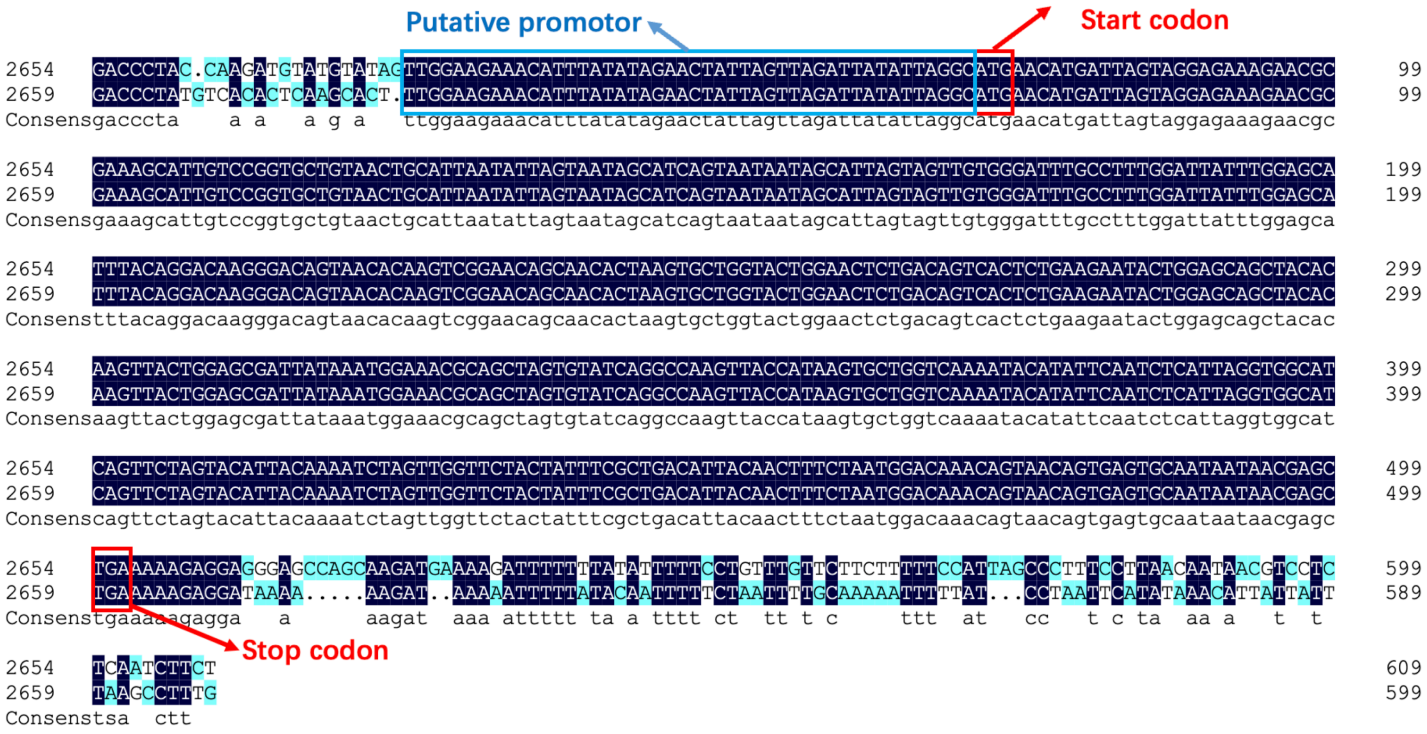
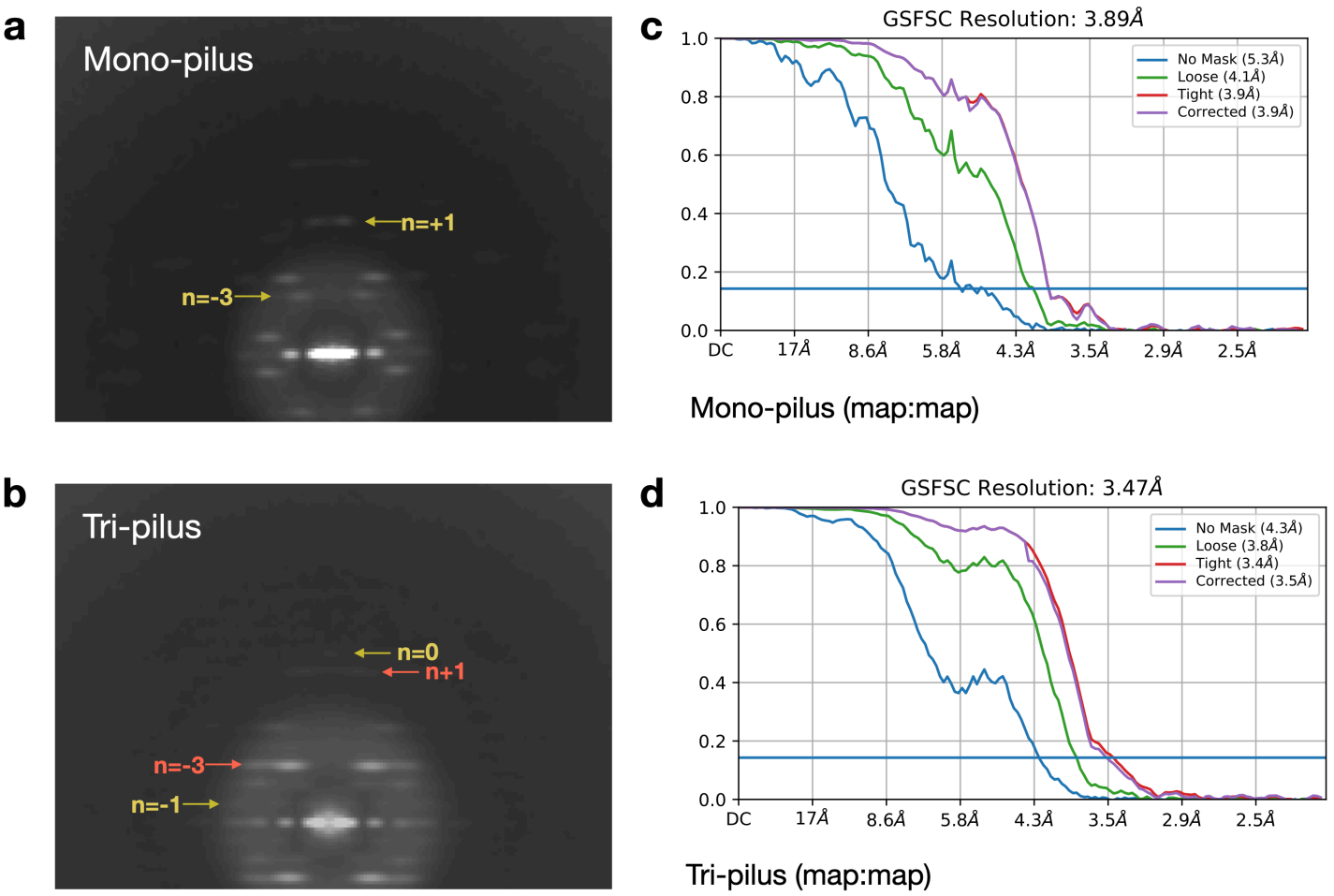
# Supplementary Information

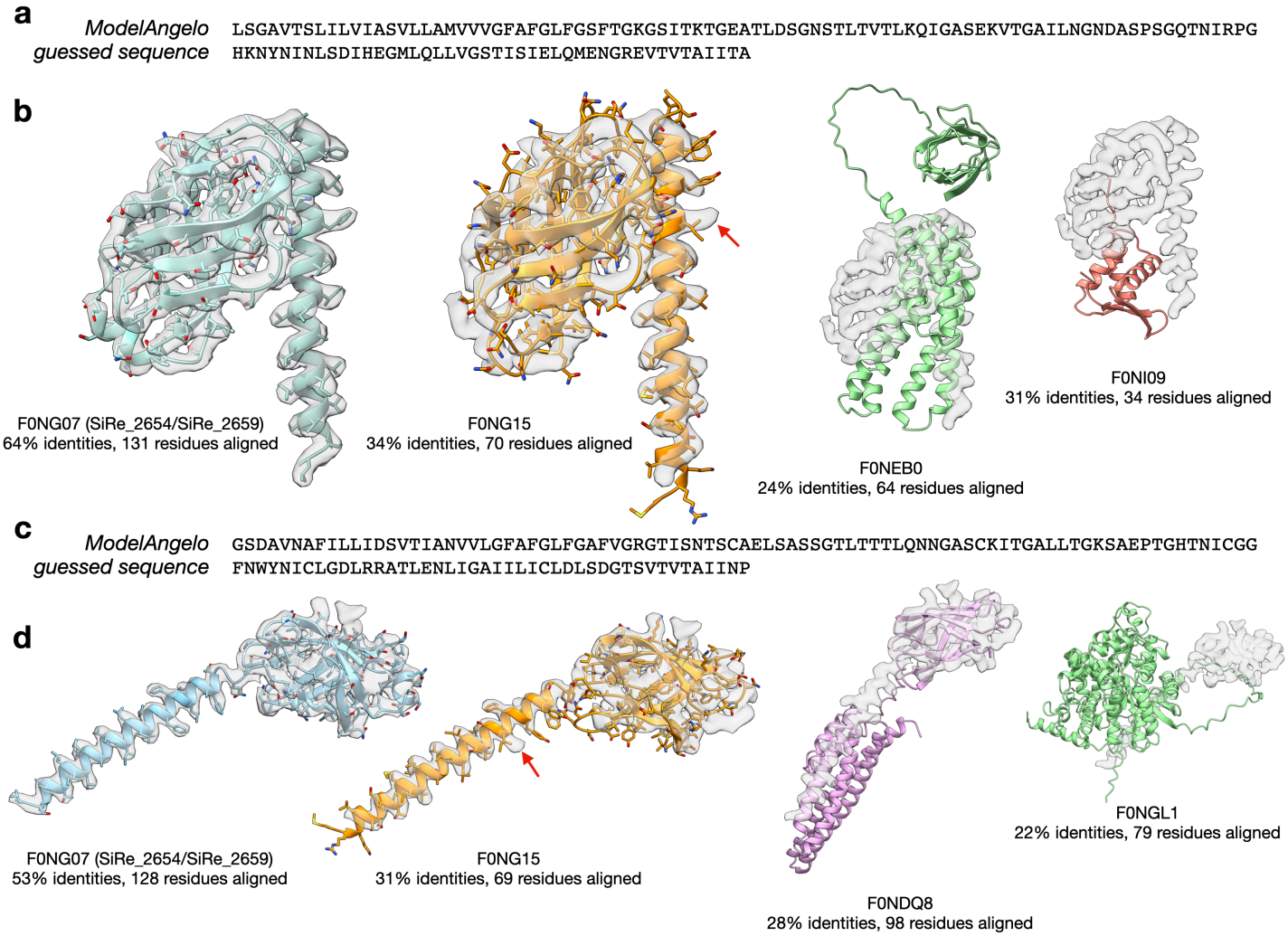



**Supplementary Figure 1. Sanger sequencing confirmed that the two pilin genes, SiRe_2654 and SiRe_2659, are identical**. The complete sequencing results are presented, with start and stop codons annotated.



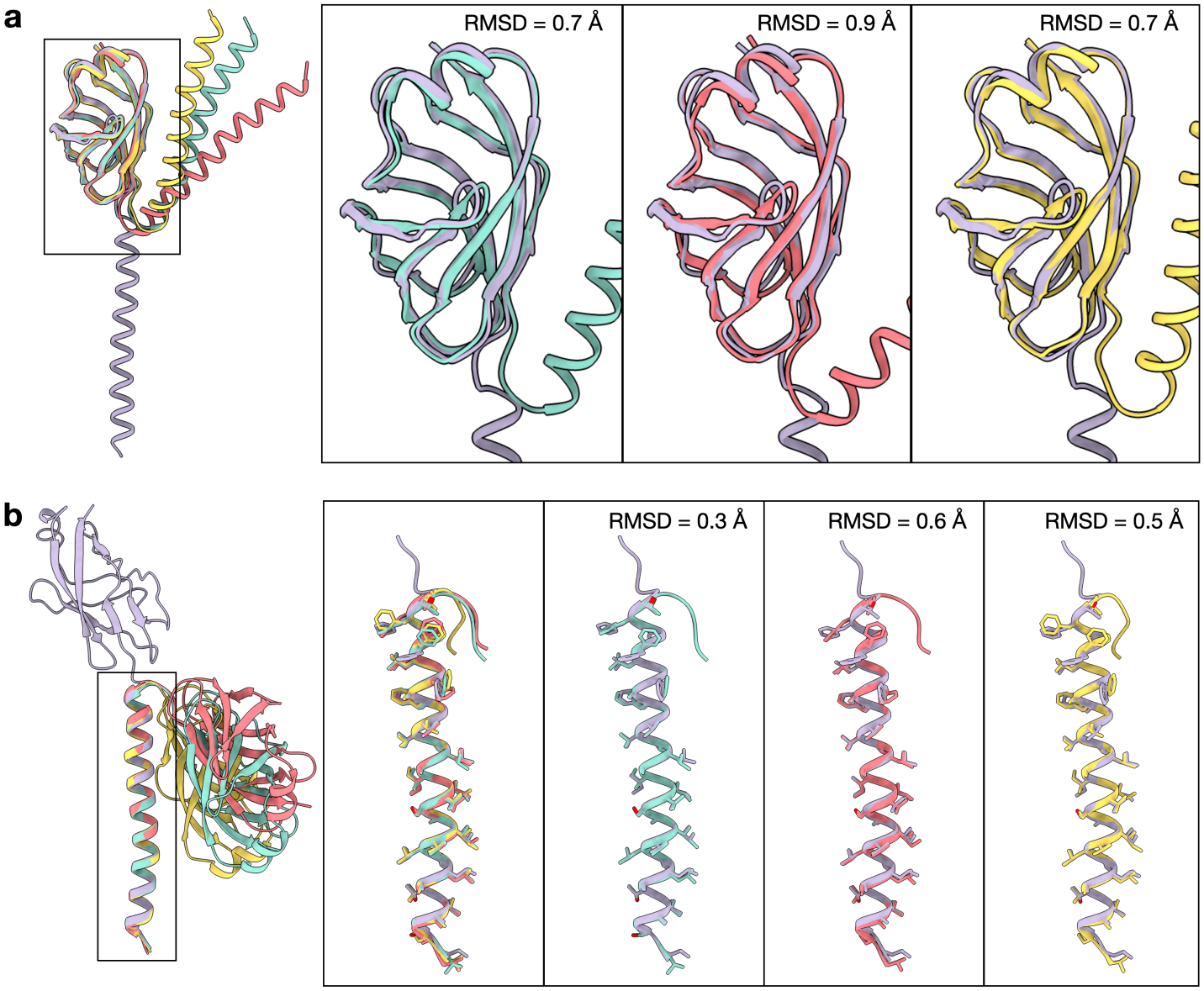

**Supplementary Figure 2. Average power spectra and Fourier Shell Correlation (FSC) calculations of mono-pilus and tri-pilus**

Average power spectra of particles from 2D class average of mono-pilus (**a**) and tri-pilus (**b**). The Bessel orders of the layer lines used to determine the global helical symmetry are labeled in yellow. For tri-pilus, the layer-lines corresponding to the features generated from the inner helices are labeled in red. The map:map "gold-standard FSC" curves are shown for the mono-pilus (**c**) and tri-pilus (**d**).




**Supplementary Figure 3. Identification of pilin using ModelAngelo and cryo-EM modeling**

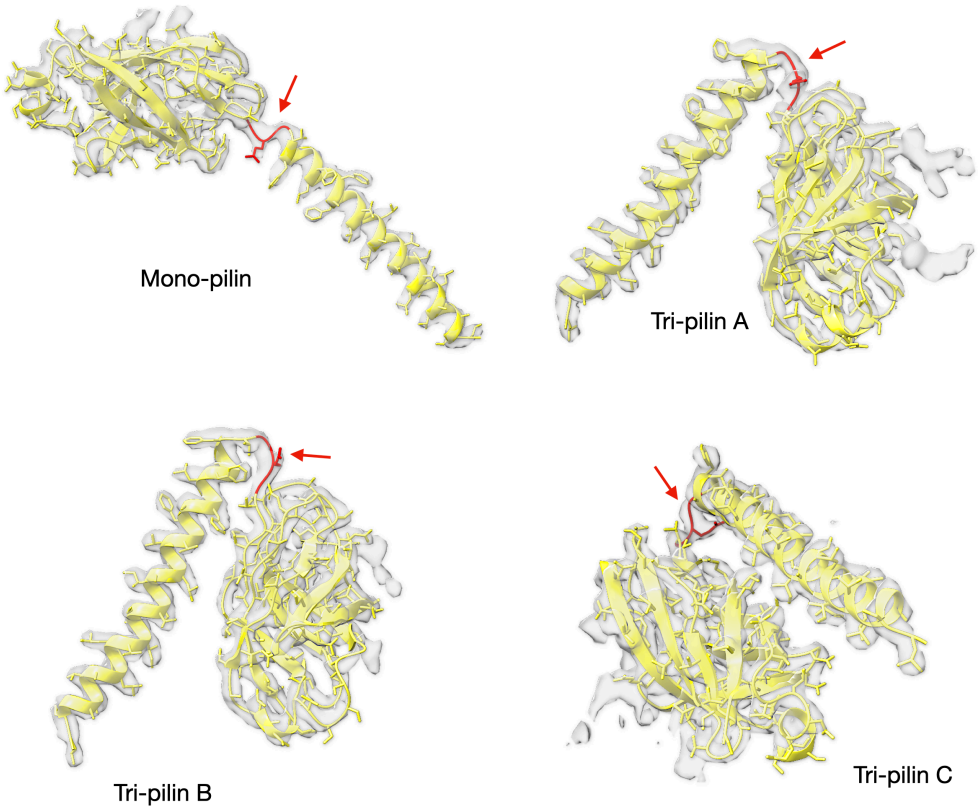
(**a**) Pilin sequence as predicted by ModelAngelo based on the tri-pilus cryo-EM map. (**b**) Top hits from the BLAST results, derived by searching the predicted sequence against *Sulfolobus islandicus REY15A*, are displayed. Both their sequence identity to the predicted sequence and the number of aligned residues are indicated. A red arrow highlights a notable disagreement between the AlphaFold model and the cryo-EM map. (**c**) Pilin sequence as predicted by ModelAngelo based on the mono-pilus cryo-EM map. (**d**) A similar analysis to (**b**) but uses the sequence predicted using the mono-pilus map.




**Supplementary Figure 4. Alignment of C-terminal and N-terminal domains of mono-pilin and tri-pilins**

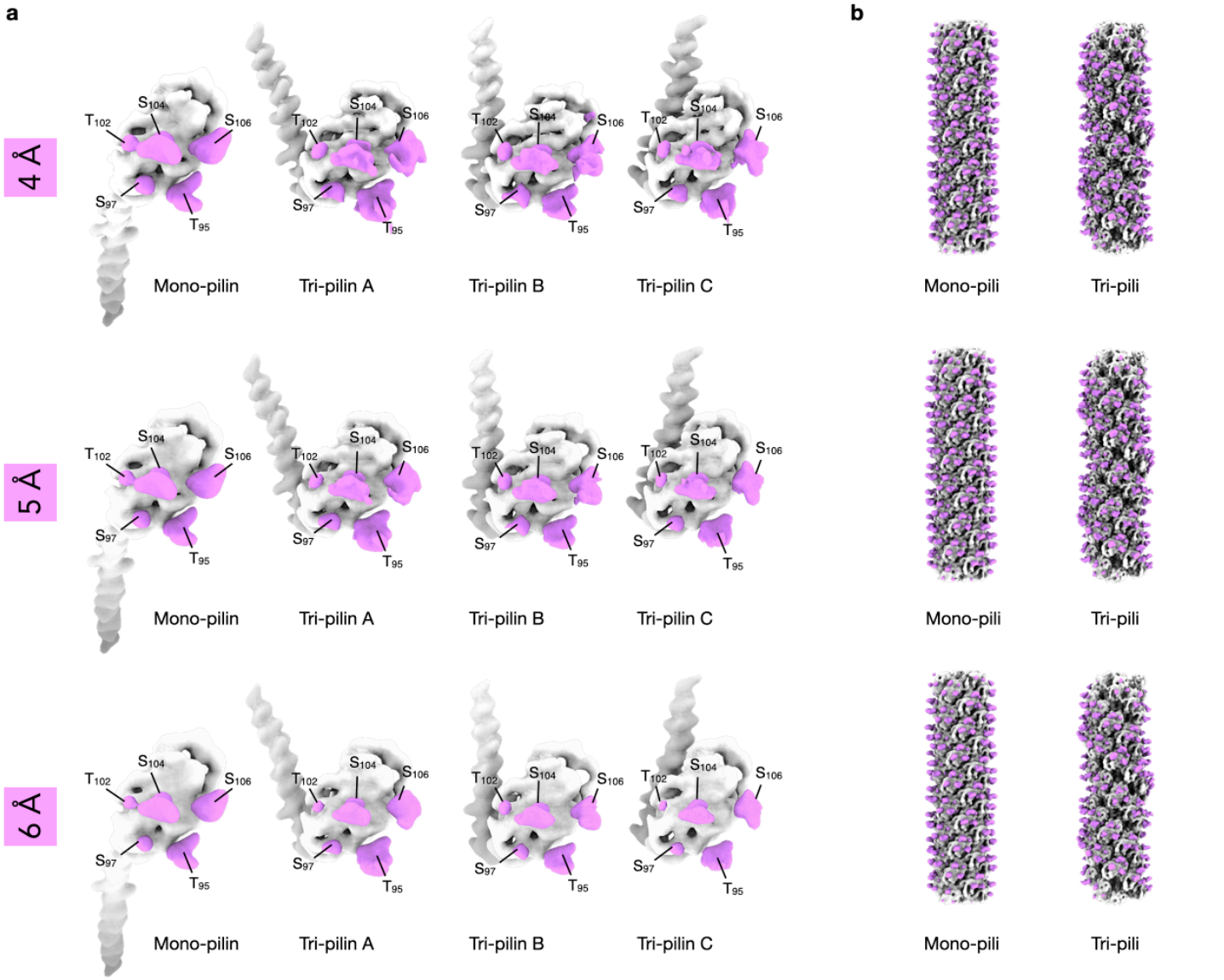
(**a**) Alignment of the C-terminal Ig-like globular domains is depicted, comparing the mono-pilin (lavender) with each of the three tri-pilins (A, cyan; B, red; C, yellow). Insets provide a magnified view of region (**a**), focusing on the mono-pilin and a single tri-pilin subunit, along with the labeled backbone RMSD (Root Mean Square Deviation).

(**b**) Alignment of each of the three tri-pilins to the mono-pilin by the N-terminal long helix, following the same visualization approach as in panel (**a**). Sidechains for residues 13-45 are additionally shown.




**Supplementary Figure 5. Cryo-EM densities of the linker region.**

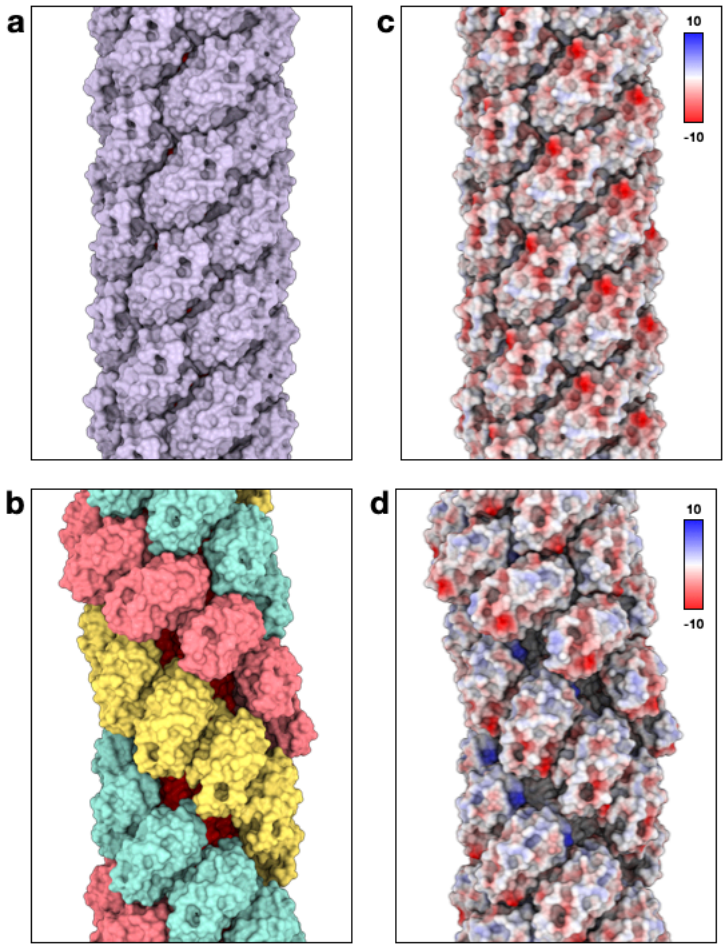
The cryo-EM densities for the pilin subunits are presented. Pilin models are shown in yellow ribbon, with side chains displayed. The linker “Gly-Gln-Gly” regions are highlighted in red.




**Supplementary Figure 6. Levels of surface glycosylation of mono and tri-pilus, filtered at different resolution.**

(**a**) Cryo-EM map densities due to post-translational modifications on pilin subunits. All volumes were filtered to 4, 5, and 6 Å, respectively. The densities accounted for the protein part (grey) were segmented out using a mask generated from the refined atomic model. The extra experimental density after subtracting the protein part, presumably coming from O-linked sugars on Ser or Thr residues, are colored in magenta. The amino acid side chains with extra densities are labeled.

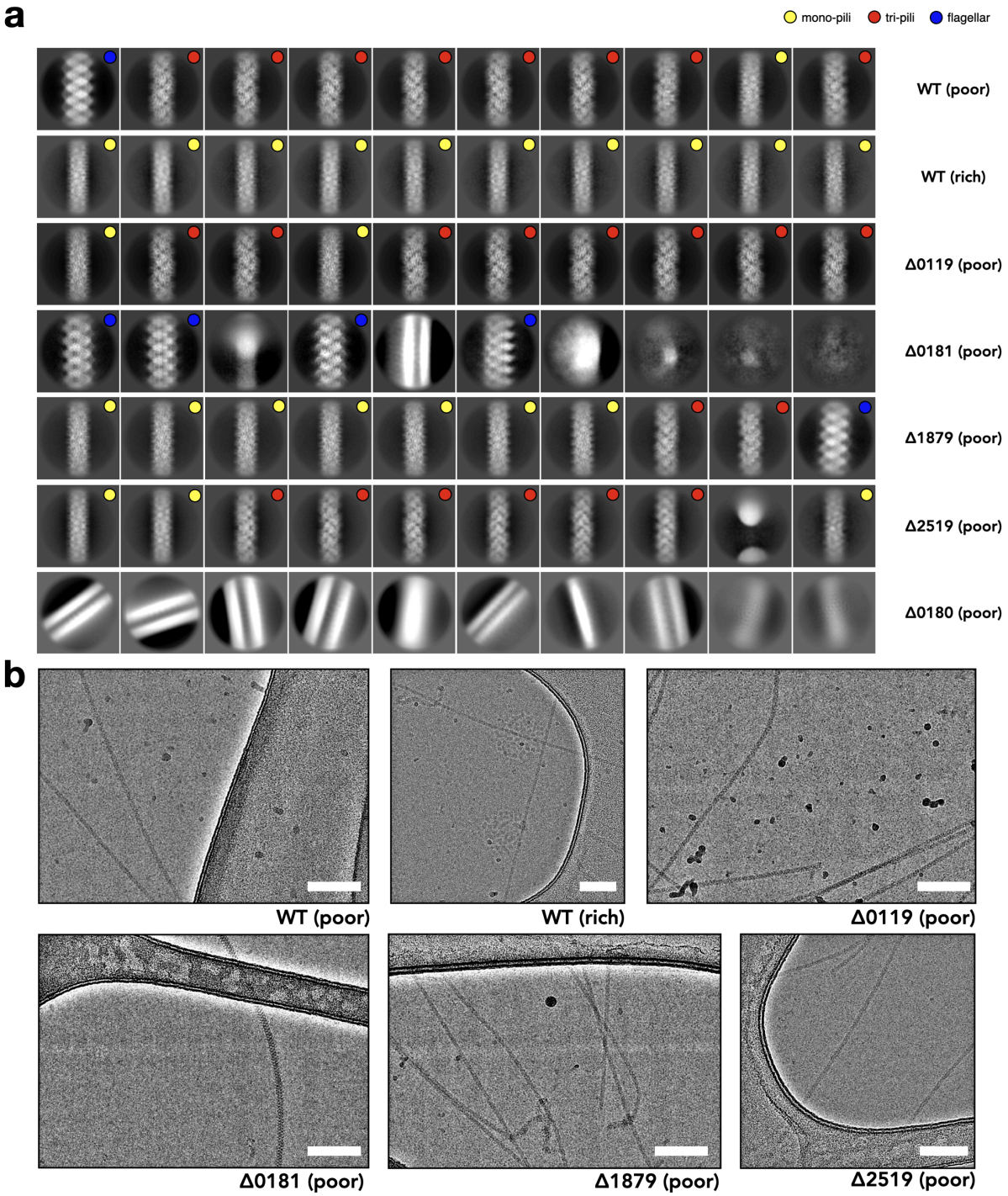
(**b**) Surface glycosylation of the mono-pilus (left) and tri-pilus (right) filtered at the same resolution as in (a).



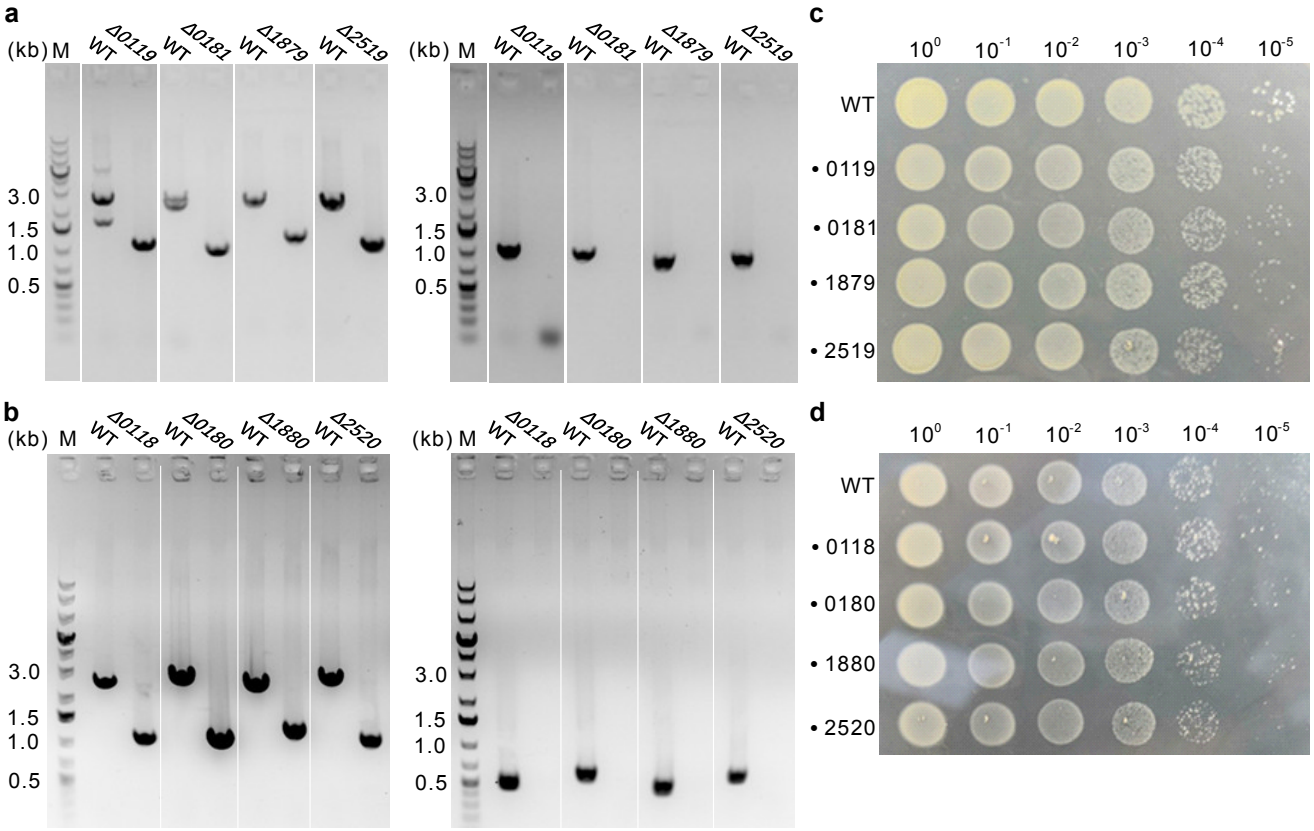

**Supplemental Figure 7. Solvent-excluded and electrostatic surfaces of the mono-pilus and tri-pilus**

(**a**) Molecular solvent-excluded surface of the mono-pilus. The inner helices are colored in red, but cannot be seen due to their shielding by the outer domains.

(**b**) Molecular solvent-excluded surface of the tri-pilus. The inner helices of all pilins are colored in red. Electrostatic surface of the mono-pilus (**c**) and the tri-pilus (**d**). Scale bar displays the color-coding of the electrostatic potential in units kcal·mol$^{-1}$·$e^{-1}$ at 298 K.




**Supplementary Figure 8. Pili production under different media and ATPase knockout strains**
(**a**) The top ten 2D classes for datasets collected in Fig 6c. In total there are 50 classes, and after the first 10, there are more classes containing carbon edges etc. Also, each class may have different number of particles. The filament species identified based on the 2D averages, and their corresponding power spectrum are labeled on the upper right.
(**b**) Representative micrographs of the strains illustrated in (**a**). Scale bars, 100 nm.




**Supplementary Figure 9. Construction of the ATPase and TadC knockout strains**

PCR verification of the ATPase (**a**) and TadC (**b**) knockout strains using the Flanking (Left) and gene specific (right) primers. Spot test to show the growth of the ATPase (**c**) and TadC (**d**) knockout strains. Knockout of the ATPase or TadC genes alone exhibited no obvious growth difference comparing with the WT strain on solid medium

## Supplemental Table 1. Plasmids used in this study

| Plasmid | Description | Reference |
|---|---|---|
| *pGE* | *Genome-editing plasmid* | Li et al[1] |
| pGE-*sire_0119* | *sire_0119* (ATPase gene) knockout | This study |
| pGE-*sire_0181* | *sire_0181* (ATPase gene) knockout | This study |
| pGE-*sire_1879* | *sire_0181* (ATPase gene) knockout | This study |
| pGE-*sire_2519* | *sire_2519* (ATPase gene) knockout | This study |
| pGE-*sire_0118* | *sire_0118* (TadC gene) knockout | This study |
| pGE-*sire_0180* | *sire_0180* (TadC gene) knockout | This study |
| pGE-*sire_1880* | *sire_1880* (TadC gene) knockout | This study |
| pGE-*sire_2520* | *sire_2520* (TadC gene) knockout | This study |

## Supplemental Table 2. Strains used in this study

| Strain | Phenotype | Reference |
|---|---|---|
| *S. islandicus REY15A* | *Wild type* | Guo et al[2] |
| Sis E2333 | REY15A *ΔpyrEFΔlacS* | Deng et al[3] |
| Sis/pGE | Control for knockout | Liu et al[4] |
| *Δsire_0119* | *sire_0119* knockout | This study |
| *Δsire_0181* | *sire_0181* knockout | This study |
| *Δsire_1879* | *sire_0181* knockout | This study |
| *Δsire_2519* | *sire_2519* knockout | This study |
| *Δsire_0118* | *sire_0118* knockout | This study |
| *Δsire_0180* | *sire_0180* knockout | This study |
| *Δsire_1880* | *sire_1880* knockout | This study |
| *Δsire_2520* | *sire_2520* knockout | This study |

## Supplemental Table 3. Spacers selected and used in this study

| Name | Sequence (5'-3') | Source |
|---|---|---|
| 0119-S-F | AAGCATACGAACAAGTTCATACCCTCGTCTAACATCATCCATA | This study |
| 0119-S-R | AGCTATGGATGATGTTAGACGAGGGTATGAACTTGTTCGTATG | This study |
| 0181-S-F | AAGAATGGCGATAGGATTGCCGCAACATTCAGACGTGAAGTAT | This study |
| 0181-S-R | AGCATACTTCACGTCTGAATGTTGCGGCAATCCTATCGCCATT | This study |
| 1879-S-F | AAGATAAGTCCTATATTATTGATTAAAAACGTATCGATAAGTT | This study |
| 1879-S-R | AGCAACTTATCGATACGTTTTTAATCAATAATATAGGACTTAT | This study |
| 2519-S-F | AAGGAAAAGCCTCTAACGATTATTGACTTGGTTTACAAGTATG | This study |
| 2519-S-R | AGCCATACTTGTAAACCAAGTCAATAATCGTTAGAGGCTTTTC | This study |
| 0118-S-F | AAGTCAGAGACTCTTTTAAGTTGCTTACTATCGCATAATTTCT | This study |
| 0118-S-R | AGCAGAAATTATGCGATAGTAAGCAACTTAAAAGAGTCTCTGA | This study |
| 0180-S-F | AAGTCTCTCTTTGGTTCAGTCCCATTTCCTATAATGGCTATTT | This study |
| 0180-S-R | AGCAAATAGCCATTATAGGAAATGGGACTGAACCAAAGAGAGA | This study |
| 1880-S-F | AAGATGATTTTACTGGCCTTTCAGTATATTTCGTCAACAATAA | This study |
| 1880-S-R | AGCTTATTGTTGACGAAATATACTGAAAGGCCAGTAAAATCAT | This study |
| 2520-S-F | AAGGTTTCGTTGGAGGTTTTACTCAGAAAGAACCTCATTCCTA | This study |
| 2520-S-R | AGCTAGGAATGAGGTTCTTTCTGAGTAAAACCTCCAACGAAAC | This study |

## Supplemental Table 4. Oligonucleotides used in this study

| Name | Sequence (5'-3') | Source |
|---|---|---|
| **Oligonucleotides used to amplify the L- and R-arm of the corresponding genes of interest** | | |
| 0119-L-arm-F-Sph I | AAGTACAATTGTGCT**GCATGC**CTATTAACCTAGCAGAATAA | This study |
| 0119-L-arm-R | TCCCTATCGAGGGCTTAACCTTCTTTATGACGAATTAAAC | This study |
| 0119-R-arm-F | GTTTAATTCGTCATAAAGAAGGTTAAGCCCTCGATAGGGA | This study |
| 0119-R-arm-R-Xho I | TAACATATTGGATG**CTCGAG**AAAAAGATGAAAGATGTAAA | This study |
| 0181-L-arm-F-Sph I | AAGTACAATTGTGCT**GCATGC**AGTAGAGTTTCCTTAATTTC | This study |
| 0181-L-arm-R | ATTGTTAATCTTGGTTTCAATATATATCACTTCTCCATCT | This study |
| 0181-R-arm-F | AGATGGAGAAGTGATATATATTTGAAACCAAGATTAACAAT | This study |
| 0181-R-arm-R-Xho I | TAACATATTGGATG**CTCGAG**TACCCTAACTGATTTATATA | This study |
| 1879-L-arm-F-Sph I | AAGTACAATTGTGCT**GCATGC**GAGATGGGTATGATTTGGTC | This study |
| 1879-L-arm-R | ATTTCTGAAATTGGTATTTCTTCGCTTACTTTATAATAGC | This study |
| 1879-R-arm-F | GCTATTATAAAGTAAGCGAAGAAATACCAATTTCAGAAAT | This study |
| 1879-R-arm-R-Xho I | TAACATATTGGATG**CTCGAG**TAGAATTCCTTATTTCCTCG | This study |
| 2519-L-arm-F-Sph I | AAGTACAATTGTGCT**GCATGC**GTTGCAATAGCTGCGGTCAG | This study |
| 2519-L-arm-R | TCCTTACTCCAGCCTAATAACTCTAAGAGATATTATTCCC | This study |
| 2519-R-arm-F | GGGAATAATATCTCTTAGAGTTATTAGGCTGGAGTAAGGA | This study |
| 2519-R-arm-R-Xho I | TAACATATTGGATG**CTCGAG**CTATGAAACCTAATCTACTG | This study |
| 0118-L-arm-F-Sph I | AAGTACAATTGTGCT**GCATGC**GATGGTCTCAATTTTAGAGA | This study |
| 0118-L-arm-R | GATATGCCAAGAATATTTAGATATATTTACCTAAAATTAC | This study |
| 0118-R-arm-F | GTAATTTTAGGTAAATATATCTAAATATTCTTGGCATATC | This study |
| 0118-R-arm-R-Xho I | TAACATATTGGATG**CTCGAG**TGTAGCATTCCAAGCAATGC | This study |
| 0180-L-arm-F-Sph I | AAGTACAATTGTGCT**GCATGC**AAAAACCGTAAGGGATTATA | This study |
| 0180-L-arm-R | AGAGTGCCGAAATAGAGATTCCTATTGTTACTTTCTCAGA | This study |
| 0180-R-arm-F | TCTGAGAAAGTAACAATAGGAATCTCTATTTCGGCACTCT | This study |
| 0180-R-arm-R-Xho I | TAACATATTGGATG**CTCGAG**TCATTTACTGGTGGTTCCAA | This study |
| 1880-L-arm-F-Sph I | AAGTACAATTGTGCT**GCATGC**GACTATCATTAAGATATAGG | This study |
| 1880-L-arm-R | ATAATCAAATCTAAGTTACCTCAAATAGCCTTTGTCTCAA | This study |
| 1880-R-arm-F | TTGAGACAAAGGCTATTTGAGGTAACTTAGATTTGATTAT | This study |
| 1880-R-arm-R-Xho I | TAACATATTGGATG**CTCGAG**TACAATATTCTTAGTCCCAT | This study |
| 2520-L-arm-F-Sph I | AAGTACAATTGTGCT**GCATGC**GGGAAGTAAGAGGGAAAGAA | This study |
| 2520-L-arm-R | ATATGAACATTTCAGCTAGGCTTCTTTCAGTCACCCCTAT | This study |
| 2520-R-arm-F | ATAGGGGTGACTGAAAGAAGCCTAGCTGAAATGTTCATAT | This study |
| 2520-R-arm-R-Xho I | TAACATATTGGATG**CTCGAG**TCTGTCCGTTAAATGTGGTT | This study |
| **Oligonucleotides used to verify the knockout strains** | | |

| | | |
|---|---|---|
| 0119-Flanking-F | GGTAAGGGAGCTATTTAATGTAGC | This study |
| 0119-Flanking-R | CTACAGAACAAACTAGCAAAGACTA | This study |
| 0119-int-F | CTGCAGGATCATATGTAAATGCTGG | This study |
| 0119-int-R | CTAAGCAAGATAAGAATATCCGTACC | This study |
| 0181-Flanking-F | GATAGCCTCAGAGAGAATATTGGA | This study |
| 0181-Flanking-R | GATATTGTAGTTTATACCTTAGCCC | This study |
| 0181-int-F | GCCGAATCCTCACATCTTTATTACC | This study |
| 0181-int-R | CTCTTGTCCTCTTATCTCACCTAC | This study |
| 1879-Flanking-F | ACGAGATTTTCGAATGGAAGGAGTTA | This study |
| 1879-Flanking-R | GTAACATAGCCTCTCTTCTTAAACC | This study |
| 1879-int-F | GAGCTAACTGTTCCCTTAGCAGAT | This study |
| 1879-int-R | CACTGGCTTGAATCTTAGTTTACC | This study |
| 2519-Flanking-F | GGCTTAGAAAACGCTTTAGTTACTG | This study |
| 2519-Flanking-R | CTCTTAGTACGCTCGCTTATCCA | This study |
| 2519-int-F | CGAACCACAGCTTGATCAGAGAGA | This study |
| 2519-int-R | CATGACCACTGGCAACAGCTTGA | This study |
| 0118-flanking-F | GTCATGGCCCAGTTTACACTA | This study |
| 0118-flanking-R | GGAGAAATTAGAGATAGAGAAGG | This study |
| 0118-int-F | GTACTTTGCTTAACATGCTCAC | This study |
| 0118-int-R | GGAACTACTCTAATAACACAGC | This study |
| 0180-flanking-F | GGAATCTCTGGAATTAGGATAG | This study |
| 0180-flanking-R | GAAGAGGTAGAGAGAGAATTAC | This study |
| 0180-int-F | CACAGCAATGCGCTACGCTAG | This study |
| 0180-int-R | GTATTTGCTGTACACCACCATTC | This study |
| 1880-flanking-F | GCTAGACAATCAATCTCTTCCC | This study |
| 1880-flanking-R | CACGTTCTGAACAACACTATACG | This study |
| 1880-int-F | GTGGCGAGTCAATAGGAATTTC | This study |
| 1880-int-R | CATCAGCGACCGATATCTTAC | This study |
| 2520-flanking-F | CAGACTCTCCTTAAGATATAGAC | This study |
| 2520-flanking-R | GGTCCAATTACTATTGCATCTCC | This study |
| 2520-int-F | GTAAGAGACAGCATCCAAGATG | This study |
| 2520-int-R | CATATATCTAGGTCTATTGCTAGC | This study |
| **Oligonucleotides used to determine the transcriptional level of the corresponding genes by RT-qPCR** | | |
| 0119-qPCR-F | GCTACATTACCTTCTCTATCTC | This study |
| 0119-qPCR-R | CAGAAGTGACTAGAGAAACAG | This study |

| | | |
|---|---|---|
| 0181-qPCR-F | GTTCTTCAGTAGTGATACGAAG | This study |
| 0181-qPCR-R | GTAACTCCTATTGACATGATACT | This study |
| 1879-qPCR-F | GTCATAGGTTCTACCGGATC | This study |
| 1879-qPCR-R | CCTTATCCAGTTATCATGAACTA | This study |
| 2519-qPCR-F | GAAAGACCACTGCATTAAACTC | This study |
| 2519-qPCR-R | CATAGTTTGAAGGTCTAGTATAG | This study |
| 0118-qPCR-F | CTATTAGGAGTTGGTCTTAGG | This study |
| 0118-qPCR-R | CTTAGTACTGCCATCAGAGAC | This study |
| 0180-qPCR-F | GAAACTTTAGCTTCTACTGCTG | This study |
| 0180-qPCR-R | GCCATTATAGGAAATGGGACTG | This study |
| 1880-qPCR-F | GTCAATAGGAATTTCCATGCC | This study |
| 1880-qPCR-R | CCAGTAAAATCATTGGAGTGAC | This study |
| 2520-qPCR-F | CTCCACTTGACGCTTTAGAG | This study |
| 2520-qPCR-R | CAACGAAACTGGAGCCAATTAAG | This study |
| TBP-F | GTGGCAACAGTTACGTTAGAG | Liu et al[5] |
| TBP-R | CCTTGGGCTGTTCTAATCTG | Liu et al[5] |

## Supplementary References

1. Li Y*, et al.* Harnessing Type I and Type III CRISPR-Cas systems for genome editing. *Nucleic Acids Res* **44**, e34 (2016).

2. Guo L*, et al.* Genome analyses of Icelandic strains of Sulfolobus islandicus, model organisms for genetic and virus-host interaction studies. *J Bacteriol* **193**, 1672-1680 (2011).

3. Deng L, Zhu H, Chen Z, Liang YX, She Q. Unmarked gene deletion and host-vector system for the hyperthermophilic crenarchaeon Sulfolobus islandicus. *Extremophiles* **13**, 735-746 (2009).

4. Liu J*, et al.* Archaeal extracellular vesicles are produced in an ESCRT-dependent manner and promote gene transfer and nutrient cycling in extreme environments. *ISME J* **15**, 2892-2905 (2021).

5. Liu J*, et al.* Virus-induced cell gigantism and asymmetric cell division in archaea. *Proc Natl Acad Sci U S A* **118**, (2021).