Supplementary Information

Cryo-EM analysis of the *Staphylococcus aureus* phenol-soluble modulin exporter PmtCD apo form in detergent micelles, nanodiscs and peptidiscs

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Table S1. Distances between Cα atoms of symmetry-related residues

Residue pair	PmtCD in nanodisc	PmtCD in LMNG	PmtCD in peptidisc	PmtCD + ADP in peptidisc
PmtD L30-L30 (extracellular side of TM1)	24.3 Å	27.2 Å	28.2 Å	39.2 Å
PmtD L19-L19 (intracellular side of TM1)	26.6 Å	29.7 Å	29.8 Å	34.9 Å
PmtD W60-W60 (extracellular side of TM2)	24.0 Å	29.2 Å	27.2 Å	25.8 Å
PmtD K72-K72 (intracellular side of TM2)	20.8 Å	24.8 Å	23.7 Å	20.7 Å
PmtD F180-F180 (extracellular side of TM5)	26.1 Å	27.3 Å	24.1 Å	5.9 Å
PmtD A165-A165 (intracellular side of TM5)	13.7 Å	15.2 Å	13.3 Å	4.2 Å

Table S2. Plasmids used in this study.

Plasmid Name	Source
pRB473 pmtCD	(*15*)
pRB473 pmtC(E145Q)D	(*15*)
pRB473 pmtCD(W60A)	(*15*)
pRB473 pmtCD(L19M)	This study
pRB473 pmtCD(L19W)	This study
pRB473 pmtCD(L30M)	This study
pRB473 pmtCD(L30W)	This study
pRB473 pmtCD(M166W)	This study
pRB473 pmtCD(I181M)	This study
pRB473 pmtCD(I181W)	This study
pTX+PSMα1-4	(*13*)
pTX17 pmtCD	(*15*)

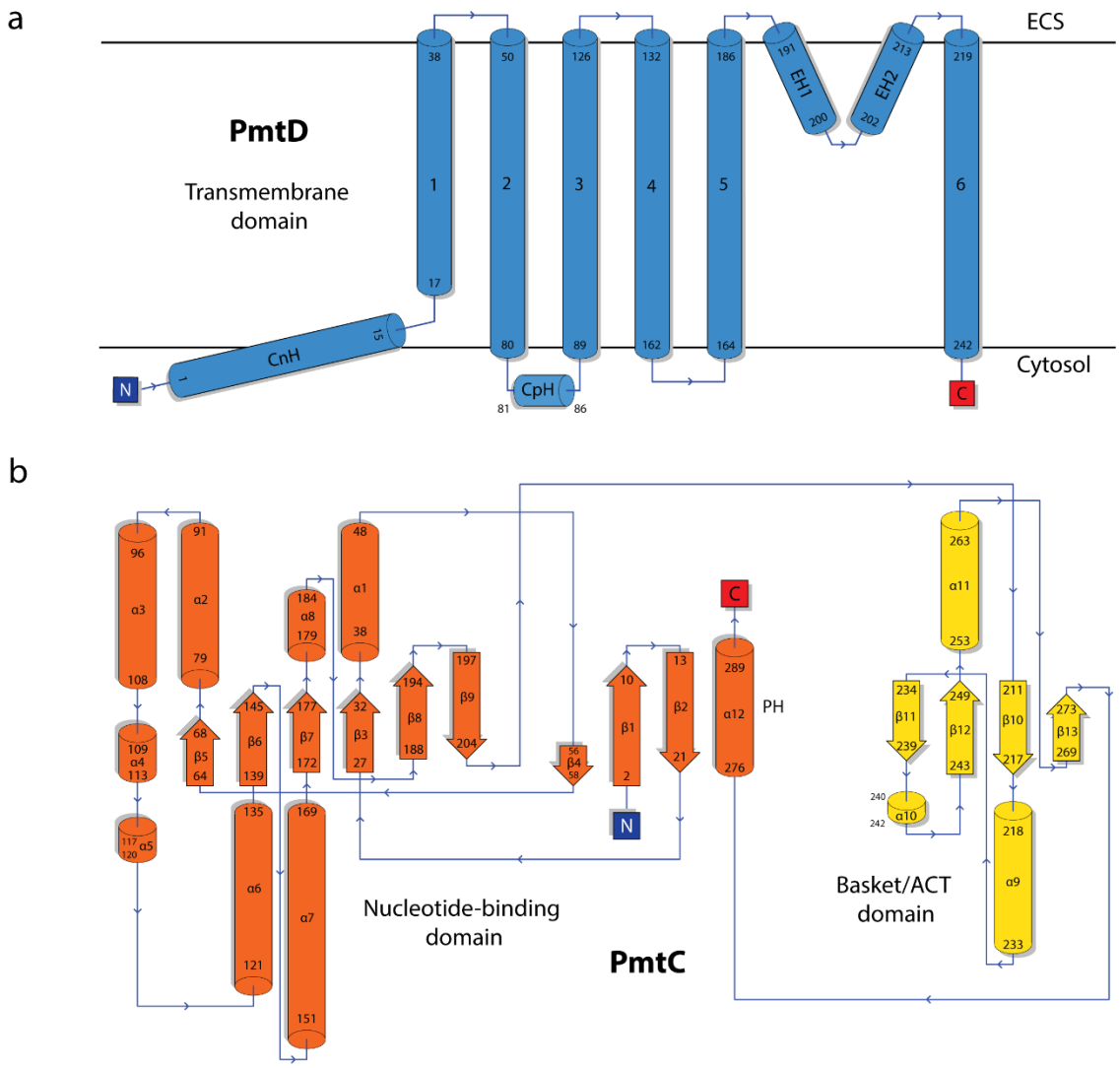

Figure S1. Secondary structure diagrams of PmtCD. (a) The exporter is comprised of two PmtD TMDs and two cytosolic protomers of PmtC. Each TMD (blue) features 6 transmembrane helices (TM1-TM6), an amphipathic connecting helix (CnH) and a coupling helix (CpH) for NBD binding, and two reentrant helices (EH1 and EH2) at the extracellular leaflet of the lipid bilayer. (b) The NBD (orange) of PmtC adopts a modified RecA-like ATPase fold as observed with most other ABC transporters. The ACT domain (yellow) between the NBD and the pinning helix (PH) of PmtC adopts the typical βαββαβ fold and dimerizes to form a basket-like structure.

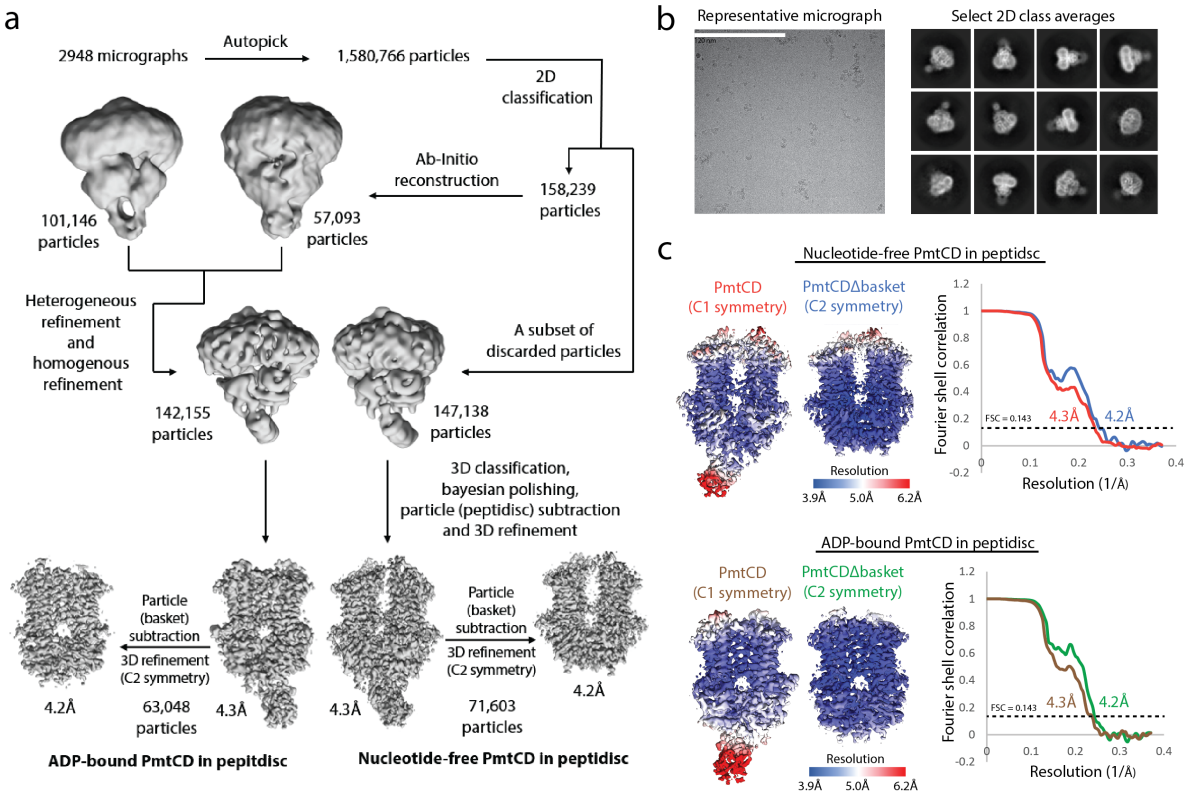

Figure S2. Cryo-EM data processing of nucleotide-free PmtCD and ADP-bound PmtCD in peptidiscs. (a) Cryo-EM data processing workflow. (b) Representative micrograph with 120 nm scale bar (white) and 2D class averages. (c) Estimated local resolution and FSC curves.

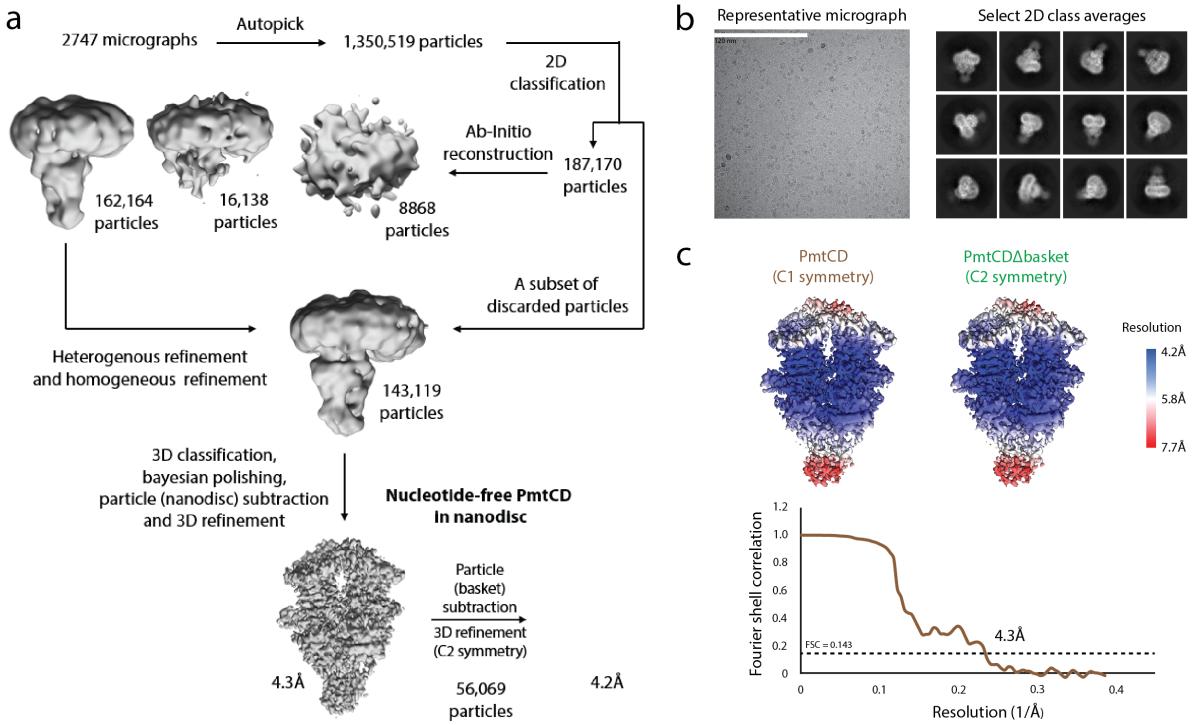

Figure S3. Cryo-EM data processing of PmtCD in nanodiscs. (a) Cryo-EM data processing workflow. (b) Representative micrograph with 120 nm scale bar (white) and 2D class averages. (c) Estimated local resolution and FSC curves.

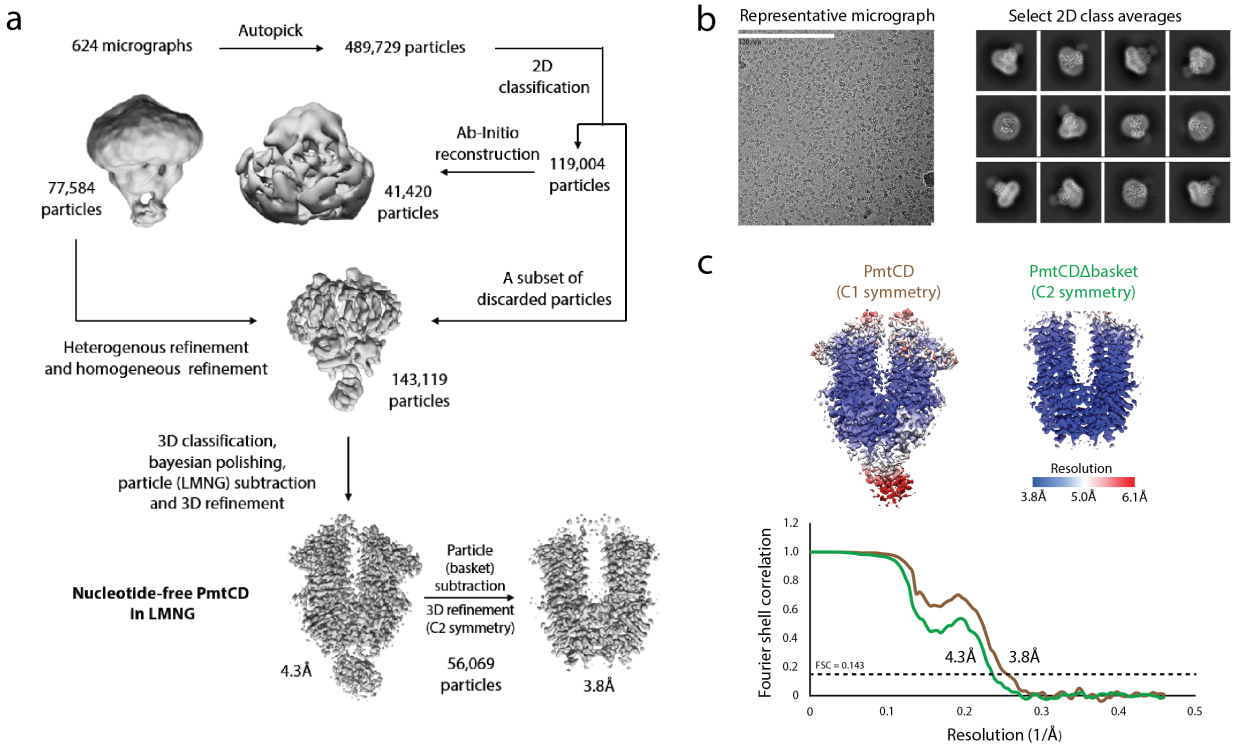

Figure S4. Cryo-EM data processing of PmtCD in LMNG. (a) Cryo-EM data processing workflow. (b) Representative micrograph with 120 nm scale bar (white) and 2D class averages. (c) Estimated local resolution and FSC curves.

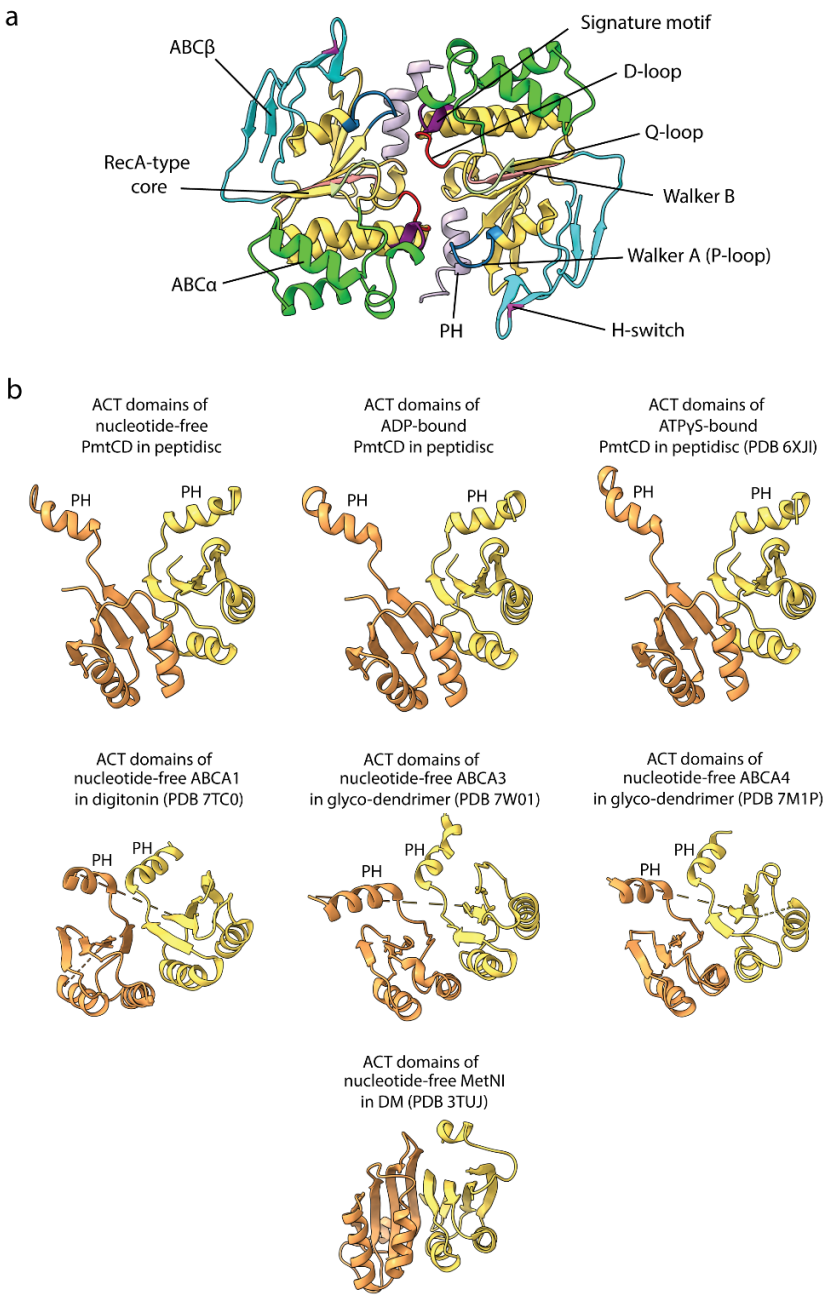

Figure S5. Structural features of the NBD and basket-like ACT domain. (a) NBD dimer of nucleotide-free PmtCD in peptidisc. Each NBD is comprised of a RecA-type core (yellow), an ABCβ subdomain (cyan), an ABCα subdomain (green), and a pinning helix (PH; light purple). Nucleotide-binding features of PmtCD include the signature motif (dark purple), D-loop (red), Q-loop (light green), Walker B motif (pink), Walker A motif (blue), and H-switch (purple). (b) The ACT domains and pinning helices of PmtCD in peptidisc (nucleotide-free, ADP-bound, and ATPγS-bound states), ABCA1 (PDB ID: 7TC0; nucleotide-free state), ABCA3 (PDB ID: 7W01; nucleotide-free state), ABCA4 (PDB ID: 7M1P; nucleotide-free state), and MetNI (PDB ID:3TUJ; nucleotide-free state).

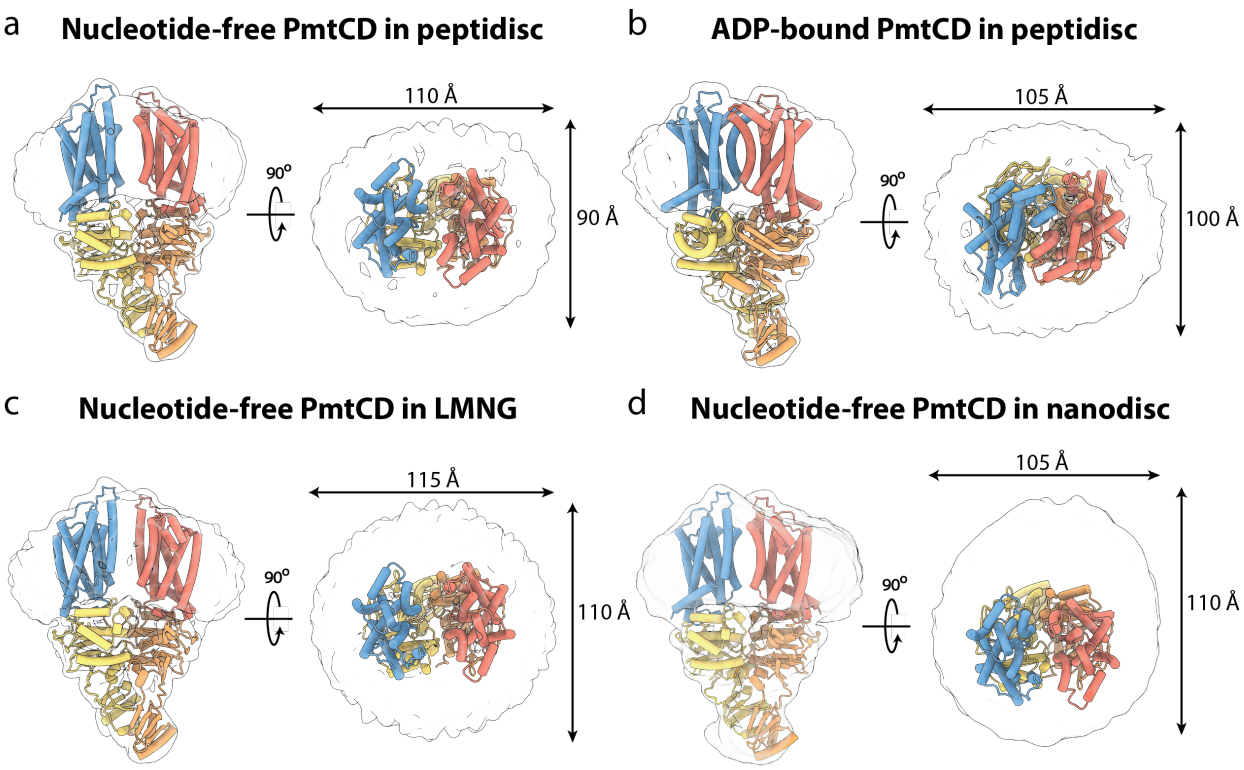

Figure S6. Cryo-EM reconstructions of PmtCD in different membrane mimetics. Unsharpened cryo-EM reconstructions of (a) nucleotide-free PmtCD in peptidisc, (b) ADP-bound PmtCD in peptidisc, (c) nucleotide-free PmtCD in LMNG, and (d) nucleotide-free PmtCD in nanodisc prior to particle subtraction. The reconstructions are shown along with cartoon representations of PmtC protomers in yellow and orange and PmtD in blue and red.

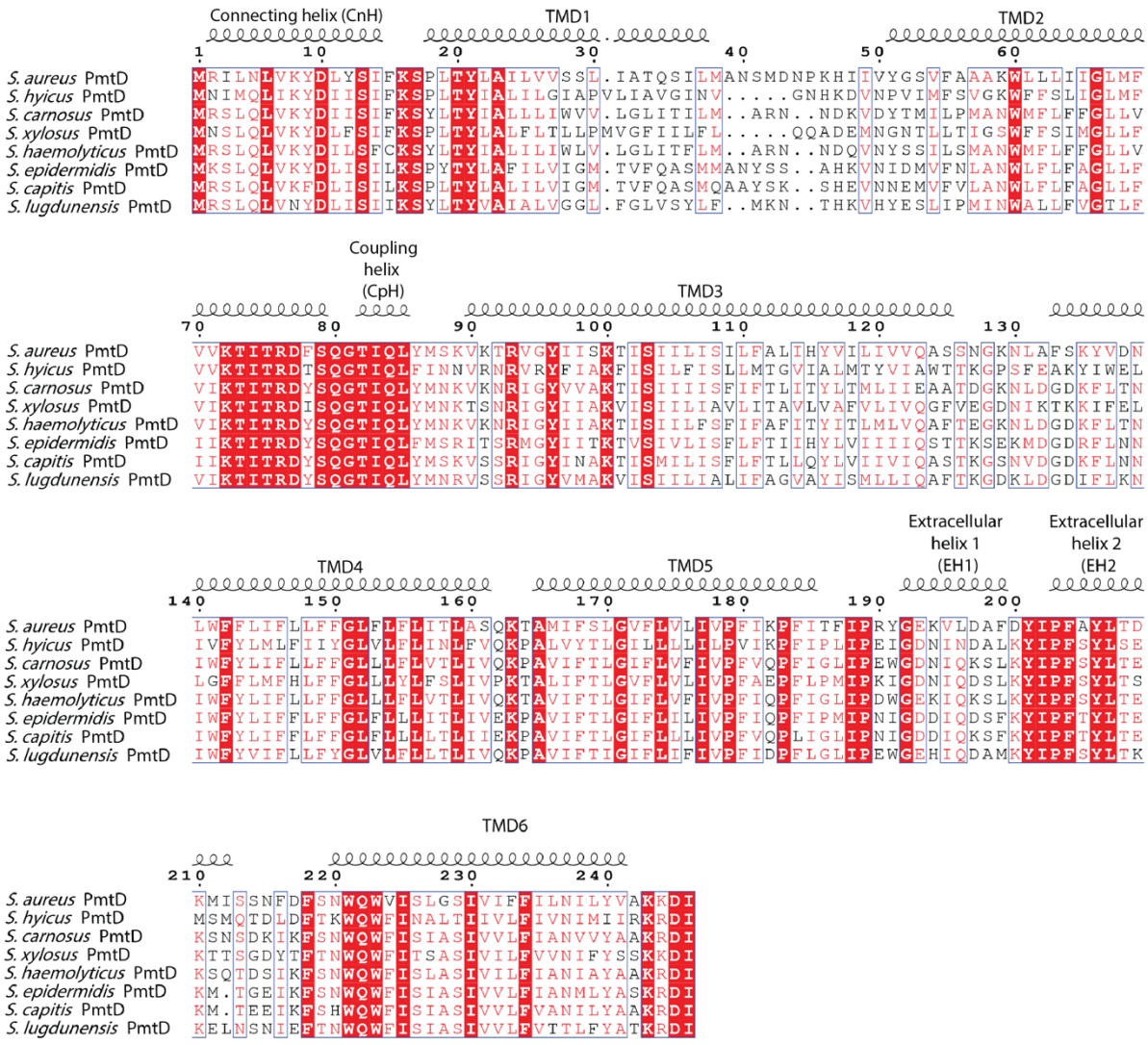

Figure S7. Sequence alignment of staphylococci PmtD. Sequences of Staphylococcus PmtD transmembrane domains were retrieved from either Uniprot or NCBI GenBank databases and aligned using clustal omega of *S. aureus* (Q2FWX0), *S. hyicus* (WP_119635425.1), *S. carnosus* (RTX89090.1) *S. xylosus* (WP_107543127.1), *S. haemolyticus* (WP_037538020.1), *S. epidermidis* (WP_001830379.1), *S. capitis* (WP_049427669.1), and *S. lugdunensis* (WP_084951693.1).

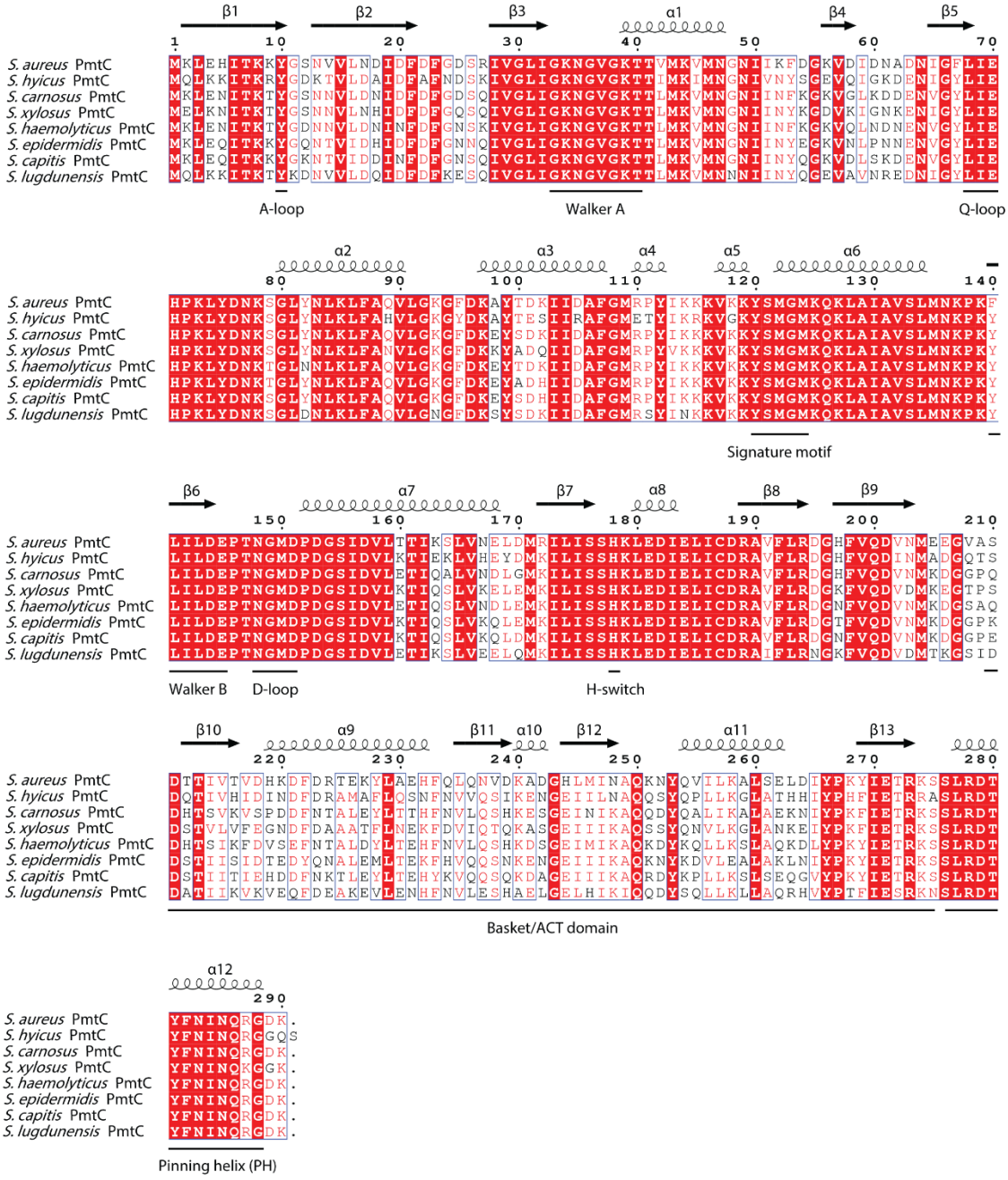

Figure S8. Sequence alignment of staphylococci PmtC. Sequences of PmtC nucleotide binding domains were retrieved from either Uniprot or NCBI GenBank databases and aligned using clustal omega of *S. aureus* (X5EJW5), *S. hyicus* (WP_039644728.1), S. carnosus (RTX89091.1) *S. xylosus* (WP_039068965.1), *S. haemolyticus* (WP_037538022.1), *S. epidermidis* (WP_075778960.1), *S. capitis* (WP_119506256.1), and *S. lugdunensis* (KAK56210.1).

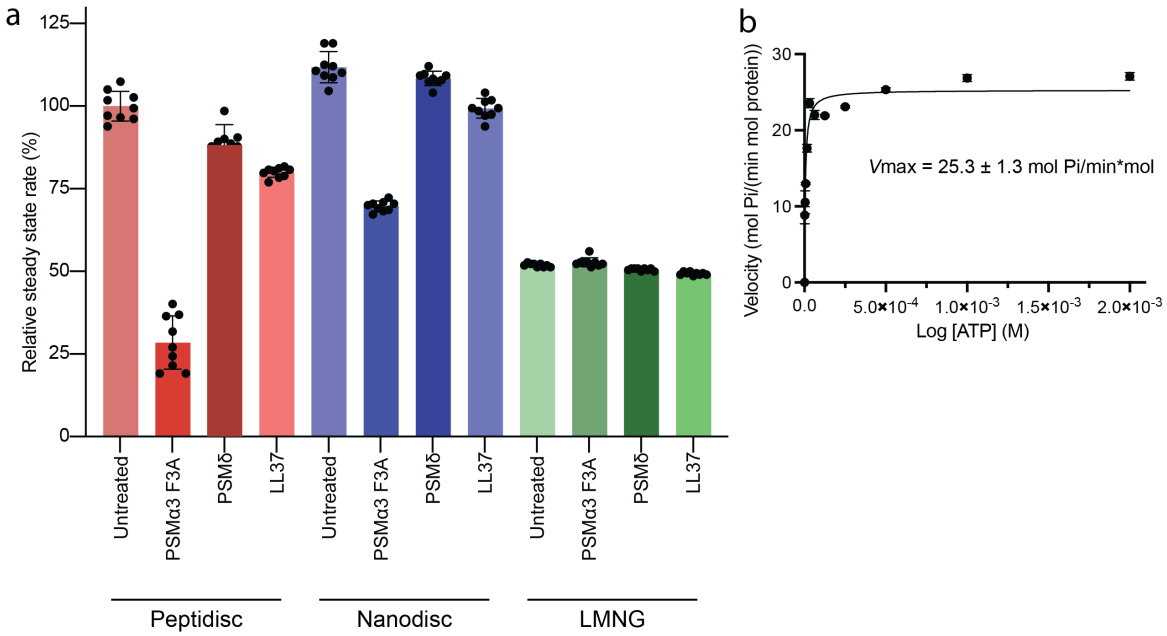

Figure S9. ATPase activity of PmtCD in different membrane mimetics. Malachite green ATPase assays of PmtCD in the presence or absence of 20 uM peptide substrates in all three mimetics (a). 595 nm absorbance of the converted to units of Pi using a K_2HPO_4 standard curve with different mimetics baseline-corrected to the untreated peptidisc reconstituted sample in GraphPad Prism 10.4.0 for relative ATPase activity comparison. Data points represent the mean of nine independent samples ± the standard deviation. (b) *V*max of the untreated peptidisc reconstituted sample determined using malachite green, data points represent the mean of three independent samples with the error reported within the 95% confidence interval.

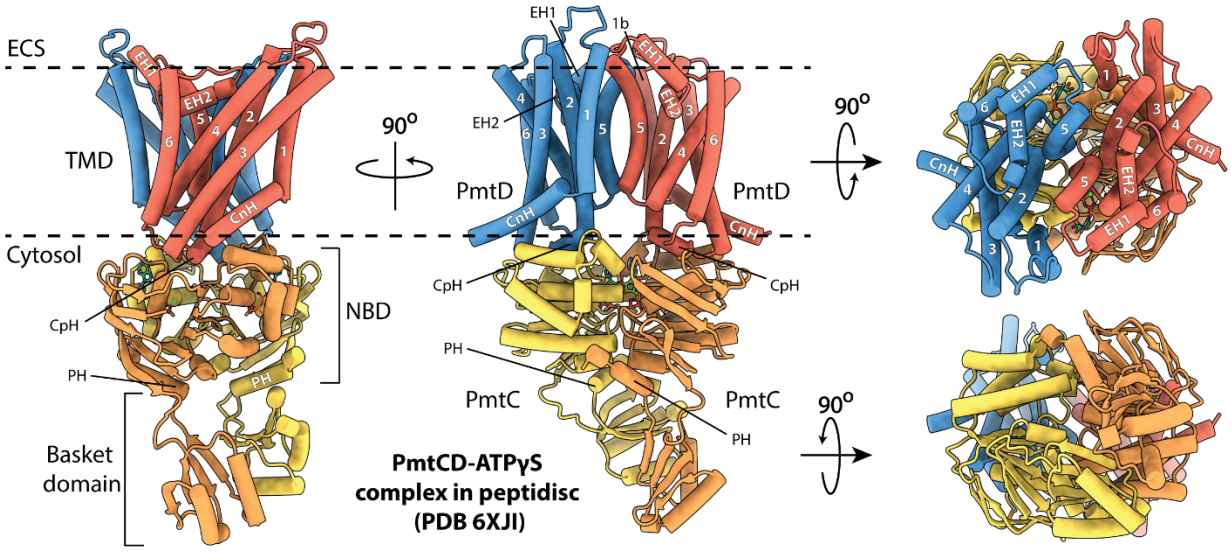

Figure S10. Cryo-EM structure of ATPγS-bound PmtCD in peptidisc (PDB ID: 6XJI). Cartoon representation of ATPγS-bound PmtCD in peptidisc (PDB ID: 6XJI). PmtC protomers are shown in yellow and orange, and PmtD protomers are shown in blue and red. Predicted membrane interfaces are indicated within dashed lines. The exporter is a heterotetramer comprised of two transmembrane domains (TMDs), two nucleotide binding domains (NBDs), and two basket domains. Transmembrane helices are labeled along with the pinning helix (PH), the two reentrant helices (EH1 and EH2), and the connecting helix (CnH) and coupling helix (CpH) for NBD binding. ATPγS in shown in green. Heteroatoms are coloured by type (O, red; P, orange; N, blue; Mg^{2+}, green).

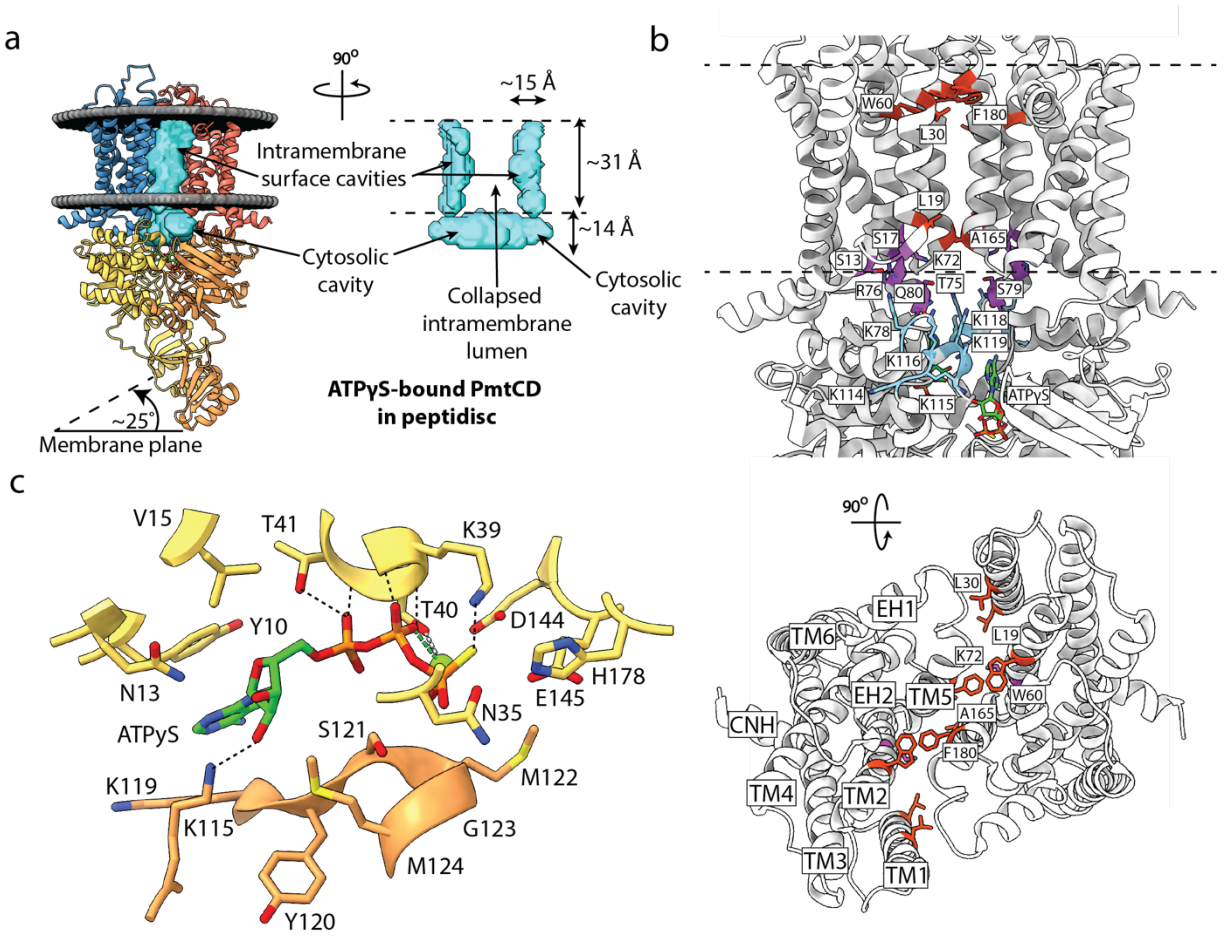

Figure S11. Structural features of ATPγS-bound PmtCD in peptidisc (PDB ID: 6XJI). (a) Cavities of ATPγS-bound PmtCD in peptidisc (PDB ID:6XJI) are shown in cyan with PmtC protomers in yellow and orange and PmtD protomers in blue and red. The predicted membrane interfaces are indicated within the grey disks, and ATPγS is shown in green. Heteroatoms are coloured by type (O, red; P, orange; N, blue; Mg^{2+}, green). (b) The cytosol-membrane interface and the intracellular regions of the two TMD-NBD modules outline a cytosolic cavity predicted to be the substrate entrance. The cavity is adjacent to a cluster of positively charged residues (blue) on the NBD and a patch of invariant polar residues at the cytosol-membrane interface (purple). Select transmembrane residues (orange) are shown. (c) The nucleotide-binding site shown with one PmtC protomer in yellow and the other in orange. Bonds between ATPγS and PmtCD are indicated by lines (salt bridges as thick dotted lines and hydrogen bonds as thin dotted lines).

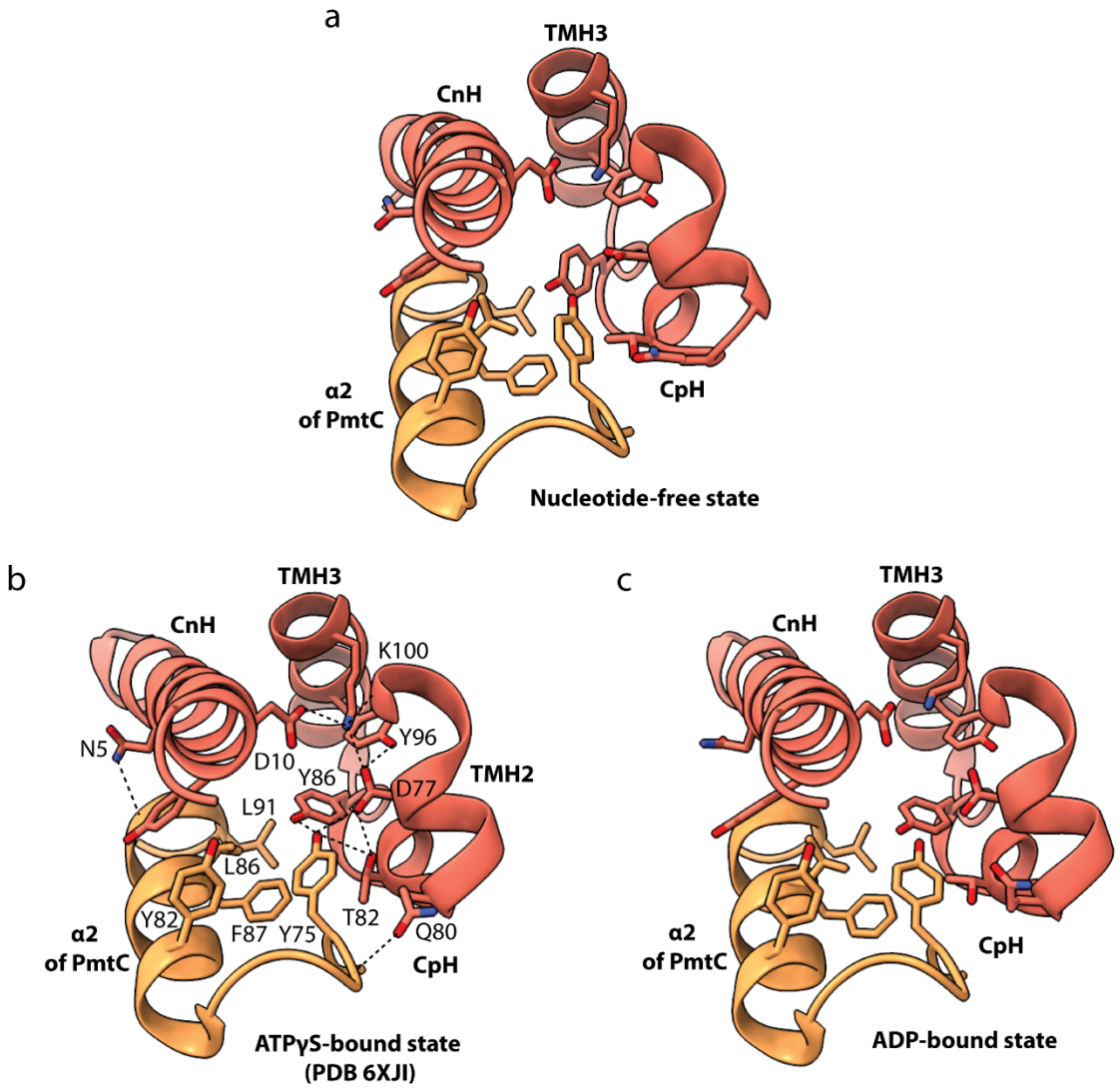

Figure S12. Interaction network between the TMD and NBD. The TMD-NBD interface of (a) nucleotide-free PmtCD in peptidisc, (b) ATPγS-bound (PDB ID:6XJI) in peptidisc, (c) ADP-bound PmtCD in peptidisc. Ribbon structures of PmtC and PmtD are shown in orange and red, respectively. Select residues are shown in stick to depict bonds and the packing of hydrophobic residues. Heteroatoms are coloured by type (O, red; N, blue). Bonds are indicated by lines (salt bridges as thick dotted lines and hydrogen bonds as thin dotted lines).

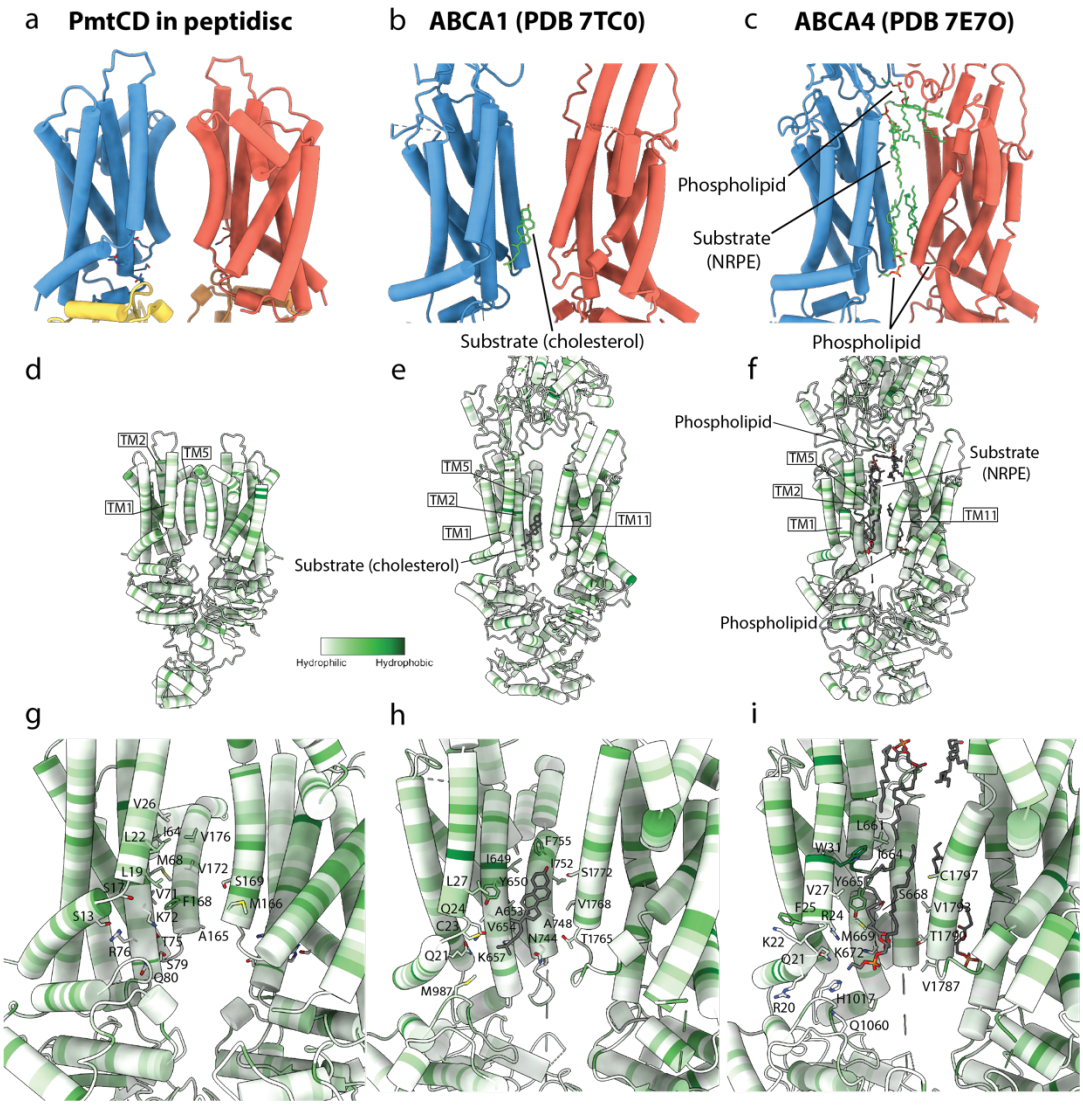

Figure S13. Substrates and lipids bound between the transmembrane domains of type V ABC transporters. Cartoon representations of (a) nucleotide-free PmtCD in peptidisc, (b) ABCA1 (PDB ID:7TC0), (c) ABCA4 (PDB ID: 7E7O). Cartoon representations coloured by hydrophobicity of (d) nucleotide-free PmtCD in peptidisc, (e) ABCA1 (PDB ID:7TC0), (f) ABCA4 (PDB ID: 7E7O), TMs forming hydrophobic pockets are labelled, and substrates or lipids present in models indicated with arrows. Hydrophobic pockets of (g) PmtCD, (h) ABCA1, and (i) ABCA4 near TMs 1, 2, 5, and 11 (ABCA transporters). Residues comprising the hydrophobic pocket where either phospholipids, substrates, or corresponding residues in PmtCD are shown and colored by hydrophobicity. Bound molecules are shown in gray and have arrows indicating their positions. Heteroatoms are coloured by type (O, red; P, orange; N, blue; S, yellow).

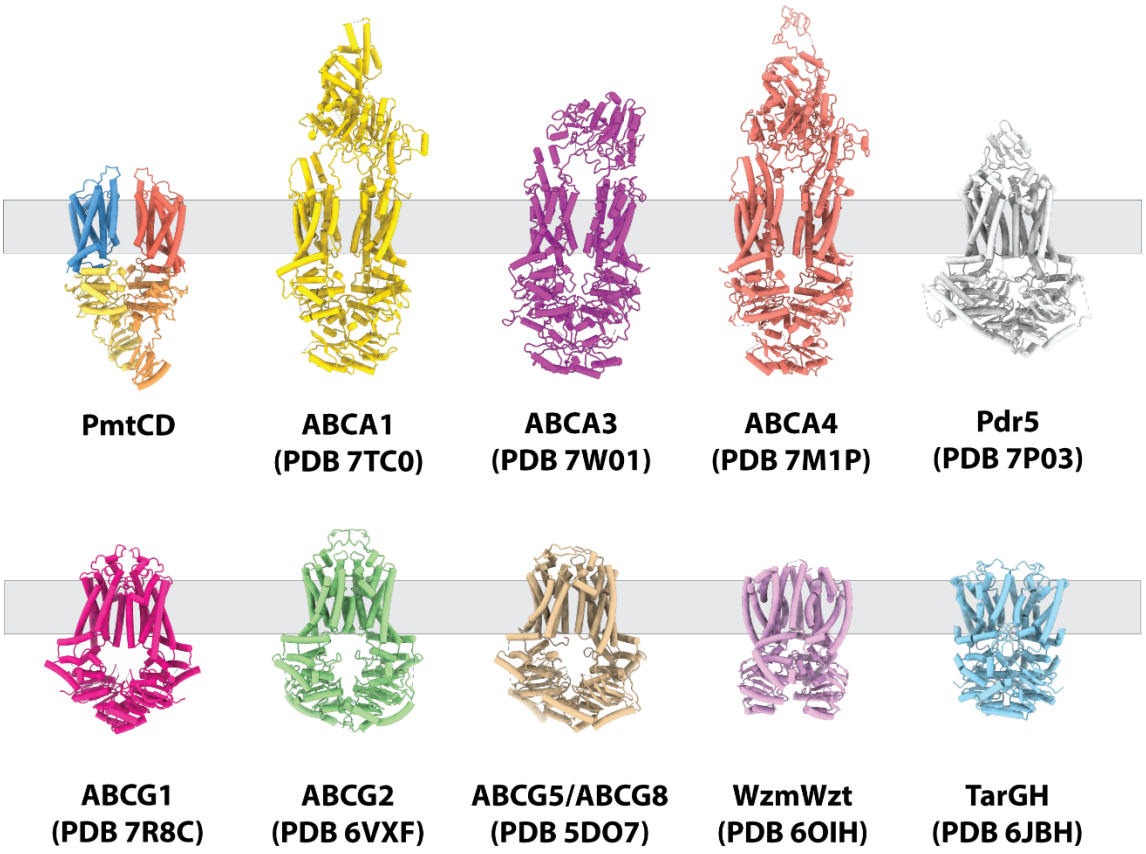

Figure S14. Structures of Type V ABC transporters. Cartoon representations of nucleotide-free PmtCD in peptidisc, ABCA1 (PDB ID: 7TC0), ABCA3 (PDB ID: 7W01), ABCA4 (PDB ID: 7M1P), Pdr5 (PDB ID: 7P03), ABCG1 (PDB ID: 7R8C), ABCG2 (PDB ID: 6VXF), ABCG5/ABCG8 (PDB ID: 5DO7), WzmWzt (PDB ID: 6OIH), and TarGH (PDB ID: 6JBH).

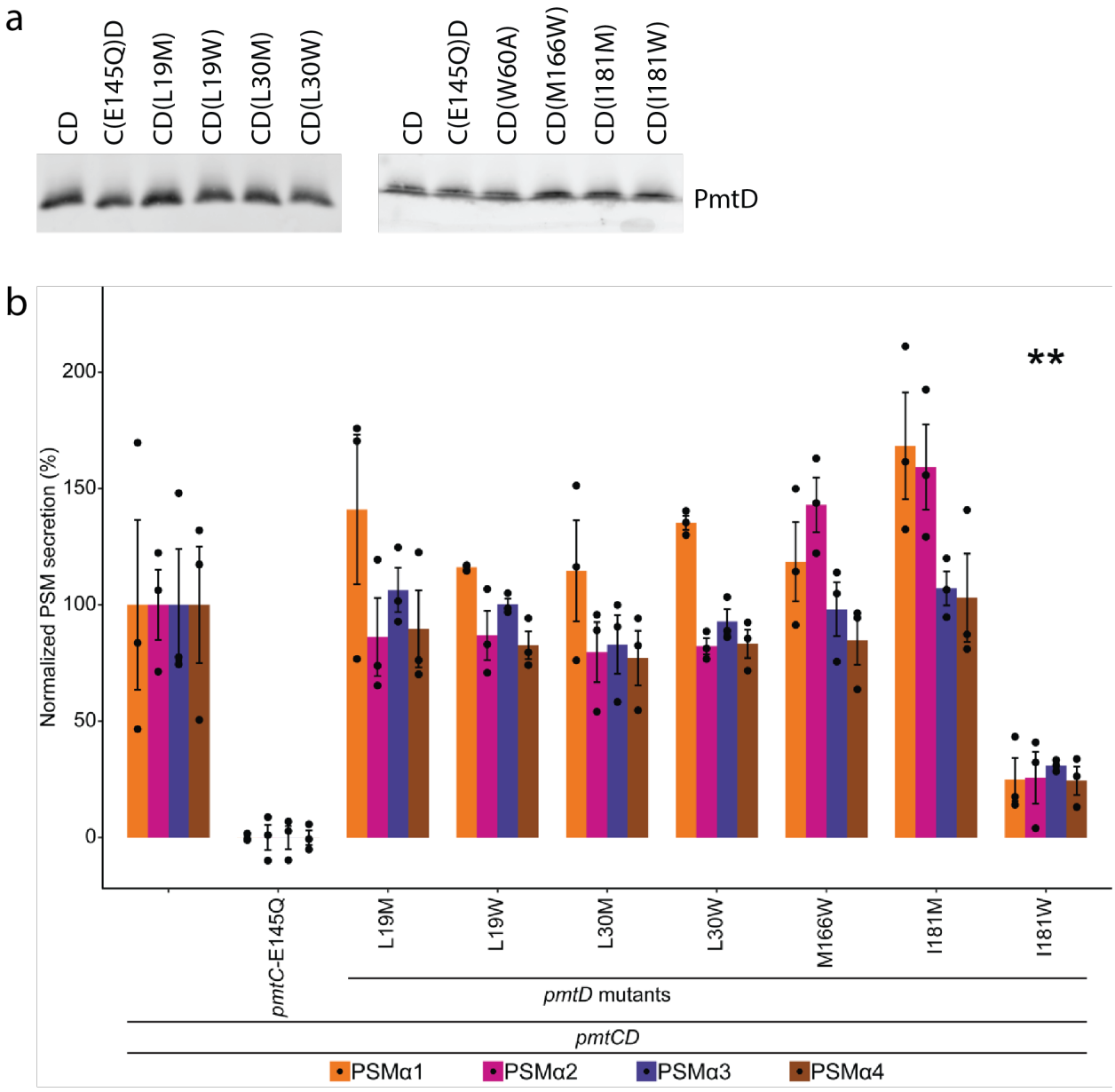

Figure S15. PSM secretion in *S. aureus* cells encoding PmtCD with the indicated amino acid substitution. (a) Western blots of PmtD from *S. aureus* cultures used to quantify PSM secretion. Bands corresponding to PmtD were quantified and normalized to the mean intensity within each replicate (n = 3). No significant differences were found across groups (ANOVA p = 0.06). (b) PSMα1-4 of S. aureus culture supernatants were quantified from the HPLC/MS extracted ion chromatogram and each PSM was normalized to between wild-type PmtCD (set to 100%) and the catalytically inactive PmtC(E145Q)D mutant (set to 0%) (n = 3 biological replicates) and with the error reported as the standard error of the mean. ** p = 0.007, Tukey's Honest Significant Difference test comparing PmtCD WT to PmtCD I181W.