

Supporting Information for

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

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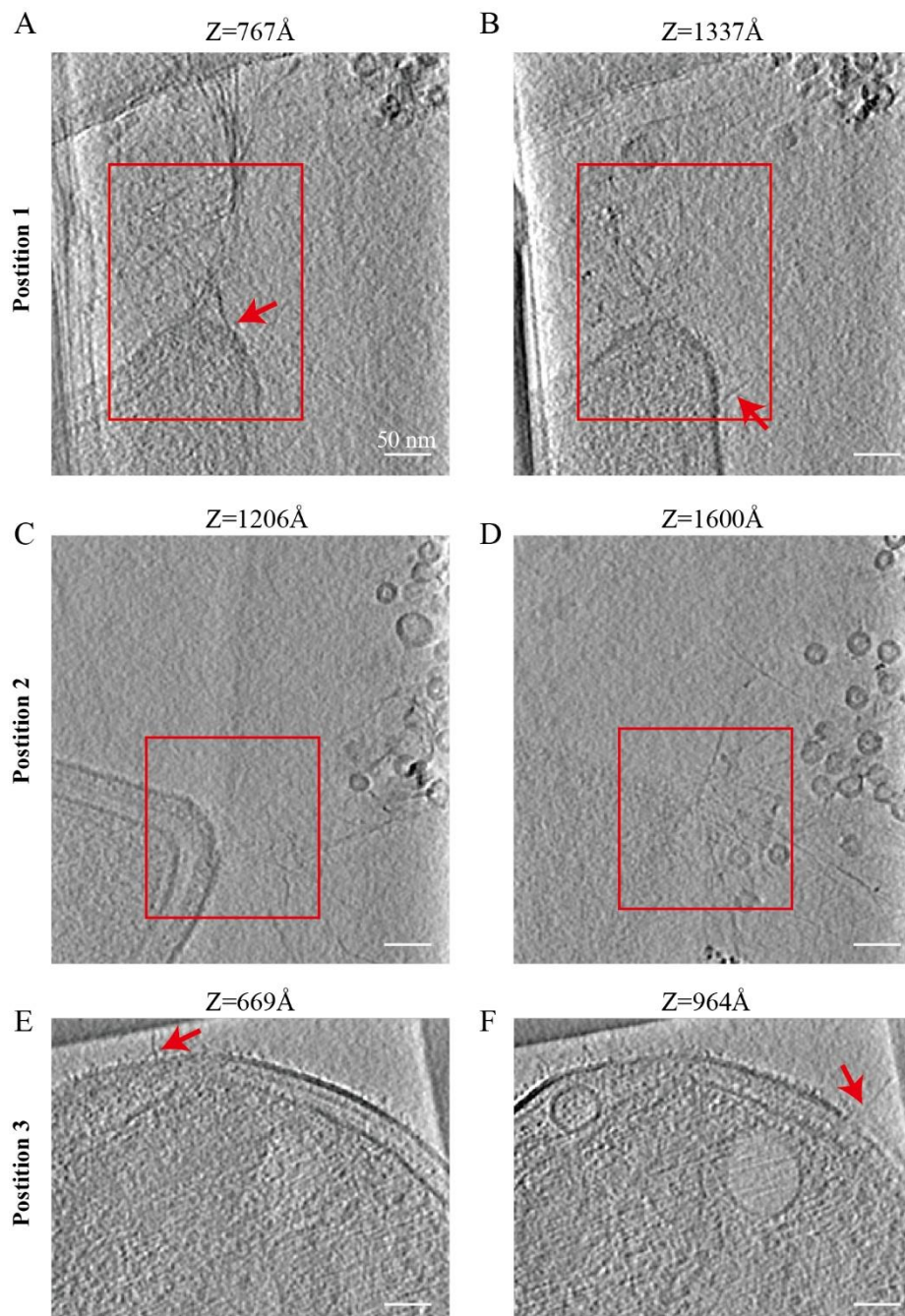
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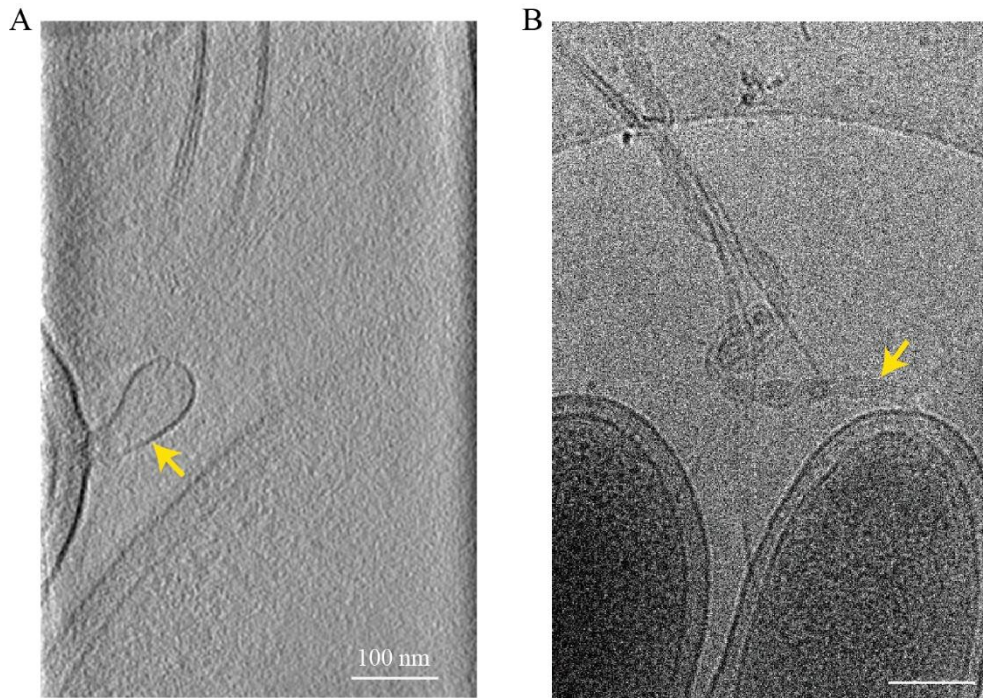
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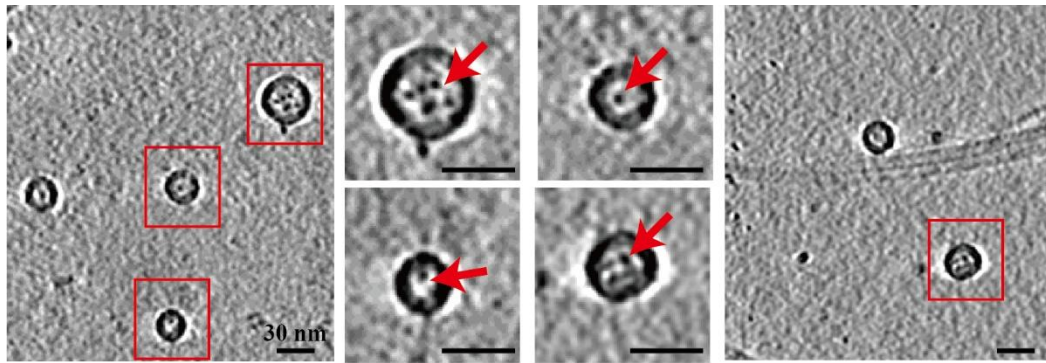
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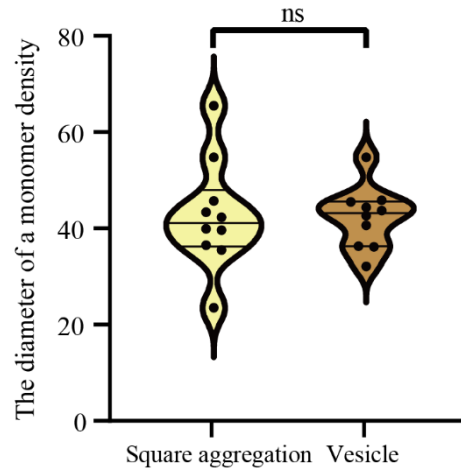
Supplementary Figure 1. Slices through tomograms of nanofilament secretion processes in *Shewanella oneidensis* MR-1 biofilms. Three representative cryo-ET multi-z slices illustrating the secretion processes of nanofilaments. The z slice values of each tomogram are labeled above the respective panels. Portions of the short ends of oval-shaped bacteria protrude and release large quantities of nanofilaments (highlighted by red boxes), while nanofilaments extend outward from the bacteria (indicated by red arrows). Scale bar: 50nm. Similar secretion events were observed in at least three independent tomograms.



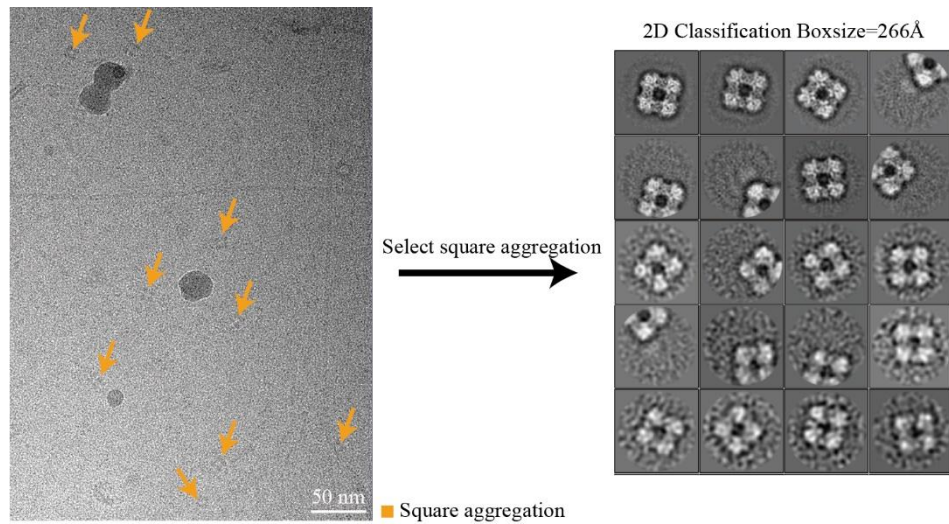
Supplementary Figure 2. Slices through tomograms of vesicle secretion processes in *Shewanella oneidensis* MR-1 biofilms. Representative slices through tomograms illustrating vesicle secretion by *Shewanella oneidensis* MR-1 bacteria within biofilms. Vesicle secretion events are indicated by yellow arrows. Scale bar: 100 nm. Similar vesicle secretion events were observed in multiple independently acquired tomograms.



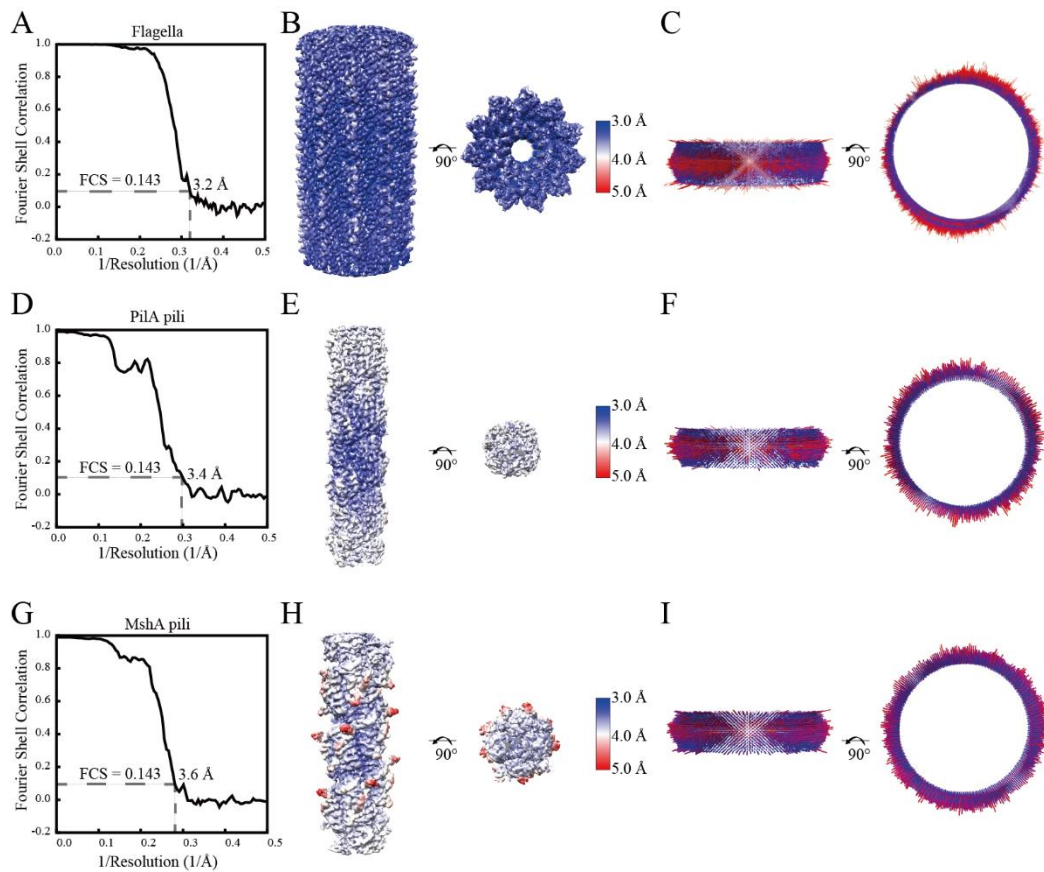
Supplementary Figure 3. Slices through tomograms of vesicles in *Shewanella oneidensis* MR-1 biofilms. Representative slices through tomograms showing vesicles in the biofilms. Densities were observed within the vesicles. Some vesicles contained single densities, and others contained multiple densities. Vesicles are outlined with red boxes, and the densities within the vesicles are indicated with red arrows. Scale bar: 30 nm. Similar vesicle-associated densities were observed in multiple independently acquired tomograms



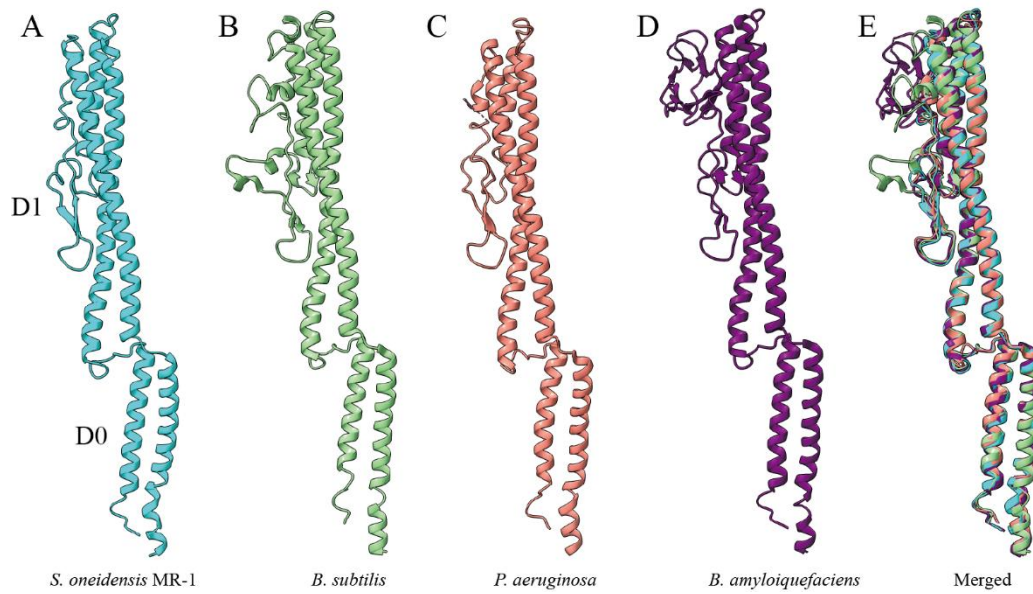
Supplementary Figure 4. Comparison of the monomer density diameters in square-shaped aggregates and vesicles. The diameters of monomer densities within square-shaped aggregates and vesicles were measured and compared. Data are presented as violin plots ($n = 10$), $n = 10$ tomographic slices per group. Statistical significance was evaluated using two-tailed unpaired Student's t -test. $P = 0.9147$, ns, not significant.



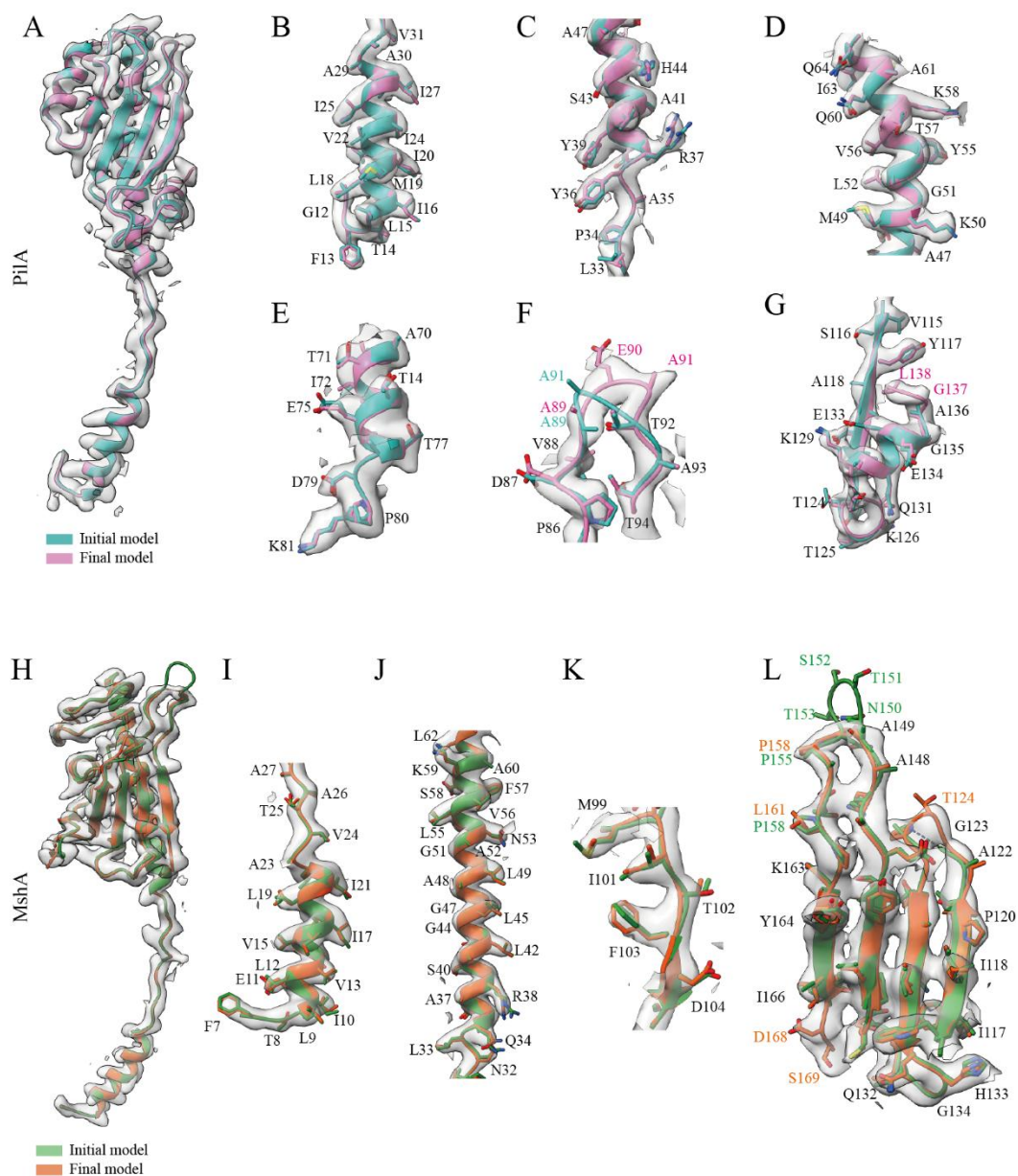
Supplementary Figure 5. 2D classification of square-shaped aggregates. Cryo-EM micrograph displaying square-shaped aggregates within the biofilm (left panel). Scale bar: 50nm. Yellow arrows indicate the locations of square-shaped aggregates, which were selected for further 2D classification. Results of the 2D classification of selected square-shaped aggregates, revealing that the square-shaped aggregates adopted a preferred orientation (right panel). The box size used for 2D classification was 266 Å.



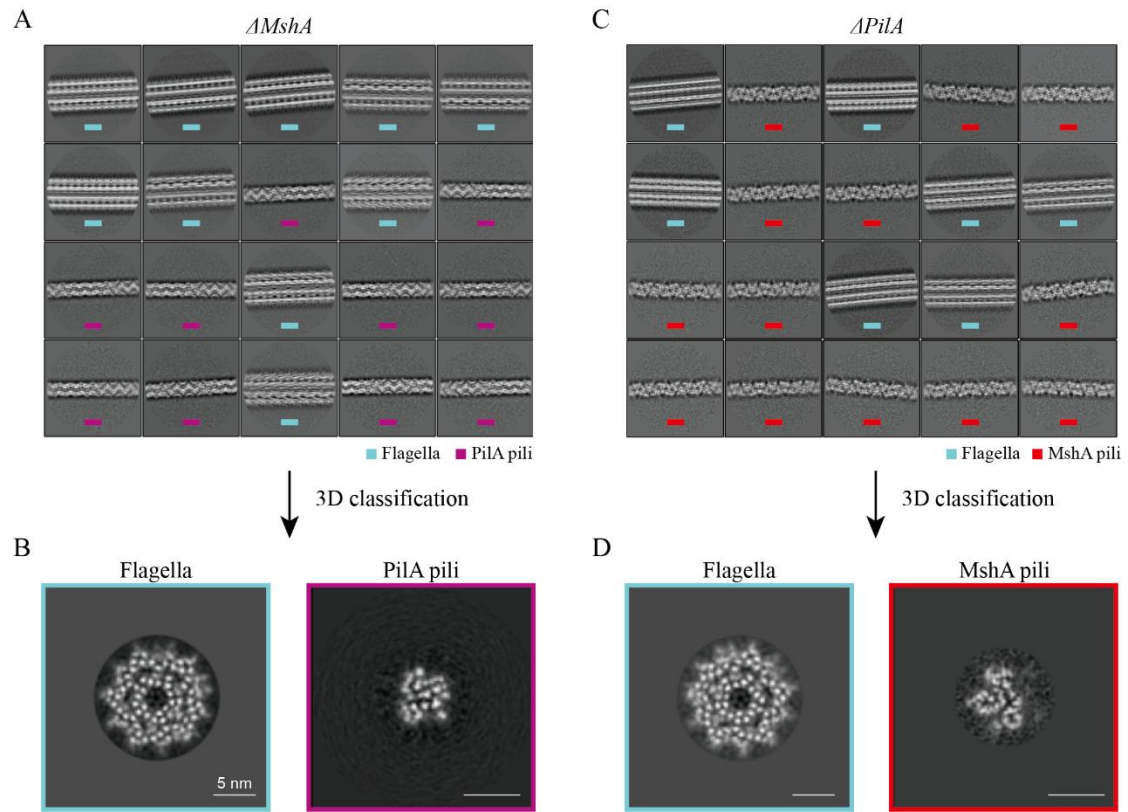
Supplementary Figure 6. Global and local resolution estimation of flagella, PilA pili and MshA pili. (A) Gold-standard Fourier shell correlation (FSC) curve for flagella. The global resolution is calculated using a FSC curve cut-off at 0.143, resulting in a global resolution of 3.2 Å. (B) Local resolution estimation of flagella. The EM reconstruction map is colored based on the local resolutions of flagella, with a color scale from 3.0 Å to 5.0 Å, showing varying resolutions across different regions of the structure. (C) Angular distribution of particles used in the final flagellar reconstruction. (D) Gold-standard FSC curve for PilA pili, with the global resolution is calculated using a FSC curve cut-off at 0.143, resulting in 3.4 Å. (E) Local resolution estimation. EM reconstruction map is colored based on the local resolutions of PilA pili, with a color scale from 3.0 Å to 5.0 Å indicating varying resolutions. (F) Angular distribution of particles used in the final PilA pilus reconstruction. (G) Gold-standard FSC curve for MshA pili, with the global resolution is calculated using a FSC curve cut-off at 0.143, resulting in 3.6 Å. (H) Local resolution estimation. EM reconstruction map is colored based on the local resolutions of MshA pili, with a color scale from 3.0 Å to 5.0 Å indicating varying resolutions. (I) Angular distribution of particles used in the final MshA pilus reconstruction.



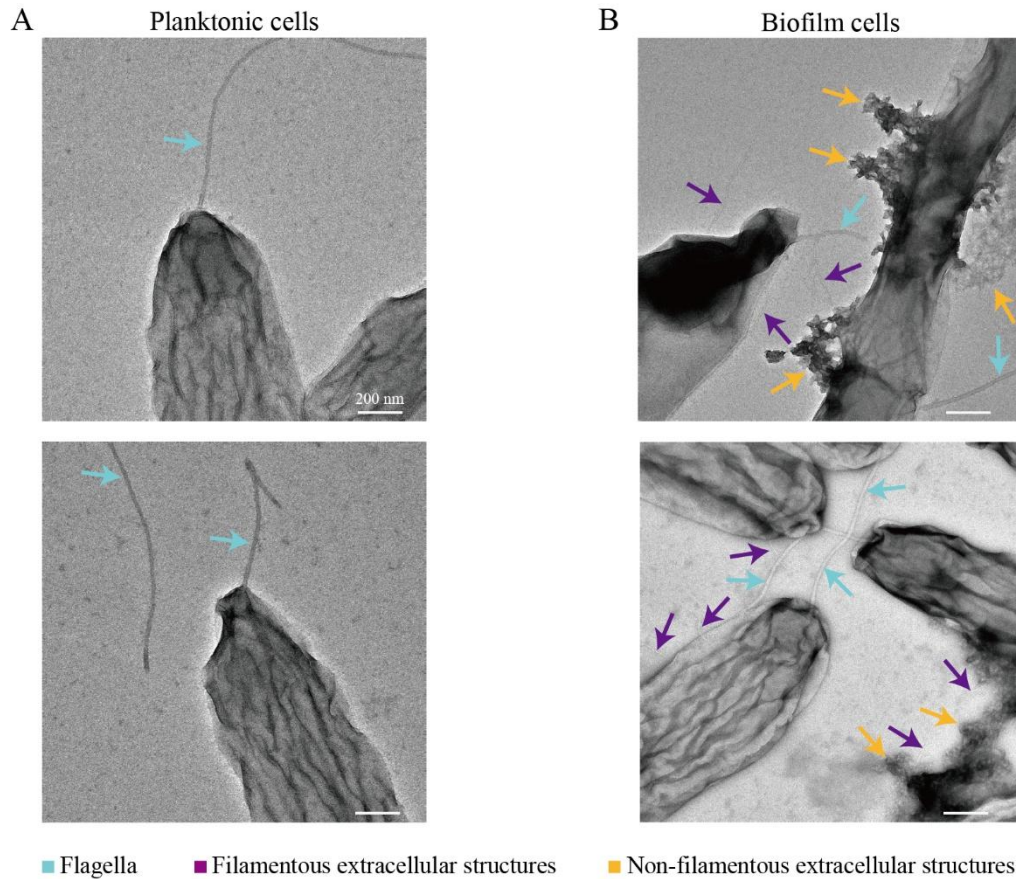
Supplementary Figure 7. Comparative arrangement of flagellar subunits in *Shewanella oneidensis* MR-1 and other bacteria. (A) A subunit of *Shewanella oneidensis* MR-1 flagella, as determined in this study. (B) A subunit of *Bacillus subtilis* flagella¹. (C) A subunit of *Pseudomonas aeruginosa* flagella¹. (D) A subunit of *Bacillus amyloiquefaciens* flagella². (E) Merged structural comparison of flagellar subunits from the four bacteria, illustrating the comparable arrangement of subunits across different species.



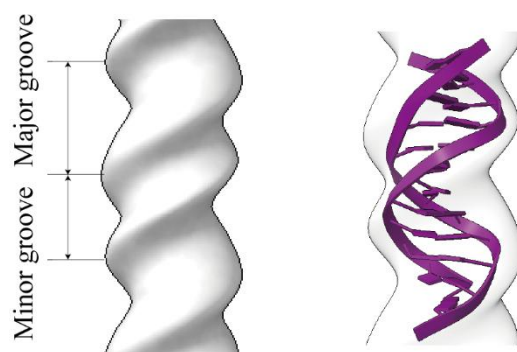
Supplementary Figure 8. Atomic model building of PilA and MshA. (A) Cryo-EM map of PilA with superimposed initial model (generated by ModelAngelo) and final refined model. (B-G) Detailed comparisons of the initial model and final model of PilA. (H) Cryo-EM map of MshA with superimposed initial model (generated by ModelAngelo) and final refined model. (I-L) Detailed comparison of the initial model and final model of MshA.



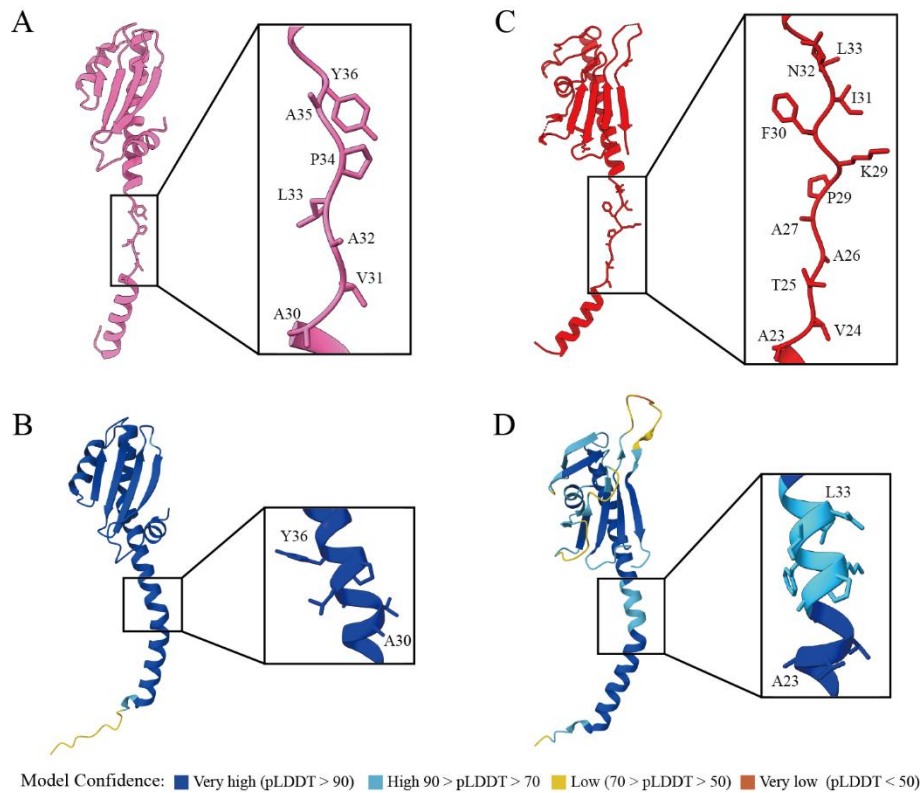
Supplementary Figure 9. Genetic validation of PilA and MshA filament assignments in *S. oneidensis* MR-1 biofilms. (A) 2D class averages of extracellular filaments from the $\Delta mshA$ mutant, with classes corresponding to flagella (cyan) and PilA pili (magenta). (B) Cross-sectional view from 3D classification of the $\Delta mshA$ dataset, showing the presence of flagella and PilA pili and the absence of MshA pili. (C) 2D class averages of extracellular filaments from the $\Delta pilA$ mutant, with classes corresponding to flagella (cyan) and MshA pili (red). (D) Cross-sectional view from 3D classification of the $\Delta pilA$ dataset, showing the presence of flagella and MshA pili and the absence of PilA pili. Scale bars, 5 nm.



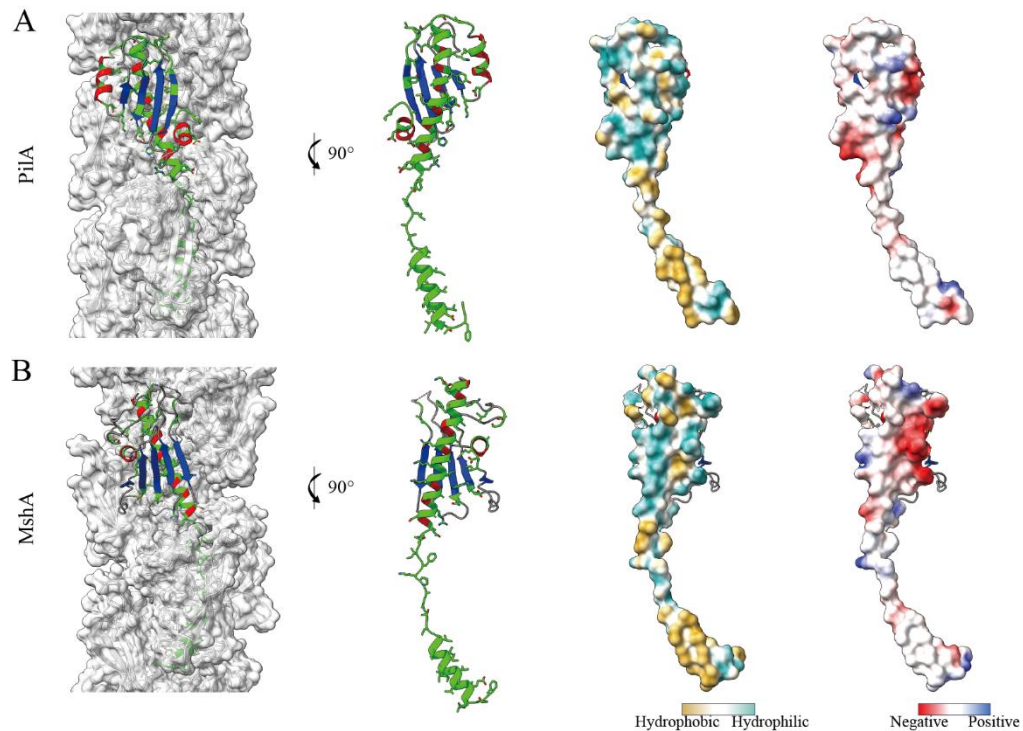
Supplementary Figure 10. Extracellular filaments are enriched in biofilm cells compared with planktonic cells. (A) Representative NS-TEM images of planktonic wild-type *S. oneidensis* MR-1 cells, showing only occasional flagella (cyan arrows). (B) Representative NS-TEM images of biofilm cells acquired 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. These imaging experiments were repeated independently at least three times with similar results.



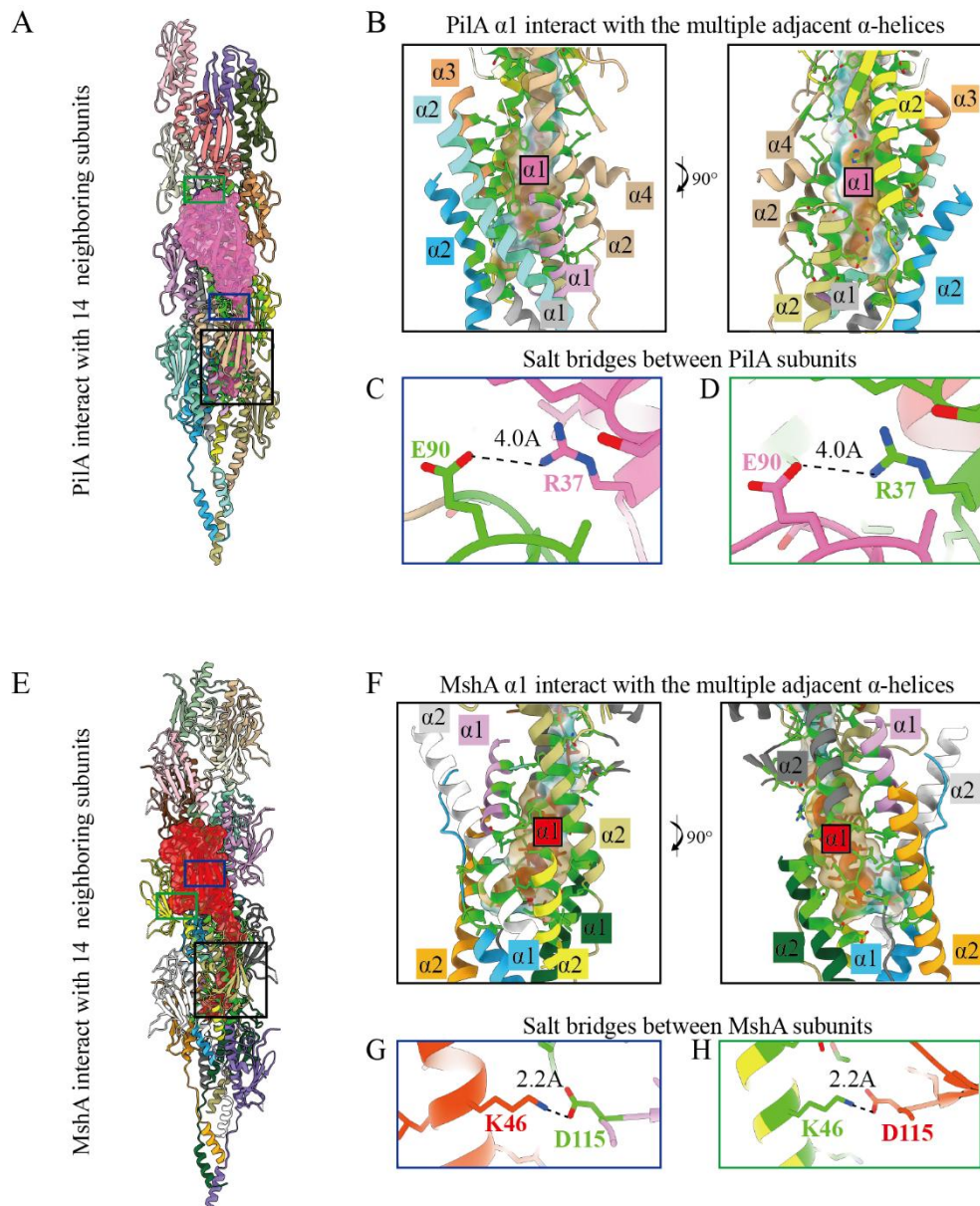
Supplementary Figure 11. Cryo-EM density map of regular filaments in 2 nm-wide filaments and their docking with DNA molecules. The major groove and minor groove are labeled (left panel). Docking of the Cryo-EM density map with a DNA molecule (PDB code 1BNA), suggesting that the filaments are composed of DNA (right panel).



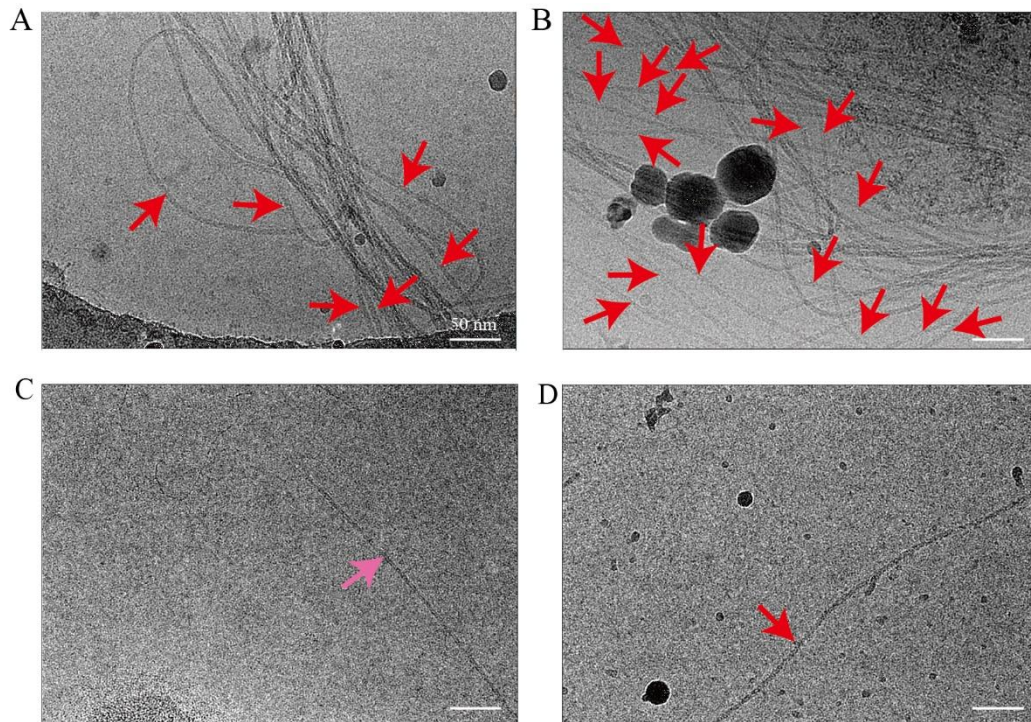
Supplementary Figure 12. Comparison of cryo-EM and AlphaFold-predicted structures of PilA and MshA Pili in *Shewanella oneidensis* MR-1. (A) Cryo-EM structure of a PilA pilus subunit, zoomed in on residues Ala30–Tyr36, which do not form an α -helix. (B) AlphaFold-predicted structure of a subunit of a PilA pilus subunit, zoomed in on residues Ala30–Tyr36, forming an α -helix. (C) Cryo-EM structure of an MshA pilus subunit, zoomed in on residues Ala23–Leu33, which do not form an α -helix. (D) AlphaFold-predicted structure of an MshA pilus subunit, highlighting residues Ala23–Leu33 forming an α -helix. The predicted local distance difference test (pLDDT) scores are displayed as color gradients within the structural images, with the score intervals represented below the structures.



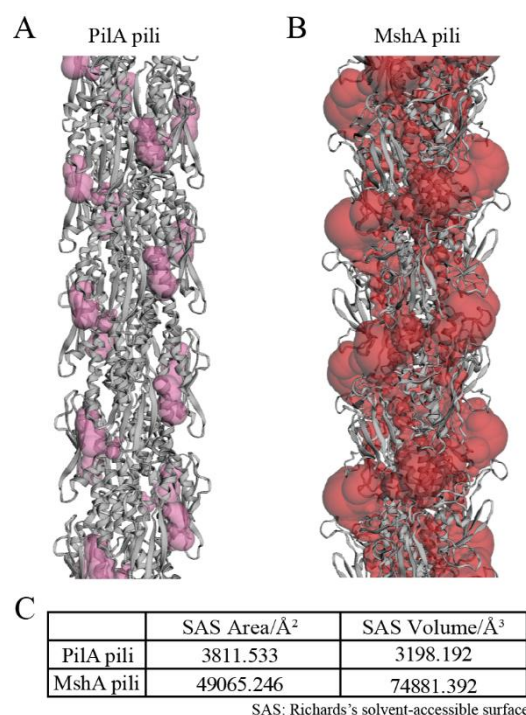
Supplementary Figure 13. Structural analysis of PilA and MshA pili. (A) Cryo-EM structure of a PilA pilus subunit depicted using a cartoon representation, while surrounding subunits are shown in surface representation. Amino acids within each subunit that engage in interactions with other subunits are highlighted in green. The hydrophobic and electrostatic properties of these interacting amino acids are illustrated. (B) Cryo-EM structure of an MshA pilus subunit depicted using a cartoon representation, while surrounding subunits are shown in surface representation. Amino acids within each subunit that engage in interactions with other subunits are highlighted in green. The hydrophobic and electrostatic properties of these interacting amino acids are illustrated.



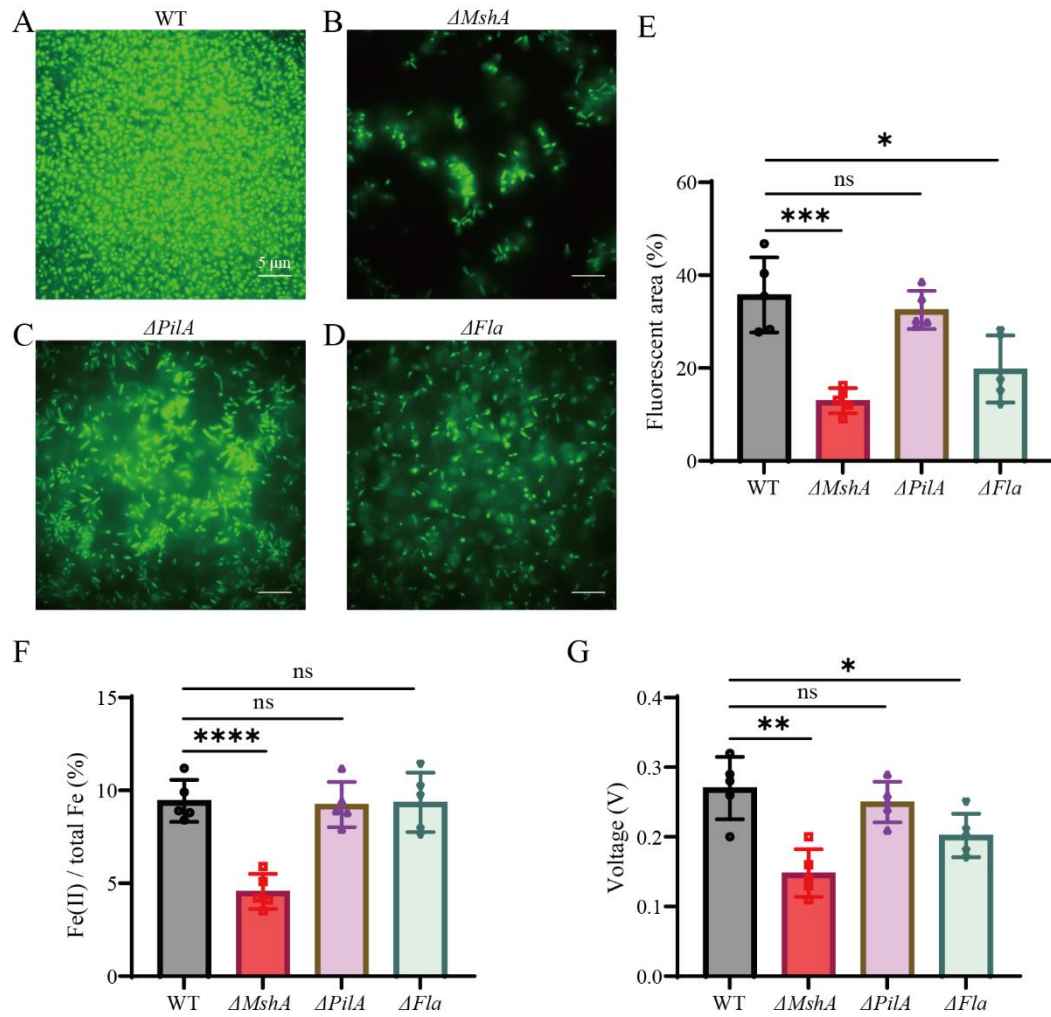
Supplementary Figure 14. Structural interactions of PilA and MshA pili. (A) Cryo-EM structure of a PilA pilus subunit, depicted with a pink surface representation. The 14 neighboring PilA subunits interacting with this subunit are shown in 14 different cartoon representations. (B) Detailed view of the N-terminal α -helices of PilA subunits, illustrating their insertion into the filament interior and interactions with adjacent subunits' α -helices. (C) and (D) Two salt bridges between PilA subunits within the filament. (E) Cryo-EM structure of a MshA pilus subunit, depicted with a red surface representation. The 14 neighboring MshA subunits interacting with this subunit are shown in 14 different cartoon representations. (F) Detailed view of the N-terminal α -helices of MshA subunits, illustrating their insertion into the filament interior and interactions with adjacent subunits' α -helices. (G) and (H) Two salt bridges between MshA subunits within the filament.



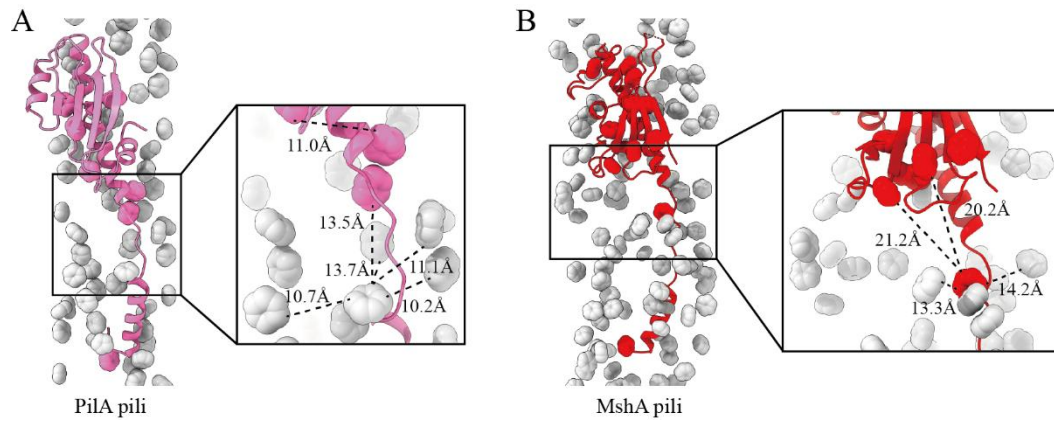
Supplementary Figure 15. Distribution of MshA and PilA Pili in the cryo-EM micrographs. (A) and (B) Cryo-EM images showing regions of the biofilm with aggregated nanofilaments, predominantly composed of MshA pili. MshA pili are marked with red arrows, and PilA pili are absent in these regions. (C) and (D) Cryo-EM images displaying regions with dispersed nanofilaments, where MshA pili and PilA pili appear individually. MshA pili are marked with red arrows, and PilA pili are marked with pink arrows.



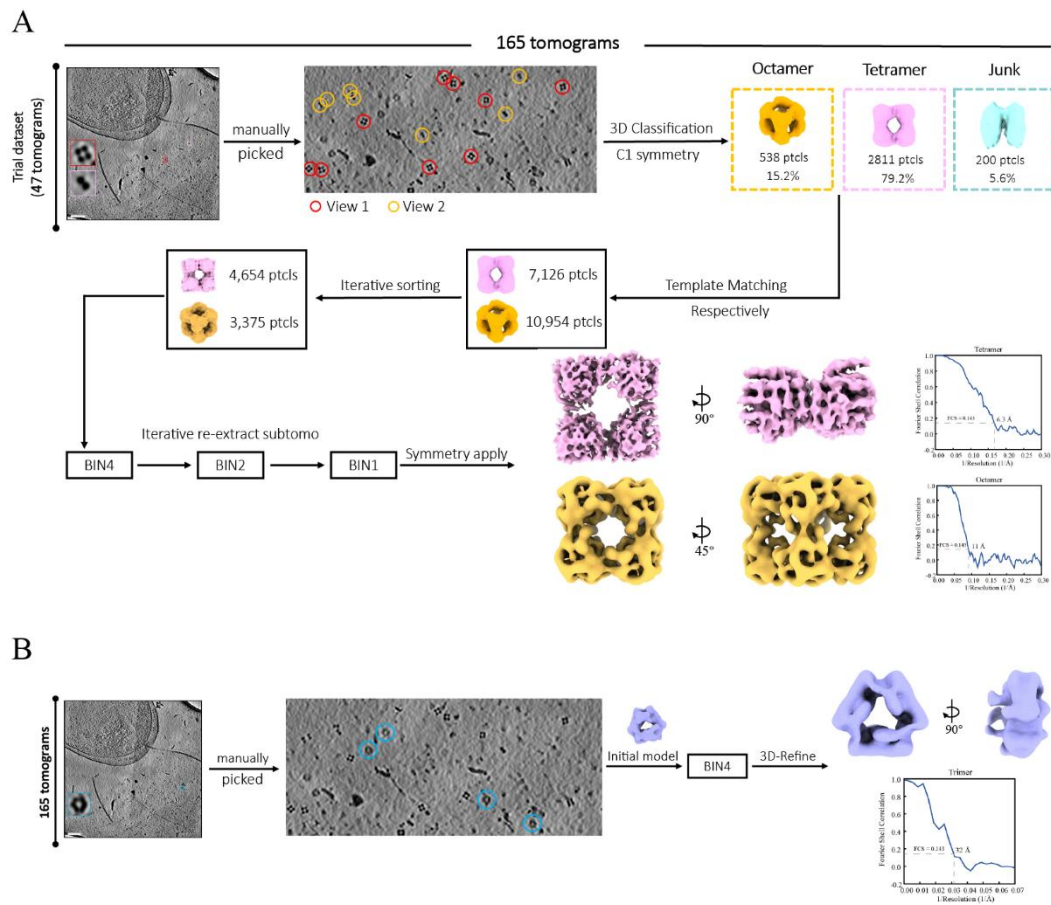
Supplementary Figure 16. Computed atlas of surface topography of PilA pili and MshA pili. (A) Structure of PilA pili depicted as a grey cartoon. Richards' solvent-accessible surface (SAS) pockets are illustrated as pink density³. (B) Structure of MshA pili depicted as a grey cartoon. SAS pockets are illustrated as red density. (C) Table presenting the calculated SAS area and SAS volume for PilA pili and MshA pili.



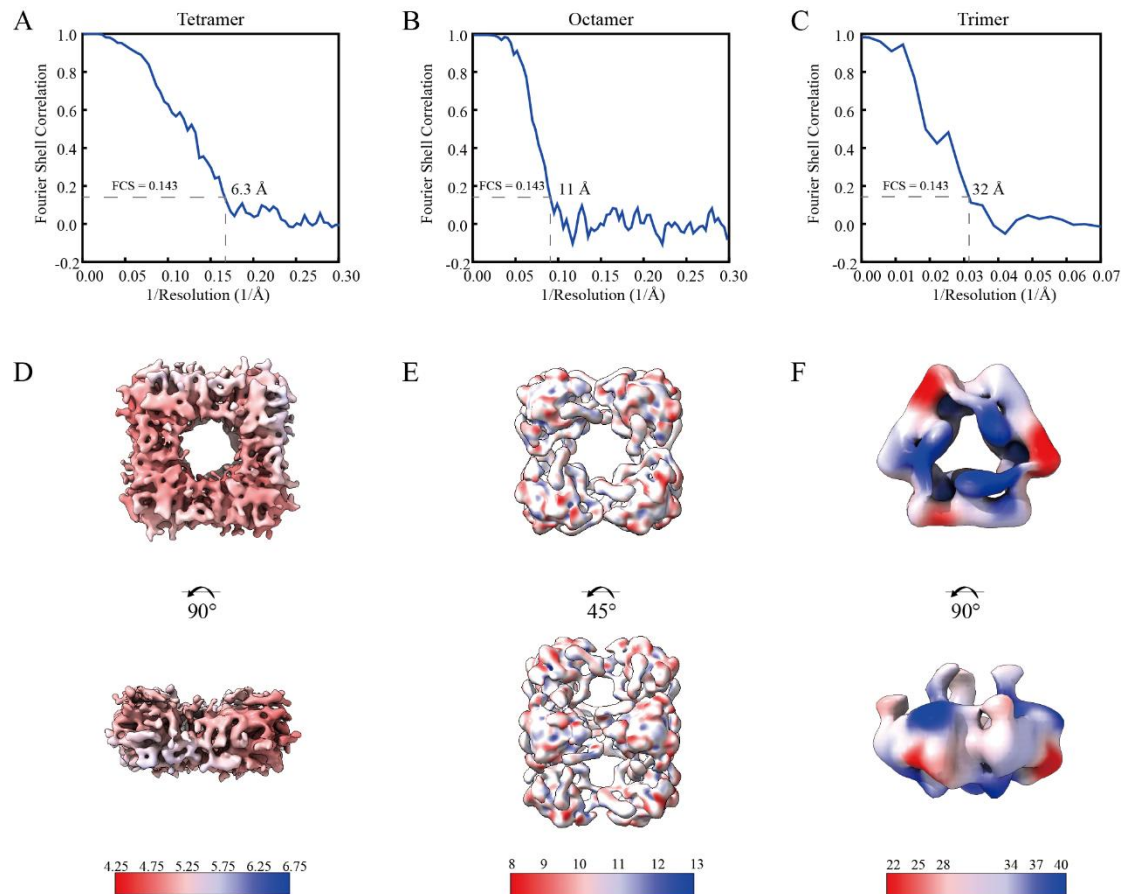
Supplementary Figure 17. Physiological phenotypes of filament deletion mutants. (A-D) Representative fluorescence images of biofilms formed by *S. oneidensis* MR-1 WT, $\Delta MshA$, $\Delta PilA$, and ΔFla strains. Scale bar, 5 μm . This imaging experiment was repeated independently at least three times with similar results. (E) Quantification of biofilm coverage, measured as fluorescent area (%). Bars represent mean $\pm$ SEM with individual data points overlaid, $n = 5$ fluorescence images per group. $\Delta MshA$, $\Delta PilA$, and ΔFla versus WT, $P = 0.0003$, 0.4516 , and 0.0110 , respectively. (F) Ferrihydrite reduction activity quantified as Fe(II)/total Fe (%). Bars represent mean $\pm$ SEM with individual data points overlaid, $n = 5$ biological replicates per group. $\Delta MshA$, $\Delta PilA$, and ΔFla versus WT, $P = 7.45 \times 10^{-5}$, 0.7948 , and 0.9294 , respectively. (G) Microbial fuel cell output voltage (V). Bars represent mean $\pm$ SEM with individual data points overlaid, $n = 5$ biological replicates per group. $\Delta MshA$, $\Delta PilA$, and ΔFla versus WT, $P = 0.0013$, 0.4265 , and 0.0236 , respectively. Two-tailed unpaired Student's *t*-test. Statistical significance is indicated as ns, not significant; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.



Supplementary Figure 18. Analysis of aromatic residues in the cryo-EM structures of PilA and MshA Pili. (A) Cryo-EM structure of PilA pili, with a PilA pilus subunit depicted as pink cartoon. Aromatic residues, represented as spherical atoms, are displayed in the structure of the subunit and its surrounding subunits. Enlarged regions show areas where the distances between benzene rings are large, with these distances labeled for clarity. (B) Cryo-EM structure of MshA pili, with an MshA pilus subunit shown as red cartoon. Aromatic residues, represented as spherical atoms, are displayed in the structure of the subunit and its surrounding subunits. Enlarged regions show areas where the distances between benzene rings are large, with these distances labeled for clarity.



Supplementary Figure 19. 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.

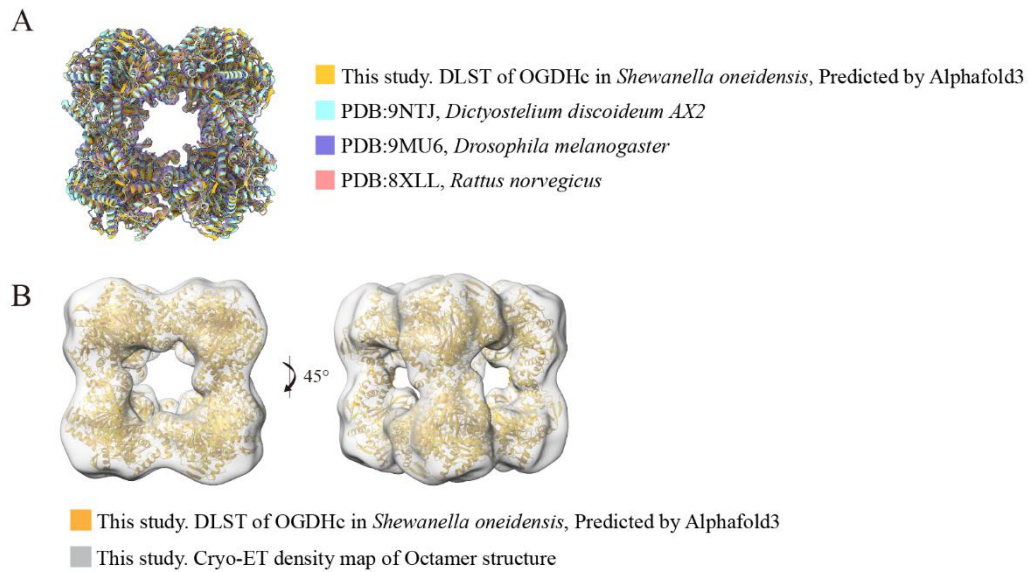


Supplementary Figure 20. Global and local resolution estimation of the tetramer, octamer, and trimer reconstructions. (A) FSC curve for the tetramer. The global resolution is calculated using a FSC curve cut-off at 0.143, resulting in a global resolution of 6.3 Å. (B) FSC curve for the octamer. Application 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. (D) Local resolution map of the tetramer reconstruction, colored according to local resolution. (E) Local resolution map of the octamer reconstruction, colored according to local resolution. (F) Local resolution map of the trimer reconstruction, colored according to local resolution. The corresponding color bars are shown in each panel.

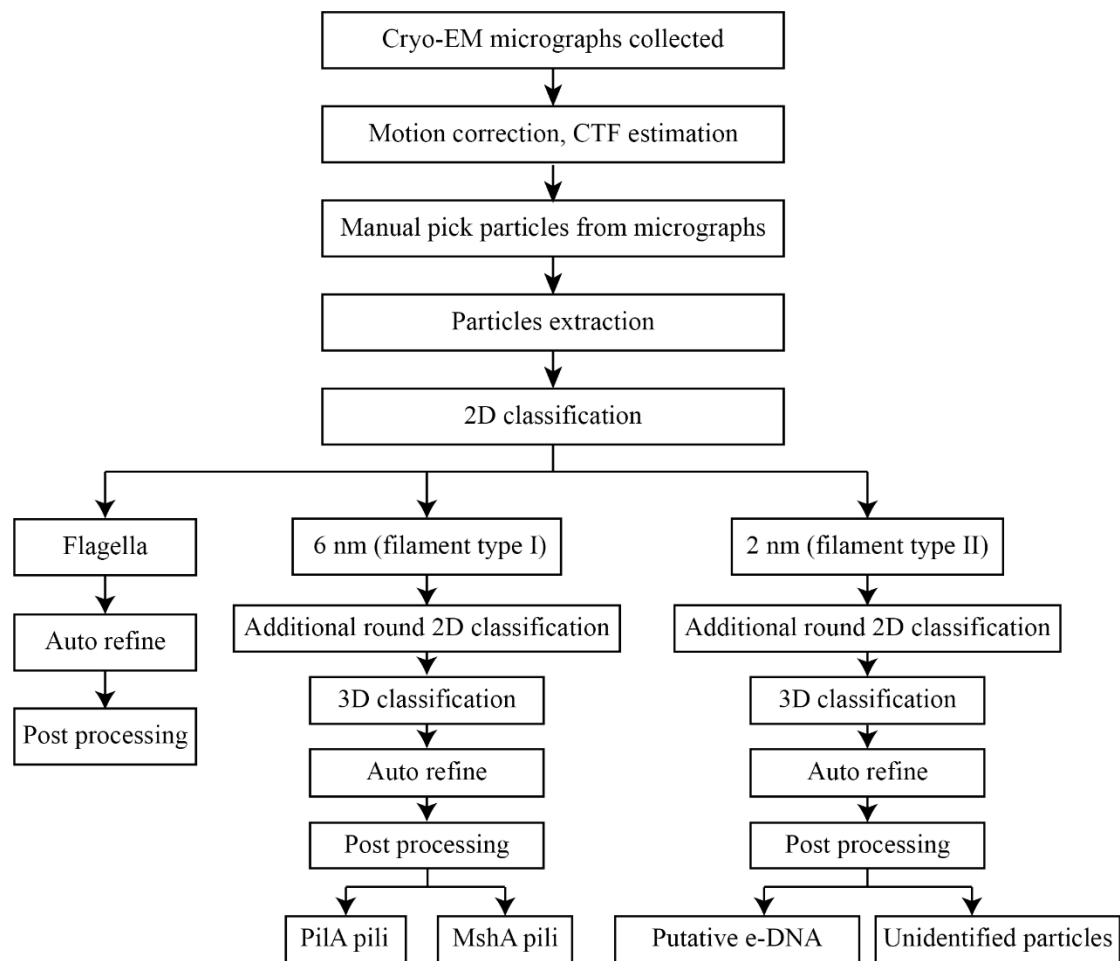
B

Mass spectrum of the protein FIA GAVS GQEVTK. The x-axis is m/z (zoom) from 100 to 1200. The y-axis is Relative Abundance from 0 to 120. The spectrum shows a series of peaks corresponding to the protein sequence. The sequence is F I A G A V S G Q E V T K. The peaks are labeled with 'y' (red) and 'b' (blue) to indicate the type of fragmentation. The base peak is at m/z 1000, labeled 'y'.

Supplementary Figure 21. 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.



Supplementary Figure 22. 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.



Supplementary Figure 23. Cryo-EM data processing workflow for extracellular filaments from *Shewanella oneidensis* MR-1 biofilms. Workflow showing cryo-EM micrograph collection, motion correction, CTF estimation, manual particle picking, particle extraction, and 2D classification. After initial classification, particles were divided into three major groups: flagella, 6 nm filaments (filament type I), and 2 nm filaments (filament type II). The flagellar dataset was refined directly, whereas the 6 nm and 2 nm filament datasets underwent additional rounds of 2D classification, followed by 3D classification, auto-refinement, and post-processing. The final classes correspond to flagella, PilA pili, MshA pili, putative e-DNA, and unidentified particles.

Supplementary Table 1. Spacer sequences of base editing for establishment of mutant strains

sgRNA	Spacer sequence
sgRNA-pilA	acaagctcagggtttacct
sgRNA-mshA	ctcagcctgttactgcagat
sgRNA-flaA	aacagactaacatgttacia
sgRNA-flaB	cagtctgaaaacggtgcaa

Supplementary Table 2. Primer sequences of PCR and sequencing

Primer name	Primer sequence
pilA-F	gataagtagccgaaacgaacac
pilA-R	cctcttcacattgtgctttgg
mshA-F	cactagtgttaaaccacacg
mshA-R	cccaatcaggataatgggtggtc
flaA-F	cagaaagcaaagacctaacc
flaA-R	cccaatcagcacgttgg
flaB-F	gctgctgccgctatatatac
flaB-R	gatgtcgtattccaaggaacc

Supplementary Movie 1. Raw cryo-ET volume corresponding to Fig. 1B and 1C. Unannotated tomographic volume of an *S. oneidensis* MR-1 biofilm region, shown without colored overlays to provide an unobstructed view of the underlying ultrastructure.

Supplementary Movie 2. Raw cryo-ET volume corresponding to Fig. 1D and 1E. Unannotated tomographic volume of a second representative *S. oneidensis* MR-1 biofilm region, displayed without rendering or colored traces.

Supplementary Movie 3. 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.

Supplementary Data 1. 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).

References

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- 2 Cheng, Y., Han, J., Song, M., Zhang, S. & Cao, Q. Serine peptidase Vpr forms enzymatically active fibrils outside *Bacillus* bacteria revealed by cryo-EM. *Nature Communications* **14**, 7503, doi:10.1038/s41467-023-43359-z (2023).
- 3 Tian, W., Chen, C., Lei, X., Zhao, J. & Liang, J. CASTp 3.0: computed atlas of surface topography of proteins. *Nucleic Acids Res* **46**, W363-w367, doi:10.1093/nar/gky473 (2018).