SUPPLEMENTARY INFORMATION

Supplementary Discussion

Description of intra-heterotrimer interactions

One prominent feature of the TraN-$TraO_{CT}$ interface in the heterotrimer is the interaction between the proline-rich region at the N-terminus of TraN and the β1-5 sheet of $TraO_{CT}$ (described in Supplementary Fig. 3c). These interactions direct the N-terminus of TraN towards the hydrophobic double-helical trans-membrane pore of $TraF_{CT}$, positioning the lipid on TraN next to the pore.

The interface between $TraF_{CT}$ and $TraO_{CT}$ in the heterotrimer is formed by residues of $TraF_{CT}$ located in the β2b and β3a strands and in the extensive loop between these two strands (Supplementary Fig. 3f). These residues interact with $TraO_{CT}$ residues located in the C-terminus of the α1 helix, and in the C-terminal region of the extended loop between the β9 and β10 strands (Supplementary Fig. 3f).

Description of the inter-heterotrimeric interactions

The interface between immediately adjacent $TraF_{CT}$ subunits will be described starting from the regions facing the outside of the ring structure (i.e. the β_n1-β1a region; Supplementary Figs. 6c and 6d) progressing towards the α2 and α3 antenna helices. The β_n1 strand in green in Supplementary Fig. 6c forms an edge strand added to the small β-sheet formed by the β7c (with which β_n1 interacts) and the β1a strands at the base of the $TraF_{CT}$ subunit in red in Supplementary Fig. 6c. The linker sequence between β_n1 and β1a of the subunit in green makes interactions (M196) with residues in both the green (P204) and red (I279) subunits at the base of the interface. Further in, the bulk of the interface is made of residues in the extended β-sheet formed by the β2a, β3b, β4, and β5 strands in the subunit in red and residues in the β1a-β1b and β7b-β7c loops of the subunit

in green. At the top of the barrel, interactions are between residues in the β1c-β2a loop of the subunit in red and residues in the β3b-β4 loop of the subunit in green. Interactions between the two antenna helices are described in the main text.

As mentioned in the main text, the extended N-terminal lever arm of $TraF_{CT}$ interacts extensively with 3 subunits. For example, and as depicted in Supplementary Fig. 6a, the arm of subunit F13 interacting with the subunit F14, F1, and F2. More generally, the arm of subunit FX ($1 \leq X \leq 14$) interacts with the subunit FX+1, FX+2, and FX+3. The first set of contacts with an immediately adjacent subunit (FX+1) is mediated by residues in the β_n1 strand of the arm (see Supplementary Fig. 6c for the F1 subunit's arm in green). The second set of contacts is between residues in the β_n1-α_n1 linker (the sequence between the β_n1 strand and the α_n1 helix) and residues in the FX+2 subunit (see Supplementary Fig. 6d, where FX-FX+2 contacts are shown between the arm of the F13 subunit and the F1 subunit). Finally, the third set of contacts is between the α_n1 helix and residues in the FX+3 subunit (see Supplementary Fig. 6d, where FX-FX+3 contacts are shown between the arm of subunit F12 and the F1 subunit).

The interface between adjacent $TraO_{CT}$ subunits consists of mostly loop residues on both sides (Supplementary Fig. 7a). The loop between the β9 and β10 strands contributes many residues to the interface (see F269, P271, R273, A275, and A277 of subunit O2 in forest-green in Supplementary Fig. 7b): these make contact with residues in the β3-β4 (A213, N214, A215) and in the β6-β7 (E239, N240) loops (in cyan in Supplementary Fig. 7b) of the adjacent $TraO_{CT}$ subunit. Additional contacts between $TraO_{CT}$ subunits include loop interactions between the β1-β2 and β8-β9 loop residues on

one side of the interface and β4-β5 loop residues on the other side of the interface (Supplementary Fig. 7b).

The interface between $TraO_{CT}$ of one heterotrimer and $TraF_{CT}$ of an adjacent heterotrimer in the tetradecameric assembly is extensive, involving 5 loops of $TraF_{CT,}$ three of which were defined in the $ComB10_{CT}$ structure as bulges (Supplementary Figs. 7a and 7c): the β2a-β2b and β4-β5 loops, and the β3a-β3b, β7a-β7b, β1b-β1c bulges. Residues in the β2a-β2b, β3a-β3b and β4-β5 loop/bulge region interact with residues in the β3-β4, β6-β7 and β8-β9 loops of $TraO_{CT}$. In the lower portion of this region (Supplementary Fig. 7c), a dense network of polar and charged residues characterises the interface (Q231, D232, D244, K245 in $TraF_{CT}$ and R241, E216, N214 in $TraO_{CT}$). The upper portion of the same region is more hydrophobic, involving residues in the $TraF_{CT}$ β7a-β7b and β1b-β1c bulges and in the $TraO_{CT}$ β4 and β5 strands as well as β5-β6 and β8-β9 loops.

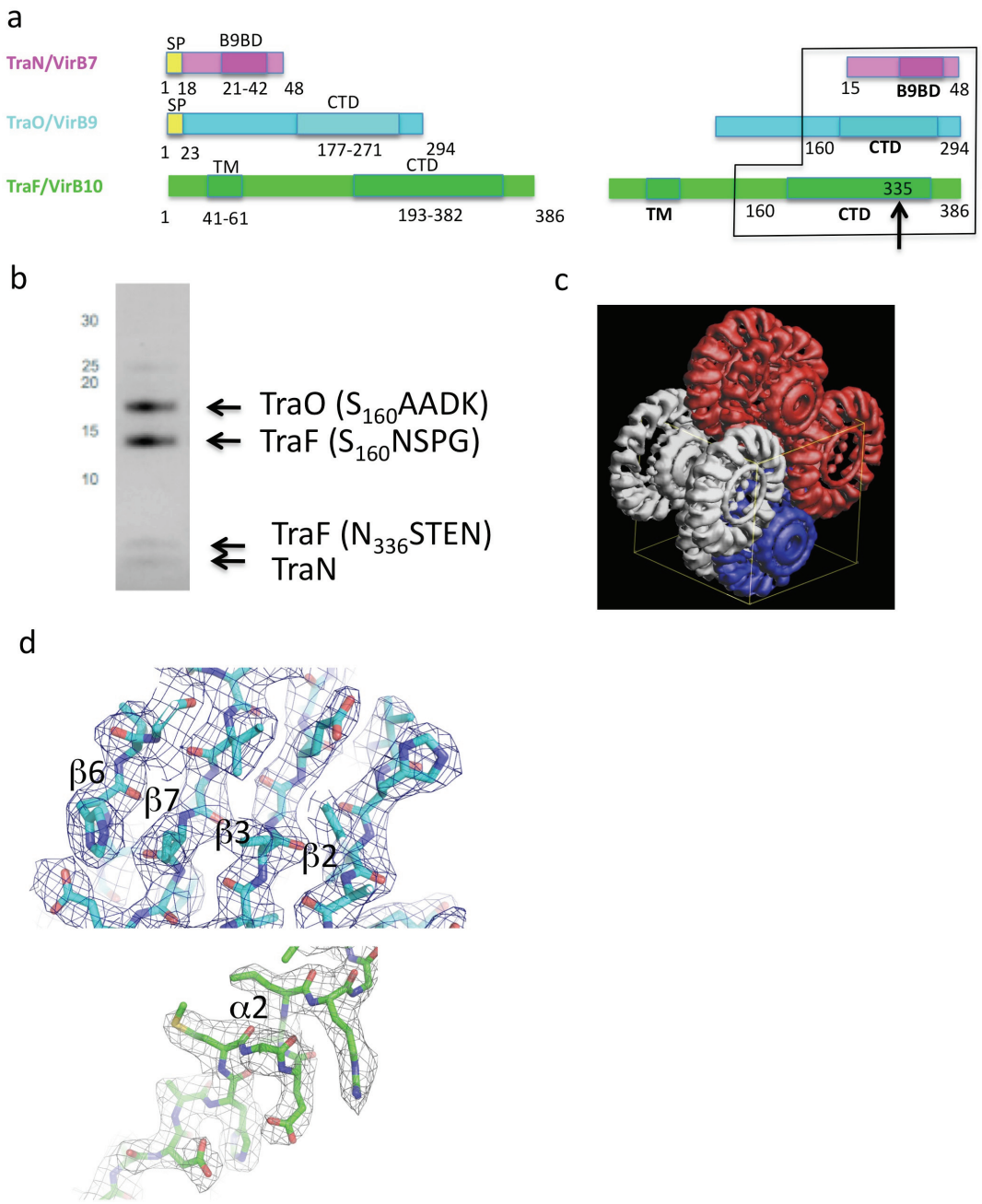
Supplementary Table 1: Data collection and refinement statistics

	ID14-4 (Native)	PROXIMA 1 (SAD)
Data collection		
Space group	$P2_12_12$	$P2_12_12$
Cell dimensions		
a, *b*, *c* (Å)	204.15, 212.58, 204.07	202.4, 211.63, 203.44
a, *b*, *g* (°)	90, 90, 90	90, 90, 90
Resolution (Å)	59.6 - 2.8 (3.0-2.8)	52.91 – 2.6 (2.8-2.6)
R_{merge}	12.3 (44.2)	4.4 (14.2)
I/sI	23.88(6.63)	12.91(3.72)
Completeness (%)	100 (100)	95.5 (94.7)
Redundancy	21.5 (17.8)	1.98(1.98)
Refinement[a]		
Resolution (Å)		52 – 2.6
No. reflections		258767
R_{work}/ R_{free}		0.227/0.259
No. atoms		39253
Protein		37854
Ligand/ion		40 (1LDAO, 3MPD)
Water		1359
B-factors		
Protein		37.3
Ligand/ion		46.3
Water		46.4
R.m.s deviations		
Bond lengths (Å)		0.019
Bond angles (°)		1.98

*Highest resolution shell is shown in parenthesis.

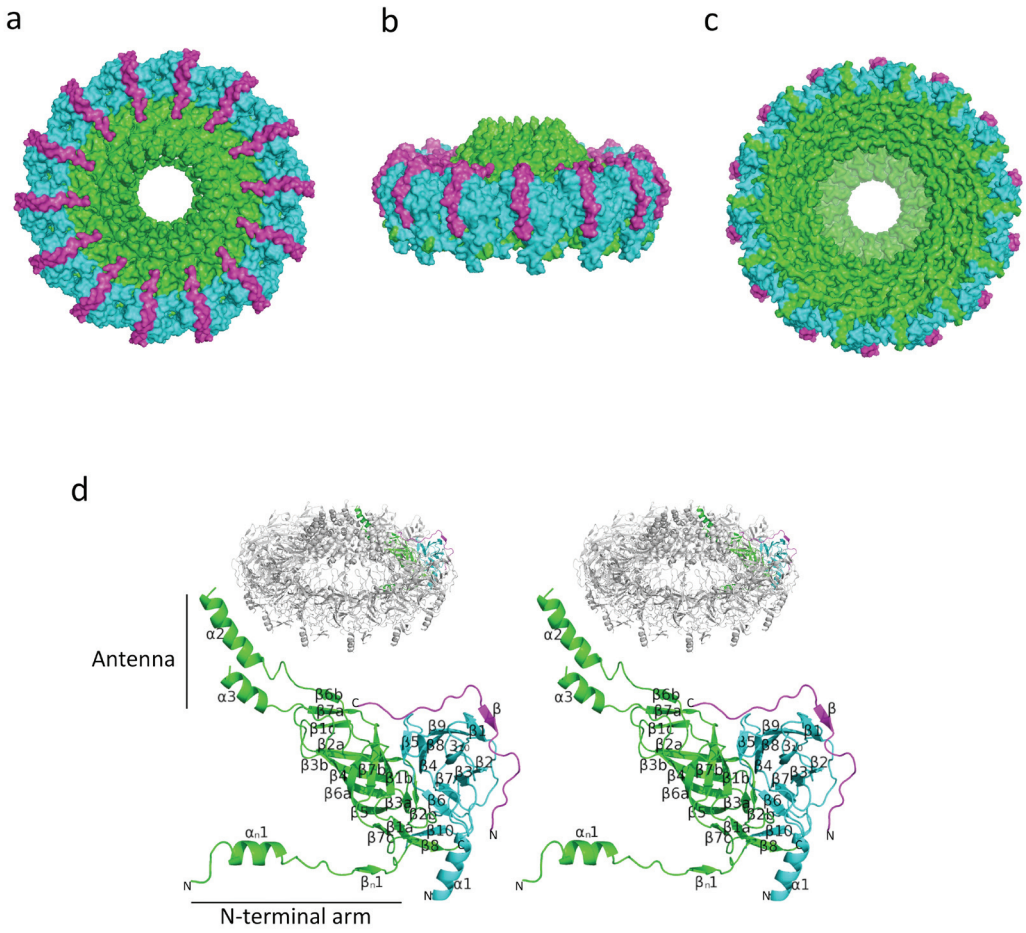
R_{merge} = Σ|I - <I>|/ΣI, where *I* = observed intensity and <*I*> = average intensity of multiple observations of symmetry-related reflections.

[a]The numbers indicated in the table are the results of the refinement using REFMAC[31]. Refinement using PHENIX[32,33] converges to R- and freeR- factors of 0.172 and 0.195 with lower Rmsd stereochemistry numbers (0.008Å for bonds and 1.22 for angles), but higher overall B-factors (39Å^2) and Rmsd B-factors between bonded atoms (2.16 for main chain atoms, and 4.0 for side chain atoms).

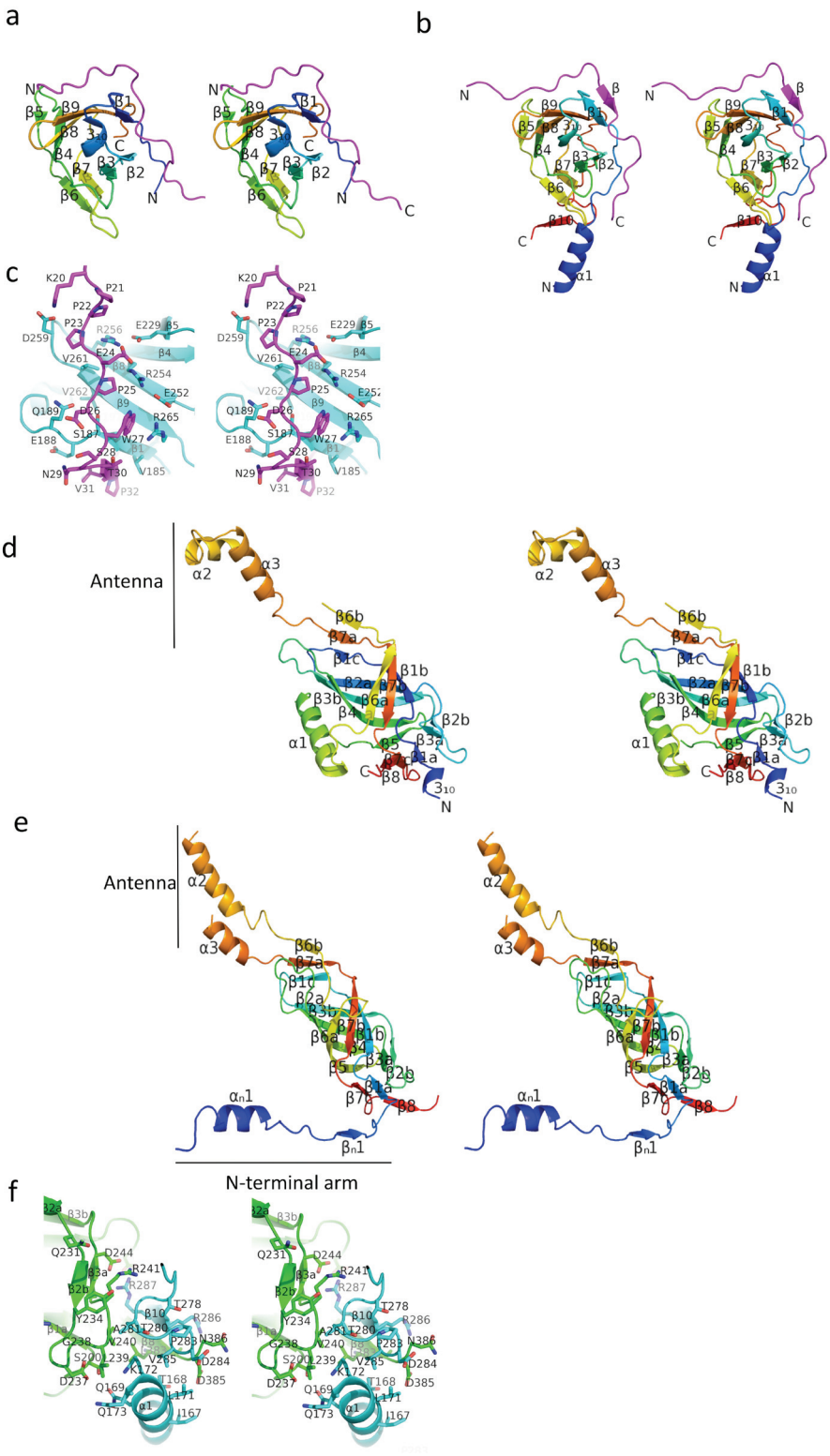


Supplementary Figure 1. Characterization of the T4SS outer membrane complex: Molecular biology, biochemistry, and structure. **a**. Schematic diagram of the constructs used in this study. Left panel: primary sequence and domain structure of TraN/VirB7,

TraO/VirB9, and TraF/VirB10. SP: signal peptide. TM: trans-inner membrane helix. B9BD: VirB9-binding domain. CTD: C-terminal domain. Right panel: schematic diagram illustrating the composition of the chymotryptic complex. The boxed area is what is left after chymotryptic cleavage of the T4SS core complex. The arrow indicates a single cut at residue 335 of TraF/VirB10. **b**. SDS-PAGE gel of the outer membrane complex. The various bands are identified and their N-terminal sequence (established using Edman degradation) is reported. **c**. Molecular Replacement-derived locations of the cryo-EM map of the trypsin-digested core complex in the unit cells of the crystals of the T4SS outer membrane complex. **d**. Representative regions of the experimental electron density map contoured at 1.5σ after phase extension to 2.8Å resolution and 14-fold NCS averaging. Upper panel: the β2-6 sheet of $TraO_{CT}$. Lower panel: the α2 helix of $TraF_{CT}$.

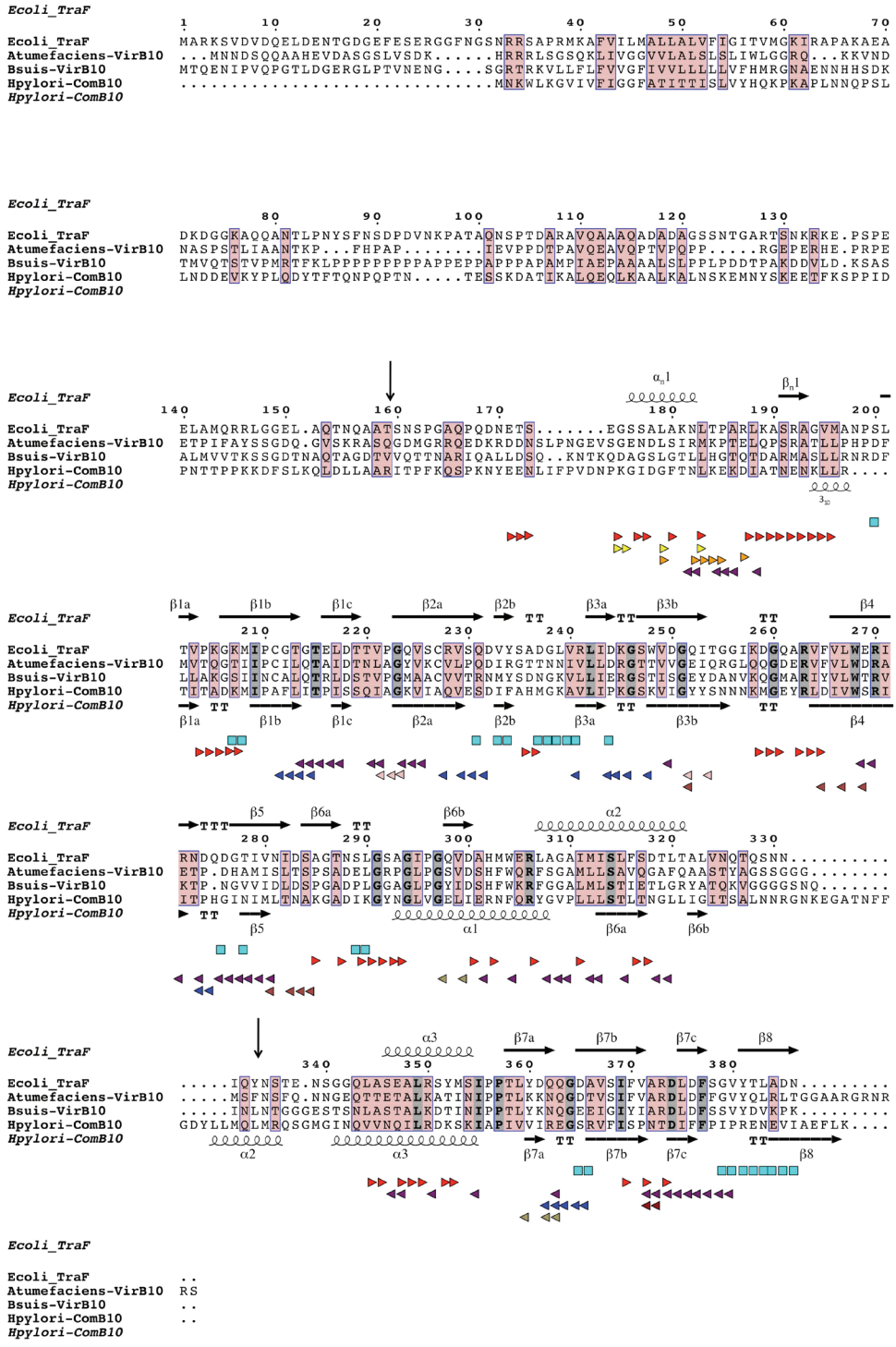


Supplementary Figure 2. Space-filling model of the T4SS outer membrane complex. **a**. Top-view from the extracellular milieu. **b**. Side view. **c**. Bottom view from the periplasm. **d**. Stereo version of Fig. 2.



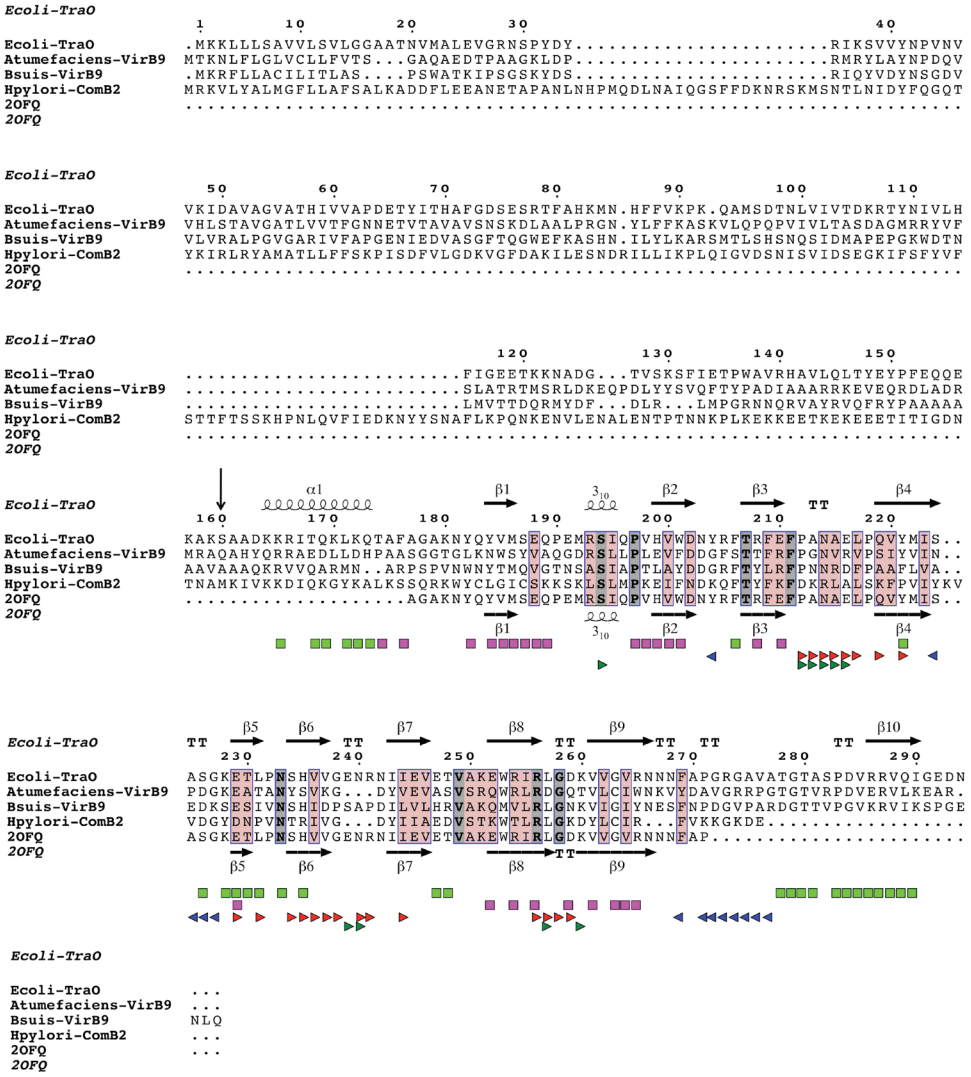
Supplementary Figure 3. Structures of and interactions between heterotrimeric components. **a**. NMR structure of the $TraO_{CT}$-TraN complex[11] in ribbon diagram

representation. **b**. Structure of $TraO_{CT}$-TraN complex in the outer membrane complex in ribbon diagram representation. **c**. Interactions of the proline-rich N-terminus of TraN (magenta) with $TraO_{CT}$. Residues are in stick representation colour-coded in magenta (TraN) and cyan ($TraO_{CT}$) for carbons, red for oxygens, blue for nitrogens and yellow for sulfurs. Residues shown are within 4.5Å from each other across the interface. **d**. Crystal structure of ComB10[14] in ribbon diagram representation. **e**. Structure of $TraF_{CT}$ in the heterotrimer (ribbon diagram representation). **f**. Example of interactions between $TraF_{CT}$ and $TraO_{CT}$. Residues within 4.5Å across the interface are shown. Stick representations of residues are the same as in panel c, but for carbons of $TraF_{CT}$ that are shown in green.



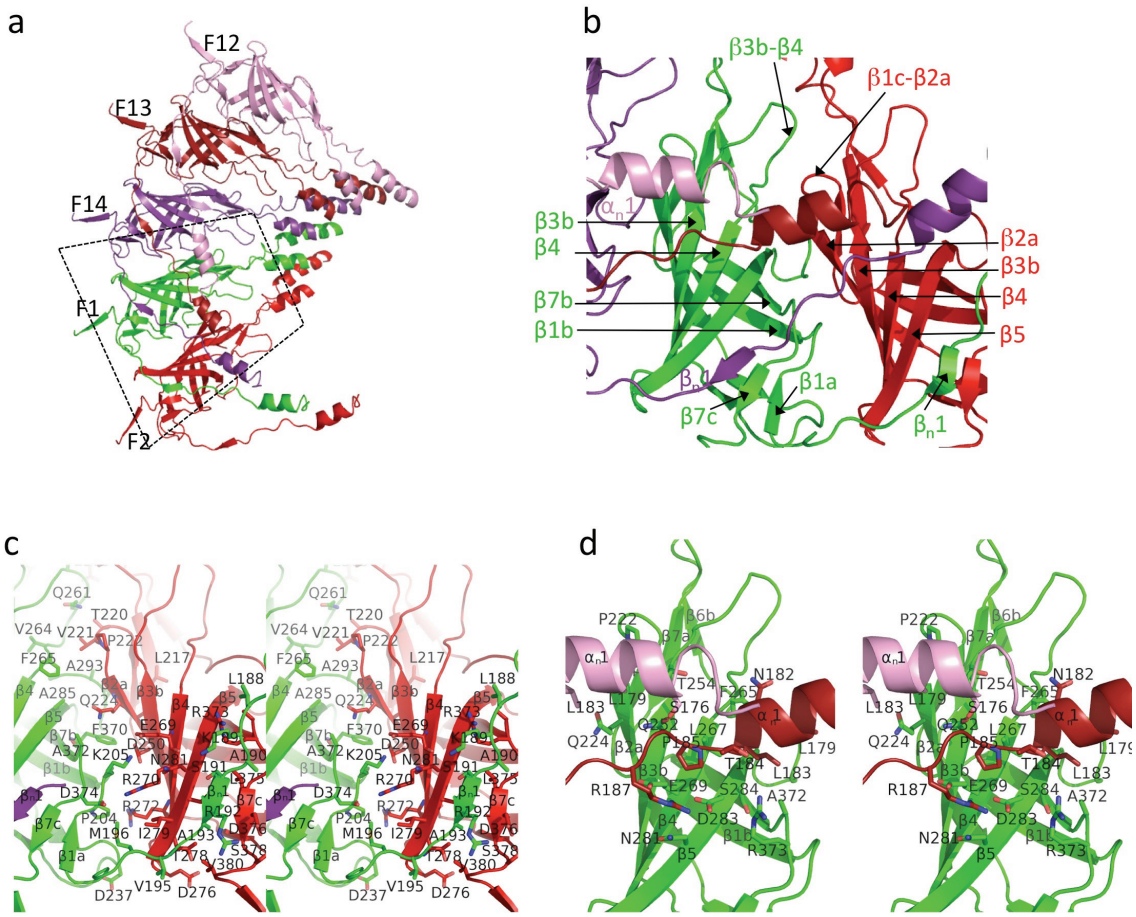
Supplementary Figure 4. Sequence alignment of VirB10 homologs. The sequences of TraF, *A. tumefaciens* VirB10, *B. suis* VirB10, and *H. pylori* ComB10 were aligned using

Clustalw[34]. The first vertical arrow above the sequence indicates the start of the TraF structure in the outer membrane complex. The second vertical arrow above the sequence indicates the location of the chymotrypsin cut between the two trans-membrane helices. Numbering above the sequence is that of TraF. Horizontal helices and arrows above the numbering indicate the positions of the secondary structures (helices and strands, respectively) as observed in the outer membrane complex. Labelling for those secondary structures is reported above the depiction of the secondary structures. Helices and arrows below the sequence alignment indicate the positions of helices and strands in the crystal structure of ComB10 (entry code 2BHV). Labelling for those secondary structures is reported under the depiction of the secondary structures. Cyan-filled square boxes indicate that the residues above them make intra-heterotrimeric interactions with $TraO_{CT}$ (i.e. interactions between subunits labelled F1 and O1 in Fig. 3a). Interactions between F1 residues and F2 residues are indicated by red forward triangles, while interactions between F1 and F14, F13, or F12 are indicated by backward triangles colour-coded with the colour used for the F14, F13, and F12 subunits in the schematic representation of Fig. 3a. This figure was made using ESPript[35].

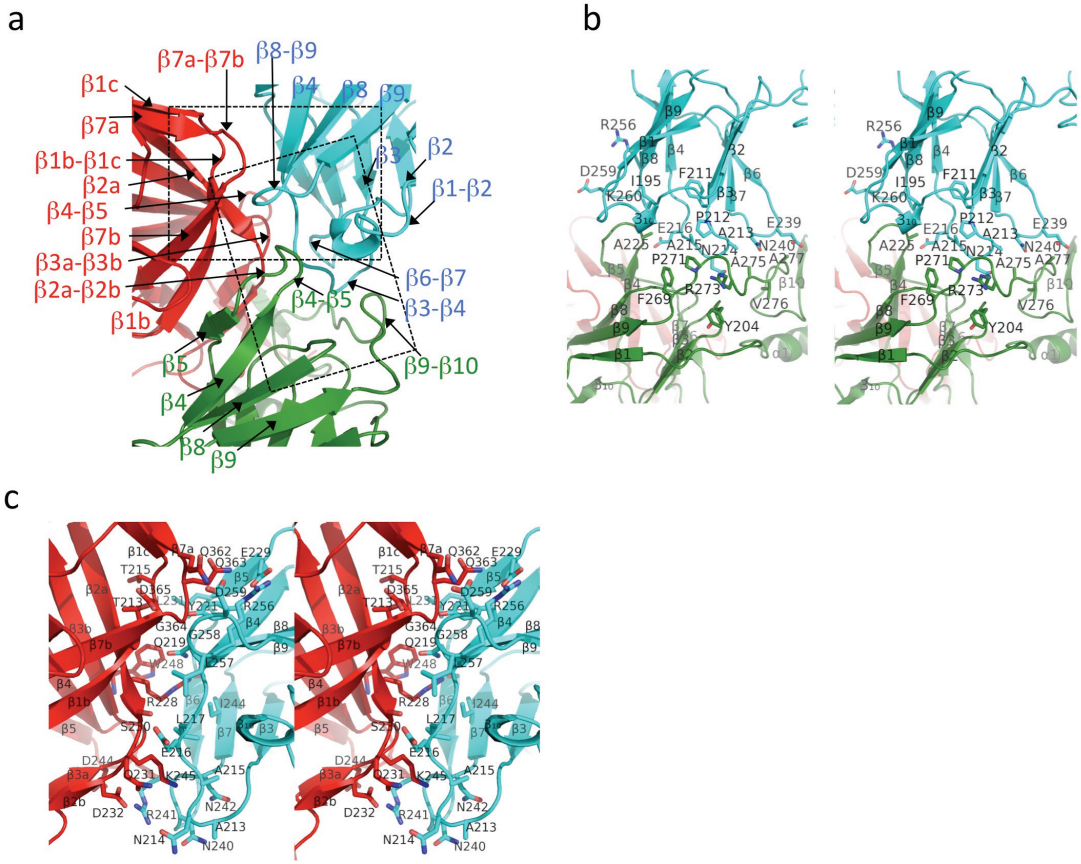


Supplementary Figure 5. Sequence alignment of VirB9 homologs. The sequences of TraO, *A. tumefaciens* VirB9, *B. suis* VirB9, and *H. pylori* ComB2 were aligned using Clustalw[34]. 2OFQ is the sequence of TraO included in the pdb coordinates file (entry code 2OFQ) for the NMR structure of the TraN/TraO complex. The vertical arrow above the sequence indicates the start of the $TraO_{CT}$ structure in the outer membrane complex. Numbering above the sequence is that of TraO. Horizontal helices and arrows above the numbering indicate the positions of the secondary structures (helices and strands, respectively) as observed in the outer membrane complex. Labelling for those secondary

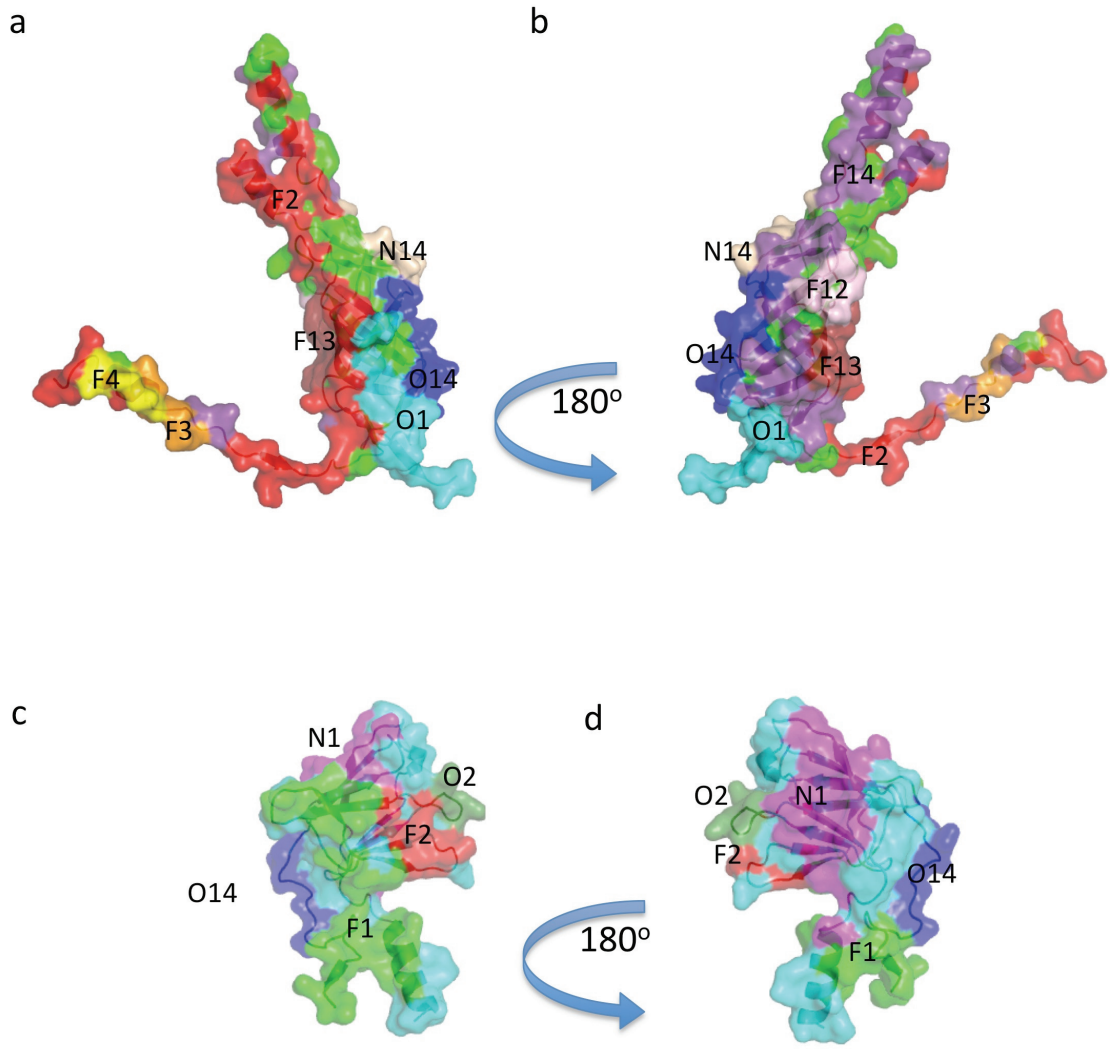
structures is reported above the depiction of the secondary structures. Helices and arrows below the sequence alignment indicate positions of helices and strands as observed in the NMR structure (entry code 2OFQ). Labelling for those secondary structures is reported under the depiction of the secondary structures. Green-and magenta-filled square boxes indicate that the residues above them make intra-heterotrimeric interactions with $TraF_{CT}$ and TraN, respectively (i.e. interactions between subunit O1 and F1 (green) and between subunit O1 and N1 (magenta) in Fig. 3e). Interactions of O1 residues with F2 residues or with O2 residues (see Fig. 3e for definition of O1, O2, and F2) are shown in red and forest-green forward triangles, respectively. Interactions of O1 residues with O14 residue (see Fig. 3e for definition of the O14 subunit) are shown in dark blue backward triangles. This figure was made using ESPript[35].



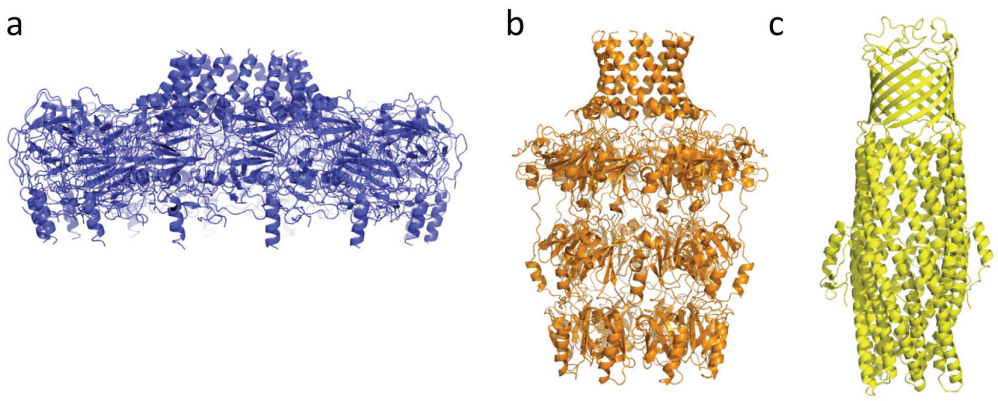
Supplementary Figure 6. Inter-heterotrimeric interactions between $TraF_{CT}$ subunits. **a**. Ribbon diagram of the F12 to F2 subunit regions of the T4SS outer membrane complex. The view is from the periplasm. The dashed line trapezoid indicates the region shown in panel b. **b**. Diagram of the region highlighted in panel a. Labels locate the secondary structures mentioned in the supplementary results. **c**. Stereo diagram of the interactions between immediately adjacent $TraF_{CT}$ subunits. Residues within 4.5Å across the interface are shown. For clarity, the N-terminal arms of F14 and F13 have been partially removed. Residues are in stick representation, colour-coded in green (F1 subunit) and red (F2 subunit) for carbons, red for oxygens, blue for nitrogen, and yellow for sulfurs. **d**. Inter-heterotrimeric interactions between residues in the $TraF_{CT}$ F1 subunit (in green in panel a) and residues of the N-terminal lever arm of $TraF_{CT}$ subunits F12 (pink) and F13 (brick red). See panel a for definition of the F1, F13, and F12 subunits. Residues within 4.5Å across the interface are shown.



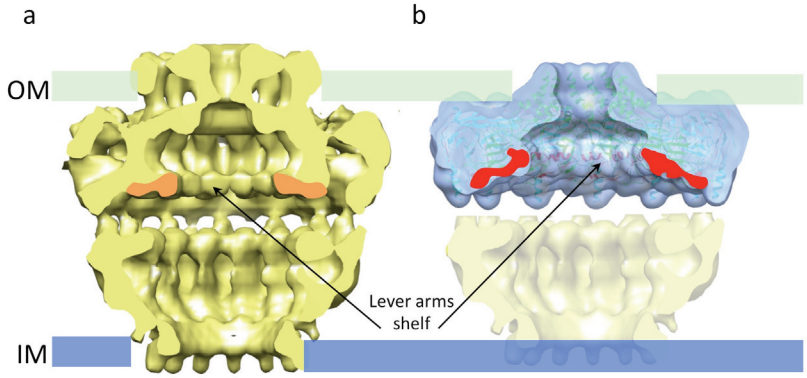
Supplementary Figure 7. Inter-heterotrimeric interactions involving the $TraO_{CT}$ subunit. **a**. Ribbon diagram and labelling of the secondary structures at the interface between O1 (cyan) and O2 (forest-green), and between O1 and F2 (red). See Fig. 3e for definition of the O1, O2, and F2 subunits. Labels containing two secondary structure names refer to the loop or bulge between the two secondary structures. The straight and slanted dash-lined rectangles indicate the regions detailed in panels b and c, respectively. **b**. Stereo diagram of interactions between adjacent $TraO_{CT}$ subunits. Residues within 4.5Å across the interface are shown. Residues are in stick representation, colour-coded in forest-green (O2) and cyan (O1) for carbons, red for oxygens, blue for nitrogens, and yellow for sulfurs. **c**. Stereo diagram of interactions between subunit O1 (in cyan) and subunit F2 (in red). Residues within 4.5Å across the interface are shown. Representation of residues is the same in panel d, except for F2 residue carbons that are in red.



Supplementary Figure 8. Interaction surfaces of $TraF_{CT}$ and $TraO_{CT}$. **a** and **b**. Two views related by 180° of the interactions surfaces of $TraF_{CT}$. The surfaces are color-coded according to the interaction diagram of Fig. 3a and labelled accordingly. **c** and **d**. Two views related by 180° of the interactions surfaces of $TraO_{CT}$. The surfaces are color-coded according to the interaction diagram of Fig. 3e and labelled accordingly.



Supplementary Figure 9. Comparison of the structures of Wza and TolC with that of the T4SS outer membrane complex. All structures are in ribbon diagram representation. **a**. T4SS outer membrane complex. **b**. Wza. **c**. TolC.



Supplementary Figure 10. Comparison of the Cryo-EM and crystal structure of the T4SS outer membrane complex. The structures superimposed in Fig. 4b of main text are here shown separately. **a**. Cut-away surface diagram of the T4SS core complex. The lever arms shelf is indicated in orange. **b**. Cut-away surface diagram of the T4SS outer membrane complex positioned where the O-layer is in the core complex. The lever arm shelf is indicated in red.

Supplementary notes

34 Thompson, J., Higgins, D., & Gibson, T., CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research* **22**, 4673-4680 (1994).

35 Gouet, P., Robert, X., & Courcelle, E. ESPript/ENDscript: extracting and rendering sequence and 3D information from atomic structures of proteins. *Nucleic Acids Research*, **31**, 3320-3323 (2003).