

Supplementary materials for

Evidence for a ‘trap-and-flip’ mechanism in a proton-dependent lipid transporter

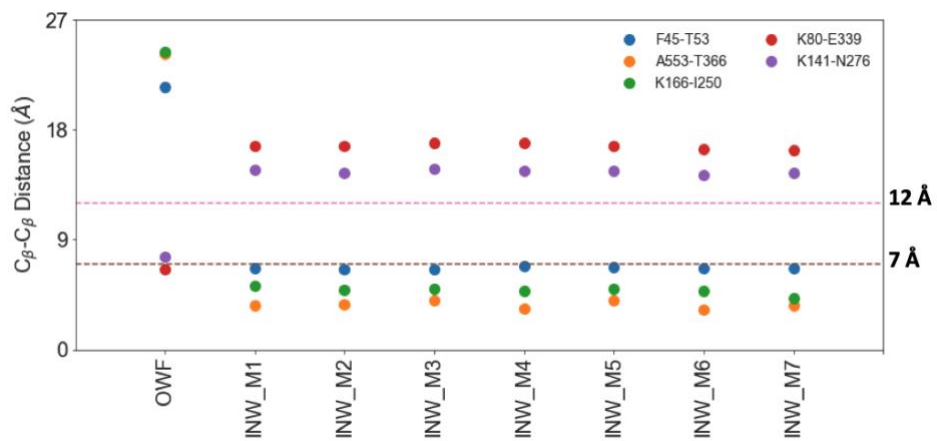
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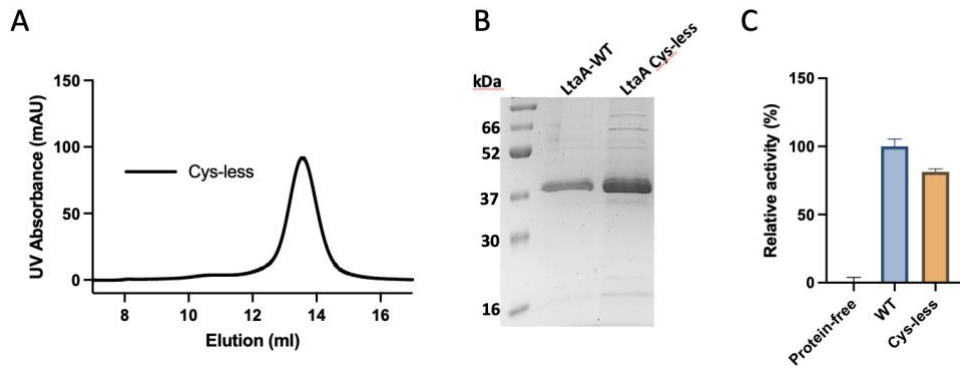
This PDF file includes:

Figs. S1 to S8

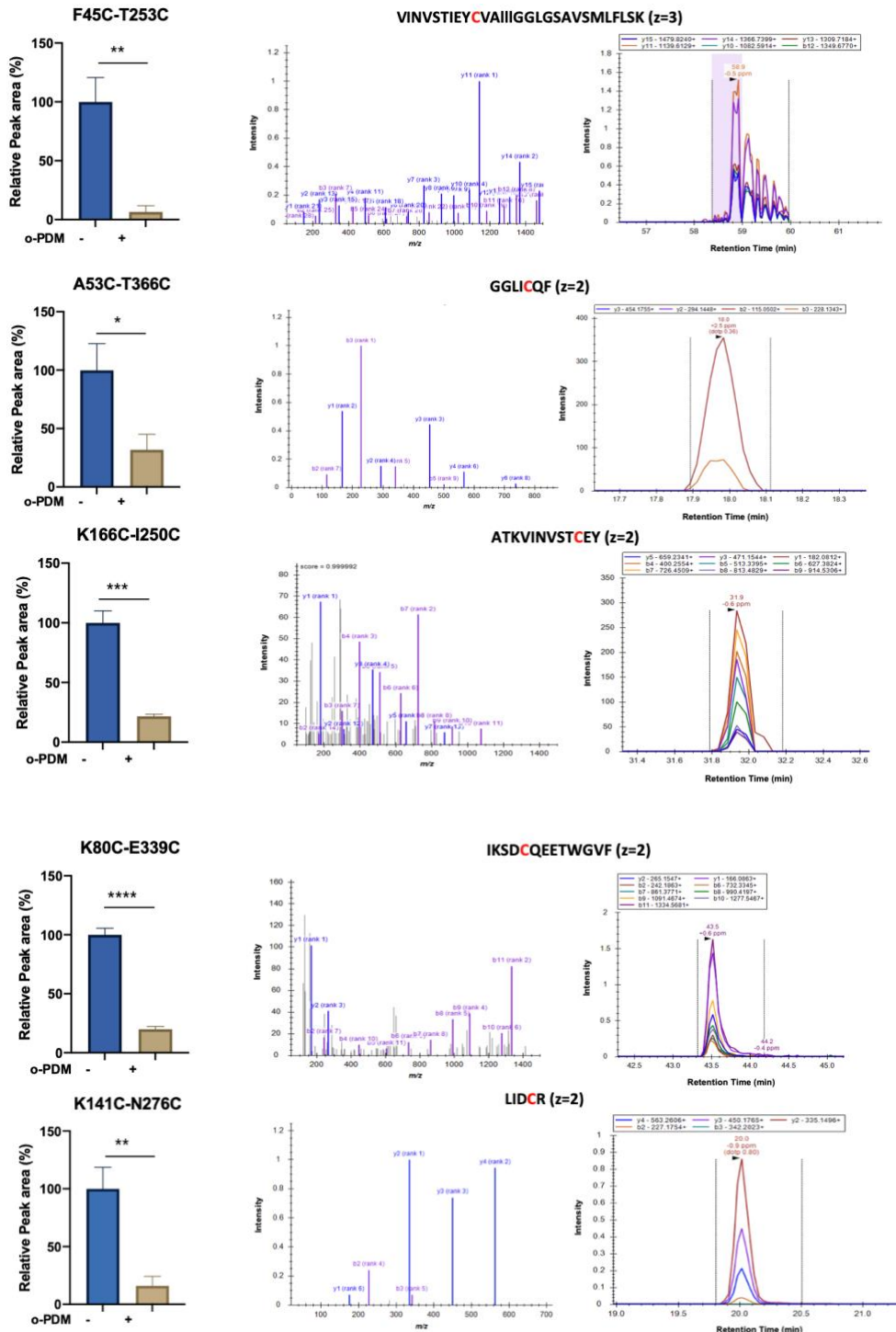
Tables S1 to S2



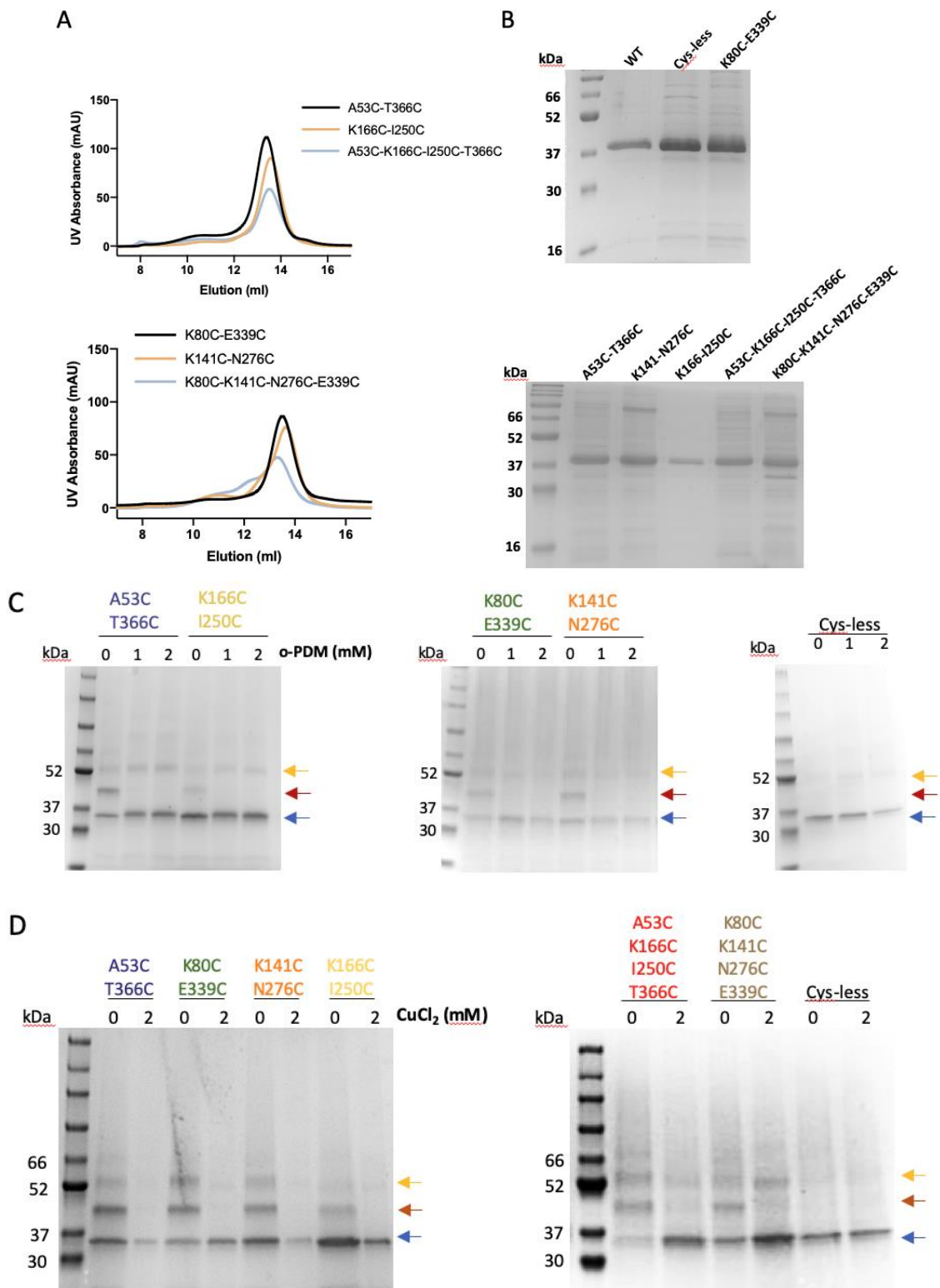
Supplementary figure 1. Distances between residues selected for cross-linking in alternating conformations of LtaA. C β -C β distances between residues selected for cysteine cross-linking in the outward-facing structure (OWF) and in the best inward-facing models (INW_M). Distance thresholds considered for selection of residues pairs are shown.



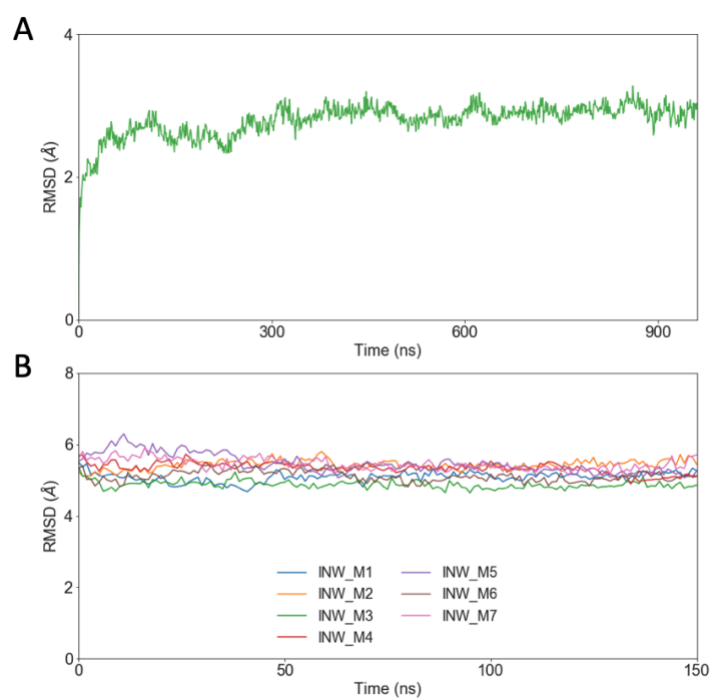
Supplementary figure 2. Cysteine-less LtaA variant can be purified and displays activity similar to LtaA-WT. **A.** Size Exclusion Chromatography profile of cysteine-less LtaA in a Superdex 200 Increase 10/300 GL column. **B.** SDS-PAGE of purified LtaA-WT and LtaA Cys-less. **C.** Relative flipping activity of LtaA Cys-less and LtaA-WT. Error bars show s.d. of technical replicates, $n \geq 3$.



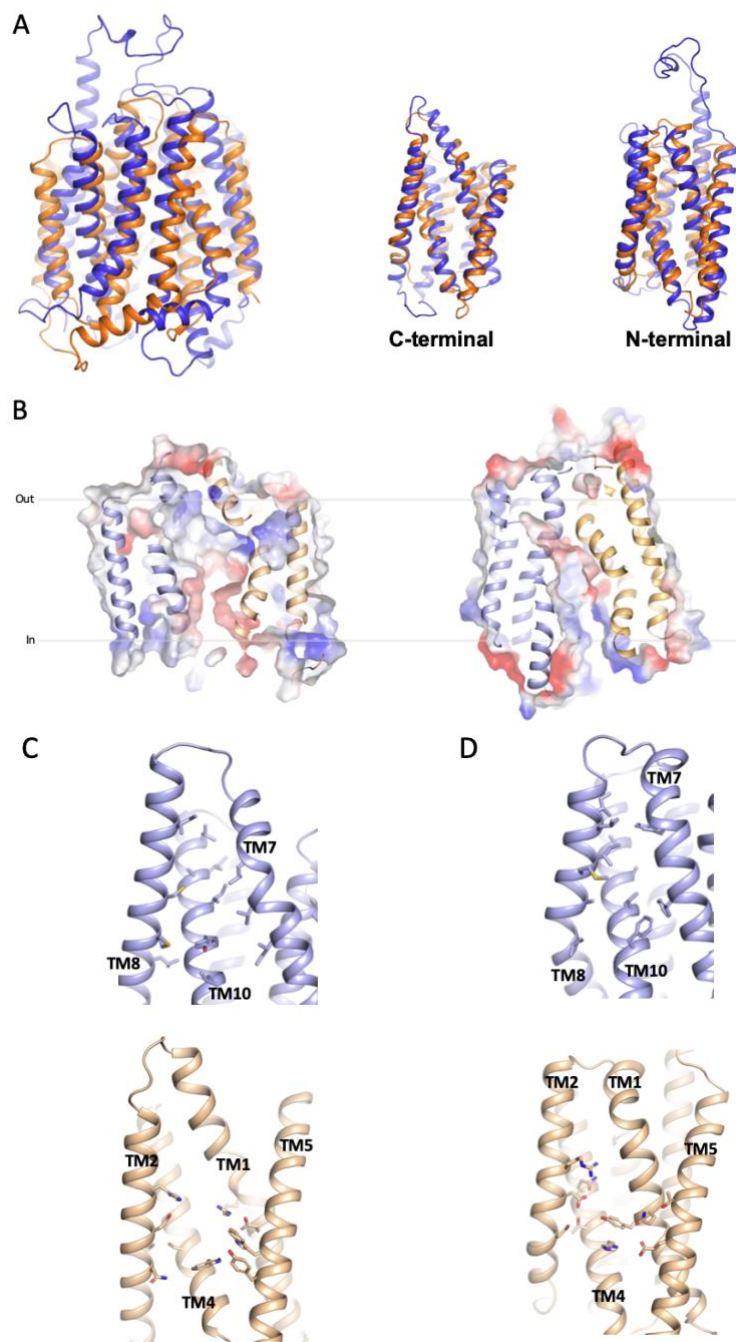
Supplementary figure 3. LC-MS analysis of cysteine cross-linking showing that LtaA adopts inward- and outward-facing states. Selected pairs of residues for cross-linking are shown in Fig. 2. *Left:* Relative abundance of cysteine containing peptides in absence (-) or presence (+) of N,N'-(o-phenylene)-dimaleimide (o-PDM). Error bars indicate s.d., $n \geq 3$. *: $P \leq 0.05$, **: $P \leq 0.01$, ***: $P \leq 0.001$, ****: $P \leq 0.0001$. *Center:* Assigned Collision-Induced Dissociation (CID) spectra obtained from cysteine containing peptides in absence of o-PDM. The position of the alkylated cysteine is indicated in red and the charge state (z) of the identified peptide ion is provided. *Right:* Elution profiles of the peptide fragments (transitions) identified used for peptide quantification by targeted LC-MS analysis.



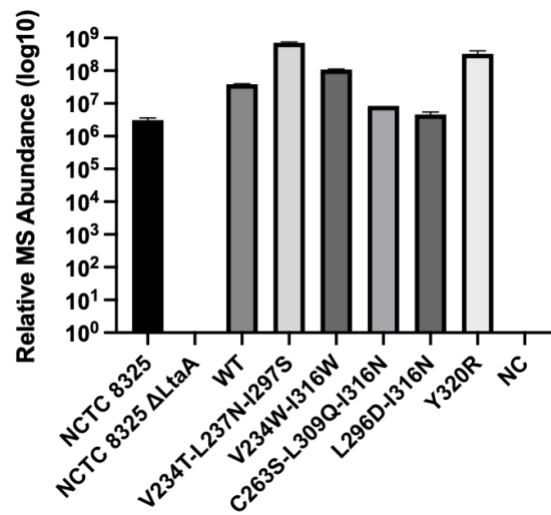
Supplementary figure 4. Purifications and cross-linking in-gel analysis of LtaA variants. A. Size Exclusion Chromatography profile of LtaA variants in a Superdex 200 Increase 10/300 GL column. **B.** SDS-PAGE of purified LtaA WT and variants. **C** and **D.** SDS-PAGE showing band shifts of samples treated with mPEG5K after cross-link with o-PDM or CuCl₂, respectively.



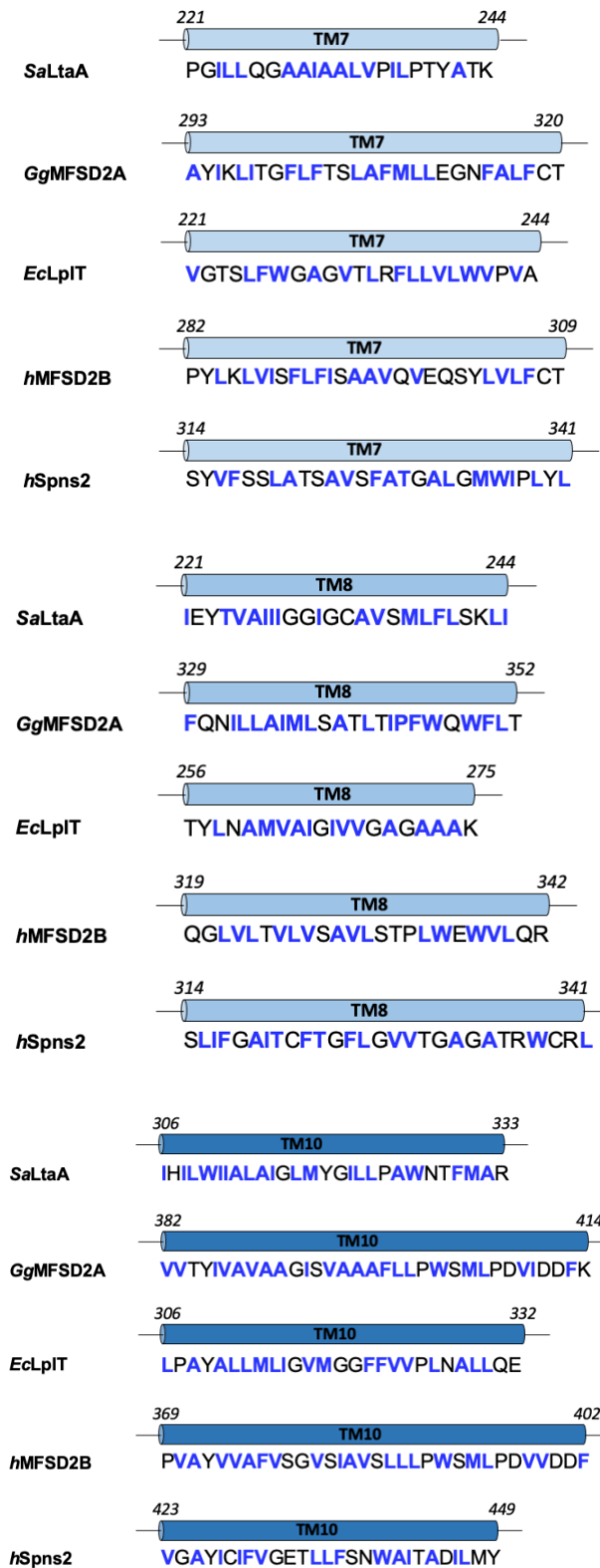
Supplementary figure 5. RMSD analysis from molecular dynamics simulations of LtaA in a membrane. Root-mean-square-deviation (RMSD) plots of C α atoms from outward-facing LtaA (**A**) and inward-facing LtaA models (**B**) during MD simulations in a membrane composed of POPS (65%), diacylglycerol (20%), cardiolipin (10%), and gentiobiosyl-diacylglycerol (5%).



Supplementary figure 6. Comparison of LtaA and MFSD2A fold and amphipathic cavity. **A.** Superposition of LtaA (orange) and MFSD2A (blue) (r.m.s.d = 4.1Å), and individual N- and C-terminal domains (r.m.s.d= 2.6Å and 2.8Å, respectively). **B.** Vacuum electrostatic surface representation of inward-facing model of LtaA (*left*) and inward-facing MFSD2A (PDB ID 7MJS) (*right*). Residues participating in formation of N- and C-terminal hydrophilic and hydrophobic pockets in LtaA (**C**) and MFSD2A (**D**). N-terminal and C-terminal domains are shown in light orange and light blue, respectively.



Supplementary figure 7. LC-MS analysis of relative abundance of LtaA-WT and variants in *S. aureus* membranes. The histogram shows relative abundances of LtaA WT and variants. Error bars show s.d. of technical replicates (n=3).



Supplementary figure 8. C-terminal TM helices participating in formation of hydrophobic pockets in MFS lipid transporters. Residues in TM7, 8, and 10 as seen in the structures of *S. aureus* LtaA (PDB ID 6S7V) and *Gallus gallus* MFSD2A (PDB ID 7MJS), and residues in predicted helical segments corresponding to the same TMs in *E. coli* LpIT, human MFSD2B, and human Spns2 are shown. Hydrophobic residues are shown in blue.

Supplementary Table 1. Validation parameters of the top seven inward-facing models. *Initial*: homology models from Modeler: *SCWRL*; the refined model by *SCWRL4*; *mini*: energy minimization of the model in a bilayer using Gromacs.

Model	Clashscore			MolProbity score			Ramachandran favoured (%)			Ramachandran outliers (%)		
	Initial	SCWRL	mini	Initial	SCWRL	mini	Initial	SCWRL	mini	Initial	SCWRL	mini
1	144.2	125.8	11.5	3.46	2.89	2.03	95.5	95.2	93.4	0.8	1.0	1.0
2	155.0	125.3	11.5	3.49	2.89	2.15	95.2	95.2	92.9	0.8	0.8	1.6
3	145.5	125.1	10.3	3.45	2.91	2.3	94.9	94.9	92.2	1.1	1.1	1.6
4	137.9	117.8	12.0	3.25	2.85	2.05	95.5	95.5	92.9	0.5	0.5	0.8
5	144.9	119.1	10.6	3.34	2.89	1.98	94.9	95.0	93.4	0.8	0.8	1.3
6	134.7	125.7	8.12	3.35	2.89	2.17	95.2	95.2	93.6	1.1	1.1	1.1
7	131.8	125.5	9.81	3.31	2.86	2.18	95.7	95.7	92.3	1.1	1.1	1.1

Supplementary Table 2. Oligo's used in this study. Mutagenesis is underlined

Oligo name	5'-3' sequence
Sa-Sall-FW	GAGAGTCGACGGTCATTCATCACAACC
Sa-V234T-L237N-FW	GGTGCCGCAATTGCTGCCCTAA <u>CC</u> CCTATAAATCCAACATATGCTACTAAGGTTAT
Sa-V234T-L237N-RV	ATAACCTTAGTAGCATATGTTGGATTATAGGGGTTAGGGCAGCAATTGCGGCACC
Sa-V234W-FW	GTGCCGCAATTGCTGCCCTATGGCCTATATTACCAACATATGCTAC
Sa-V234W-RV	GTAGCATATGTTGGTAATATAGGCCATAGGGCAGCAATTGCGGCACC
Sa-C263S-FW	CATTATTGGTGGTATCGGCTCTGCAGTTTCGATGCTATTT
Sa-C263S-RV	AAATAGCATCGAAACTGCAGAGCCGATACCACCAATAATG
Sa-L296D-FW	TCTAAGCGGATTTATTTTATACATGATAGATATTTTTACTCTATCTATGATTGTTAATATTCACA
Sa-L296D-RV	TGTGAATATTAACAATCATAGATAGAGTAAAAATATCTATCATGTATAAAAATAAATCCGCTTAGA
Sa-I297S-FW	GATTTATTTTATACATGATATTAAGTTTTACTCTATCTATGATTGTTAATATTC
Sa-I297S-RV	GAATATTAACAATCATAGATAGAGTAAAACTTAATATCATGTATAAAAATAAATC
Sa-L309Q-I316N-FW	CTATGATTGTTAATATTCACATCAATGGATTATCGCTTTAGCTAATGGTCTAATGTATGGCATCTTATTAC
Sa-L309Q-I316N-RV	GTAATAAGATGCCATACATTAGACCAATTAGCTAAAGCGATAATCCATTGGATGTGAATATTAACAATCATAG
Sa-I316N-FW	CTTGTGGATTATCGCTTTAGCTAATGGTCTAATGTATGGCATCTTATTAC
Sa-I316N-RV	GTAATAAGATGCCATACATTAGACCAATTAGCTAAAGCGATAATCCACAAG
Sa-I316W-FW	CTTGTGGATTATCGCTTTAGCTGGGGTCTAATGTATGGCATCTTATTAC
Sa-I316W-RV	GTAATAAGATGCCATACATTAGACCCCAAGCTAAAGCGATAATCCACAAG
Sa-Y320R-FW	TTAGCTATCGGTCTAATGCGTGGCATCTTATTACCAGCATG
Sa-Y320R-RV	CATGCTGGTAATAAGATGCCACGCATTAGACCGATAGCTAA
LtaA-NotI-RV	GAGAGCGGCCGCAATAGTATTGTTAATCGTAGTATGTTTGAATTAATAAGA
F45C-FW	GAGCTACATTAAGTGTCTGCCACCG
F45C-RV	CGGTGGCAGACAGTTAATGTAGCTC
A53C-FW	CCGTGACATCGATTGTGTGCTATTACATC

A53C-RV	GATGTAATAGCGACACAAATCGATGTCACGG
K80C-FW	CGTTATCGGCTTCTATTATGTAAGTTTGAAGCTAAGATC
K80C-RV	GATCTTAGTTCCAACTTACATAATAAGAAGCCGATAACG
K141C-FW	GAGGATAAACCGCGCTGTCAAATGGGCTACGTG
K141C-RV	CACGTAGCCCATTTGACAGCCGCGTTTATCCTC
K166C-FW	CTTTATGAATTTGCTGATCTGTGTTTCATCCTACTCGCTTCG
K166C-RV	CGAAGCGAGTAGGATGAACAACAGATCAGCAAATTCATAAAG
I250C-FW	CAAAAGTCATTAATGTATCAACGTGTGAATATACCGTGGCTATTATC
I250C-RV	GATAATAGCCACGGTATATTCAACGTTGATACATTAATGACTTTTG
T253C-FW	GTATCAACGATTGAATATTGTGTGGCTATTATCATCGGCGG
T253C-RV	CCGCCGATGATAATAGCCACACAATATTCAATCGTTGATAC
C263S-FW	CGGCGGCATTGGATCGGCCGTTAGTATGTTG
C263S-RV	CAACATACTAACGGCCGATCCAATGCCGCCG
N276C-FW	CTTAGTAAGTTGATCGACTGCCGTTTCGCGTAACTTTATGTAC
N276C-RV	GTACATAAAGTTACGCGAACGGCAGTCGATCAACTTACTAAG
E339C-FW	GCTTCATCAAGAGCGACTGTCAAGAGGAGACCTGG
E339C-RV	CCAGGTCTCCTCTTGACAGTCGCTCTTGATGAAGC
T366C-FW	GTTTGCGGATTGATTGCCAGTTCACCAAC
T366C-RV	GTTGGTGAAGTGGCAATCAATCCGCCAAAC
LtaA-V234T-L237N-FW	GATTGCGGCTTTGACTCCTATCAACCCTACGTATGCCACA
LtaA-V234T-L237N-RV	TGTGGCATACTAGGGTTGATAGGAGTCAAAGCCGCAATC
LtaA-V234W-FW	GATTGCGGCTTTGTGGCCTATCCTGCCTAC
LtaA-V234W-RV	GTAGGCAGGATAGGCCACAAAGCCGCAATC
LtaA-C263S-FW	GCGGCATTGGATCGGCCGTTAGTATG
LtaA-C263S-RV	CATACTAACGGCCGATCCAATGCCGC
LtaA-L296D-FW	GGTTTTATTCTGTATATGATTGATATCTTCACGTTGTCTATG
LtaA-L296D-RV	CATAGACAACGTGAAGATATCAATCATATACAGAATAAAACC
LtaA-I297S-FW	TTATTCTGTATATGATTCTTAGTTTACGTTGTCTATGATTGTC
LtaA-I297S-RV	GACAAATCATAGACAACGTGAAACTAAGAATCATATACAGAATAA
LtaA-L309Q-FW	GTCAACATCCATATCCAATGGATTATTGCCTTGG
LtaA-L309Q-RV	CCAAGGCAATAATCCATTGGATATGGATGTTGAC
LtaA-I316N-FW	TGCCTTGCGAACGGCCTGATG
LtaA-I316N-RV	CATCAGGCCGTTCCGCAAGGCA
LtaA-I316W-FW	ATTATTGCCTTGGCGTGGGGCCTGATGTAT
LtaA-I316W-RV	ATACATCAGGCCCCACGCCAAGGCAATAAT
LtaA-Y320R-FW	GATCGGCCTGATGCGTGGGATTCTGTTGCCA
LtaA-Y320R-RV	GATCGGCCTGATGCGTGGGATTCTGTTGCCA