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Architecture of the *Vibrio cholerae* toxin-coregulated pilus machine revealed by electron cryotomography

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Supplementary Tables 1 to 3

Supplementary References

Other supplementary material for this manuscript includes the following:

Supplementary Video 1
Supplementary Discussion:

Evidences supporting that the structures analyzed in this study were TCPMs

In addition to the TCP, *V. cholerae* O395 N1 also encodes two T4aP systems, the mannose-sensitive hemagglutinin (MSHA) pilus and the chitin-regulated competence pilus (ChiRP), as well as a T2SS. To test whether the structures observed were TCPMs or one of these other secretin-containing systems, we imaged a strain missing TcpQ, a component which is known to be crucial for stabilizing TcpC channels\(^1\). No similar structures were seen in 21 cryotomograms (Supplementary Table 2), indicating that the other secretin-containing systems are either not expressed under our laboratory conditions, or look substantially different from the putative TCPM structures. Additionally, the pilus fibres visualized in our cryotomograms have a diameter of 8 nm, which matches a three dimensional EM reconstruction of purified *V. cholerae* TCP\(^2\). Based on the numbers of pilin residues present, MSHA and ChiRP pili are expected to be thinner (≈6 nm) similar to the T4aP of *Neisseria* spp.\(^3,4\), and the T2SS is not expected to assemble a extracellular pilus\(^5\). Furthermore, pili bundling has never been observed for MSHA and ChiRP pili, overexpression of ToxT downregulates expression of MSHA pili\(^6,8\), and chitin is required for expression of ChiRP under laboratory conditions\(^9,10\). Finally, as will be described later in the main text, in the course of this project we imaged mutants lacking the TCPM components TcpB, TcpD and TcpS and calculated sub-tomogram average structures. In each case we observed particles that resembled the putative TCPMs but lacked specific densities (Fig. 3), and the pore size and vestibule diameter of the mutants and wild-type were consistently larger than the cryo-EM single particle reconstruction of a purified T2SS secretin channel from the same species\(^11\). For these reasons, we conclude that the particles analyzed here were in fact TCPMs rather than any other known or unknown similar structure.
**Supplementary Figure 1** | Examples of slices through sub-tomograms containing (a) piliated and (b) non-piliated wild-type TCPM basal body structures. Red asterisks: OM- and IM-associated densities. Images are representative of 111 piliated and 115 non-piliated particles. Scale bars, 10 nm.
Supplementary Figure 2 | Generation of the composite TCPM sub-tomogram averages.

**a,b**, Sub-tomogram averages of wild-type piliated TCPM independently aligned on the OM and IM complexes, respectively. The distribution of the blue dots in **b** indicates the translations imposed on the OM complexes to align the IM complexes. We did not observe any pattern in the flexibility between the two complexes. **c**, Composite sub-tomogram average using the upper and lower halves of **a** and **b**, respectively, with the red dashed line indicating the interface. **d**, Local
resolution of e calculated by Resmap$^{12}$. e-h, As in a-d but of the non-piliated TCPM. Scale bars, 10 nm.
Supplementary Figure 3 | Comparison of T4PM in situ structures.

a, From left to right: sub-tomogram averages of the V. cholerae wild-type TPCM non-piliated basal body (this work), the M. xanthus ΔpilB T4aPM non-piliated basal body (EMD-3260)\textsuperscript{13}, and the T. thermophilus T4aPM non-piliated basal body (EMD-3022)\textsuperscript{14}. The sub-tomogram averages are aligned by the OM. The conserved gate and periplasmic ring structures are indicated by...
colored arrowheads. The known locations of *M. xanthus* and *T. thermophilus* secretin N domains are labeled with I to VI. Scale bars, 10 nm. 

**b,** Domain architectures of the secretin protein in *V. cholerae* TCPM (TcpC), *M. xanthus* T4aPM (PilQ), and *T. thermophilus* T4aPM (PilQ) determined based on their protein sequences using CDvist\textsuperscript{15} with HMMER and HHsearch against Pfam 29.0. The protein regions corresponding to different density features in **a** are indicated by the arrowheads with corresponding colors. The AMIN domains in *M. xanthus* PilQ are known to have irregular binding on the peptidoglycan layer and not contribute to densities in the sub-tomogram average\textsuperscript{13}. Asterisk indicates the secretin N domain described in the previous report\textsuperscript{14} but could not be identified by CDvist.
Supplementary Figure 4 | The conserved domain between *M. xanthus* PilP and *V. cholerae* TcpS suggests that TcpS likely interacts with TcpC in a similar fashion as PilP interacts with PilQ.

PilP and TcpS were used as query sequences against sequences in the MiST database\(^1\) using blastp search with an E-value cutoff of 1E-5 to retrieve their homologs. All homologs were aligned using L-INS-I from MAFFT package. The sequences of PilP and TcpS were then extracted from the alignment result and submitted to CDvist. Both sequences show significant homology to the CDD domain model TIGR03021 (pilP_fam). The identified sequence region also coincides with the binding site of T2SS GspC (homolog to PilP) to GspD (homolog to PilQ) in a complex crystal structure\(^1\), suggesting that PilP and TcpS likely bind the secretin channel in their systems in a similar fashion. The gaps of the alignment (dotted lines) and aligned regions (solid lines) are shown for each sequence.
Supplementary Figure 5 | The upper- and mid-periplasmic rings of the TCPM are composed of more than the secretin channel protein TcpC.

a, Outer membrane portion of the *V. cholerae* TCPM sub-tomogram average. b, 2D projection average of purified EPEC BfpB\(^1\). c, Superposition of TCPM density in a with the outline of BfpB density in b on the gate shows extra densities in the TCPM (arrows). d, Domain architecture of the secretin proteins *V. cholerae* TcpC and EPEC BfpB as predicted by HMMER and HHsearch using the Pfam 29.0 database via CDvist. The domain models of Secretin_N and Secretin_N_2 are denoted as N and N2 respectively. Scale bars, 5 nm.
Supplementary Figure 6 | Identifying domain architectures and available atomic homology models of TCPM components and placing the models into the TCPM *in vivo* molecular envelopes.

**a**, Each panel shows a TCPM component with labels indicating regions conserved either in proteins with determined structures deposited in the Protein Data Bank database, or in domains in the Pfam or CDD database found in other T4P systems. The gene name and locus number of each TCPM component are shown in the upper-left corner of each panel. The component function is shown in the upper-right corner of each panel. Each domain box contains the PDB code of the most significant hit to the Protein Data Bank database with the exception of the TcpC and TcpS panels in which the domain boxes contain the names of the most significant hit to the Pfam 29.0 and CDD databases, respectively. The Secretin_N Pfam domain model is denoted as N. The sequence identity is displayed above each domain when there is a PDB structure available for homology modeling. The probability of true positive is displayed above the identified domains from Pfam and CDD databases. The sequence coverage of the identified homologous structures and domains of the TCP proteins is denoted by residue numbers above the edges of each domain box. Atomic models are shown under the domain boxes. Predicted transmembrane regions are represented by grey shaded boxes. **b**, Available atomic models of TcpA, TcpE, TcpT, TcpQ and TcpB are placed in the envelopes of non-piliated (left) and piliated (right) TCPMs.
Supplementary Figure 7 | Examples of slices through TCPM structures in ΔtcpR cells.

a,b, piliation-stalled and non-piliated ΔtcpR TCPM basal body structures, respectively. Images are representative of 115 particles. White arrows in a indicate stem densities extending from the IM to the OM vestibule. Scale bars, 10 nm.
**Supplementary Table 1 | ECT data collection parameters.**

| Parameter                                      | Value                        |
|------------------------------------------------|------------------------------|
| Microscope                                     | FEI Polara (FEG, Gatan energy filter) |
| Voltage (kV)                                   | 300                          |
| Camera                                         | Gatan K2 summit              |
| Magnification (x)                              | 27,500                       |
| Defocus (µm)                                   | -6                           |
| Pixel size (after binning for analysis) (Å)    | 7.8                          |
| Tilt series angle coverage (°)                 | −60 to +60                   |
| Tilt series increment angle (°)                | 1                            |
| Electron dose per tomogram (e⁻/Å³)             | 160                          |
Supplementary Table 2 | Number of cryotomograms collected on different strains and TCPM structures used for generating the sub-tomogram averages.

|                   | Wild-type | ΔtcpB | ΔtcpD | ΔtcpQ | ΔtcpR | ΔtcpS |
|-------------------|-----------|-------|-------|-------|-------|-------|
| Tomograms         | 74        | 73    | 42    | 21    | 35    | 41    |
| Piliated TCPM     | 111       | np    | np    | np    | np    | np    |
| Non-piliated TCPM | 115       | 240   | 325   | np    | 115   | 222   |

np: none present
## Supplementary Table 3 | Component relationships between the TCPM and other machines.

| TCPM (known function) | T4aPM analog | Other analogs | Evidence of analogy |
|-----------------------|--------------|---------------|---------------------|
| TcpA (major pilin)    | PilA         |               | Functional          |
| TcpB (minor pilin)    |              | CofB          | HHsearch - Protein Data Bank structures |
| TcpC (secretin pore)  | PilQ         |               | HHsearch - Pfam 29.0 domain models |
| TcpQ (secretin pore stabilization) |              | VirB7         | HHsearch - Protein Data Bank structures |
| TcpT (assembly ATPase) | PilB         |               | BLAST - 2nd best hit (E-value: 5E-35) |
| TcpR (TcpT IM-tethering) |              |               |                     |
| TcpD (unknown)        |              |               |                     |
| TcpS (unknown)        | PilP         |               | HHsearch - CDD domain models |
| TcpE (IM platform)    | PilC         |               | BLAST - 2nd best hit (E-value: 2E-7) |
| TcpF (colonization factor) |              |               |                     |
| TcpN/ToxT (expression regulator) |              |               |                     |
| TcpJ (prepolin peptidase) |              | GspO          | BLAST - Best hit (E-value: 5E-15) |

TcpA (major pilin) - PilA
TcpB (minor pilin) - CofB (ETEC T4bPM minor pilin)
TcpC (secretin pore) - PilQ
TcpQ (secretin pore stabilization) - VirB7 (X. citri T4SS OM-complex component)
TcpT (assembly ATPase) - PilB
TcpR (TcpT IM-tethering)
TcpD (unknown)
TcpS (unknown) - PilP
TcpE (IM platform) - PilC
TcpF (colonization factor)
TcpN/ToxT (expression regulator)
TcpJ (prepolin peptidase) - GspO (M. xanthus T2SS prepolin peptidase)
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