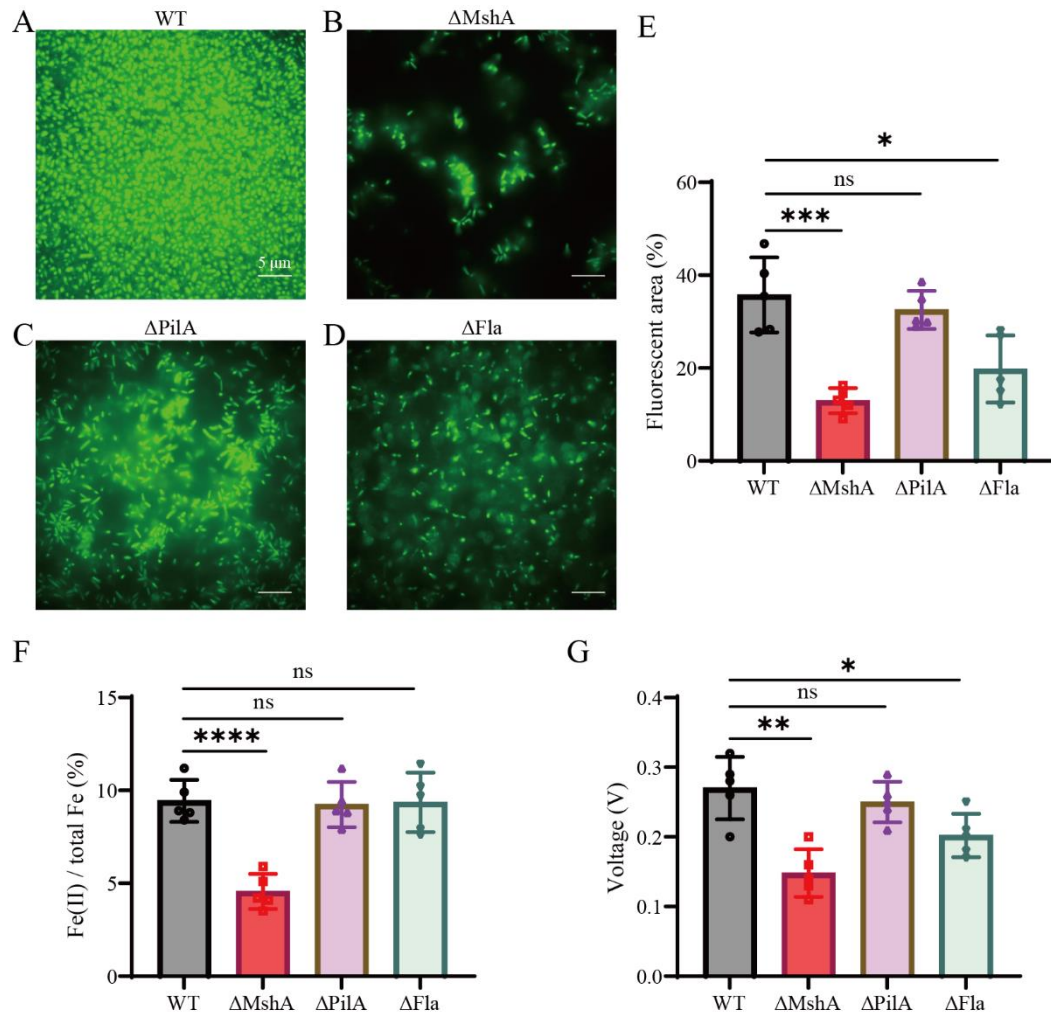


Figure R1-1 (Figure S18 in the revised manuscript). Physiological phenotypes of filament deletion mutants. (A-D) Representative fluorescence images of biofilms formed by wild-type *S. oneidensis* MR-1 WT, Δ MshA, Δ PilA, and Δ Fla strains. Scale bar, 5 μ m. (E) Quantification of biofilm coverage, measured as fluorescent area (%). (F) Ferrihydrite reduction activity quantified as Fe(II)/total Fe (%). (G) Microbial fuel cell output voltage (V). Bars represent mean $\pm$ SEM; individual data points are overlaid. Statistical significance is indicated as ns, not significant; *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.

In the revised Discussion, we more directly connect the native-context PilA and MshA structures to their roles in biofilm formation and extracellular electron transfer. While prior genetic studies established that both filament types contribute to biofilm development and are upregulated under oxygen limitation, the structural basis for their distinct contributions remained unclear. Here we highlight that MshA pili display a more balanced, patchy surface charge distribution, greater solvent-accessible surface area, and a stronger tendency

to form bundles in situ than PilA, providing a structural rationale for MshA's dominant role in stabilizing biofilm architecture and mediating filament–matrix cross-linking.

We also note that the atomic models help address the long-standing question of whether *Shewanella* type IV pili act as intrinsically conductive nanowires. In both PilA and MshA, aromatic residues do not form a continuous π -stacked pathway and are generally spaced beyond 4 Å, and together with our C-AFM measurements this supports a model in which these pili primarily function as interactive scaffolds that can organize other redox-active components rather than serving as the main conductive elements.

Finally, we outline how these structures can inform future applications: the defined inter-subunit interfaces and exposed surface patches provide concrete targets for mutagenesis or engineering to tune pilus bundling, surface chemistry, and interactions with electrodes or synthetic polymers. In combination with the oxygen-dependent remodeling we observe, these insights offer a framework for structure-guided optimization of *Shewanella* biofilm architecture for microbial fuel cells and bioremediation.

To make these points clear in the manuscript, we added corresponding content in the Discussion section paragraph 2, as follows:

“Building on these observations, our data help explain how PilA and MshA support biofilm function. MshA pili not only display a more balanced, patchy surface charge distribution and larger solvent-accessible surface area than PilA, but are also enriched in bundled regions of the matrix, consistent with a role as cross-linking hubs that engage neighboring filaments and matrix components. At the same time, the aromatic residues in both PilA and MshA are arranged without a continuous π -stacked pathway and are generally spaced beyond 4 Å, which, together with our C-AFM measurements, supports a model in which these pili act as interactive scaffolds rather than intrinsically conductive nanowires. The defined inter-subunit interfaces and exposed surface

patches therefore provide concrete targets for mutagenesis or protein engineering to tune pilus bundling, surface chemistry, and interactions with electrodes or synthetic polymers in future bioenergy and bioremediation applications.”

Q2: What controls were run to confirm that the filaments were what they are hypothesized to be? e.g., were any pilA or mshA mutants tested to see if these structures disappeared as expected? Or could planktonic cells drop cast onto a microgrid and frozen to assess non-biofilm cells?

REPLY: We thank the reviewer for this important suggestion regarding controls. Following the reviewer’s comments, we performed both genetic and planktonic controls to validate the identity of the filaments.

For the genetic controls, we used Δ pilA and Δ mshA strains in which PilA or MshA is not expressed. Biofilms formed by these mutants were imaged by cryo-EM under the same conditions as the wild-type strain. In biofilms formed by the Δ pilA strain, the PilA filaments were no longer observed, whereas the MshA and the flagellar filaments were still present. Conversely, in Δ mshA biofilms, the MshA filaments disappeared, while the PilA and flagella remained (Fig. R1-2). These are consistent with our assignments of the three filament types to PilA pili and MshA pili, respectively.

As an additional control, we examined planktonic cells. In the planktonic samples, we detected only occasional flagella and essentially no pili-like ~6 nm filaments around the cells (Fig. R1-3), indicating that PilA and MshA pili are enriched under biofilm-forming conditions and are not general contaminants of the preparation.

We added corresponding content in the Results section “Structural determination of biofilm filaments”, paragraph 3, as follows:

“For genetic validation, we generated Δ PilA and Δ MshA mutants and

imaged their biofilms under the same cryo-EM conditions as the wild-type strain, focusing on the ~12 nm and ~6 nm filament populations. In Δ MshA biofilms, MshA filaments were absent, whereas PilA filaments and flagella remained. Conversely, in Δ PilA biofilms, PilA filaments were no longer observed, while MshA filaments and flagella persisted (Fig. S10). These genotype-dependent changes, together with the structural fits, are consistent with our assignment of the filament classes to PilA pili and MshA pili, respectively. We also examined planktonic wild-type cells and observed only occasional flagella and virtually no ~6 nm pilus-like filaments, indicating that PilA and MshA pili are enriched under biofilm-forming conditions (Fig. S11).”

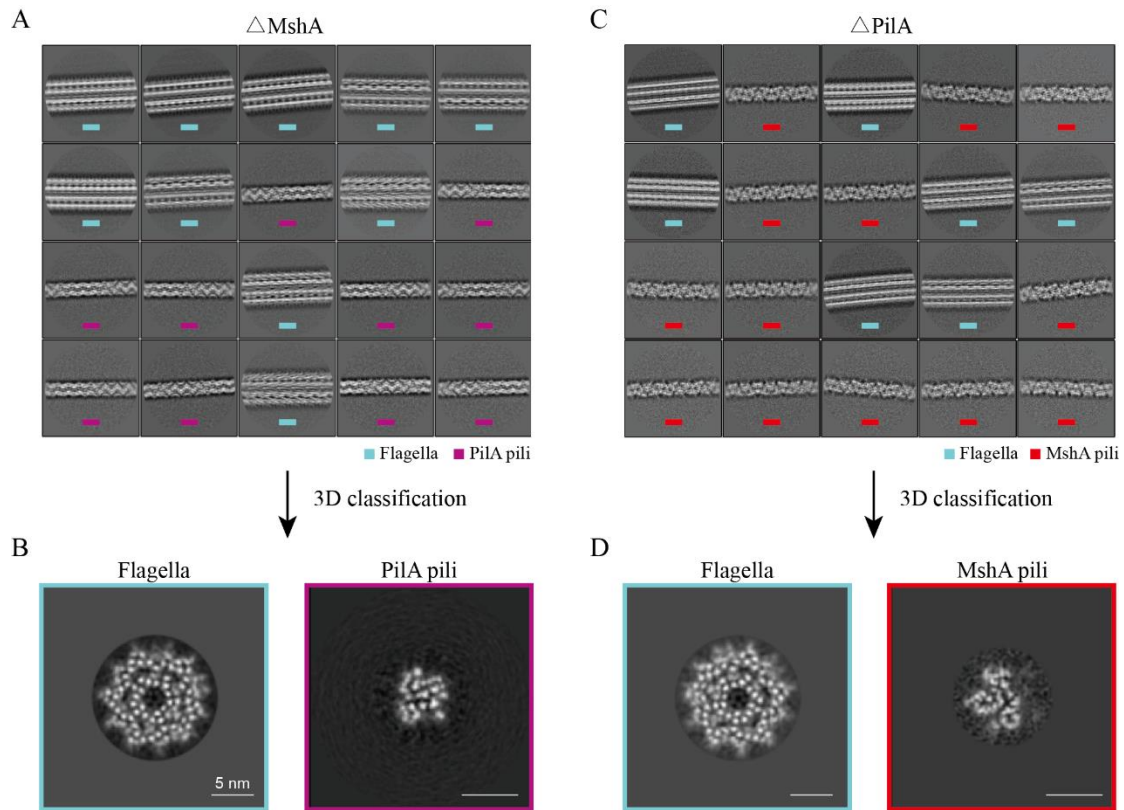


Figure R1-2 (Figure S10 in the revised manuscript). Genetic validation of PilA and MshA filament assignments in *S. oneidensis* MR-1 biofilms. (A) 2D class averages of extracellular filaments from the Δ mshA mutant, with classes corresponding to flagella (cyan) and PilA pili (magenta). (B) Cross-sectional view from 3D classification of the Δ mshA dataset, showing the presence of flagella and PilA pili and the absence of MshA pili. (C) 2D class averages of extracellular filaments from the Δ pilA mutant, with classes corresponding to flagella (cyan) and MshA pili (red). (D) Cross-sectional view from 3D classification of the Δ pilA dataset, showing the presence of flagella and MshA pili and the absence of PilA pili. Scale bars, 5 nm.

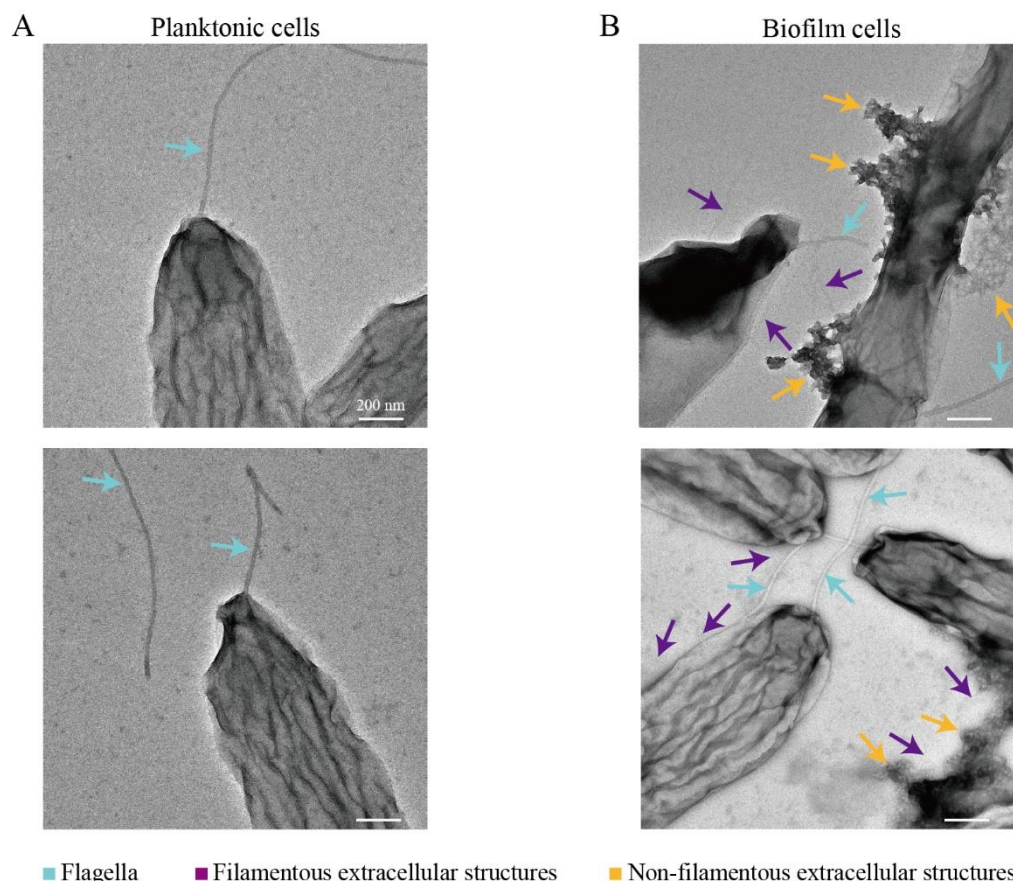


Figure R1-3 (Figure S11 in the revised manuscript). Extracellular filaments are enriched in biofilm cells compared with planktonic cells. (A) Representative NS-TEM images of planktonic wild-type *S. oneidensis* MR-1 cells, showing only occasional flagella (cyan arrows). (B) Representative NS-TEM images of biofilm cells acquired under the same imaging conditions, highlighting abundant extracellular filamentous structures (purple arrows) and non-filamentous extracellular material (yellow arrows), together with flagella (cyan arrows). Scale bars, 200 nm.

Q3: Regarding the discussion of the conductive properties of the filaments elucidated via AFM (around L265), is it possible that the conductivity of the filaments is affected by the absence of an electrochemical driving force on the growth substrate (i.e. the cells are not grown on a poised electrode)?

REPLY: We thank the reviewer for raising this important point about the electrochemical context. Our C-AFM measurements were performed using an experimental workflow established in prior studies for probing charge transport along individual extracellular filaments⁵. During C-AFM, a defined bias is

applied between the conductive AFM tip and the underlying conductive support, so an electrochemical driving force is present during the measurement itself. Accordingly, the C-AFM readout is best interpreted as a filament-level transport measurement under a controlled local bias.

At the same time, we recognize that electrode-grown biofilms represent a distinct experimental context, and implementing filament-resolved C-AFM under poised-electrode growth can be technically challenging. In practice, electrode-grown samples can introduce additional variability in surface topography and biofilm thickness, and the electrode surface (and associated deposits) can complicate reliable filament identification and stable tip – sample contact, making reproducible single-filament measurements more difficult. To avoid overinterpretation, we have clarified in the Discussion that our conductivity measurements were performed on biofilms grown without an externally applied potential, and that extending these measurements to electrode-grown conditions is an important direction for future work.

We added corresponding content in the Discussion section paragraph 2, as follows:

“Here, our C-AFM measurements follow established single-filament assays and probe charge transport along individual filaments under a defined local bias during scanning⁵. In future work, this filament-resolved readout can be extended to filaments harvested from biofilms grown under additional electrochemical conditions, including growth on poised electrodes, to enable direct comparisons across growth environments.”

Q4: Regarding the putative eDNA filament conductivity results/discussion, there is precedence for eDNA enabling electron transfer in other organisms that produce redox shuttles (see: doi.org/10.1016/j.cell.2020.07.006). *S. oneidensis* also produces redox active compounds, could a similar mechanism be at play here?

REPLY: We thank the reviewer for this thoughtful suggestion and for drawing our attention to the study by Saunders *et al.* (Cell, 2020). Following the reviewer's suggestion, we have considered these results in the context of our system and have added a discussion of this possibility in the revised manuscript. In *Pseudomonas aeruginosa*, Saunders *et al.* showed that phenazine redox shuttles bind to eDNA in the biofilm matrix and can participate in DNA-mediated charge transfer, allowing eDNA-bound phenazines to support efficient extracellular electron transfer while remaining retained in the biofilm.

Shewanella oneidensis MR-1 has been reported to secrete flavin-based redox shuttles⁶, and it will be important in future work to determine whether these small molecules associate with eDNA in the matrix and, if so, whether such complexes measurably affect charge transport. Our C-AFM data showing that a subset of ~2 nm filaments (some of which are likely eDNA) can carry measurable current are consistent with the idea that eDNA-containing structures may participate in local electron transfer, for example by organizing secreted shuttles into a percolating network. At the same time, our main conclusion from the present work is based on the atomic structures of the PilA and MshA pili, which lack a continuous π -stacked pathway and contain relatively large spacings between aromatic residues, indicating that these type IV pili are unlikely to function as intrinsically conductive "nanowires" regardless of the precise contribution of matrix-associated shuttles.

To better reflect this, we have added corresponding content in the Discussion paragraph 4, as follows:

"Extracellular DNA may also contribute to electron transfer in a more indirect manner. A study in Pseudomonas aeruginosa showed that extracellular DNA can bind phenazine redox shuttles and support DNA-mediated charge transfer within biofilms⁷. S. oneidensis MR-1 has been reported to release flavin-based redox-active compounds⁶, raising the possibility that matrix-associated shuttles could be retained locally and participate in charge transport

within MR-1 biofilms. In the present study, we focus on the native structural context and do not directly test eDNA shuttle interactions or their impact on electron transfer. This possibility could be examined in future work using DNase treatment and controlled flavin modulation together with electrical readouts. More broadly, how vesicle trafficking, extracellular multimer assembly, eDNA-containing filaments, and overall biofilm conductivity are functionally connected will require further investigation.”

Q5: L367: Throughout the materials and methods, there needs to be some description of how many microgrids were used for each analysis and how many separate images were used for the analysis. I am currently left wondering if this work was all done on one image from one grid per condition. Table 1 says 40 frames, but are all frames from 1 grid? My question stems from biological variability. If you inoculated another serum bottle with the same conditions, would you see the same thing?

REPLY: We appreciate the reviewer’s concern about sampling and biological variability. The structures were not determined from a single grid or a single image per condition. For each condition, we prepared at least three independent *S. oneidensis* biofilm cultures grown under the same conditions.

Specifically, each condition was performed in at least three separate serum bottles, and at least one cryo-EM grid was prepared from each serum-bottle culture. For each grid, we collected on the order of 3,000-4,000 images (micrographs). The reconstructions used the numbers of micrographs listed in Table 1: 4,000 micrographs for the flagellum and 9,000 micrographs each for the PilA and MshA pili. Because the flagellar filament was relatively straightforward to reconstruct and its helical parameters are already well established in other bacteria, we used a subset of 4,000 micrographs for the final map to reduce computational load. We then processed the remaining

5,000 flagellar micrographs and obtained a reconstruction that was indistinguishable from the original within our resolution limits, indicating that repeating the analysis on additional grids and datasets yields the same structural result.

The 40 frames entry in Table 1 refers to dose fractionation on the direct electron detector. In the setup, the direct electron detector records each micrograph as a series of 40 short frames from the same field of view. These 40 frames are aligned and summed to correct for beam-induced motion, and the resulting single motion-corrected image is what we refer to as one micrograph for particle picking and reconstruction. Thus: a frame is one of the 40 sub-images recorded during one exposure, a micrograph (image) is the final motion-corrected sum of those 40 frames, and the micrograph counts in Table 1 correspond to these final motion-corrected images collected.

We added corresponding content in the Methods section “Bacteria culture” and “Cryo-EM data collection”, as follows:

“From each independent culture, at least three cryo-EM grids were prepared and screened.”

“Each exposure was recorded as a 40-frame movie and motion-corrected into one summed micrograph; micrograph counts in Table 1 refer to these motion-corrected images.”

Finally, the Δ pilA and Δ mshA strains described in our response to Q2 were grown as independent biofilm cultures and consistently showed the expected selective loss of the corresponding filament type while the other filaments remained. This agreement across multiple cultures, grids, and strains supports both the reproducibility of the observations and the robustness of the structural assignments.

Minor Comments:

Q1: Were the grids dehydrated before freezing? I think not based on how I am

reading the methods, but please be explicit here as that can cause structural changes in samples.

REPLY: We thank the reviewer for raising this point. In line with this suggestion, we have clarified in the Methods that the grids were not dehydrated at any stage prior to vitrification. Biofilms on the grids were kept in aqueous buffer, briefly blotted under controlled humidity, and then directly plunge-frozen in liquid ethane without any intermediate drying or chemical dehydration steps.

We have now added the sentence to the “Cryo-EM data collection” section in the Methods as followings:

“Cryo-EM grids were carefully removed from the bottles using tweezers. Each grid was blotted with filter paper under controlled humidity, and grids were not dehydrated at any stage; samples were maintained in aqueous buffer and vitrified directly by plunge-freezing in liquid ethane”

Q2: L124: “data collection was focused on cell-free regions of the grids enriched in extracellular material”. I understand the reason for doing this. How far from the cells were these measurements taken?

REPLY: We thank the reviewer for this helpful comment. In response to this suggestion, we have clarified in the Results how the imaging areas were selected relative to the cells.

During automated cryo-EM data collection using EPU (Thermo Fisher Scientific), targets were chosen based on image contrast to identify regions with optimal ice thickness. Fields in which the beam passed predominantly through thick *S. oneidensis* cell bodies or visibly poor ice were excluded, and micrographs were then collected from the surrounding extracellular regions across the grid. In the raw micrographs, the outlines of bacterial cells are sometimes visible at the edges of the recorded fields, indicating that the

analyzed filaments were sampled from extracellular zones that remain within the biofilm environment.

Accordingly, the sentence in the Results section “Structural determination of biofilm filaments” paragraph 1 has been revised as follows:

“Because intact cells rendered parts of the biofilm too thick for high-resolution imaging, we focused data collection on cell-free grid regions enriched in extracellular material and excluded views dominated by thick cell bodies or poor ice. This strategy enabled high-resolution sampling of the surrounding biofilm matrix, including regions immediately adjacent to the cell surface.”

And we added corresponding content in the Methods section “Cryo-EM data collection” as follows:

“For high-resolution imaging, data were collected using EPU (Thermo Scientific™). Target areas were selected by screening atlas and grid-square images and then choosing regions with suitable ice thickness judged from image contrast. Regions dominated by thick cell bodies or visibly poor ice (e.g., crystalline or contaminated areas) were excluded, and micrographs were collected from extracellular regions enriched in filamentous material across the grid, including regions in close proximity to cells.”

Q3: L311: Small typo: “we the first atomic-resolution structure...”.

REPLY: We thank the reviewer for pointing out this typo. Following the reviewer’s suggestion, we have corrected the sentence at line 311 from “...we the first atomic-resolution structure...” to “...we present the first atomic-resolution structure...”

Q4: L367: Worded confusingly. Makes it sound like the cells were added to the serum bottle and then autoclaved.

REPLY: We thank the reviewer for pointing out this confusing wording. In the original text, “sealed, and autoclaved” referred only to the preparation of the serum bottles and medium prior to inoculation, not to autoclaving live cells. Following the reviewer’s suggestion, we have revised this section of the Methods to make the sequence of steps explicit:

“Shewanella oneidensis MR-1 was grown overnight in LB broth at 30 °C to an OD₆₀₀ of 2.0-2.5. Separately, replicate 100-mL serum bottles containing 60 mL of defined medium were sealed and autoclaved, allowed to cool, and then used for inoculation⁸.”

Q5: L369: I am not understanding the experimental setup here. Are the serum bottles the reactors? Are they open to the atmosphere in the InvivoO2 workstation? Was there any agitation or were the bottles stagnant?

REPLY: We thank the reviewer for raising this important point about the culture configuration. To avoid any ambiguity, we have clarified in the Methods that the 100 mL serum bottles served as the culture vessels and were incubated inside the InvivoO₂® 400 Physoxia Workstation. Under our setup, the headspace gas in the bottles equilibrated with the workstation-controlled atmosphere, and oxygen levels were regulated by adjusting the N₂/air ratio in the workstation. During incubation, the bottles were maintained under gentle orbital shaking (50 rpm) to provide mild mixing while allowing biofilm formation on the grids.

Accordingly, we added the following text to the Methods section “Bacteria culture”, as follows:

“The inoculated serum bottles were then incubated inside an InvivoO₂® 400 Physoxia Workstation, in which the gas environment within the bottles was continuous with the workstation-controlled atmosphere. Oxygen levels were regulated by adjusting the N₂/air ratio in the workstation. During incubation, bottles were kept under gentle orbital shaking (50 rpm).”

Q6: Figure 1: I would like to see the raw image without all of the colored traces, especially for B and C. I understand why the Authors chose to include the colored lines to point out the filament, but seeing both the raw and processed images side by side would be valuable.

REPLY: We thank the reviewer for this helpful suggestion. In response, we now provide the unannotated raw cryo-ET slices corresponding to Fig. 1B and Fig. 1C in the Supporting Information (Fig. R1-4), together with two accompanying movies (Movie R1-1 and Movie R1-2) showing the same regions.

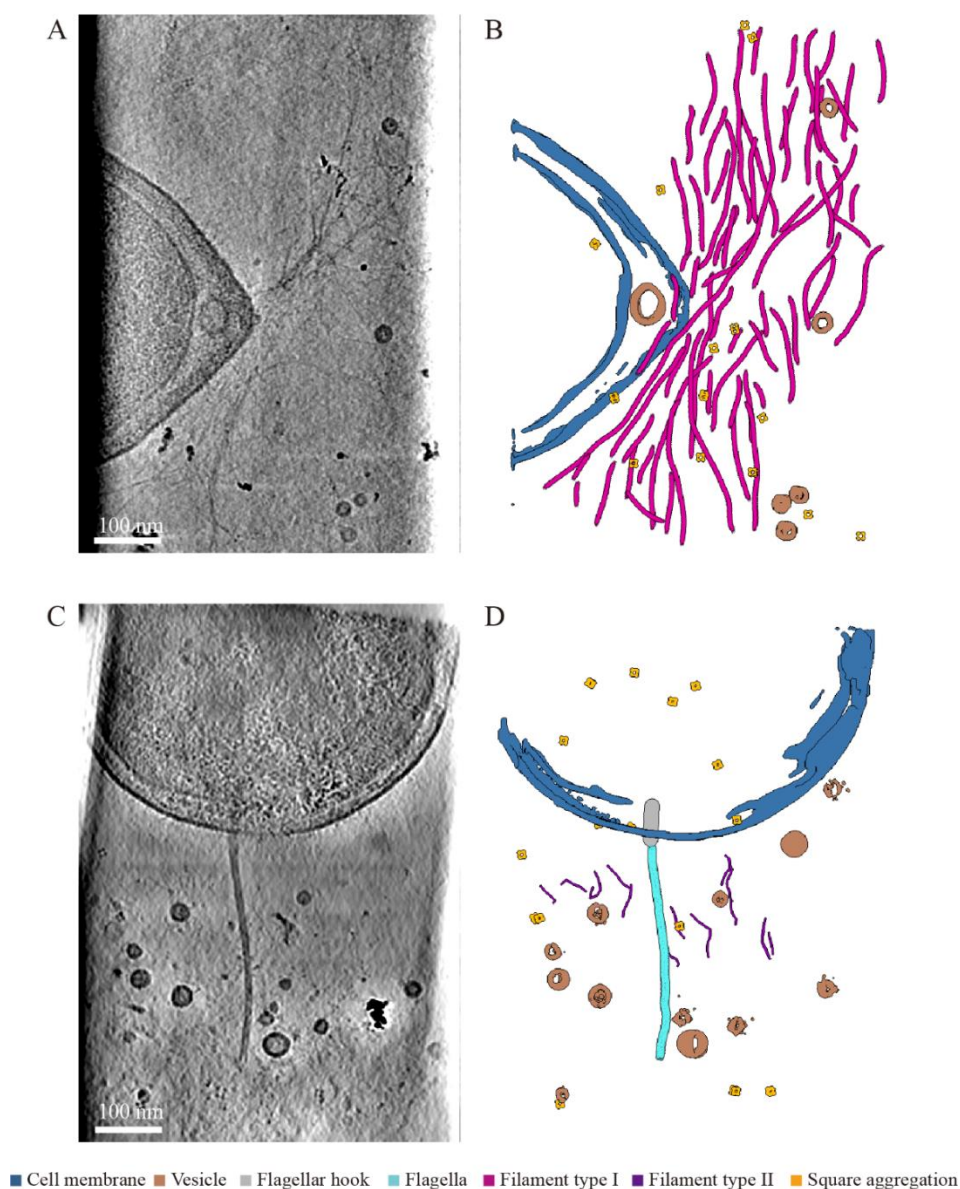


Figure R1-4 (Figure S1 in the revised manuscript). Tomographic slices corresponding to Fig. 1B and Fig. 1C. (A) Unannotated tomographic slice showing extracellular filaments

and particles surrounding an *S. oneidensis* MR-1 cell. (B) 3D rendering of the same view shown in (A), highlighting the major extracellular features. (C) Unannotated tomographic slice showing a second example with abundant extracellular material around the cell envelope. (D) 3D rendering of the same view shown in (C). Scale bars, 100 nm.

Movie R1-1 (Movie S1 in the revised manuscript). Raw cryo-ET volume corresponding to Fig. 1B.

Unannotated tomographic volume of an *S. oneidensis* MR-1 biofilm region, shown without colored overlays to provide an unobstructed view of the underlying ultrastructure. Related still images are shown in Fig. S1A-B.

Movie R1-2 (Movie S2 in the revised manuscript). Raw cryo-ET volume corresponding to Fig. 1C.

Unannotated tomographic volume of a second representative *S. oneidensis* MR-1 biofilm region, displayed without rendering or colored traces. Related still images are shown in Fig. S1C-D.

Q7: I understand why the Authors are labelling the unidentified structure as eDNA based on the modelling work, but I would be more conservative and say “putative eDNA” or something to that effect since I did not see other evidence presented that it was eDNA.

REPLY: We thank the reviewer for this careful and important comment. Following the reviewer’s suggestion, we have revised the manuscript to describe the ~2 nm filaments as “putative extracellular DNA (eDNA)” in the text and figure.

Reviewer #2 (Remarks to the Author)

Dear authors,

Your study provides valuable structural insights into *Shewanella oneidensis* MR-1 biofilms, notably delivering the first high-resolution structure of an MSHA-type pilus and native-context models of PilA pili and flagella. The combination of cryo-EM, cryo-ET, and functional assays is impressive and advances the field significantly.

REPLY: We thank the reviewer for the encouraging and thoughtful assessment of our study. We have carefully considered all of the reviewer's suggestions and made detailed revisions throughout the manuscript to improve clarity, strengthen the evidence supporting our conclusions, and enhance the presentation of the results. And we respond to the reviewer's questions in detail below.

However, several points could strengthen your manuscript:

Q1: The identity and function of the ~2 nm flexible filaments and the newly observed non-periodic filaments under anaerobic conditions remain unclear. Additional biochemical or genetic validation is needed to better characterize these components.

REPLY: We thank the reviewer for raising this important point. Following the reviewer's suggestion, we added a complementary biochemical analysis to help narrow down candidate molecular components associated with the unknown filaments observed in our study.

Specifically, we performed LC-MS/MS on extracellular-enriched material from anaerobic biofilm cultures, with aerobically grown cultures processed in parallel as a reference. Comparative proteomics identified multiple proteins enriched or preferentially detected under anaerobic conditions, providing an

initial candidate set associated with the condition in which these previously unassigned filament populations become prominent (Table R2-1).

We emphasize that this proteomic survey is intended to provide a practical starting point for targeted validation rather than to assign filament composition directly, because proteomics reports proteins present in the extracellular milieu and does not by itself establish which specific components assemble into a given filament. Resolving the molecular identity and physiological role of these flexible, non-periodic filaments will require dedicated, hypothesis-driven follow-up experiments, such as prioritized genetic perturbation or epitope tagging coupled to cryo-EM/cryo-ET readouts, together with enzyme-sensitivity assays (e.g., DNase/RNase, protease, polysaccharidase) to probe the biochemical nature of the filaments. We view these targeted validations as a natural next step building on the structural framework established here, and we are pursuing this direction in ongoing work.

These additions have been incorporated into the revised Results “Oxygen limitation enhances filament abundance and aggregate formation” paragraph 1, as follows:

“To complement these structural observations, we performed LC-MS/MS on extracellular-enriched material from anaerobic biofilm cultures, with aerobically grown cultures processed in parallel as a reference. Comparative proteomics identified multiple proteins enriched or preferentially detected under anaerobic conditions (Table S1), providing an initial candidate set associated with the condition in which these unknown filaments become prominent. Importantly, this proteomic survey is intended to guide subsequent, hypothesis-driven validation rather than to assign filament composition directly, as definitive molecular identification will require dedicated targeted experiments, for example, prioritized genetics tagging with cryo-EM readouts and enzyme-sensitivity assays. Such focused follow-up work will be an important direction for future studies aimed at resolving the molecular basis and physiological roles

of these non-periodic extracellular architectures.”

Table R2-1 (Table S1 in the revised manuscript). Comparative proteomics of extracellular material from aerobic and anaerobic *S. oneidensis* MR-1 biofilms. LC-MS/MS quantification was performed on extracellular fractions prepared from anaerobic cultures, with aerobically grown cultures processed in parallel. The table lists proteins detected in both conditions and shows their integrated MS signal intensities and the corresponding enrichment ratio (Anaerobic/Aerobic).

Protein IDs	Anaerobic Intensity	Aerobic Intensity	Anaerobic/Aerobic
Q8EJM1	1.754E+07	1.747E+07	1.004
Q8EAX6	4.296E+09	4.269E+09	1.006
Q8ECA6	4.232E+09	4.197E+09	1.008
Q8CX36	1.350E+08	1.328E+08	1.016
Q8EAE7	1.488E+07	1.459E+07	1.020
Q8ECJ7	6.154E+07	6.009E+07	1.024
Q8EC41	1.971E+07	1.908E+07	1.033
K4PU16	2.460E+08	2.363E+08	1.041
Q8EJW1	3.119E+07	2.984E+07	1.045
Q8EFP2	4.767E+07	4.518E+07	1.055
Q8E9X8	7.859E+07	7.440E+07	1.056
Q8EGN2	1.496E+07	1.409E+07	1.061
Q8E9V0	1.385E+07	1.304E+07	1.062
Q8EG15	1.089E+08	1.021E+08	1.066
Q8CX48	4.826E+09	4.472E+09	1.079
Q8E8W6	3.066E+07	2.821E+07	1.087
Q8E864	3.123E+07	2.866E+07	1.090
Q8E8J8	1.009E+08	9.189E+07	1.098
Q8EHF3	2.431E+07	2.159E+07	1.126
Q8EFN8	2.123E+08	1.879E+08	1.130
Q8ECT0	1.232E+08	1.082E+08	1.139
Q8EB81	4.678E+07	4.094E+07	1.143
Q8EBP3	7.974E+07	6.962E+07	1.145
Q8EIN9	2.319E+07	2.016E+07	1.150
Q8ECK4	1.141E+09	9.836E+08	1.160
Q8EHM9	7.086E+08	6.089E+08	1.164
Q8EDA2	1.186E+07	1.018E+07	1.165
Q8EAQ4	4.777E+08	4.037E+08	1.183
Q8EAG0	3.236E+08	2.703E+08	1.197
Q8EK61	4.062E+08	3.328E+08	1.221
Q8EG16	3.894E+09	3.177E+09	1.226
Q8EK51	3.485E+08	2.785E+08	1.251
Q8E8U5	2.153E+07	1.715E+07	1.255
P83223	1.328E+09	1.055E+09	1.258
Q8EJX3	2.253E+08	1.783E+08	1.263
Q8EAG5	5.944E+07	4.702E+07	1.264
Q8EE24	1.799E+07	1.416E+07	1.270
Q8EB96	1.766E+07	1.377E+07	1.283
Q8EF71	2.882E+07	2.244E+07	1.285
Q8EDY6	3.072E+07	2.375E+07	1.293
Q8EDD0	1.547E+07	1.180E+07	1.311
K4PW07	2.197E+07	1.641E+07	1.339
P59166	2.731E+08	2.015E+08	1.355
Q8ECT8	1.030E+08	7.593E+07	1.356
Q8EAW7	2.976E+07	2.175E+07	1.368
Q8EC97	2.323E+07	1.694E+07	1.371
Q8EHZ8	3.372E+07	2.456E+07	1.373
Q8E8H1	6.694E+07	4.860E+07	1.377
Q8CX49	2.178E+09	1.574E+09	1.384
Q8EGG6	5.421E+08	3.903E+08	1.389
Q8EAC7	3.837E+07	2.752E+07	1.394

Table R2-1 (Table S1 in the revised manuscript) (continued).

Protein IDs	Anaerobic Intensity	Aerobic Intensity	Anaerobic/Aerobic
Q8EDH7	7.282E+06	5.114E+06	1.424
Q8EFY6	2.927E+08	2.054E+08	1.425
Q8EBU8	4.457E+07	3.122E+07	1.428
Q8EHY9	1.452E+07	1.004E+07	1.446
Q8E8Z7	1.051E+07	7.228E+06	1.453
Q8EK69	3.681E+08	2.528E+08	1.456
Q8EBS3	2.376E+08	1.603E+08	1.483
Q8CX47	5.710E+07	3.702E+07	1.542
Q8EBH3	2.497E+09	1.608E+09	1.553
Q8EAU2	8.215E+07	5.222E+07	1.573
Q8EJK7	2.094E+08	1.260E+08	1.662
Q8EDB4	2.501E+07	1.484E+07	1.685
Q8EK56	2.692E+07	1.554E+07	1.733
Q8EAS7	5.044E+07	2.908E+07	1.734
Q8EK52	3.095E+07	1.728E+07	1.791
Q8EIU3	5.849E+06	3.190E+06	1.833
Q8EG54	4.569E+07	2.459E+07	1.858
Q8ECJ2	1.028E+07	5.430E+06	1.894
Q8EAL4	1.406E+08	7.416E+07	1.895
Q8CVD5	3.554E+08	1.865E+08	1.905
Q8EFP1	1.828E+07	8.868E+06	2.062
Q8EK72	1.170E+08	5.551E+07	2.107
Q8E9L6	2.231E+07	1.044E+07	2.137
Q8E8C1	3.099E+07	1.442E+07	2.149
Q8EBH9	6.012E+07	2.747E+07	2.189
Q8EJ69	3.840E+07	1.750E+07	2.195
Q8EK63	1.160E+08	5.113E+07	2.268
Q8EGC4	6.129E+07	2.561E+07	2.393
Q8E8E6	3.377E+09	1.409E+09	2.398
Q8EEV9	3.483E+07	1.167E+07	2.984
Q8EA03	1.094E+08	3.186E+07	3.433
Q8ED73	6.207E+07	1.580E+07	3.927
Q8EH72	3.926E+07	9.025E+06	4.350
Q8EJP5	8.932E+07	2.042E+07	4.375
Q8EHD7	1.139E+08	2.024E+07	5.625
Q8EDK0	1.179E+07	1.545E+06	7.630
Q8EA04	6.271E+08	1.678E+07	37.380
P42272	9.171E+06	0	-
Q8EAH4	4.153E+07	0	-
Q8EAI9	8.295E+06	0	-
Q8EAP2	1.009E+07	0	-
Q8ECI8	7.542E+06	0	-
Q8ECL7	3.577E+06	0	-
Q8EDW6	3.909E+07	0	-
Q8EJM8	1.602E+07	0	-
A0A7D5HPU4	1.467E+07	0	-
K4PST1	8.787E+06	0	-
Q8E8Z0	2.224E+07	0	-
Q8E9B5	3.433E+07	0	-
Q8EA39	6.438E+06	0	-
Q8EAF5	1.749E+07	0	-
Q8EAJ5	3.915E+07	0	-
Q8EB30	1.130E+08	0	-
Q8EBE7	2.134E+07	0	-
Q8EF24	5.227E+06	0	-
Q8EGD4	2.393E+07	0	-
Q8EGJ6	1.847E+07	0	-
Q8EGL1	1.243E+07	0	-
Q8EH73	1.377E+07	0	-
Q8EJB3	5.627E+06	0	-
Q8EKI1	3.646E+07	0	-

Q2: The multimeric protein aggregates in the biofilm matrix were resolved only at limited resolution, preventing definitive identification. Higher-resolution structural analysis or complementary biochemical studies would improve understanding of their.

REPLY: We thank the reviewer for this important suggestion. Following the reviewer's suggestion, we added a summary flowchart of the subtomogram averaging processing pipeline for the multimeric aggregates to improve transparency and reproducibility, and expanded our analyses by combining structure guided fitting against reported cube-like multimeric assemblies with LC-MS/MS to provide constrained, testable hypotheses for particle identity.

Using the subtomogram averaging workflow, we obtained representative reconstructions for the three prevalent multimeric forms. These maps robustly capture the overall 3D architecture, enabling informed comparisons to known multimeric assemblies. However, they do not provide sufficient high-frequency detail for de novo model building or definitive identification. To strengthen transparency and reproducibility, we have expanded the particle-level 3D description and classification of the aggregates with enlarged tomogram views (Fig.R2-1 and Movie R2-1) and representative multimer reconstructions added a flowchart summarizing the processing pipeline for the multimeric particles in the revised manuscript (Fig. R2-2 and R2-3). In addition, to extract maximal insight from the available maps, we performed structure-guided comparisons and fitting against several reported cube-like multimeric assemblies (PDB: 9NTJ, 9MU6, 8XLL), and complemented these comparisons with anaerobic LC-MS/MS (Fig. R2-4 and R2-5). Based on this integrated analysis, we provide a tentative assignment that the octameric particle is consistent with the *S. oneidensis* MR-1 dihydrolipoyllysine-residue succinyltransferase (E2) component of the 2-oxoglutarate dehydrogenase complex (UniProt Q8EFN9). We emphasize that this remains a working hypothesis given the current low

resolution map. For the tetrameric and trimeric particles, our present fitting attempts do not yield a unique or convincing match, which may reflect mixed composition, partial assemblies, or conformational variability in the extracellular environment. We have clarified these points in the revised manuscript and included the corresponding analyses.

Going forward, definitive identification will benefit from complementary approaches that increase particle homogeneity and enable higher-resolution structure determination, such as biochemical purification of the extracellular aggregates, optimized sample preparation to boost particle counts and reduce heterogeneity, and targeted genetic or labeling strategies (e.g., epitope tagging and knockouts of prioritized candidates) coupled with cryo-ET validation.

These additions are described in the revised Results section “Oxygen limitation enhances filament abundance and aggregate formation” paragraph 4 and 5, as follows:

“To objectively characterize these assemblies in three dimensions (3D), we performed a particle-level analysis directly in the cryo-ET volumes. Individual multimeric particles were extracted from the tomograms and reconstructed to assess their 3D morphology in situ. Closer inspection of these proteinaceous aggregates revealed that they are not uniform squares, but instead comprise discrete multimers with defined sizes and symmetries (Fig. 5H-J and Movie S3), including roughly square-shaped tetramers and octamers, as well as triangular trimers. Inspection of the particles in the tomographic volumes indicates that they fall into three dominant morphological classes, with two relatively flattened forms and one clearly thicker form, highlighting pronounced structural heterogeneity within the aggregate population. To reduce user bias and quantify this heterogeneity, we then applied a reference free subtomogram averaging workflow to obtain representative reconstructions for the three prevalent multimeric forms (Fig. S20). The tetramer, octamer, and trimer reconstructions reached approximate resolutions of 6.3 Å, 11 Å, and 32 Å, respectively (Fig.

S21). At these resolutions, the maps robustly define overall shape and symmetry but do not support unambiguous *de novo* atomic model building or definitive protein identification.”

“To obtain preliminary insight into the molecular identity of these assemblies, we performed structure-guided comparisons and rigid-body fitting against several reported cube-like multimeric architectures (PDB: 9NTJ, 9MU6, 8XLL), and considered these results together with our anaerobic extracellular proteomics LC-MS/MS. This integrated analysis supports a tentative assignment that the octameric particle is most consistent with the dihydrolipoyllysine-residue succinyltransferase (E2) component of the 2-oxoglutarate dehydrogenase complex in *S. oneidensis* MR-1 (UniProt Q8EFN9) (Fig S22 and S23). Given the current resolution and extracellular context, we present this assignment as tentative rather than definitive. For the tetrameric and trimeric particles, our current fitting attempts do not yield a unique, high-confidence match, and their molecular identities remain unresolved.”

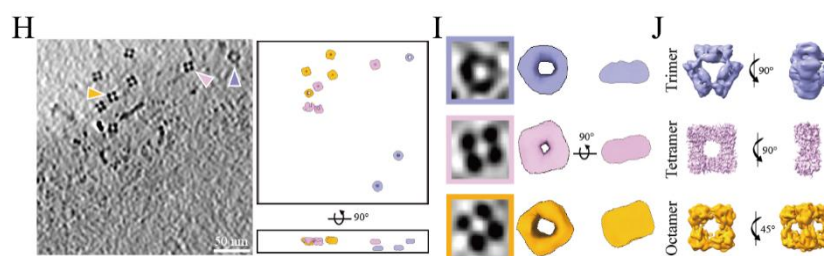


Figure R2-1 (Figure 5 H-J in the revised manuscript). (H) Representative tomographic slice showing multimeric extracellular aggregates (arrowheads). Scale bar, 50 nm. (I) Zoomed-in views of representative individual aggregates *in situ*, showing three distinct particle morphologies together with their corresponding 3D reconstructions. (J) Subtomogram-averaged density maps of the tetramer, octamer, and trimer.

Movie R2-1 (Movie S3 in the revised manuscript). 3D visualization of extracellular multimeric particles *in situ*.

Tomographic views highlighting aggregates in *S. oneidensis* MR-1 biofilms, including trimer-, tetramer-, and octamer-like particles. The movie shows representative particles in the tomographic context and the corresponding 3D reconstructions. Related panels are shown in Fig. 5H.

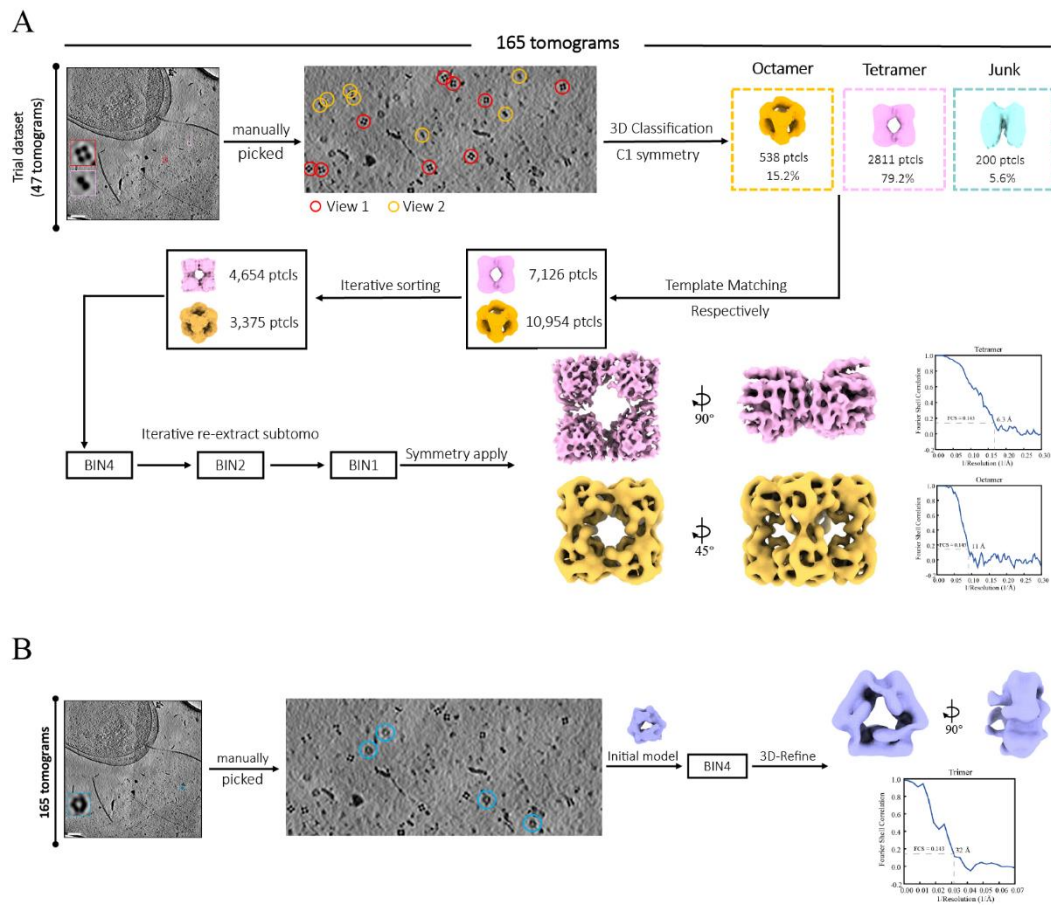


Figure R2-2. (Figure S20 in the revised manuscript). Subtomogram averaging workflow for reconstructing heterogeneous extracellular multimers in situ. (A) Workflow for identifying and reconstructing tetramer- and octamer-like particles from cryo-ET data. Candidate particles were manually picked from a trial subset of tomograms, extracted as subtomograms, and subjected to reference-free 3D classification (C1 symmetry) to separate octamer-like, tetramer-like, and junk classes. The resulting initial 3D model was low-pass filtered and then used to generate templates for template matching across the full dataset separately for each class. This was followed by iterative sorting and re-extraction for high-resolution refinement. Final refinements were performed with symmetry applied, yielding representative 3D reconstructions for the tetramer- and octamer-like assemblies, along with their corresponding Fourier shell correlation (FSC) curves. (B) Workflow for the trimer-like particles. Candidate trimers were manually picked from the full dataset, used to generate an initial model, and refined by subtomogram averaging, producing a representative trimer-like reconstruction with the corresponding FSC curve.

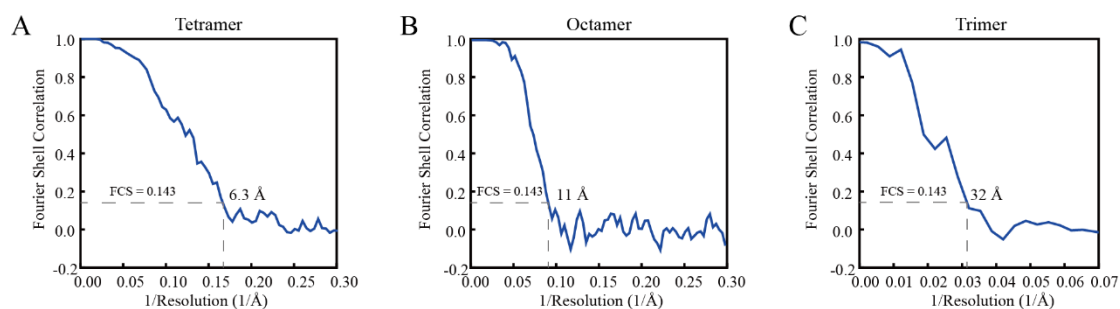


Figure R2-3 (Figure S21 in the revised manuscript). Global resolution estimation of the (A) tetramer, (B) octamer, and (C) trimer using gold-standard Fourier shell correlation (FSC) analysis. (A) FSC curve for the tetramer. The global resolution is calculated using a FSC curve cut-off at 0.143, resulting in a global resolution of 6.3 Å. (B) FSC curve for the octamer. Application of the 0.143 FSC cut-off criterion produced a global resolution of 11 Å. (C) FSC curve for the trimer. Application of the 0.143 FSC cut-off criterion produced a global resolution of 32 Å.

Q8EFN9_Dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex

```

MSIEIKVPVLPESVADATIAIWHVKVGQVSRDQNLVDIETDKVVLEVVPEDGHIGEFLLFHEGDTVLGEQVIAKFTAGA 80
VSGQEVTKAQAEAAAPAVASDESNDALSPSVRRLLAEHNVDASKVKGTVGGRTKEDVEAFIKSAPKAAASAPVVQPL 160
AAGRSEKRVPMTRLRKTIANRLLLEAKNSTAMLTTFNEVNMKPIMDIRKQYQDIFEKRHGIRLGFMSFYVKAVTEALKRFP 240
EVNASIDGDDIVYHNYFDVSIHAVSTPRGLVTPVLRDITDMSLADIEKAVRDLAIKGRDGLKLTVADMTGGNFTVTNGGVFG 320
SLMSTPILNLPQSAILGMHAIKDRPMAVNGQVEILPMMYLALSYDHRIIDGRESVGFLVAIKDFLEDPTRLLLDL 395

```

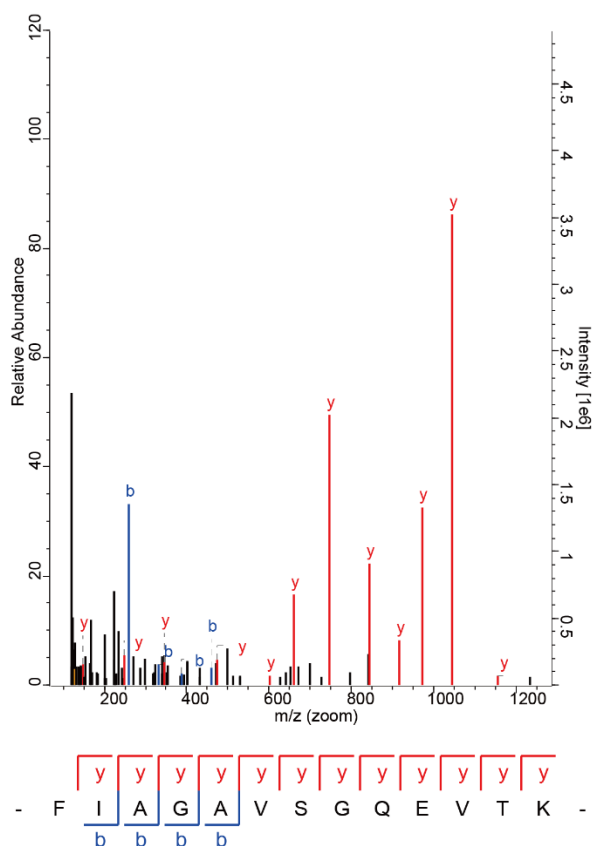


Figure R2-4 (Figure S22 in the revised manuscript). LC-MS/MS evidence supporting DLST (OGDHc E2; UniProt Q8EFN9) as a candidate component associated with the

octameric particles. (A) Amino-acid sequence of *S. oneidensis* MR-1 DLST (Q8EFN9). Peptide regions detected by LC-MS/MS are highlighted in blue. The peptide shown in (B) is additionally marked with a red background. (B) Representative MS/MS spectrum for one DLST-derived peptide fragment. Assigned b and y fragment ions are labeled in blue and red, respectively, and the corresponding peptide sequence is shown below with matched ion series indicated.

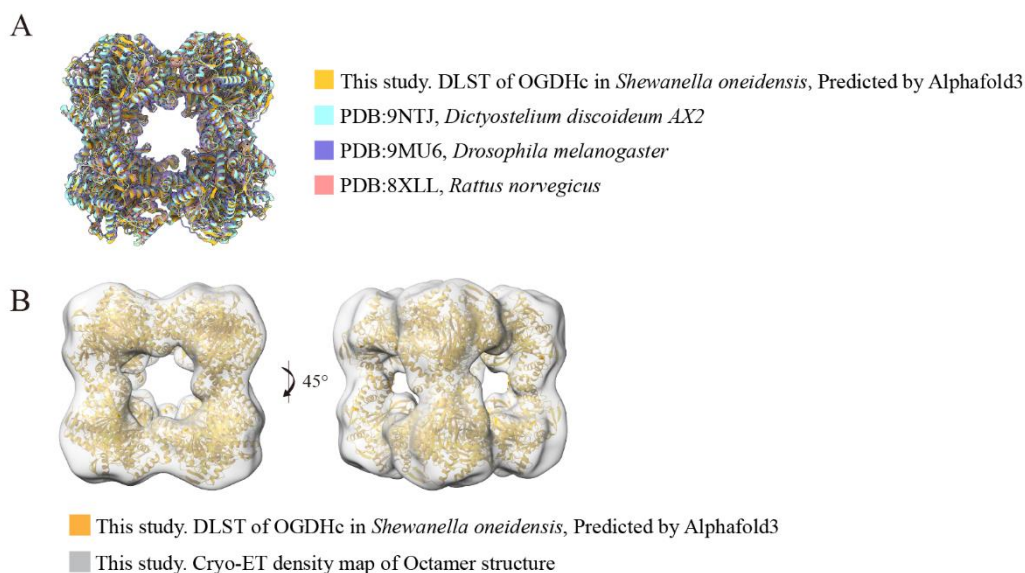


Figure R2-5 (Figure S23 in the revised manuscript). Structural comparison and tentative fitting of the octameric particle to the OGDHc E2 (DLST) scaffold. (A) Superposition of the AlphaFold3-predicted *S. oneidensis* DLST (E2) oligomer with representative DLST/OGDHc E2 structures from other species (PDB: 9NTJ, PDB: 9MU6 and PDB: 8XLL), illustrating the conserved overall architecture. (B) Docking of the AlphaFold3-predicted *S. oneidensis* DLST into the octamer *in situ* cryo-ET density map, shown in two orientations.

Q3: While your structural data and conductivity assays argue against pili-mediated electron conduction, clarifying these findings and discussing alternative electron transfer mechanisms in more depth would benefit readers and avoid confusion given prior literature.

REPLY: We thank the reviewer for this helpful suggestion. Following this advice, we revised the manuscript to more clearly define the scope of our conclusions from the filament structures and C-AFM measurements, and to place these results in the context of prior nanowire reports and alternative extracellular electron transfer mechanisms proposed for *S. oneidensis*.

In the revised text, we clarify that our central conclusion concerns the intrinsic conductivity of the type IV pili resolved in the manuscript. The atomic structures of PilA and MshA show no continuous π -stacked pathway, and aromatic clusters are generally separated by distances that are not consistent with efficient long-range hopping. In line with this, the ~6 nm filaments do not exhibit measurable conductivity in our C-AFM assays under the tested conditions. Taken together, these observations argue against PilA and MshA functioning as intrinsically conductive nanowires, helping to reduce potential ambiguity with earlier studies that associated filamentous structures with conductivity in *Shewanella* biofilms.

At the same time, we emphasize that these findings do not exclude important roles for pili in EET indirectly, for example as structural scaffolds that organize or spatially position redox-active components within the biofilm matrix. To provide readers with a clearer framework, we expanded the discussion of EET routes that remain fully compatible with our observations, including outer-membrane and periplasmic extensions enriched in electron-transfer components, vesicle-associated redox complexes, and soluble flavin shuttles that can mediate electron transfer within the matrix.

We also incorporated prior work suggesting that matrix components such as eDNA can support electron transfer by retaining and organizing secreted redox shuttles, analogous to the eDNA–phenazine mechanism reported in *Pseudomonas aeruginosa*⁷. Under this model, even if PilA and MshA are not intrinsically conductive, biofilm conductivity could still emerge from composite networks involving shuttles, eDNA, and other extracellular assemblies, particularly under anaerobic conditions where we observe increased extracellular structures.

These clarifications and the expanded discussion have been incorporated into the revised Discussion paragraph 2 and 4, as follows:

“Building on these observations, our data help explain how PilA and MshA

support biofilm function. MshA pili not only display a more balanced, patchy surface charge distribution and larger solvent-accessible surface area than PilA, but are also enriched in bundled regions of the matrix, consistent with a role as cross-linking hubs that engage neighboring filaments and matrix components. At the same time, the aromatic residues in both PilA and MshA are arranged without a continuous π -stacked pathway and are generally spaced beyond 4 Å, which, together with our C-AFM measurements, supports a model in which these pili act as interactive scaffolds rather than intrinsically conductive nanowires. Here, our C-AFM measurements follow established single-filament assays and probe charge transport along individual filaments under a defined local bias during scanning. In future work, this filament-resolved readout can be extended to filaments harvested from biofilms grown under additional electrochemical conditions, including growth on poised electrodes, to enable direct comparisons across growth environments. The defined inter-subunit interfaces and exposed surface patches therefore provide concrete targets for mutagenesis or protein engineering to tune pilus bundling, surface chemistry, and interactions with electrodes or synthetic polymers in future bioenergy and bioremediation applications.”

“Extracellular DNA may also contribute to electron transfer in a more indirect manner. A study in *Pseudomonas aeruginosa* showed that extracellular DNA can bind phenazine redox shuttles and support DNA-mediated charge transfer within biofilms⁷. *S. oneidensis* MR-1 has been reported to release flavin-based redox-active compounds⁶, raising the possibility that matrix-associated shuttles could be retained locally and participate in charge transport within MR-1 biofilms. In the present study, we focus on the native structural context and do not directly test eDNA shuttle interactions or their impact on electron transfer. This possibility could be examined in future work using DNase treatment and controlled flavin modulation together with electrical readouts. More broadly, how vesicle trafficking, extracellular multimer assembly, eDNA-

containing filaments, and overall biofilm conductivity are functionally connected will require further investigation.”

Q4: Functional correlations between structure and biofilm physiology are currently limited. Consider integrating or discussing potential genetic or mutational experiments to link filament structure with biofilm formation and activity.

REPLY: We thank the reviewer for this important suggestion. Following the reviewer’s advice, we strengthened the structure and function connection by adding genetic perturbation experiments targeting the major extracellular filaments and quantifying the resulting biofilm and redox and electrochemical phenotypes.

Specifically, we compared wild-type MR-1 with deletion mutants lacking MshA pili (Δ MshA), PilA pili (Δ PilA), or flagella (Δ Fla). We evaluated biofilm formation by fluorescence imaging (fluorescent area coverage), ferrihydrite reduction activity as Fe(II)/total Fe (%), and microbial fuel cell performance as output voltage (V) (Fig. R2-6). Across these assays, Δ MshA showed significant defects in all three readouts, with reduced biofilm coverage, strongly decreased Fe(II)/total Fe, and lower MFC voltage. In contrast, Δ PilA did not differ significantly from wild type in biofilm coverage, Fe(II)/total Fe, or output voltage under our conditions. Δ Fla exhibited a modest reduction in biofilm coverage and MFC voltage, while ferrihydrite reduction remained comparable to wild type (Fig. R2-6).

These functional phenotypes are consistent with prior genetic studies indicating that MshA plays a dominant role in *S. oneidensis* biofilm development and can influence electrochemical activity, whereas PilA typically contributes more weakly, and flagella primarily affect surface colonization and motility-related biofilm development. Notably, the strong Δ MshA defects also align with

our structural analysis showing that MshA pili present a more balanced, patchy surface charge distribution and a larger solvent-accessible surface area than PilA, features expected to promote filament–filament and filament–matrix interactions that stabilize biofilm architecture. And we note that the atomic models reported here also enable future structure-guided mutagenesis, for example, targeting key surface charge patches or inter-subunit interfaces identified in PilA and MshA, to directly test determinants of bundling, matrix interactions, and biofilm activity.

We added the following paragraph at the end of the Results section, “Structural analysis of flagella and pili” :

*“To connect the filament structures to biofilm physiology, we compared wild-type MR-1 with deletion mutants lacking each filament (Δ MshA, Δ PilA, and Δ Fla). Fluorescence imaging showed that biofilm coverage was markedly reduced in Δ MshA relative to wild type, whereas Δ PilA displayed no significant difference and Δ Fla exhibited a modest decrease (Fig. S18A-E). Consistent with these trends, ferrihydrite reduction activity, quantified as Fe(II)/total Fe (%), was strongly impaired in Δ MshA, but remained comparable to wild type in Δ PilA and Δ Fla (Fig. S18F). In microbial fuel cell measurements, Δ MshA produced a significantly lower output voltage and Δ Fla showed a modest decrease, while Δ PilA again was not significantly different from wild type (Fig. S18G). These phenotypes are in line with prior genetic studies showing that MshA plays a dominant role in *S. oneidensis* biofilm formation and can impact electrochemical activity, whereas PilA typically has a more limited contribution, and flagella mainly influence surface colonization and motility-related biofilm development¹⁻⁴. And the strong Δ MshA defects also align with our structural analysis, in which MshA pili exhibit a more balanced, patchy surface charge distribution and larger solvent-accessible surface area than PilA, features that are well suited to promote filament–filament and filament-matrix interactions that stabilize biofilm architecture.”*

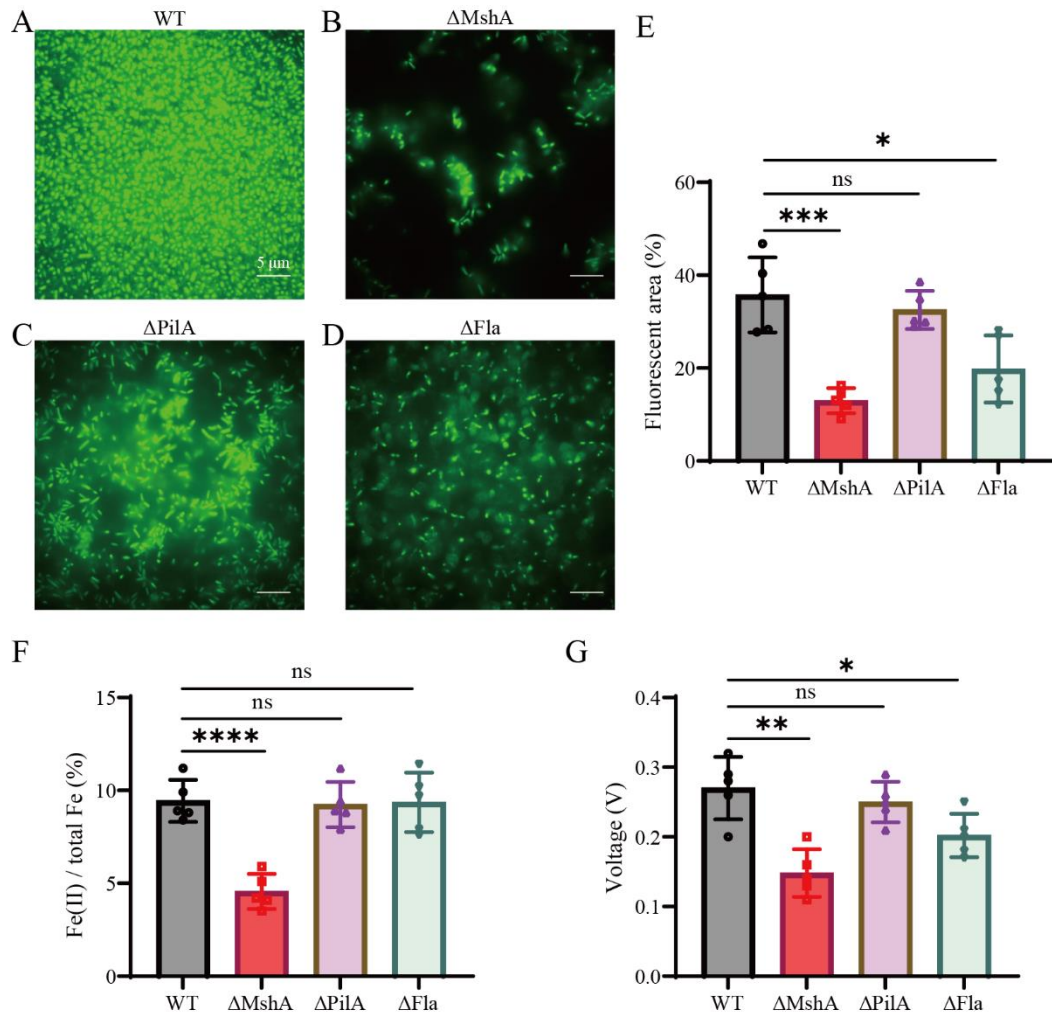


Figure R2-6 (Figure S18 in the revised manuscript). Physiological phenotypes of filament deletion mutants. (A-D) Representative fluorescence images of biofilms formed by wild-type *S. oneidensis* MR-1 WT, $\Delta MshA$, $\Delta PilA$, and ΔFla strains. Scale bar, 5 μm . (E) Quantification of biofilm coverage, measured as fluorescent area (%). (F) Ferrihydrite reduction activity quantified as Fe(II)/total Fe (%). (G) Microbial fuel cell output voltage (V). Bars represent mean $\pm$ SEM; individual data points are overlaid. Statistical significance is indicated as ns, not significant; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

In addition, we added a corresponding statement to the second paragraph of the Discussion section, as follows:

“The defined inter-subunit interfaces and exposed surface patches therefore provide concrete targets for mutagenesis or protein engineering to tune pilus bundling, surface chemistry, and interactions with electrodes or synthetic polymers in future bioenergy and bioremediation applications.”

Q5: The biofilms were grown under controlled lab conditions on EM grids. A brief discussion addressing how these conditions might differ from natural environments and the possible impact on filament expression or biofilm architecture would add valuable context.

REPLY: We thank the reviewer for this helpful suggestion. Based on the reviewer's comment, we added a discussion to provide environmental context for our grid-based laboratory biofilm growth conditions and to clarify how these conditions may influence filament expression and biofilm architecture.

Biofilms formed on EM grids develop under defined geometry and controlled media and oxygen conditions, whereas natural *S. oneidensis* biofilms typically form on heterogeneous mineral or sediment surfaces with spatially variable nutrient and redox gradients and community interactions. These differences could influence the abundance, bundling, and spatial organization of extracellular filaments and other matrix components. We therefore present the relative filament abundance and spatial arrangement observed here as representative of our defined conditions, while the near-atomic architectures of PilA pili, MshA pili, and flagella describe their core filament assemblies.

We also note that extending these measurements to more environmentally relevant settings will be an important direction for future work, for example by growing biofilms on mineral substrates or in gradient-forming systems, and by examining how changing redox conditions and mixed-community contexts reshape extracellular structures.

These points have been incorporated into the revised Discussion paragraph 3, as follows:

*"In this study, biofilms were cultivated directly on EM grids, which enabled rapid vitrification and minimized perturbation from handling. Because natural *S. oneidensis* habitats are more heterogeneous, future work could extend these observations by growing biofilms on more native-like substrates and under*

spatially structured redox conditions to assess how environmental complexity influences filament expression and matrix organization.”

Q6: Figure 1's heavy overlay of segmentation colors obscures underlying structural details; alternative visualization strategies could improve clarity.

REPLY: We thank the reviewer for this helpful suggestion. Following the reviewer's advice, we added additional Supporting material to improve visualization clarity without obscuring underlying structural details.

Specifically, we provide a new Supplementary figure showing the corresponding raw tomographic slices with no segmentation overlay (Fig. R2-7), together with two Supplementary movies that displays the tomogram through z-slices to allow readers to inspect the native densities and spatial relationships directly (Movie R2-2 and R2-3).

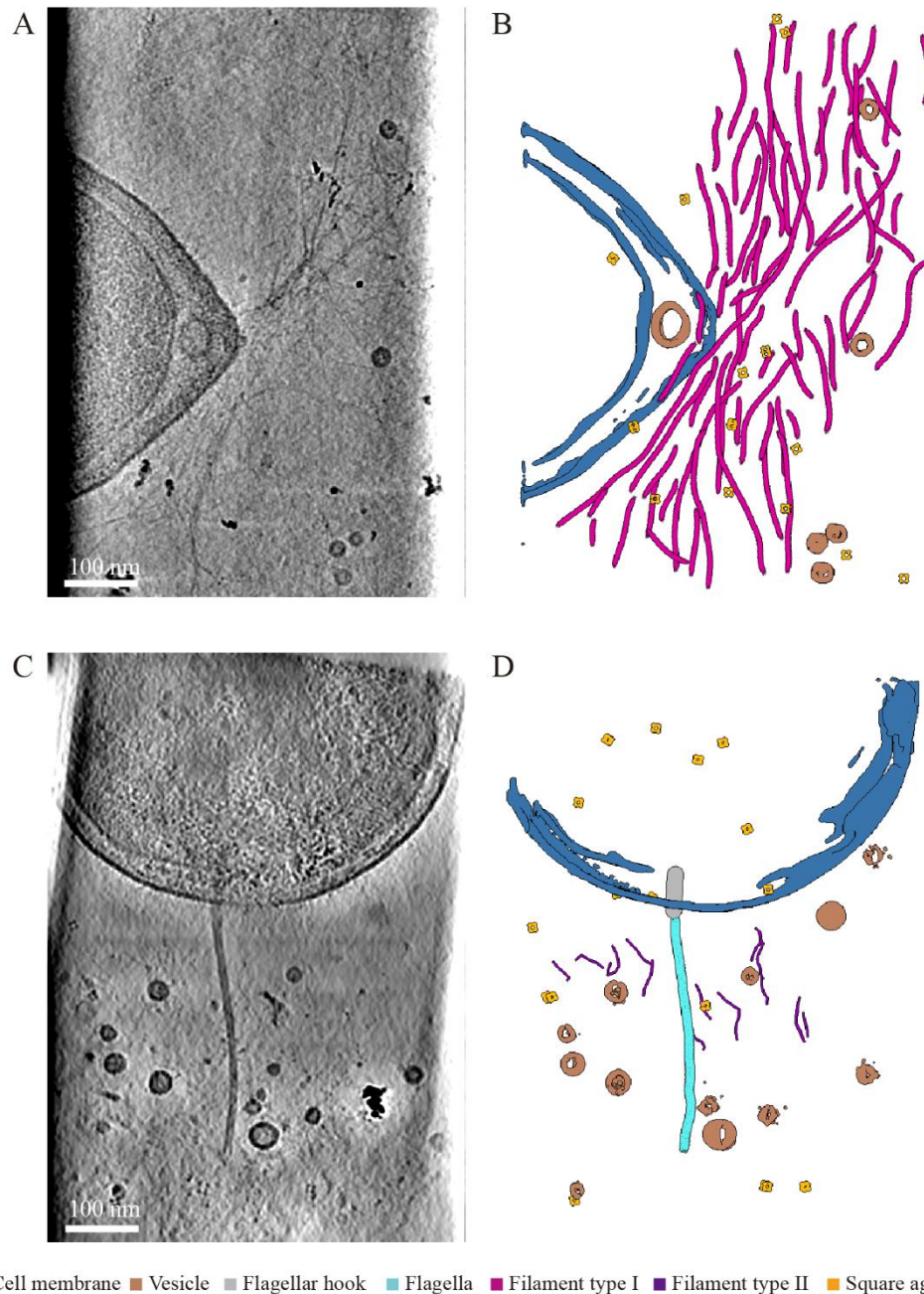


Figure R2-7 (Figure S1 in the revised manuscript). Tomographic slices corresponding to Fig. 1B and Fig. 1C. (A) Unannotated tomographic slice showing extracellular filaments and particles surrounding an *S. oneidensis* MR-1 cell. (B) 3D rendering of the same view shown in (A), highlighting the major extracellular features. (C) Unannotated tomographic slice showing a second example with abundant extracellular material around the cell envelope. (D) 3D rendering of the same view shown in (C). Scale bars, 100 nm.

Movie R2-2 (Movie S1 in the revised manuscript). Raw cryo-ET volume corresponding to Fig. 1B.

Unannotated tomographic volume of an *S. oneidensis* MR-1 biofilm region, shown without colored overlays to provide an unobstructed view of the underlying ultrastructure. Related still images are shown in Fig. S1A-B.

Movie R2-3 (Movie S2 in the revised manuscript). Raw cryo-ET volume corresponding to Fig. 1C.

Unannotated tomographic volume of a second representative *S. oneidensis* MR-1 biofilm region, displayed without rendering or colored traces. Related still images are shown in Fig. S1C-D.

Q7: Emphasizing current limitations in functional assignment of unidentified structures will help prevent overinterpretation of preliminary data.

REPLY: We thank the reviewer for this important point. Following the reviewer's suggestion, we revised the Discussion to more explicitly state the current limitations in molecular and functional assignment of the unidentified structures. We now present these interpretations as working hypotheses based on the available evidence and avoid definitive functional language where direct validation is not yet available.

We added the following statement to the paragraph 3 of the Discussion, as follows:

"Importantly, the identities and physiological roles of these non-periodic filaments and multimeric aggregates cannot yet be assigned definitively from the current data. Accordingly, we interpret these structures cautiously and treat proposed mechanisms as working hypotheses that will require targeted biochemical and genetic validation."

Overall, the methodology and data analysis are sound, and your findings merit publication after minor revisions addressing these points. These enhancements would increase the clarity, biological relevance, and impact of your work.

Thank you for your important contribution.

REPLY: We thank the reviewer for this positive assessment and for the constructive recommendations. Based on the reviewer's comments, we have

made the requested revisions throughout the manuscript to improve clarity, strengthen the biological context, and better define the scope and limitations of our conclusions. We appreciate the reviewer's time and thoughtful input, which have helped to improve the quality and impact of the work.

Reviewer #3 (Remarks to the Author):

Li et al., Ultrastructural and atomic characterization of biofilm-associated extracellular filaments in *Shewanella oneidensis* MR-1

The authors present a structural study by cryo-ET into the extracellular matrix of biofilm-producing bacteria (*Shewanella oneidensis*). They find several types of filamentous structures which they analysed in detail on high-resolution 3-D structures obtained by cryo-ET rendering (on whole cells) and cryo-EM 3D-averaging. However, for obvious reasons, the cryo-EM studies were conducted on cell-free areas with enriched filamentous material. They claim that the surface structures and electrostatic patterns of these extracellular filaments play a crucial role in stabilizing biofilms, and in metal reduction, which may have biomedical implications. Their study on oxygen limitation is remarkable, though, figure 5 is hard to interpret for an outside reader, esp. the micrographs.

Overall, this is a well-executed study on extracellular matrix of bacteria in biofilms. I cannot see any major flaws with the methods applied here. These biofilms are indeed a current topic of many research labs (including labs dealing with space flight!), and their implications in our life are not well studied yet. The novelty aspect of this work is a bit overrated. E.g., bacterial flagella and pili have been studied since quite some time, but typically in isolation and not within the context of a bacterial biofilm. Hence, I believe this paper deserves publication after some revision.

REPLY: We thank the reviewer for the careful assessment of our manuscript and for the constructive suggestions regarding data interpretation and figure presentation. We have revised the text and figures to improve transparency in density assignment for cellular cryo-ET and to make the key observations more accessible to non-specialist readers.

Some comments:

Q1: One of the big issues always with cellular cryo-ET is the correct identification of densities in the micrographs. Hence, I am not always fully convinced that the structures indicated (e.g., Fig. 5) are indeed what the authors want to claim, or if these are simply unidentified impurities (e.g., from lysed cells). This is much less of an issue with isolated material, or the detached filaments discussed in figure 2.

REPLY: We thank the reviewer for the helpful comment. Based on the reviewer's concern about density assignment in cellular cryo-ET, we revised Fig. 5 and the associated text by adding an individual particle-level 3D characterization of the square-shaped aggregates, together with a clearer presentation of the subtomogram processing workflow and its outputs.

Utilizing 3D tomographic information, we extracted and inspected individual multimeric particles within the tomographic volumes. Through automated segmentation of individual subtomograms using unbiased, convolutional neural network-based approaches, this analysis demonstrated that the aggregates consistently group into three dominant morphological classes., including two relatively flattened forms and a clearly thicker form (Fig. R3-1 and Movie R3-1). We then applied a reference-free subtomogram workflow and now show the computational procedure and representative results in detail (Fig. R3-2), following commonly used cryo-ET approaches⁹⁻¹¹. Together, these additions provide an objective 3D description of the aggregates and clarify the structural heterogeneity present in the dataset, strengthening the basis for interpreting the highlighted structures in Fig. 5.

The corresponding additions have been incorporated into the 4 paragraph of the Results section, “Oxygen limitation enhances filament abundance and aggregate formation” , as follows:

“To objectively characterize these assemblies in three dimensions, we

performed a particle-level analysis directly in the cryo-ET volumes. Individual multimeric particles were extracted from the tomograms and reconstructed to assess their 3D morphology *in situ*. Closer inspection of these proteinaceous aggregates revealed that they are not uniform squares, but instead comprise discrete multimers with defined sizes and symmetries (Fig. 5H, I and Movie S3), including roughly square-shaped tetramers and octamers, as well as triangular trimers. Inspection of the particles in the tomographic volumes indicates that they fall into three dominant morphological classes, with two relatively flattened forms and one clearly thicker form, highlighting pronounced structural heterogeneity within the aggregate population. To reduce user bias and quantify this heterogeneity, we then applied a reference free subtomogram averaging workflow to obtain representative reconstructions for the three prevalent multimeric forms (Fig. S20). The tetramer, octamer, and trimer reconstructions reached approximate resolutions of 6.3 Å, 11 Å, and 32 Å, respectively (Fig. S21).”

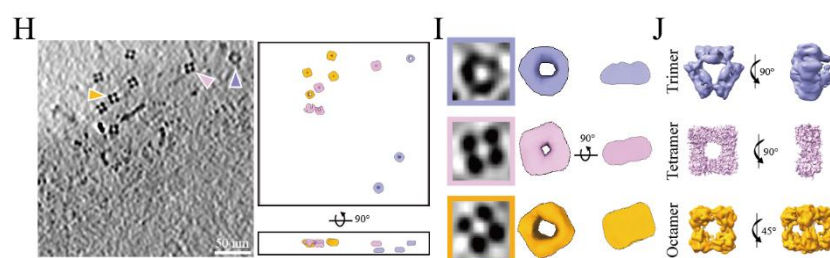


Figure R3-1 (Figure 5 H-J in the revised manuscript). (H) Representative tomographic slice showing multimeric extracellular aggregates (arrowheads). Scale bar, 50 nm. (I) Zoomed-in views of representative individual aggregates *in situ*, showing three distinct particle morphologies together with their corresponding 3D reconstructions. (J) Subtomogram-averaged density maps of the tetramer, octamer, and trimer.

Movie R3-1 (Movie S3 in the revised manuscript). 3D visualization of extracellular multimeric particles *in situ*.

Tomographic views highlighting aggregates in *S. oneidensis* MR-1 biofilms, including trimer-, tetramer-, and octamer-like particles. The movie shows representative particles in the tomographic context and the corresponding 3D reconstructions. Related panels are shown in Fig. 5H.

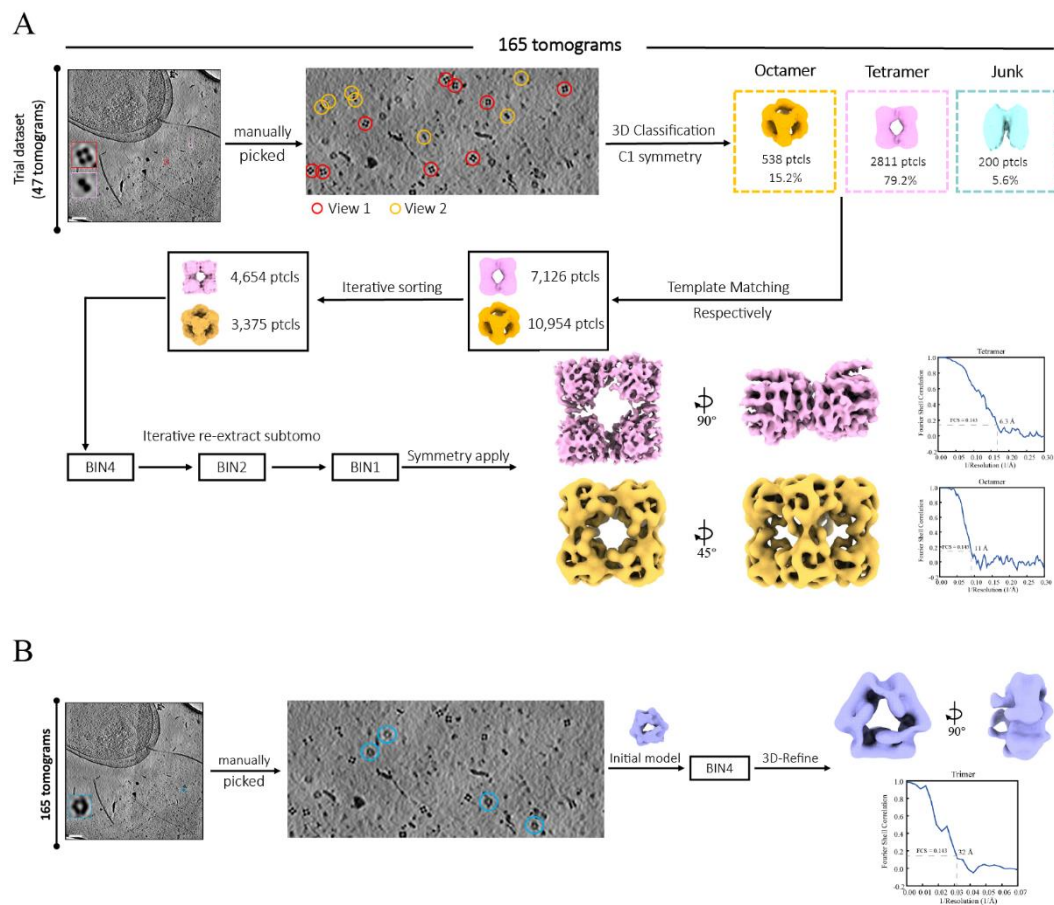


Figure R3-2 (Figure S20 in the revised manuscript). Subtomogram averaging workflow for reconstructing heterogeneous extracellular multimers in situ. (A) Workflow for identifying and reconstructing tetramer- and octamer-like particles from cryo-ET data. Candidate particles were manually picked from a trial subset of tomograms, extracted as subtomograms, and subjected to reference-free 3D classification (C1 symmetry) to separate octamer-like, tetramer-like, and junk classes. The resulting initial 3D model was low-pass filtered and then used to generate templates for template matching across the full dataset separately for each class. This was followed by iterative sorting and re-extraction for high-resolution refinement. Final refinements were performed with symmetry applied, yielding representative 3D reconstructions for the tetramer- and octamer-like assemblies, along with their corresponding Fourier shell correlation (FSC) curves. (B) Workflow for the trimer-like particles. Candidate trimers were manually picked from the full dataset, used to generate an initial model, and refined by subtomogram averaging, producing a representative trimer-like reconstruction with the corresponding FSC curve.

Q2: The authors conducted AFM studies, but they are not presented in the main text, only in the supplementary data. The point of supplementary data is to further explain what has been shown in the main text, NOT to add additional data of a completely different kind since they base a good portion of their

conclusion on these measurements, discussed in the results. This may reflect my own antiquated view, but, nevertheless, I would like to see an AFM figure in the main text.

REPLY: We thank the reviewer for this helpful suggestion on improving data presentation and readability. Based on the reviewer’s comment, we moved representative AFM results from the Supplementary Information into the main text as an updated figure panel (Fig R3-3), so that the AFM evidence supporting our Results and conclusions is directly visible in the primary figures.

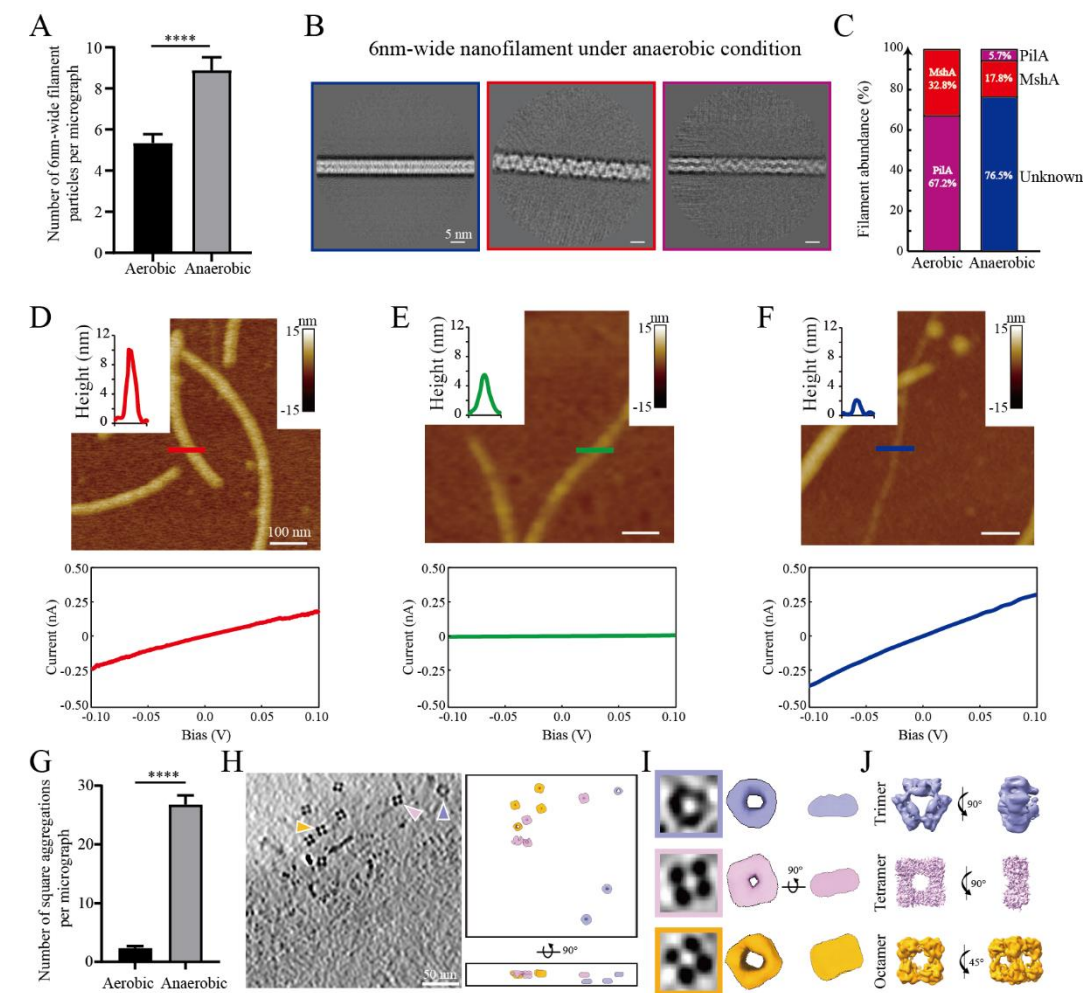


Figure R3-3 (Figure 5 in the revised manuscript). Impact of oxygen limitation on the extracellular composition of *Shewanella oneidensis* MR-1 biofilms. (A) Comparison of 6 nm-wide filament particle number per micrograph under aerobic and anaerobic conditions. Data are presented as mean $\pm$ SEM. $n = 4000$. Student’s t test, **** $P < 0.0001$. (B) 2D classification of 6 nm-wide nanofilaments under anaerobic cultivation, categorized into three classes: Non-periodic nanofilaments (blue outlined box), MshA pili (red outlined

box), PilA pili (magenta outlined box). (C) Filament abundance under aerobic and anaerobic conditions, illustrating the distribution changes in nanofilament categories. (D-F) Representative AFM height images (top) and corresponding line-section height profiles (insets) for three extracellular filament classes, with mean heights of 10.0 ± 0.3 nm (D), 5.8 ± 0.2 nm (E), and 2.1 ± 0.3 nm (F) (mean $\pm$ SD, $n = 10$). Bottom, current-voltage (I-V) curves measured by conductive AFM (C-AFM) on the corresponding filaments. Scale bars, 100 nm. (G) Comparison of square-shaped aggregates per micrograph under aerobic and anaerobic conditions. Data are presented as mean $\pm$ SEM. $n = 50$. Student's t test, **** $P < 0.0001$. (H) Representative tomographic slice showing multimeric extracellular aggregates (arrowheads). Scale bar, 50 nm. (I) Zoomed-in views of representative individual aggregates *in situ*, showing three distinct particle morphologies together with their corresponding 3D reconstructions. (J) Subtomogram-averaged density maps of the tetramer, octamer, and trimer.

Q3: The figures are well made and informative. I would add a side-by-side image of areas with and without rendering (esp. figure 1). Currently, the rendering hides the actual structures underneath.

REPLY: We thank the reviewer for this helpful suggestion. Following the reviewer's advice, we added an additional Supplementary figure (Fig. R3-4) and two movies (Movie R3-2 and R3-3) that present the same regions shown in Fig. 1 both without and with segmentation. These matched views are shown at the same orientation and scale to allow direct, side-by-side comparison of the underlying raw structures and the corresponding rendered interpretation.

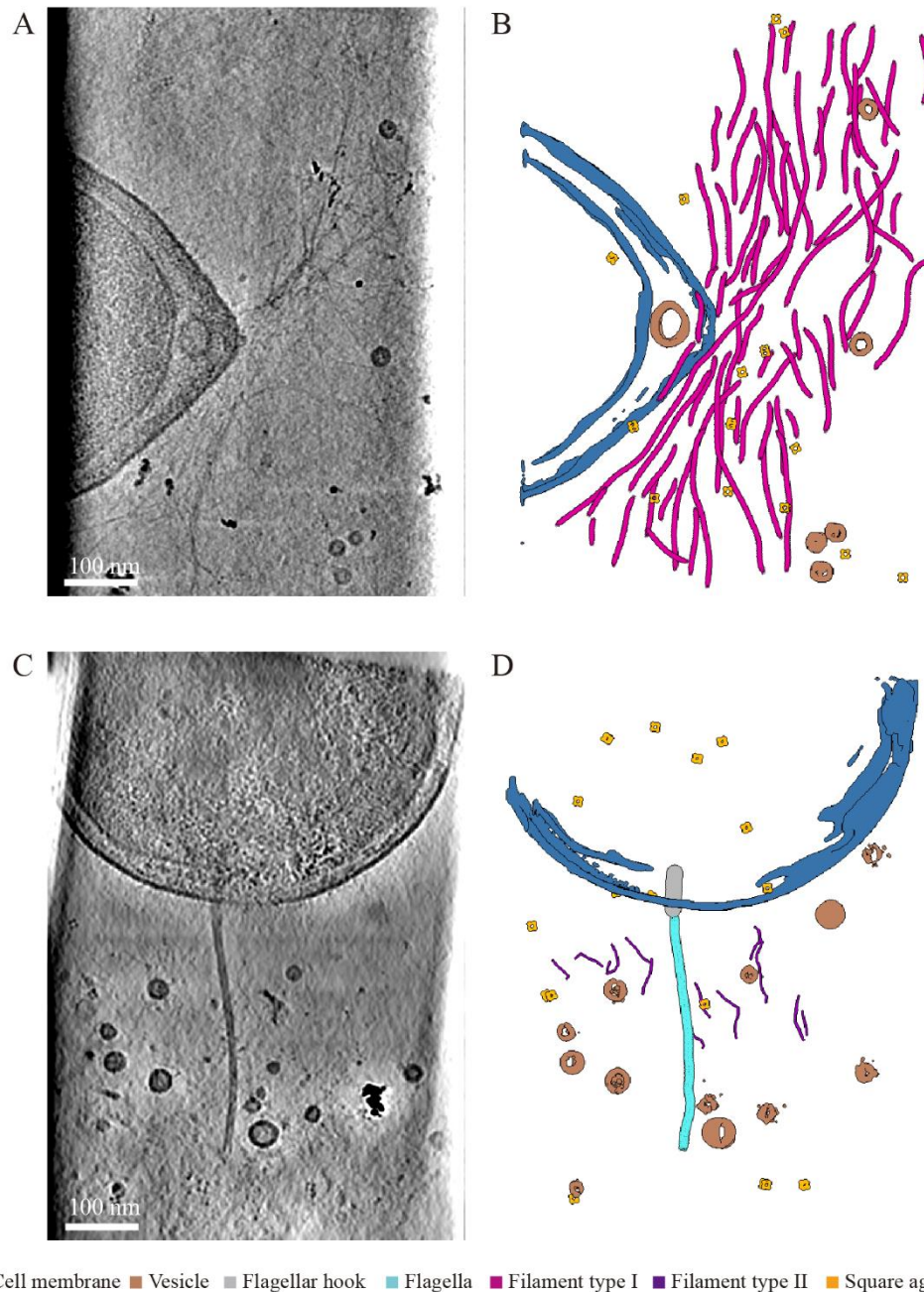


Figure R3-4 (Figure S1 in the revised manuscript). Tomographic slices corresponding to Fig. 1B and Fig. 1C. (A) Unannotated tomographic slice showing extracellular filaments and particles surrounding an *S. oneidensis* MR-1 cell. (B) 3D rendering of the same view shown in (A), highlighting the major extracellular features. (C) Unannotated tomographic slice showing a second example with abundant extracellular material around the cell envelope. (D) 3D rendering of the same view shown in (C). Scale bars, 100 nm.

Movie R3-2 (Movie S1 in the revised manuscript). Raw cryo-ET volume corresponding to Fig. 1B.

Unannotated tomographic volume of an *S. oneidensis* MR-1 biofilm region, shown without colored overlays to provide an unobstructed view of the underlying ultrastructure. Related still images are shown in Fig. S1A-B.

Movie R3-3 (Movie S2 in the revised manuscript). Raw cryo-ET volume corresponding to Fig. 1C.

Unannotated tomographic volume of a second representative *S. oneidensis* MR-1 biofilm region, displayed without rendering or colored traces. Related still images are shown in Fig. S1C-D.

Q4: As already mentioned above, figure 5E is essentially asking the reader to believe what the authors claim. In my opinion, there is no way to distinguish octamers from tetramers or even other particles. This panel has to be presented at a much larger scale.

REPLY: We thank the reviewer for this important comment on the interpretability of Fig. 5E. Following the reviewer's suggestion, we revised the presentation by providing a substantially enlarged view with additional tomographic context and the updated figures and corresponding text are included in our response to Q1 (Fig. R3-1 Movie R3-1 and Fig. R3-2).

In addition, to address the broader concern that stoichiometry cannot be reliably inferred from a single tomogram slice, we adjusted the wording in the main text to avoid over-interpreting the 2D appearance in Fig. 5E. Instead, as described in our response to Q1, to classify these assemblies, we now leverage direct 3D characterization of individual, non-averaged particles within cryo-ET volumes, combined with a reference-free subtomogram averaging workflow. This added analysis objectively groups the aggregates into three dominant morphological classes and provides representative reconstructions for each class, together with a detailed depiction of the subtomogram processing pipeline.

Q5: Figure 6 is mostly imagination and guess-work, at best representing a single momentary shot. It should be accompanied by an overview micrograph where these biofilm arrangement can be seen unobstructed.

REPLY: We thank the reviewer for this constructive comment. Following the reviewer’s suggestion, we added an unobstructed overview micrograph that shows the biofilm organization, so readers can directly see the context that the schematic in Fig. 6 is summarizing.

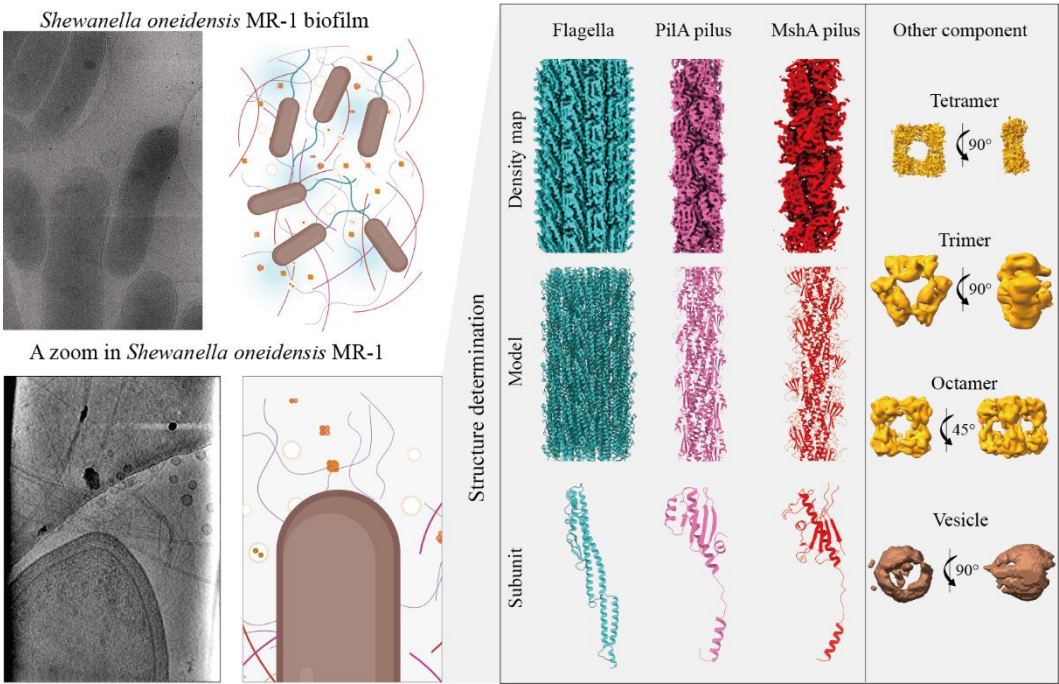


Figure R3-5 (Figure 6 in the revised manuscript). Schematic summary of *Shewanella oneidensis* MR-1 biofilms. Left, overview and zoomed cryo-EM images showing the biofilm context. Middle, schematic cartoon summarizing extracellular features observed by cryo-ET/cryo-EM. Right, density maps and fitted models for three filaments (flagella, PilA pili, and MshA pili), and representative extracellular particles (trimer-, tetramer-, and octamer-like multimers) and vesicles.

Created in BioRender. Liu, C. (2026) <https://BioRender.com/c93vg13>

References

1. Saville, R.M., Dieckmann, N. & Spormann, A.M. Spatiotemporal activity of the mshA gene system in *Shewanella oneidensis* MR-1 biofilms. *FEMS Microbiol Lett* **308**, 76–83 (2010).
2. Thormann, K.M., Saville, R.M., Shukla, S., Pelletier, D.A. & Spormann, A.M. Initial Phases of biofilm formation in *Shewanella oneidensis* MR-1. *J Bacteriol* **186**, 8096–104 (2004).
3. Fitzgerald, L.A. et al. *Shewanella oneidensis* MR-1 Msh pilin proteins are involved in extracellular electron transfer in microbial fuel cells. *Process Biochemistry* **47**, 170–174 (2012).
4. Bouhenni, R.A. et al. The Role of *Shewanella oneidensis* MR-1 Outer Surface Structures in Extracellular Electron Transfer. *Electroanalysis* **22**, 856–864 (2010).
5. Liu, X., Walker, D.J.F., Nonnenmann, S.S., Sun, D. & Lovley, D.R. Direct Observation of Electrically Conductive Pili Emanating from *Geobacter sulfurreducens*. *mBio* **12**, e0220921 (2021).
6. Marsili, E. et al. *Shewanella* secretes flavins that mediate extracellular electron transfer. *Proc Natl Acad Sci U S A* **105**, 3968–73 (2008).
7. Saunders, S.H. et al. Extracellular DNA Promotes Efficient Extracellular Electron Transfer by Pyocyanin in *Pseudomonas aeruginosa* Biofilms. *Cell* **182**, 919–932.e19 (2020).
8. Pirbadian, S. et al. *Shewanella oneidensis* MR-1 nanowires are outer membrane and periplasmic extensions of the extracellular electron transport components. *Proc Natl Acad Sci U S A* **111**, 12883–8 (2014).
9. Chen, M. et al. A complete data processing workflow for cryo-ET and subtomogram averaging. *Nature Methods* **16**, 1161–1168 (2019).
10. Tegunov, D., Xue, L., Dienemann, C., Cramer, P. & Mahamid, J. Multi-particle cryo-EM refinement with M visualizes ribosome-antibiotic complex at 3.5 Å in cells. *Nature Methods* **18**, 186–193 (2021).
11. Watson, A.J.I. & Bartesaghi, A. Advances in cryo-ET data processing: meeting the demands of visual proteomics. *Current Opinion in Structural Biology* **87**, 102861 (2024).

Reviewer 1:

In Figure 1B and C, my impression from the response to comments is that the overlay would be removed and put next to the image so that the raw structure in the images could be observed. That does not seem to have happened here.

REPLY: Thank the reviewer for this helpful suggestion. We apologize that the previous revision did not make this change sufficiently clear. In the revised Figure 1, we have now incorporated the unannotated tomographic slice and the 3D rendering of the same view that were previously shown in Figure S1, and arranged them adjacent to the corresponding annotated views. This presentation separates the raw image from the overlay and allows the underlying structure to be visualized more clearly. The figure legend has also been updated accordingly.