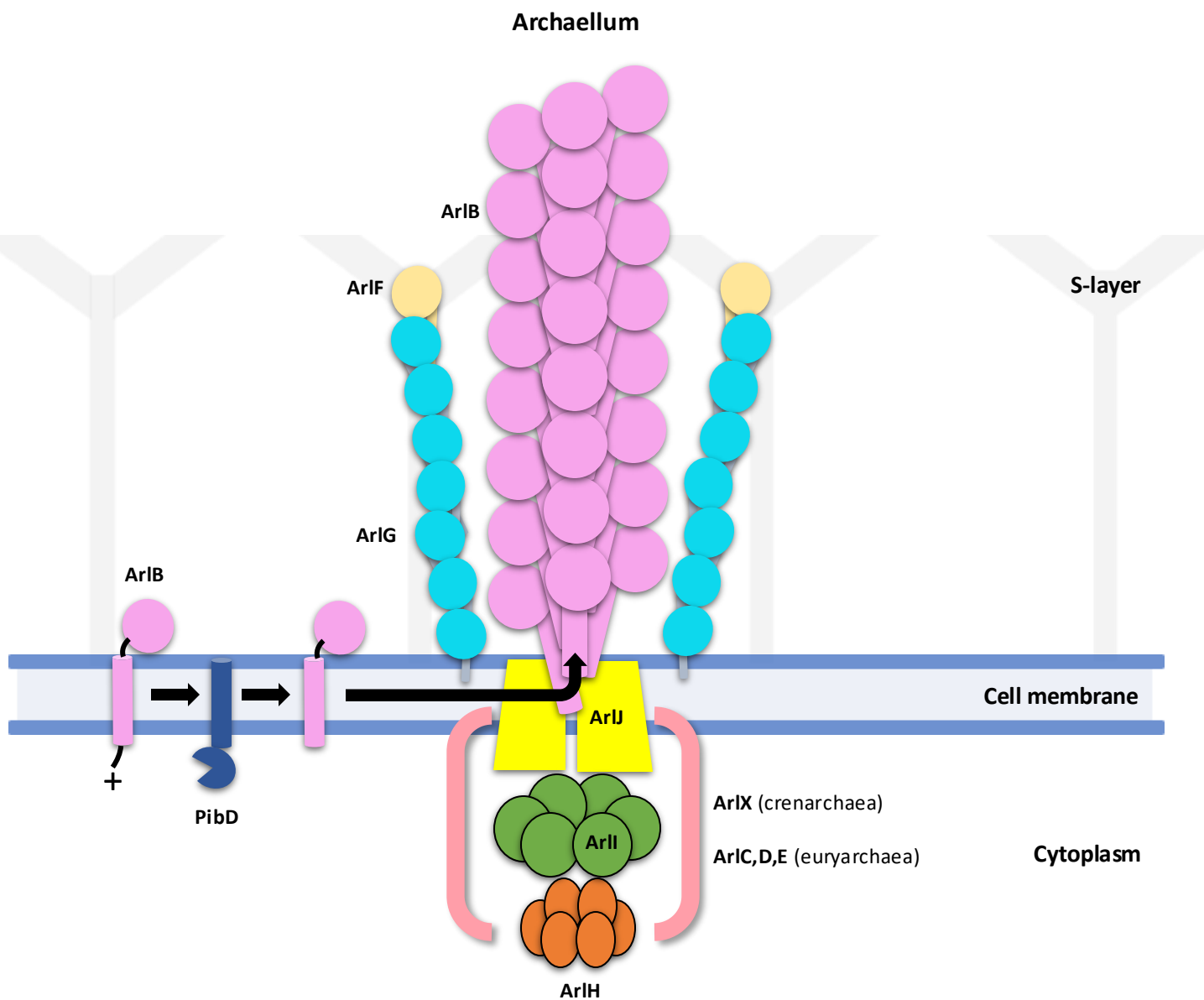
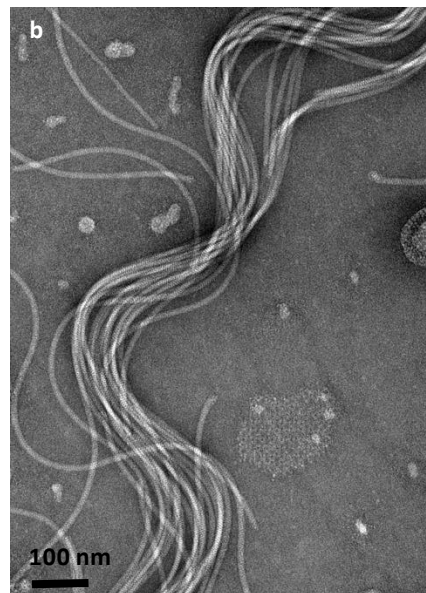
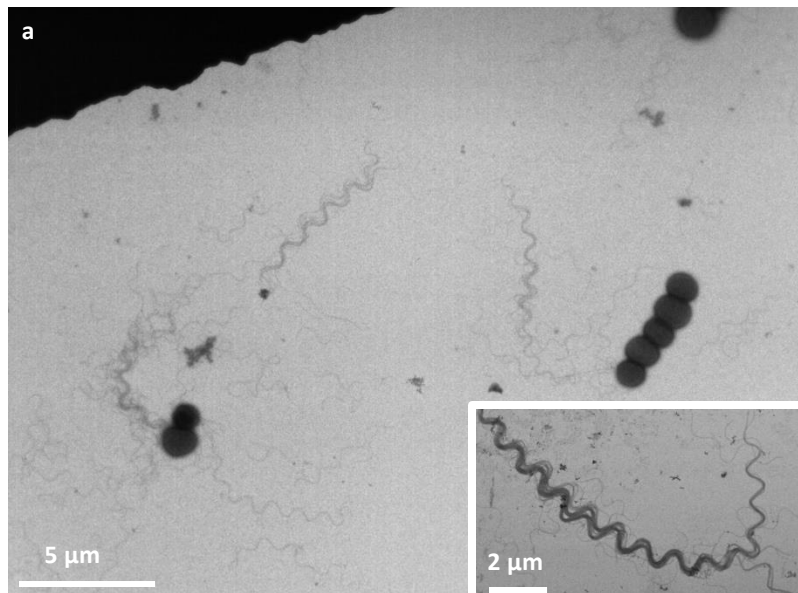




### Supplementary Figure 1 – Model of the archaellum machinery

The archaellum machinery is integrated into the cell membrane and S-layer. The archaellum filament is composed of archaellins (pink; ArlB in case of *Sulfolobus acidocaldarius*). The archaellins are initially expressed as membrane-integral proteins via the Sec translocon (not shown). These proteins are then processed by a type-III signal peptidase (PibD), which cleaves the positively-charged N-terminal leader peptide from the archaellin. This primes the archaellin for the integration into the nascent filament, which is assembled by the membrane-integral platform protein ArlJ (yellow). The energy for the assembly of the filament is provided by ATP hydrolysis catalysed by the ATPase ArlI (green). ArlI interacts with ArH, which is thought to act as a regulator that (once the filament is assembled) switches the machinery from assembly to rotation mode. Surrounding ArlJ,I and H is a ring of ArlX in crenarchaea and ArlC,D,E in euryarchaea, which is thought to have stator function. In euryarchaea, ArlC,D,E is also thought to transmit signals from chemoreceptors to the archaellum machinery. In the periplasm, filaments of ArlG, capped by ArlF, integrate the archaellum machinery into the S-layer and likely act as stators<sup>1</sup>.

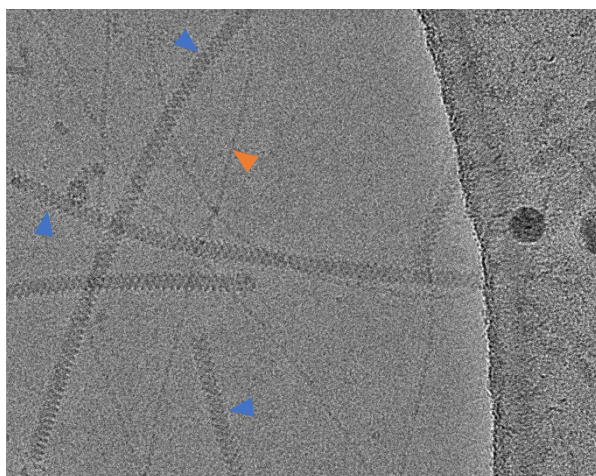
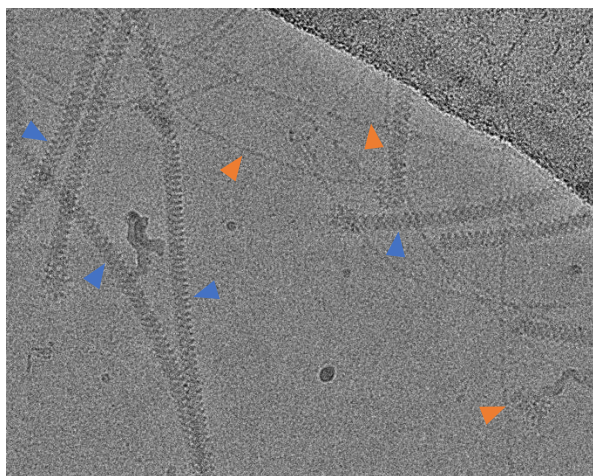


**Supplementary Figure 2 – Example negative stain micrographs of the hyper-archaellated strain MW2106.**

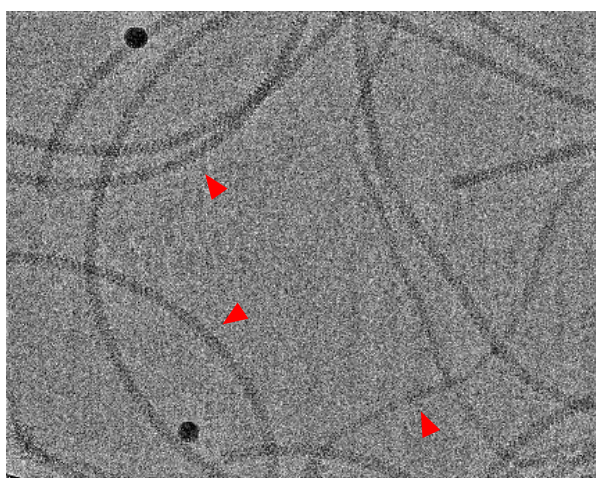
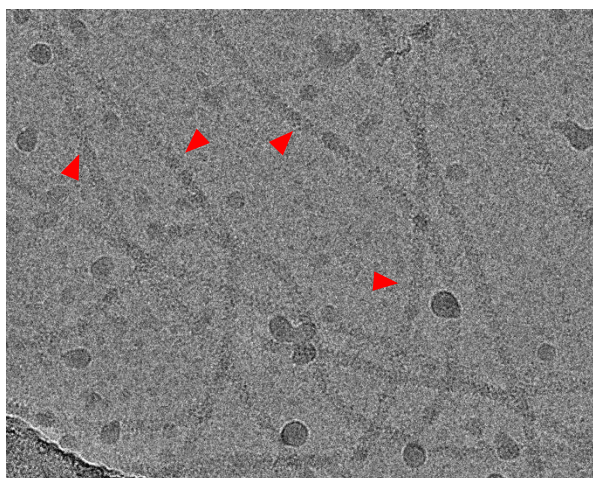
**a**, overview showing various negatively stained cells on the TEM grid. Insert and **b**, magnified views of archaellar bundles associated with the cells.



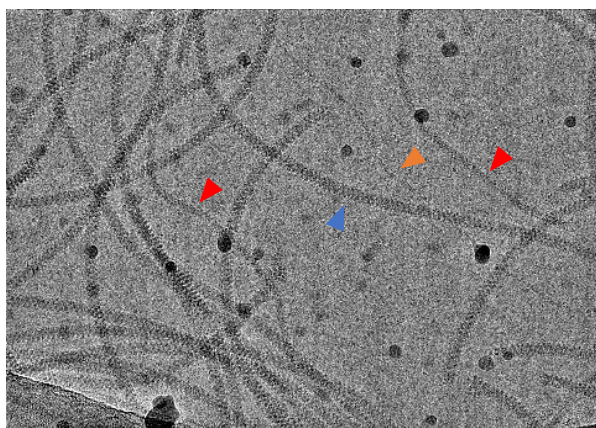
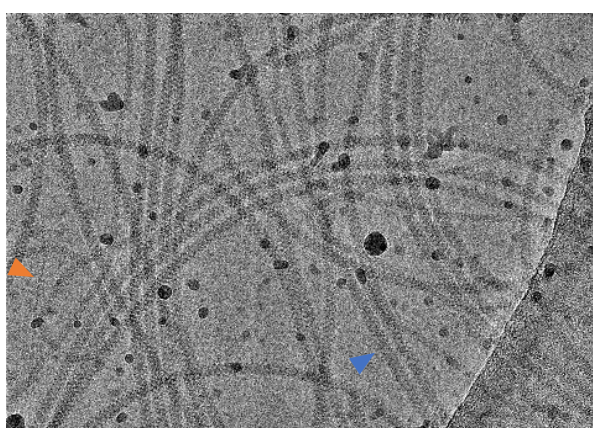
**a** Archaeella and threads with full-length glycans isolated from strain MW2106



**b** Aap with full-length glycans isolated from strain MW158

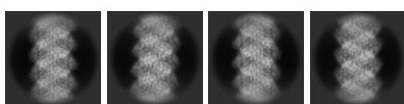


**c** Aap, archaeella and threads with truncated glycans isolated from the  $\Delta agl3$  mutant (MW039)

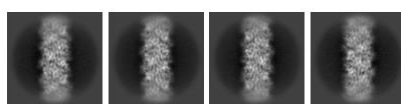


**d**

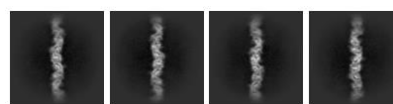
Archaeellum wt



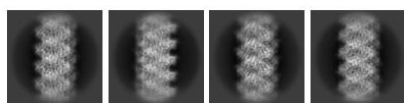
Aap wt



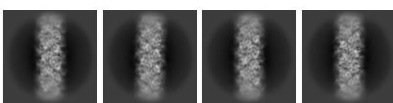
Thread wt



Archaeellum  $\Delta agl3$



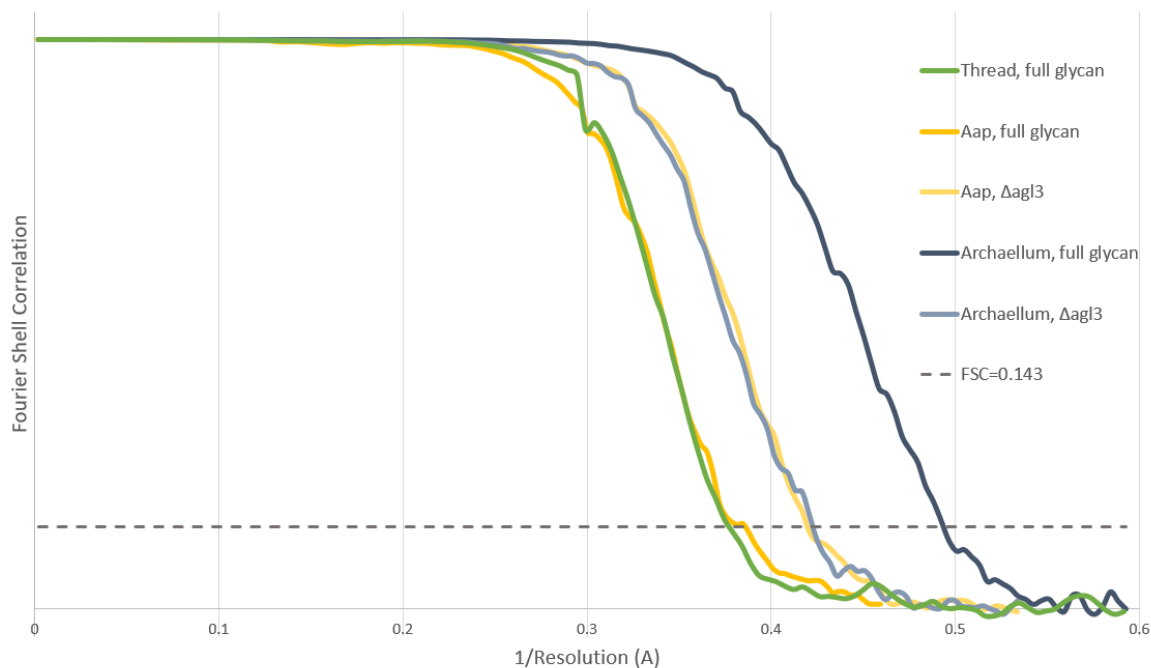
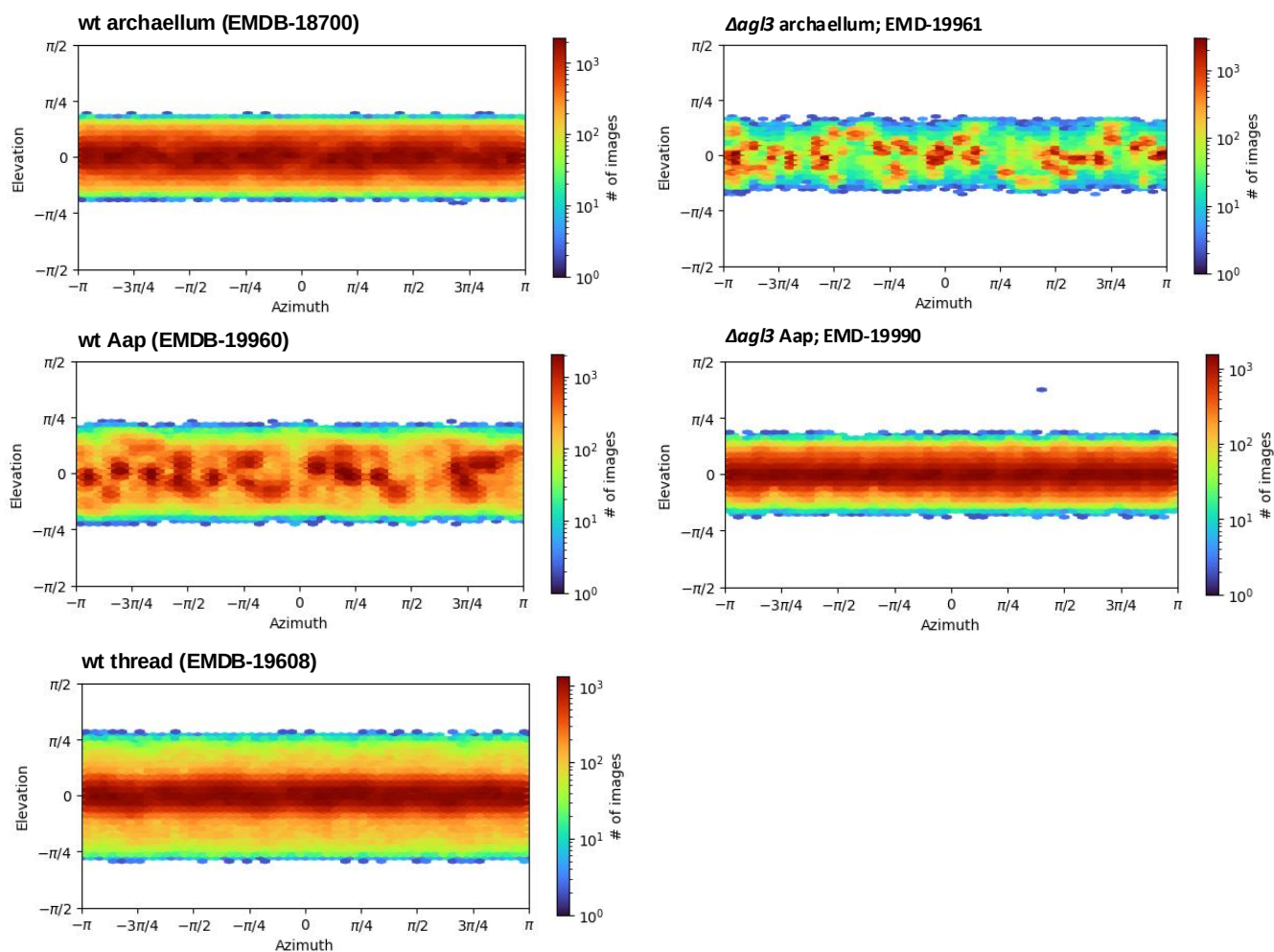
Aap  $\Delta agl3$



**Supplementary Figure 3 – Example cryoEM micrographs and representative 2D classes of isolated filaments from *S. acidocaldarius*.**

**a-c** micrographs of archaella and threads isolated from strain MW2106 (**a**); Aap isolated from strain MW158 (**b**); Aap, archaella and threads isolated from the  $\Delta agl3$  mutant (strain MW039) (**c**). Blue arrowheads, archaella; red arrowheads, Aap; orange arrowheads, threads; **b**, representative 2D classes of wt/ $\Delta agl3$  archaella, wt/ $\Delta agl3$  Aap, and wt threads;



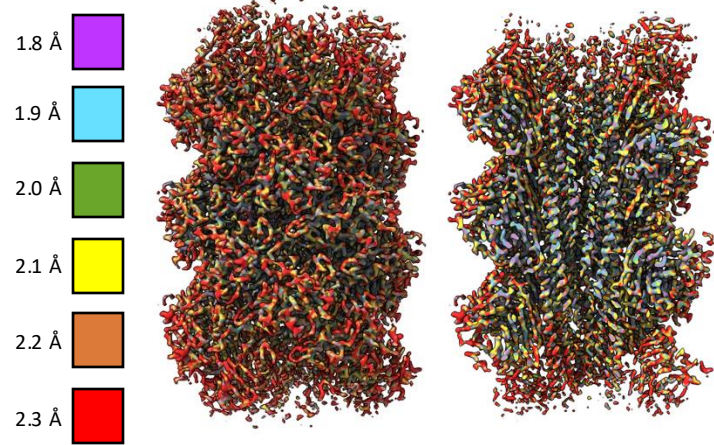
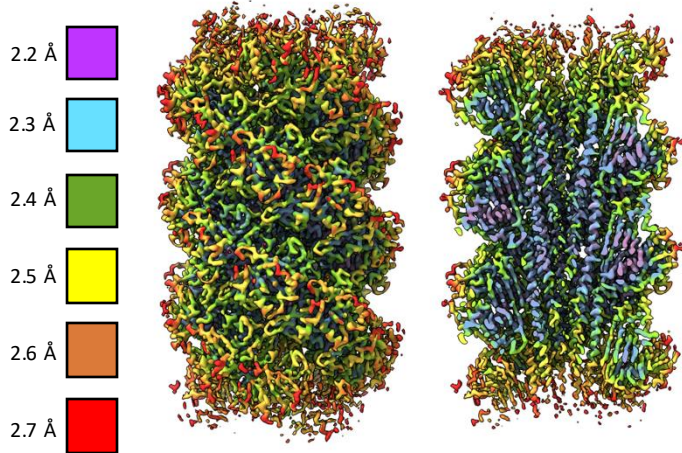
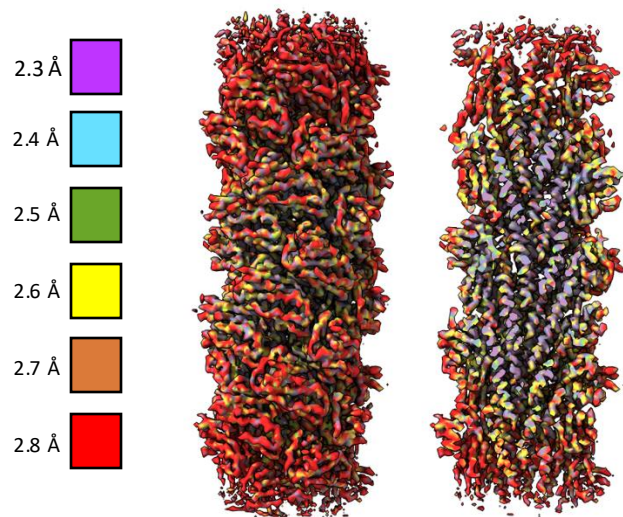
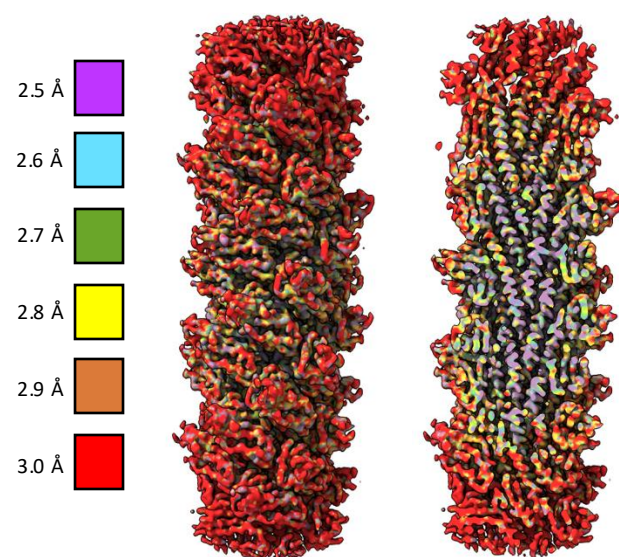
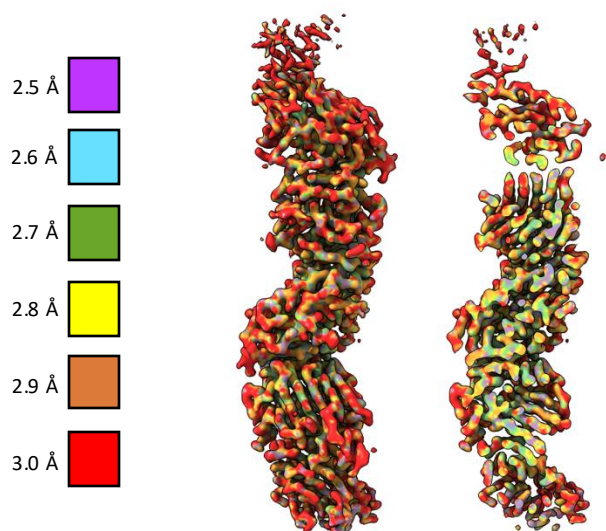
**a****b**

**Supplementary Figure 4 – 2D classes, angular sampling and global resolution estimation via Gold Standard Fourier Shell Correlation (FSC).**

**a**, FSC curves for each of the reported cryoEM maps. Filament types are grouped by colour. Thread, green; AAP wt/*Δag/3* mutant, shades of yellow; archaellum wt/*Δag/3* mutant, shades of blue.

**b**, angular distribution of particles used in the final reconstructions.



**a****Archaeellum full glycan****Archaeellum  $\Delta agl3$** **b****Aap full glycan****Aap  $\Delta agl3$** **c****Thread full glycan**

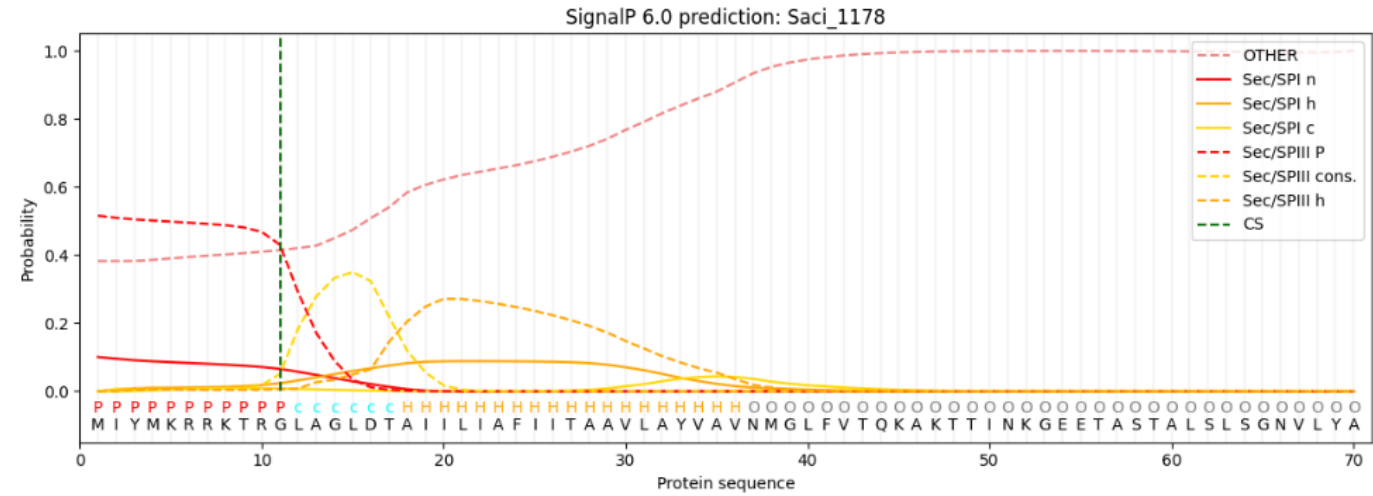
### Supplementary Figure 5 – Local Resolution estimation

**a**, maps in side view and cross section of the wt archaellum with the full glycan (left) and the archaellum from the  $\Delta agl3$  mutant (right). **b**, maps in side view and cross section of the wt Aap with the full glycan (left) and the Aap from the  $\Delta agl3$  mutant (right). **c**, map in side view and cross section of the wt thread with the full glycan

a

Signal peptide  
MIYMKRRKTRGLAGLDTAIIILIAFIITAAYVAVNMGLFVTQKAKTTINKGEETASTA  
LSLSGNVLYAVNYPTNTKSYWMYFTVSPSSGVSSVDLSPSTTAISFTAASRGVSLSNIIQ  
FSLLSVLPSQVNNKVQVKLGTSIINLTAFSSNSAGQTYVYSDPNYALLALNYTLGQEV  
KGGQLTSSPLYIISNTSIVASKPWLKNDNVFTFNISVNGTEVEYYAVVNKTFAFTYPVSG  
FPLAGSDIAPAGSVIGWMILFGPGEATNVFQYETVTIQITPNIGSPLTISQYIYQPDGKV  
TVIG

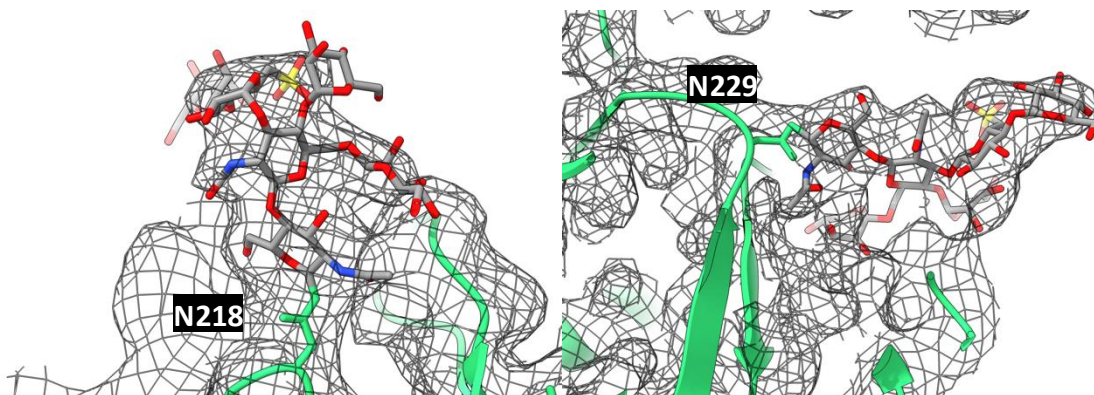
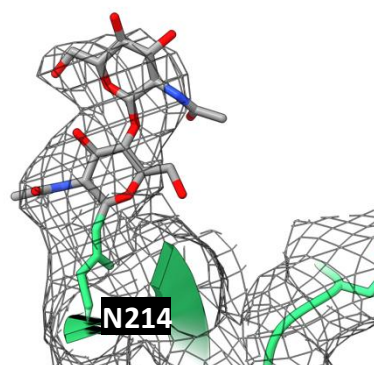
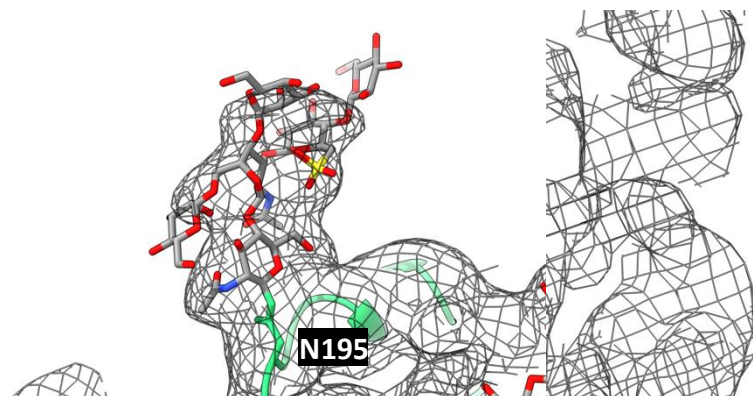
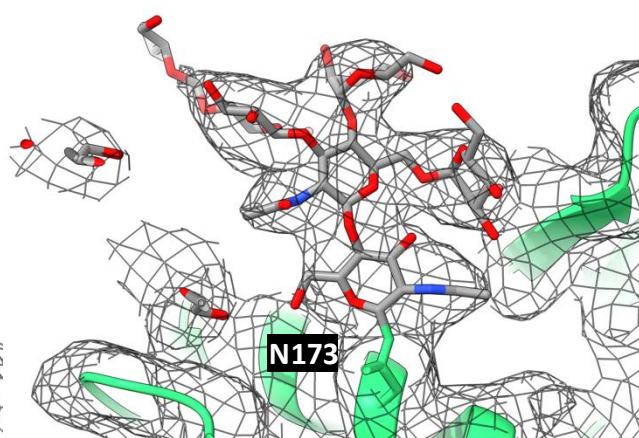
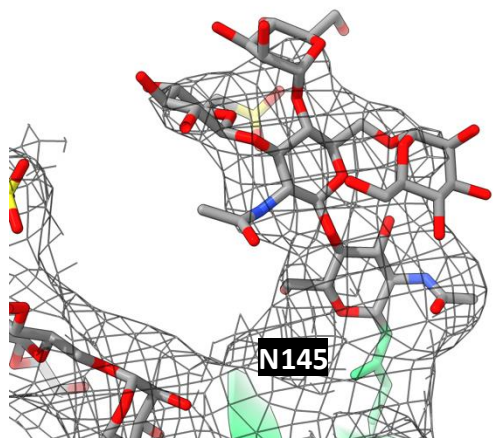
b



### Supplementary Figure 6 – ArlB sequence and Sec-III peptidase

**a**, amino acid sequence of ArlB. The signal peptide is indicated. The sequence of the mature protein is coloured by domain. The  $\alpha$ -helix is red, the main head domain is grey, and the glycosylation sub-domain is cyan. **b**, the SignalP 6.0 server <sup>2</sup> predicts a signal-III peptidase cleavage site.



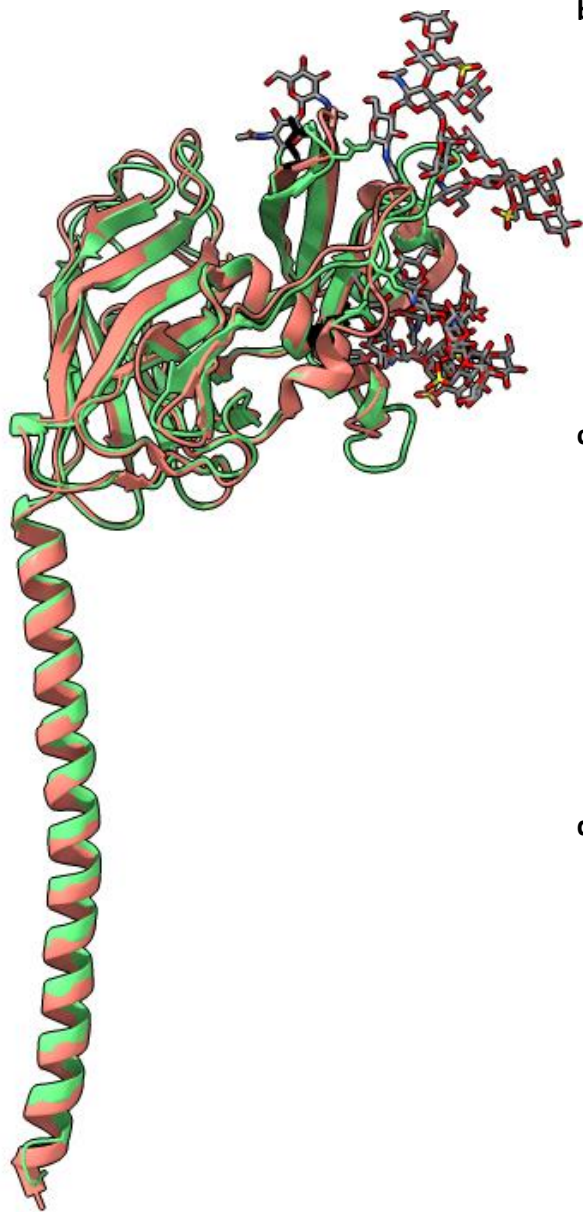


### **Supplementary Figure 7 – Glycosylation sites within the archaellum**

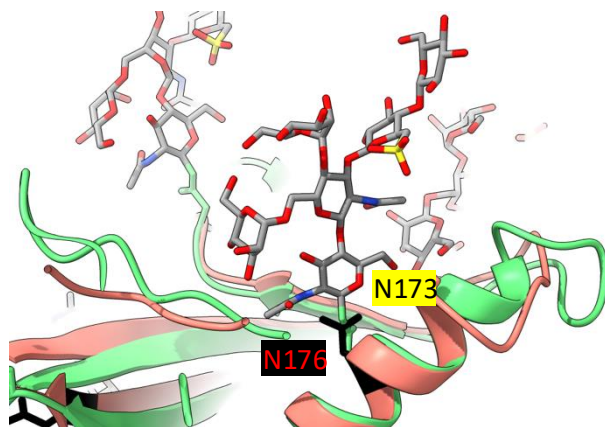
Close-ups of the six glycosylation sites (N145, N173, N195, N214, N218 and N229) within the *S. acidocadarius* archaellum. Protein is shown as green ribbons and glycans as sticks, coloured by element. The cryoEM map is shown as grey mesh.



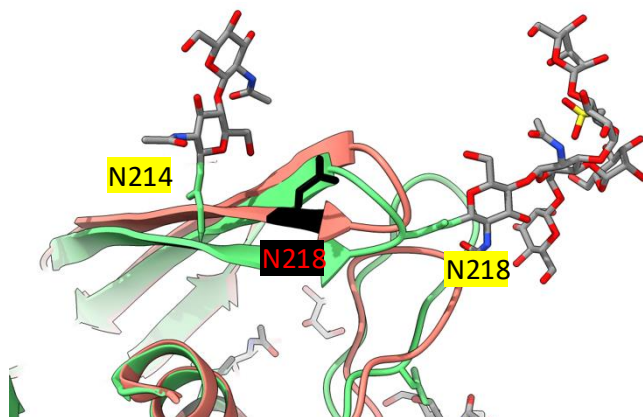
a



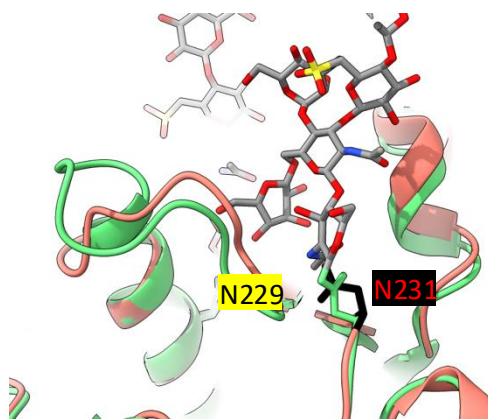
b



c

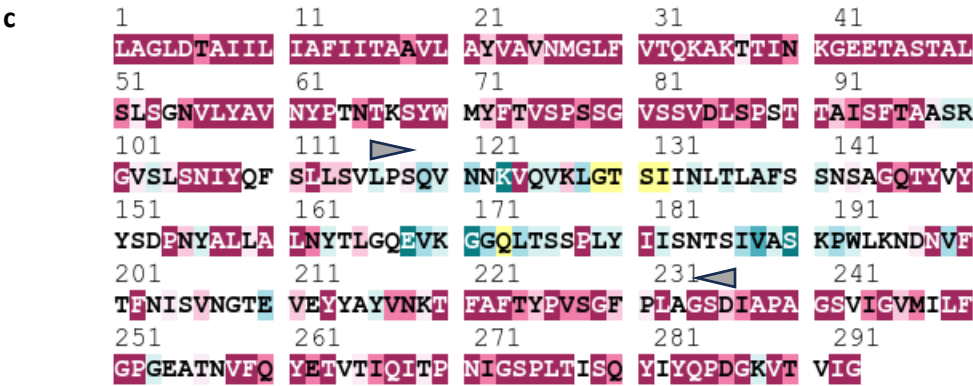
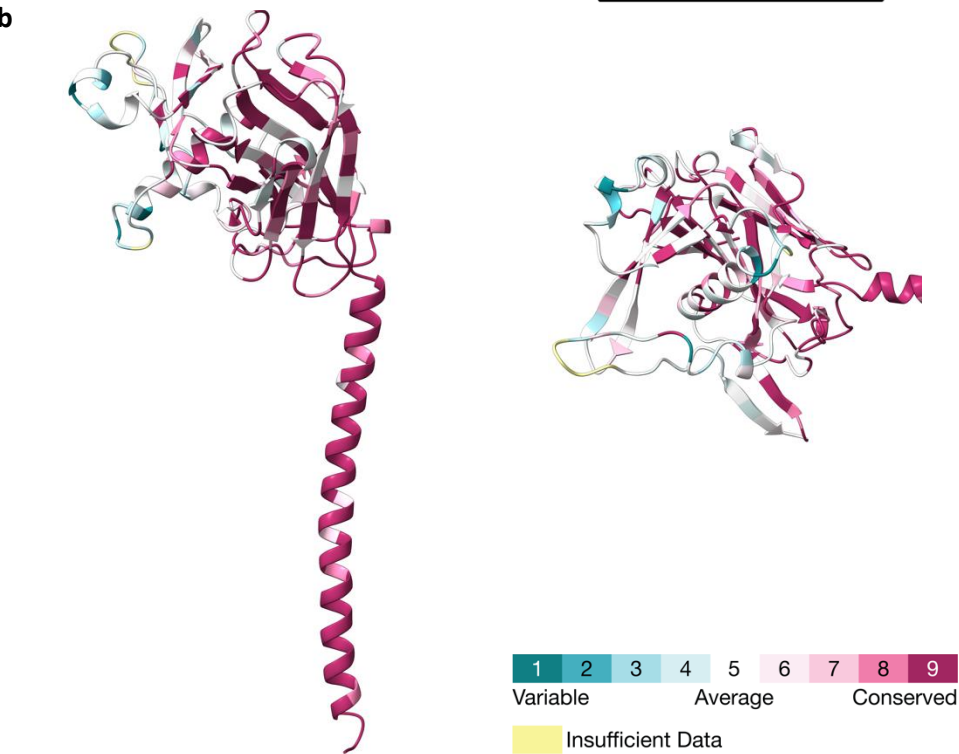
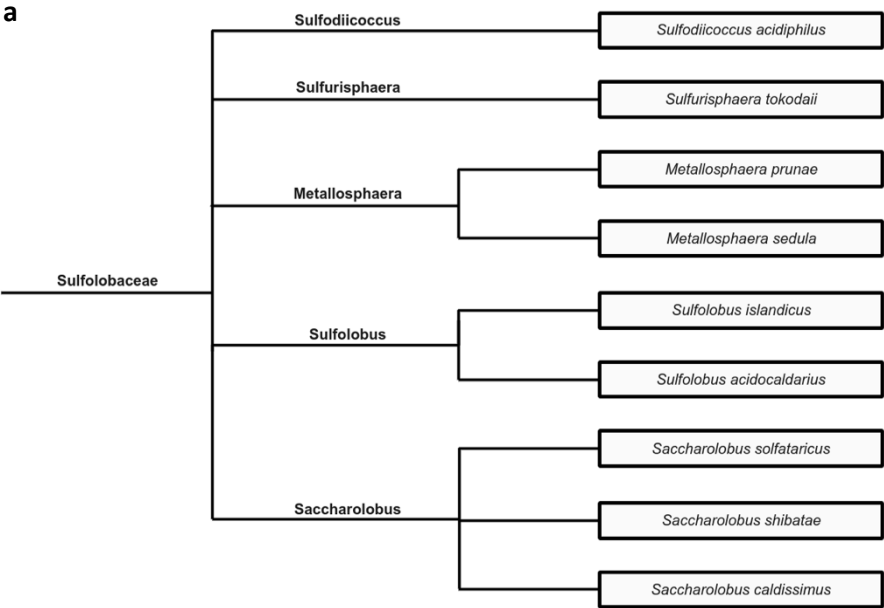


d



**Supplementary Figure 8: Conservation between *S. acidocaldarius* and *S. islandicus* ArlB**

**a**, superimposition of the archaellins from *S. acidocaldarius* (green) and *S. islandicus*<sup>3</sup> (PDB-8CWM; salmon) showing almost identical protein structures. However, the glycosylation sites are divergent. All three glycosylation sites in *S. islandicus* (red text on black background) line up with the corresponding sites in *S. acidocaldarius* (black text on yellow background). However, the *S. acidocaldarius* archaellum has three additional glycosylation sites (N145, N195 and 214) that are not found in *S. islandicus*.



### Supplementary Figure 9: Conservation of ArlB homologs across Sulfolobales

**a**, phylogenetic tree showing 9 Sulfolobales species sharing homologous ArlB proteins. **b**, ConSurf<sup>4</sup>, shows the degree of conservation in ArlB. The ArlB structure is coloured by similarity, based on the species in **a**. **c**, amino acid sequence of *S. acidocaldarius* ArlB coloured by conservation. In **b** and **c**, regions of high conservation are maroon and regions of low conservation are cyan. Note that the glycosylation domain is the most divergent part of the protein. In **c**, the glycosylation domain is indicated by grey arrowheads.

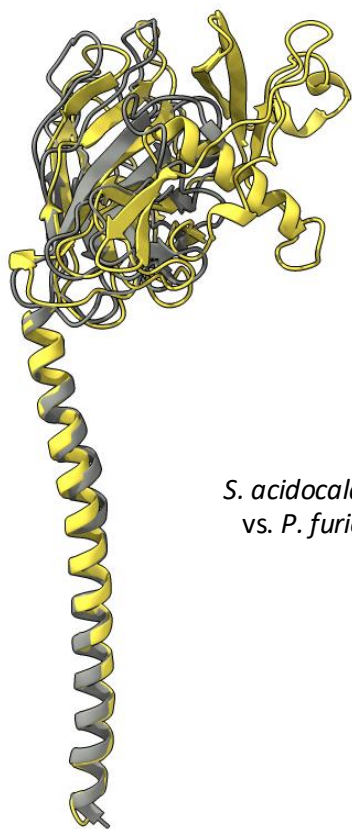


### Supplementary Figure 10: ArlB homologs in sulfolobales

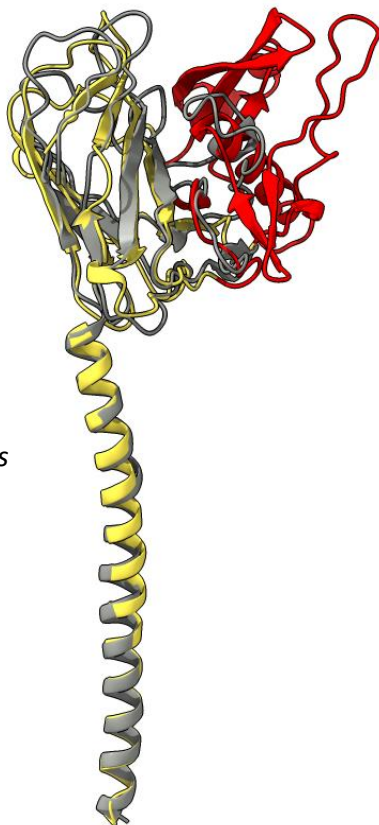
Multisequence alignment of archaellins from 9 sulfolobales species (as in Supplementary Figure 9). Sequences highlighted in yellow show full residue conservation amongst all 9 species, red amino acids are glycosylation sites, magenta boxes show residues overlapping with metal binding sites in euryarchaeota. Note that the analogous residues in crenarchaeota are not capable of coordinating metals. Grey arrowheads with interconnecting dashed lines delineate the glycosylation domain in *S. acidocaldarius*.



**a**

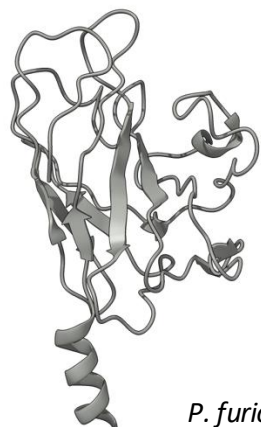


**b**

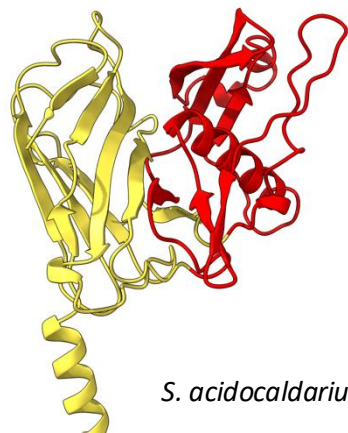


*S. acidocaldarius*  
vs. *P. furiosus*

**c**



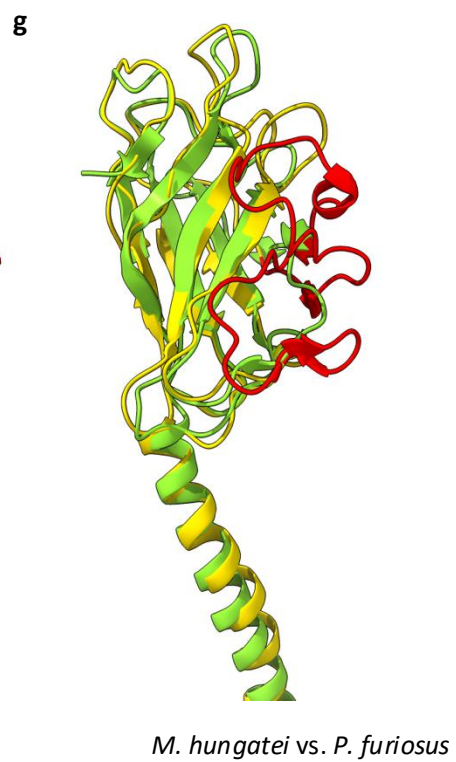
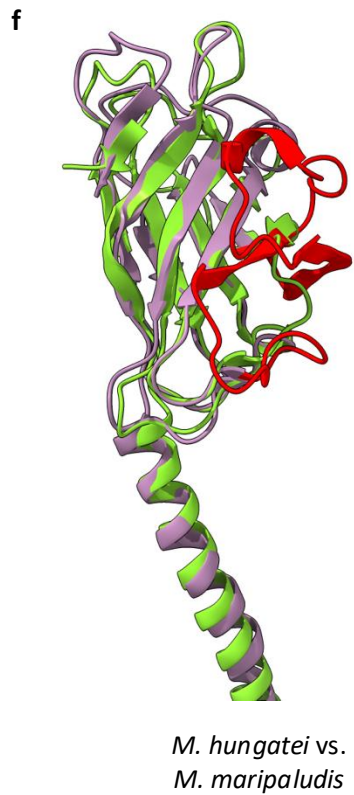
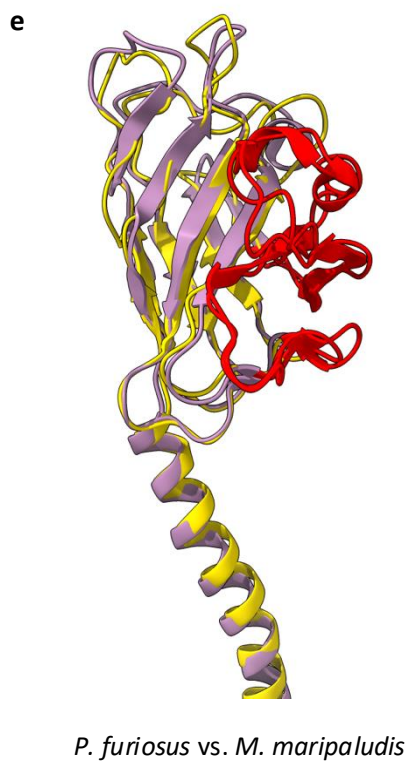
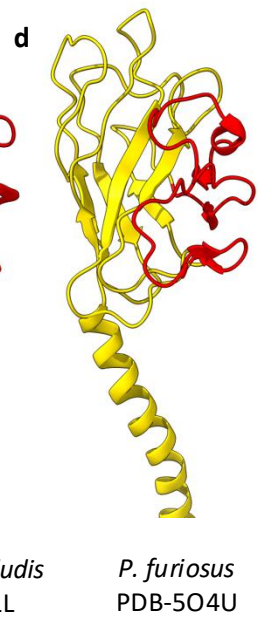
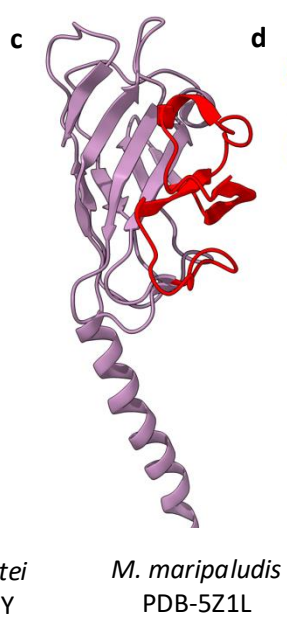
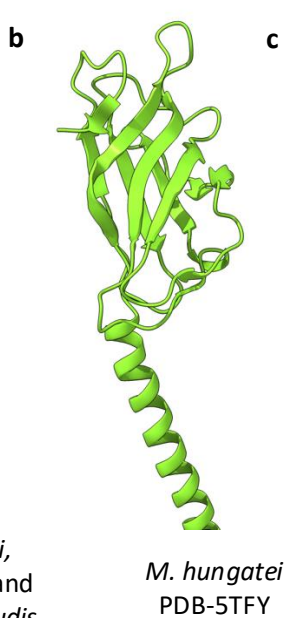
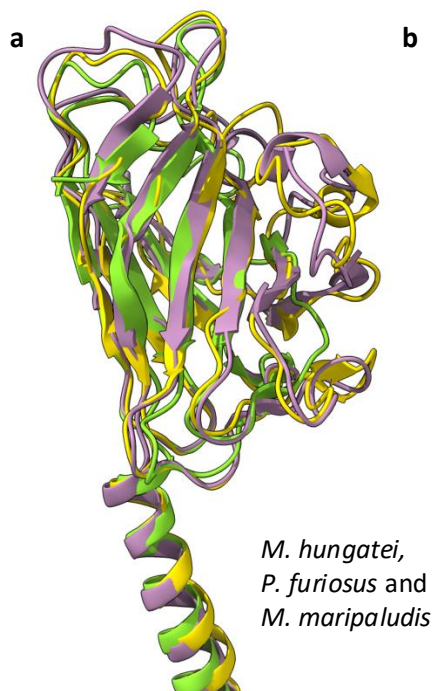
*P. furiosus*



*S. acidocaldarius*

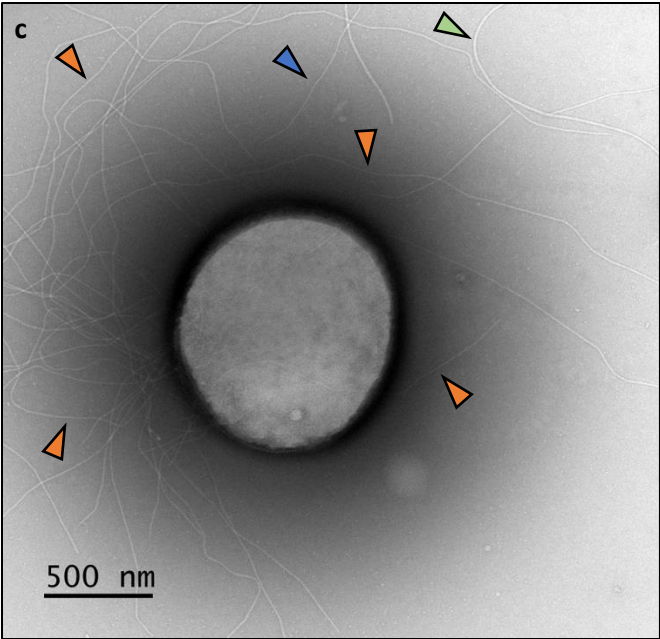
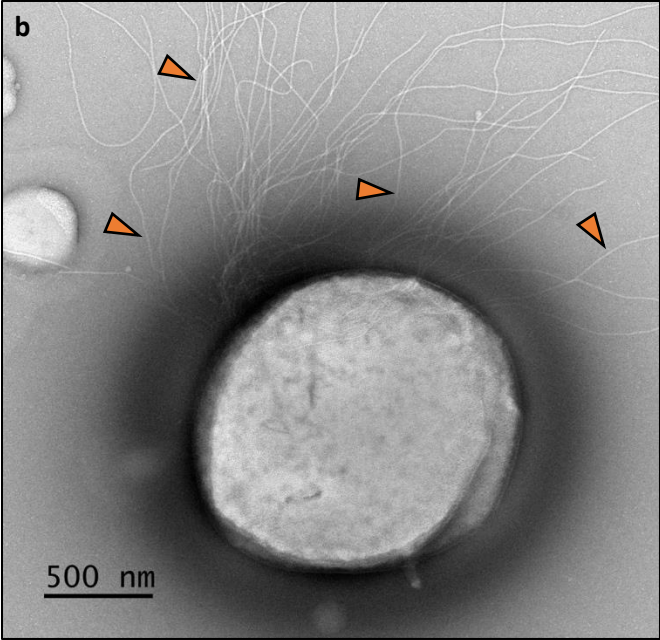
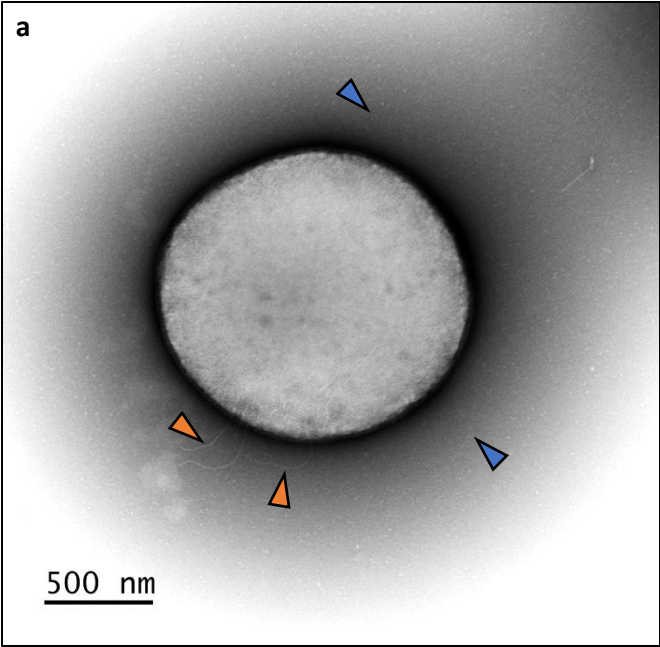
**Supplementary Figure 11: Glycosylation subdomain in crenarchaeota**

**a**, superimposition of the archaellin ArlB from *S. acidocaldarius* (yellow) with FlaB0 from *P. furiosus* <sup>5</sup> (PDB-5O4U; grey). **b**, highlights the glycosylation sub-domain of *S. acidocaldarius* ArlB in red. **c**, archaellin head domains of *P. furiosus* and *S. acidocaldarius* side-by-side.



### Supplementary Figure 12: Structural divergence of archaellins in euryarchaea

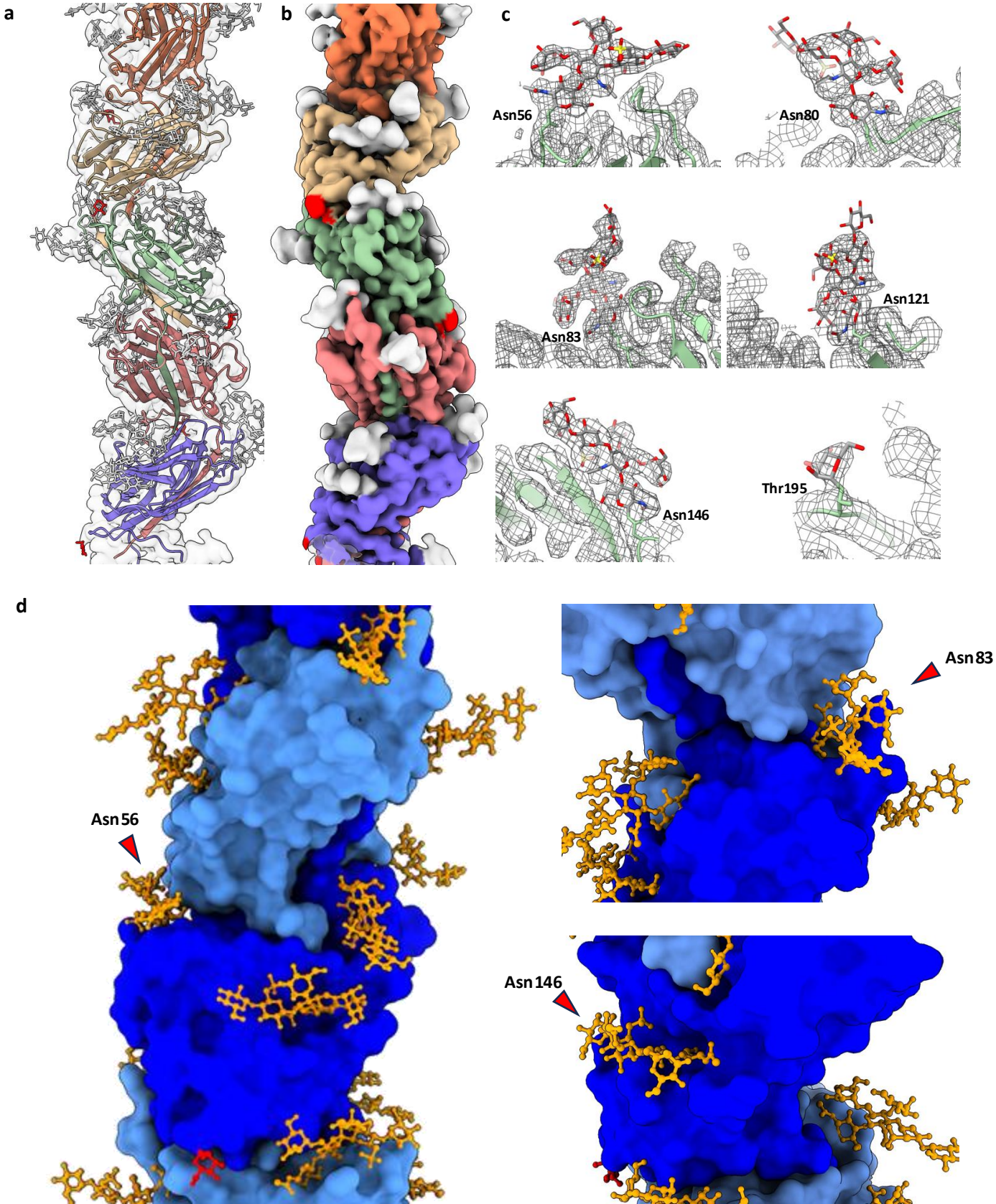
**a**, superimposition of the archaellins of *M. hungatei* (PDB-5TFY<sup>6</sup>; green), *M. maripaludis* (PDB-5Z1L<sup>7</sup>, purple) and *P. furiosus* (PDB-5O4U<sup>5</sup>, yellow). **b-d**, archaellins from (a) shown side-by-side. Red backbone sections visualise areas of structural variability. **e-g**, pairwise superimposition of *P. furiosus* and *M. maripaludis* (**e**); *M. hungatei* and *M. maripaludis* (**f**); and *M. hungatei* vs. *P. furiosus* (**g**).



### **Supplementary Figure 13: Growth-phase dependent expression of filaments**

*S. acidocaldarius* DSM639 at (a) early lag; (b) late logarithmic; and (c) stationary growth phase. Cells in early lag phase produce mainly threads (blue arrowheads). Aap (orange arrowheads) are produced soon after - from late log to stationary phase. Archaeella (green arrowheads) appear mostly in stationary phase with 0-5 archaeella per cell.

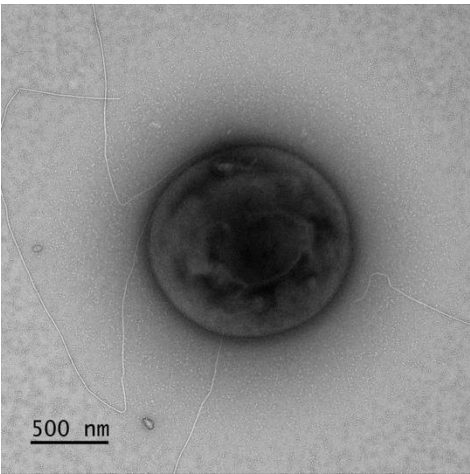
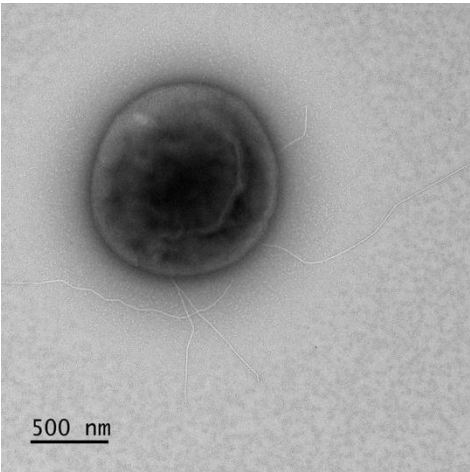
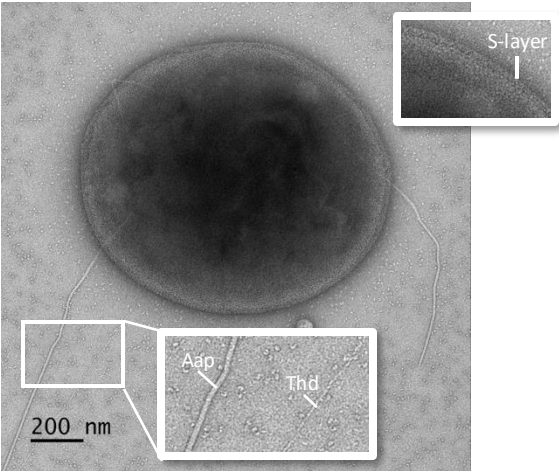
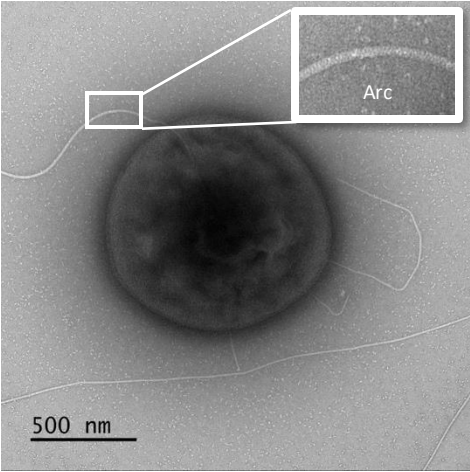




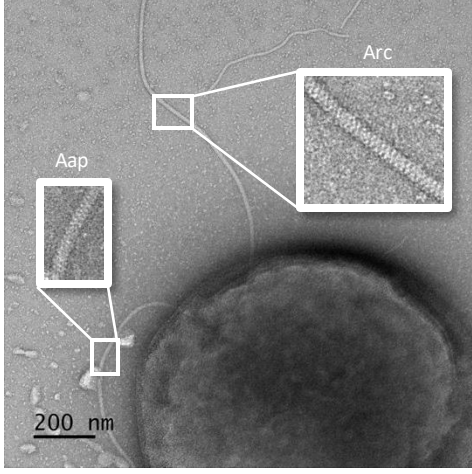
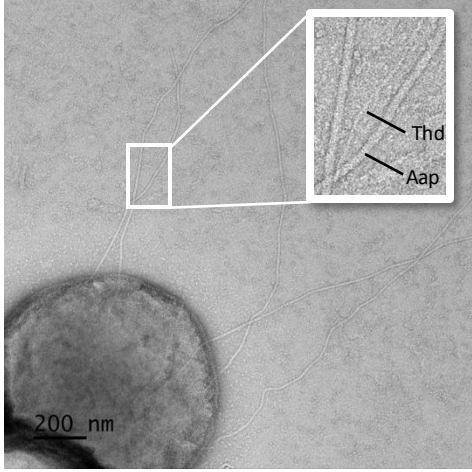
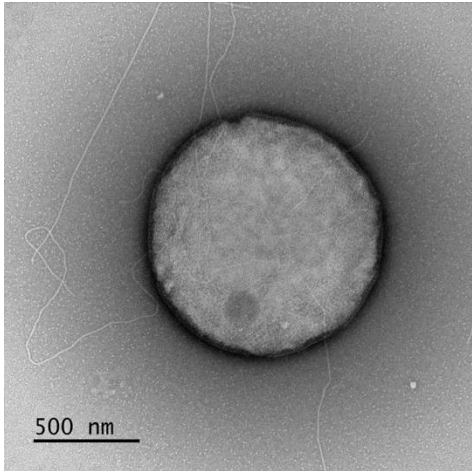
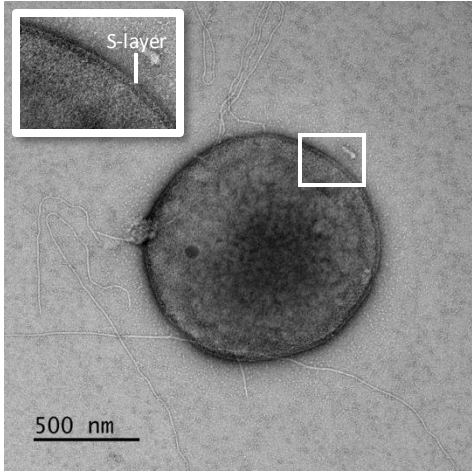
**Supplementary Figure 14: 2.7 Å resolution cryoEM map and model of the thread with full length glycans**

**a**, map of the *S. acidocaldarius* thread at 2.7 Å resolution (transparent grey) and atomic model in ribbon representation (multicolour). N-glycans are white sticks, O-glycans are red sticks. **b**, cryoEM map of the thread coloured according to the model in a. A high contour level was set to visualize the weaker glycan densities. **c**, closeups of the five N-glycans (bound to Ans56, Ans80, Ans83, Ans121 and Ans146), and one O-glycan (bound to Thr195) of the thread subunit Saci\_0406. Protein is shown as green ribbons and glycans are coloured by element. **d**, closeups showing how glycans in the thread are wedged into clefts between subunits (Asn56 and Asn83) or within the same subunit (Asn146). Protein is shown as surface (alternating subunits are coloured mid and light blue). N-glycans as orange balls and sticks and O-glycans red balls and sticks.

*S. acidocaldarius* wt (MW001)



*S. acidocaldarius*  $\Delta$ agl3

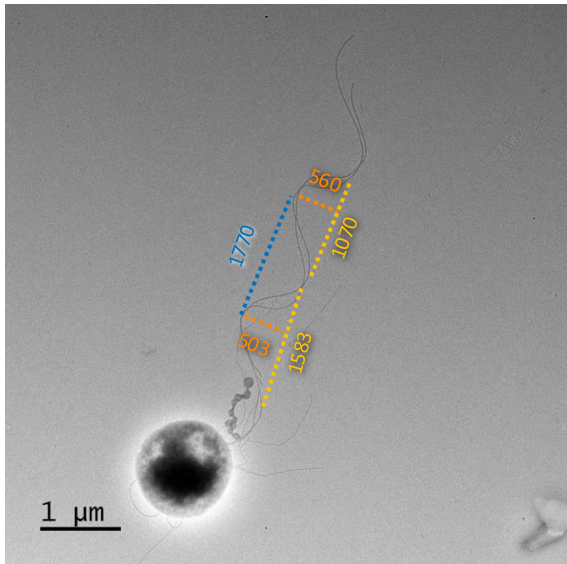
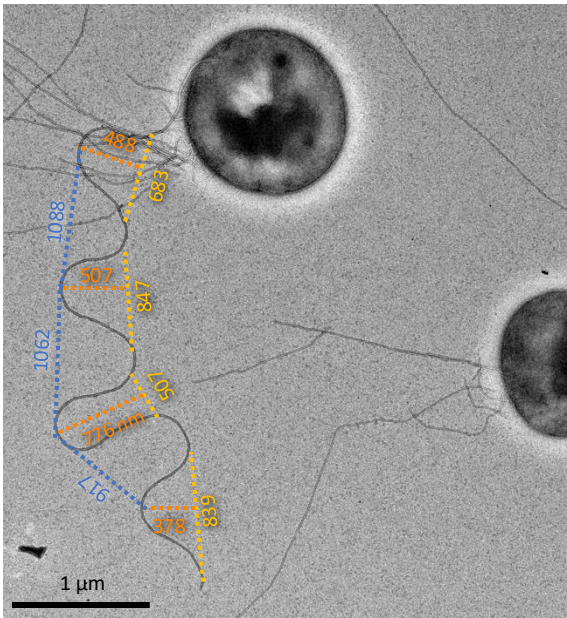
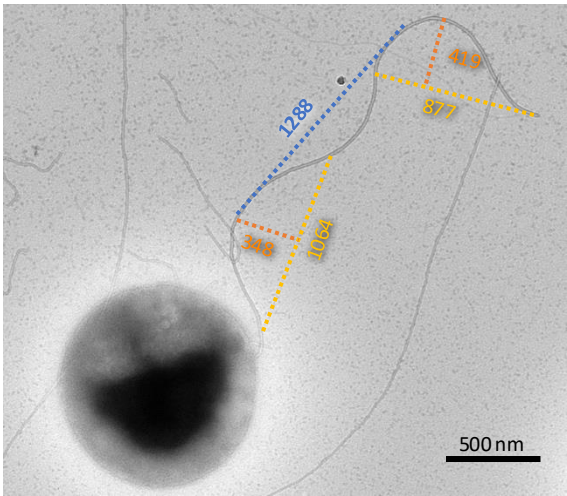


### Supplementary Figure 15: Morphology of the $\Delta$ agl3 mutant

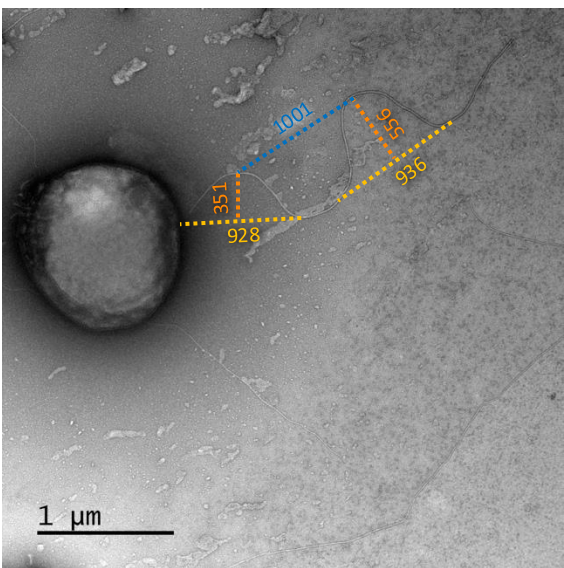
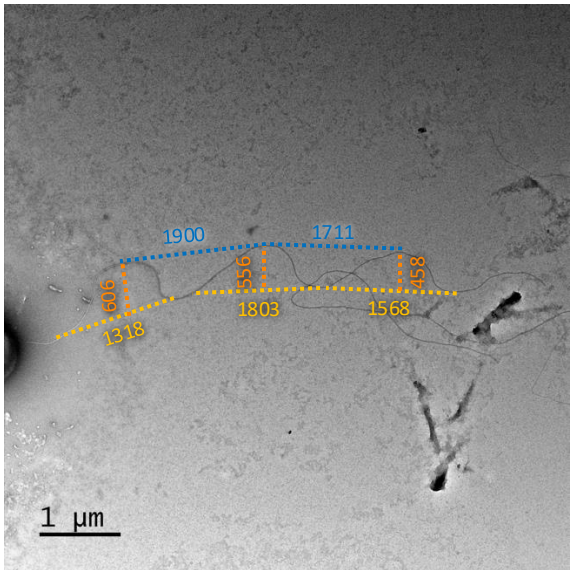
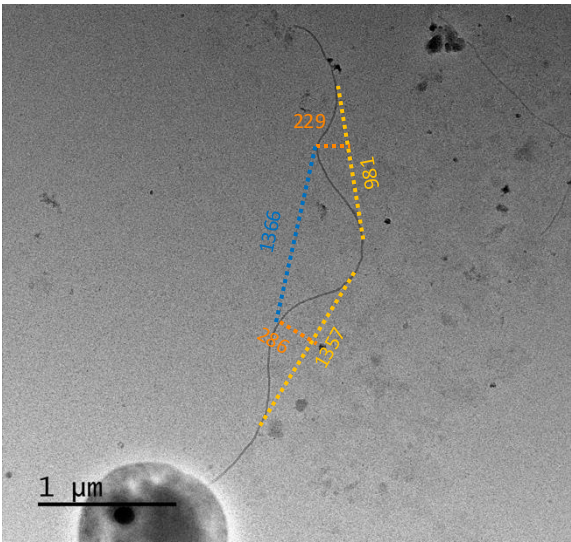
Negative stain images of *S. acidocaldarius* wt (MW001) (left panel) and the  $\Delta$ agl3 mutant cells (right panel) do not show obviously distinct phenotypes. Arc, archaellum; Thd, thread.



*S. acidocaldarius* wt (MW001)



*S. acidocaldarius*  $\Delta$ agl3 (MW039)



### **Supplementary Figure 16: Archaela of the wt and $\Delta$ agl3 mutant in comparison**

Negative stain images of *S. acidocaldarius* wt (MW001) (left panel) and the  $\Delta$ agl3 mutant cells (MW039) (right panel) displaying the whole lengths of their archaela. The apparent lengths and waveforms of the archaela vary significantly within the same sample. Therefore, clear conclusions about differences in these parameters between WT and the mutant cells cannot be drawn. All measurements in nm.

**Supplementary Table 1 - Cryo-EM data collection, refinement and validation statistics for *S. acidocaldarius* wild type filaments with full-length glycans**

|                                | wt archaeellum<br>(EMDB-18700)<br>(PDB 8QX4) | wt thread<br>(EMDB-19608)<br>(PDB 8RZL) | wt Aap<br>(EMDB-19960)<br>(PDB 9ETS) |
|--------------------------------|----------------------------------------------|-----------------------------------------|--------------------------------------|
| Data collection and processing |                                              |                                         |                                      |
| Magnification                  | 105 k                                        | 105 k                                   | 105 k                                |
| Voltage (kV)                   | 300                                          | 300                                     | 300                                  |
| Electron exposure (e-/Å²)      | 43.27                                        | 43.27                                   | 40                                   |
| Defocus range (µm)             | -1.0 to -2.5                                 | -1.0 to -2.5                            | -1.0 to -2.1                         |
| Pixel size (Å)                 | 0.829                                        | 0.829                                   | 1.07                                 |
| Symmetry imposed               | Twist: 107.923°<br>Rise: 5.43 Å              | Twist: -103.281°<br>Rise: 31.650 Å      | Twist: -39.859°<br>Rise: 15.524 Å    |
| Initial particle images (no.)  | 1,705,100                                    | 2,852,968                               | 7,833,953                            |
| Final particle images (no.)    | 1,059,736                                    | 626,078                                 | 505,862                              |
| Map resolution (Å)             | 2.0                                          | 2.7                                     | 2.6                                  |
| FSC threshold                  | 0.143                                        | 0.143                                   | 0.143                                |
| Map resolution range (Å)       | 1.8 – 2.3                                    | 2.5-3.0                                 | 2.3-2.8                              |
| Refinement                     |                                              |                                         |                                      |
| Initial model used (PDB code)  | Ab initio                                    | 7PNB                                    | 8Q30                                 |
| Map/Model resolution (Å)       | 2.2                                          | 2.8                                     | 2.8                                  |
| FSC threshold                  | 0.5                                          | 0.5                                     | 0.5                                  |
| Map sharpening B factor (Å²)   | n/a                                          | n/a                                     | n/a                                  |
| Model composition              |                                              |                                         |                                      |
| Non-hydrogen atoms             | 51,960                                       | 8,895                                   | 42,941                               |
| Protein residues               | 5,860 (293x20)                               | 915 (183x5)                             | 5,217 (141x37)                       |
| Ligands (glycans)              | 620 (31x20)                                  | 155 (31x5)                              | 456                                  |
| B factors (Å²)                 |                                              |                                         |                                      |
| Protein                        | 77.8                                         | 89.3                                    | 91.95                                |
| Ligand                         | 159.5                                        | 171.3                                   | 224.9                                |
| R.m.s. deviations              |                                              |                                         |                                      |
| Bond lengths (Å)               | 0.008                                        | 0.008                                   | 0.008                                |
| Bond angles (°)                | 1.493                                        | 1.498                                   | 1.390                                |
| Validation                     |                                              |                                         |                                      |
| MolProbity score               | 1.76                                         | 1.69                                    | 1.06                                 |
| Clashscore                     | 3.44                                         | 1.93                                    | 2.15                                 |
| Poor rotamers (%)              | 0.40                                         | 3.21                                    | 1.23                                 |
| Ramachandran plot              |                                              |                                         |                                      |
| Favored (%)                    | 97.94                                        | 95.3                                    | 99.05                                |
| Allowed (%)                    | 2.06                                         | 4.97                                    | 0.95                                 |
| Disallowed (%)                 | 0.0                                          | 0.0                                     | 0.0                                  |



**Supplementary Table 2 - Cryo-EM data collection, refinement and validation statistics for *S. acidocaldarius*  $\Delta$ agl3 filaments with truncated glycans**

|                                                     | $\Delta$ agl3 Archaeellum<br>(EMDB-18700)<br>(PDB 8QX4) | $\Delta$ agl3 Aap<br>(EMDB-19608)<br>(PDB 8RZL) |
|-----------------------------------------------------|---------------------------------------------------------|-------------------------------------------------|
| <b>Data collection and processing</b>               |                                                         |                                                 |
| Magnification                                       | 130 k                                                   | 130 k                                           |
| Voltage (kV)                                        | 300                                                     | 300                                             |
| Electron exposure (e <sup>-</sup> /Å <sup>2</sup> ) | 40                                                      | 40                                              |
| Defocus range (μm)                                  | -1.8 to -2.2                                            | -0.8 to -2.2                                    |
| Pixel size (Å)                                      | 0.829                                                   | 0.829                                           |
| Symmetry imposed                                    | Twist: 107.923°<br>Rise: 5.399 Å                        | Twist: -103.281°<br>Rise: 15.282 Å              |
| Initial particle images (no.)                       | 1,174,572                                               | 3,560,700                                       |
| Final particle images (no.)                         | 256,869                                                 | 691,479                                         |
| Map resolution (Å)<br>FSC threshold                 | 2.4<br>0.143                                            | 2.4<br>0.143                                    |
| Map resolution range (Å)                            | 2.2 – 2.7                                               | 2.5-3.0                                         |
|                                                     |                                                         |                                                 |
| <b>Refinement</b>                                   |                                                         |                                                 |
| Initial model used (PDB code)                       | 8QX4                                                    | 8Q30                                            |
| Map/Model resolution (Å)<br>FSC threshold           | 2.6<br>0.5                                              | 2.5<br>0.5                                      |
|                                                     |                                                         |                                                 |
| Map sharpening B factor (Å <sup>2</sup> )           | n/a                                                     | n/a                                             |
| Model composition                                   |                                                         |                                                 |
| Non-hydrogen atoms                                  | 48,800                                                  | 33,540                                          |
| Protein residues                                    | 5,860 (293x20)                                          | 4230 (141x30)                                   |
| Ligands (glycans)                                   | 360 (18x20)                                             | 260 (9x30)                                      |
| B factors (Å <sup>2</sup> )                         |                                                         |                                                 |
| Protein                                             | 81.1                                                    | 61.4                                            |
| Ligand                                              | 170.9                                                   | 131.4                                           |
| R.m.s. deviations                                   |                                                         |                                                 |
| Bond lengths (Å)                                    | 0.008                                                   | 0.008                                           |
| Bond angles (°)                                     | 1.540                                                   | 1.596                                           |
| <b>Validation</b>                                   |                                                         |                                                 |
| MolProbity score                                    | 1.07                                                    | 1.28                                            |
| Clashscore                                          | 2.31                                                    | 5.26                                            |
| Poor rotamers (%)                                   | 1.21                                                    | 0.30                                            |
| <b>Ramachandran plot</b>                            |                                                         |                                                 |
| Favored (%)                                         | 98.26                                                   | 99.52                                           |
| Allowed (%)                                         | 1.74                                                    | 0.48                                            |
| Disallowed (%)                                      | 0.0                                                     | 0.0                                             |

## Supplementary References

1. Nuno de Sousa Machado J, Albers SV, Daum B. Towards Elucidating the Rotary Mechanism of the Archaellum Machinery. *Front Microbiol.* 2022 Mar 21;13:848597. doi: 10.3389/fmicb.2022.848597
2. Teufel F, Almagro Armenteros JJ, Johansen AR, et al. SignalP 6.0 predicts all five types of signal peptides using protein language models. *Nature Biotechnology* 2022 40:7. 2022;40(7):1023-1025. doi:10.1038/s41587-021-01156-3
3. Kreutzberger MAB, Sonani RR, Liu J, et al. Convergent evolution in the supercoiling of prokaryotic flagellar filaments. *Cell.* 2022;185(19):3487-3500.e14. doi:10.1016/J.CELL.2022.08.009
4. Ashkenazy H, Abadi S, Martz E, et al. ConSurf 2016: an improved methodology to estimate and visualize evolutionary conservation in macromolecules. *Nucleic Acids Res.* 2016;44(W1):W344-W350. doi:10.1093/NAR/GKW408
5. Daum B, Vonck J, Bellack A, et al. Structure and in situ organisation of the pyrococcus furiosus archaellum machinery. *Elife.* 2017;6. doi:10.7554/ELIFE.27470
6. Poweleit N, Ge P, Nguyen HH, Loo RRO, Gunsalus RP, Zhou ZH. CryoEM structure of the Methanospirillum hungatei archaellum reveals structural features distinct from the bacterial flagellum and type IV pilus. *Nature Microbiology* 2016 2:3. 2016;2(3):1-12. doi:10.1038/nmicrobiol.2016.222
7. Meshcheryakov VA, Shibata S, Schreiber MT, et al. High-resolution archaellum structure reveals a conserved metal-binding site. *EMBO Rep.* 2019;20(5):e46340. doi:10.15252/EMBR.201846340