



32 **Transport of lipids across membranes is fundamental for diverse biological pathways in cells.**  
33 **Multiple ion-coupled transporters participate in lipid translocation, but their mechanisms**  
34 **remain largely unknown. Major facilitator superfamily (MFS) lipid transporters play central**  
35 **roles in cell wall synthesis, brain development and function, lipids recycling, and cell**  
36 **signaling. Recent structures of MFS lipid transporters revealed overlapping architectural**  
37 **features pointing towards a common mechanism. Here we used cysteine disulfide trapping,**  
38 **molecular dynamics simulations, mutagenesis analysis, and transport assays *in vitro* and *in***  
39 ***vivo*, to investigate the mechanism of LtaA, a proton-dependent MFS lipid transporter**  
40 **essential for lipoteichoic acids synthesis in the pathogen *Staphylococcus aureus*. We reveal**  
41 **that LtaA displays asymmetric lateral openings with distinct functional relevance and that**  
42 **cycling through outward- and inward-facing conformations is essential for transport activity.**  
43 **We demonstrate that while the entire amphipathic central cavity of LtaA contributes to lipid**  
44 **binding, its hydrophilic pocket dictates substrate specificity. We propose that LtaA catalyzes**  
45 **lipid translocation by a ‘trap-and-flip’ mechanism that might be shared among MFS lipid**  
46 **transporters.**

47  
48 Major facilitator superfamily (MFS) transporters are found in all kingdoms of life and move a  
49 large variety of molecules across biological membranes [1-8]. Structural characterization of  
50 MFS transporters that participate in the uptake of water-soluble molecules and extrusion of  
51 drugs has contributed to a broad understanding of their transport mechanism [4, 8-17].  
52 However, multiple reports have attributed alternative functions to MFS transporters, such as  
53 translocation of lipids associated with fundamental biological pathways. Some examples  
54 include the bacterial lysophospholipid transporter LpIT, involved in lipids recycling in Gram-  
55 negative bacteria [7, 18]; the human transporter MFSD2A, expressed at the blood-brain- and  
56 blood-retinal-barrier, contributing to major uptake of docosahexaenoic acid (DHA) [5, 6, 19-  
57 21]; the human transporters Spns2 [22, 23], and MFSD2B [24], which contribute to transport  
58 of sphingosine 1-phosphate (S1P) in endothelial cells and erythrocytes; and the gentiobiosyl-  
59 diacylglycerol transporter LtaA, involved in cell wall synthesis in *Staphylococcus aureus* [25,  
60 26]. However, despite their well described cellular roles, the mechanisms of MFS lipid  
61 transporters remain insufficiently understood.

62 We have previously shown that LtaA is a proton-dependent MFS lipid antiporter [26].  
63 It contributes to adaptation of *S. aureus* to acidic conditions, common in the skin and

64 nasopharynx of the human host [26-28]. LtaA takes part in the assembly of lipoteichoic acids,  
65 phosphate-rich polymers important for control of bacterial cell division, protection from  
66 environmental stress, host cell adhesion, antibiotic resistance, biofilm formation, and immune  
67 evasion [29-32]. *S. aureus* lipoteichoic acids display a polymer of 1,3-glycerol-phosphate  
68 repeat units attached to C-6 of the non-reducing glucosyl of the glycolipid gentiobiosyl-  
69 diacylglycerol [31-33]. This glycolipid is synthesized at the cytoplasmic leaflet of the  
70 membrane by the glycosyltransferase YpfP, and is translocated to the outer leaflet by the  
71 activity of LtaA [25, 26]. The essential role of LtaA in adjusting the pool of glycolipids available  
72 at the extracellular side of the membrane, makes this protein a central player for lipoteichoic  
73 acids assembly and a potential target for drugs aiming to counteract antimicrobial resistant *S.*  
74 *aureus* strains e.g., methicillin-resistance *S. aureus* (MRSA) and vancomycin-resistant *S. aureus*  
75 (VRSA) [30].

76 Two different general models of transporter-catalyzed lipid translocation have been  
77 proposed in the past [34-42]. A ‘trap-and-flip’ model, in which the lipid substrate is retrieved  
78 from one leaflet of the membrane, enclosed into a central cavity, and then delivered to the  
79 other leaflet [40, 43, 44], and a ‘credit-card’ model that departs from the classical alternating-  
80 access model and involves translocation of the lipid head-group across a hydrophilic cleft or  
81 cavity in the transport protein, while aliphatic chains remain embedded in the membrane [36-  
82 38, 41, 42, 45, 46]. However, it is not known which of these two models describe better the  
83 mechanism of MFS lipid transporters. Answering this question is not only important to  
84 understand the basis of the processes catalyzed by these proteins, but could also provide a  
85 foundation for the design of drugs and/or lipid-linked-bioactive molecules targeting cells or  
86 organs expressing pharmacologically relevant proteins from this superfamily.

87 Until now, only the high-resolution structures of outward-facing LtaA and inward-  
88 facing MFSD2A have been elucidated [21, 26]. Both transporters displayed the canonical MFS  
89 fold of 12 transmembrane (TM) helices and an amphipathic central cavity that has not been  
90 observed in any MFS transporter of water-soluble molecules. The similar architectural  
91 features observed in the structures of LtaA and MFSD2A indicate common elements in their  
92 transport mechanisms and likely among all MFS lipid transporters. Here, we used cysteine  
93 disulfide trapping of outward- and inward-facing LtaA, in combination with molecular  
94 dynamics simulations, mutagenesis analysis, and transport assays *in vitro* and *in vivo*, and  
95 showed that cycling through outward- and inward-facing conformations is essential for LtaA

96 activity. We demonstrate that LtaA displays membrane exposed lateral openings with distinct  
97 functional relevance and characterized the architecture and biochemical properties of the  
98 amphipathic central cavity during alternating-access. Our results indicate that while the  
99 hydrophilic pocket of the amphipathic central cavity dictates substrate specificity, the  
100 hydrophobic pocket is only relevant for aliphatic chains binding. We describe critical  
101 mechanistic elements revealing that LtaA adopts a ‘trap-and-flip’ mechanism that might be  
102 shared among MFS lipid transporters.

103

## 104 **Results**

### 105 ***Model of inward-facing LtaA and validation by cysteine cross-linking***

106 To investigate whether LtaA uses a ‘trap-and-flip’ or a ‘credit-card’ mechanism, we first  
107 aimed to establish a system that allowed us to perform cysteine disulfide trapping of end-  
108 point conformations of LtaA during its transport cycle. The architecture of the previously  
109 solved structure of LtaA [26], facilitates cysteine disulfide trapping of outward-facing states,  
110 whereas there is no structural information to guide trapping of inward-facing states. Thus, we  
111 first generated an inward-facing model of LtaA using ‘repeat-swap’ modeling [47]. Like other  
112 transporters from the MFS superfamily, the topology of LtaA consists of two domains, a N-  
113 terminal domain (TM1-TM6; domain-1), and a C-terminal domain (TM7-TM12; domain-2),  
114 each of which contains two structural repeats with inverted-topology related by a pseudo-  
115 rotational two-fold symmetry axis parallel to the plane of the membrane (**Fig. 1A,B**). After  
116 swapping the conformations of the inverted repeats observed in the outward-facing structure  
117 of LtaA (PDB ID 6S7V) [26, 47], we constructed a large set of models *in silico* that were refined  
118 aiming to improve side chains packing, stereochemistry, and modeling scores. The models  
119 with the best scores converged to one conformation (**Fig. 1C and Suppl. Table. 1**), which  
120 displayed multiple interactions between the extracellular parts of TM1-TM7, TM2-TM11, and  
121 TM5-TM8, sealing the entrance to the central cavity (**Fig. 1C**). In contrast, the cytoplasmic  
122 regions of helices TM2-TM11, TM5-TM8, and TM4-TM10, lining the entrance to the central  
123 cavity from the cytoplasm, are away from each other about  $16.0 \pm 0.1 \text{ \AA}$ ,  $16.0 \pm 0.1 \text{ \AA}$ , and  
124  $17.6 \pm 0.2 \text{ \AA}$ , respectively (**Fig. 1C**). The helical loop between TM6 and TM7 that connects the  
125 N- and C-terminal domains was modeled based on the conformation observed in the outward-  
126 facing structure.

127 To validate the inward-facing model, we selected pairs of residues among the  
128 extracellular regions for which C $\beta$ -C $\beta$  distances were less than 7 Å, but which present C $\beta$ -C $\beta$   
129 distances of over 12 Å in the outward-facing structure (**Suppl. Fig. 1**). Based on these criteria,  
130 we identified the pairs F45-T253, A53-T366, and K166-I250 (**Fig. 2A and Suppl. Fig. 1**),  
131 occupying three different positions that provide good coverage of the conformational change  
132 predicted by our models. We then introduced cysteine residues at these positions on a starting  
133 construct in which the one native cysteine in LtaA was replaced with serine. The cysteine-less  
134 LtaA variant effectively performed glycolipid flipping in proteoliposomes (**Suppl. Fig. 2**). The  
135 three mutants F45C-T253C, A53C-T366C, and K166C-I250C were then irreversibly crosslinked  
136 with N,N'-(o-phenylene)-dimaleimide (o-PDM), which has a spacer arm length of 6 Å.  
137 Crosslinked and non-crosslinked LtaA mutants were digested with either trypsin or  
138 chymotrypsin, and analyzed by high-resolution liquid chromatography–mass spectrometry  
139 (LC–MS) to evaluate the presence of non-crosslinked cysteine containing peptides. The  
140 peptides abundance was normalized against an internal reference peptide. We successfully  
141 identified non-crosslinked peptides in untreated samples of the three mutants F45C-T253C,  
142 A53C-T366C, and K166C-I250C (**Fig. 2A and Suppl. Fig. 3**). The abundance of these peptides  
143 was clearly diminished in the crosslinked protein samples (**Fig. 2A**), demonstrating that the  
144 selected pairs of residues are in close proximity as predicted in the inward-facing model.

145 As a control, we performed a similar experiment but with pairs of residues that were  
146 shown to interact at the cytoplasmic region of the outward-facing structure (**Fig. 2B**). Thus,  
147 we introduced cysteine residues at the positions K80-E339 and K141-N276, present at the  
148 cytoplasmic ends of TM2-TM11 and TM5-TM8, respectively. C $\beta$ -C $\beta$  distances between these  
149 residues are smaller than 7.5 Å in the outward-facing structure, but larger than 12 Å in the  
150 inward-facing models (**Suppl. Fig. 1**). LC-MS analysis of the double mutants K80C-E339C and  
151 K141C-N276C confirmed the proximity of these residues as non-crosslinked peptides are more  
152 abundant in untreated samples, whereas in the presence of the cross-linking agent their  
153 abundances decrease substantially (**Fig. 2B**). In summary, our cross-linking analysis support  
154 the predicted conformation and interactions reported by the inward-facing model of LtaA and  
155 indicate the position of residues to guide cysteine disulfide trapping of LtaA conformations.

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159 ***Alternating conformations in proteoliposome membranes***

160 We investigated the conformations displayed by LtaA in membranes by evaluating the  
161 cross-linking of double cysteine mutants reconstituted in proteoliposomes (**Fig. 2C**). The  
162 cysteine pairs reported on the conformation of the TM helices that line the lateral openings,  
163 TM2-TM11 and TM5-TM8 (**Fig. 1C**). We screened for successful cross-links by using a gel-shift  
164 assay in which we first incubated with the o-PDM cross-linker, followed by treating the  
165 proteoliposomes with 5-kDa PEG-maleimide (mPEG5k) [48]. This treatment generates a  
166 substantial shift in the protein mobility in polyacrylamide gel electrophoresis as mPEG5k  
167 irreversibly binds free cysteines. However, if the introduced cysteines are cross-linked by o-  
168 PDM, then they will not react with mPEG5k and no shift in gel mobility would be observed.  
169 We evaluated the cross-linking of residues A53C-T366C (TM2-TM11) and K166C-I250C (TM5-  
170 TM8), positioned at the extracellular region, and K80C-E339C (TM2-TM11) and K141C-N276C  
171 (TM5-TM8), located at the cytoplasmic region (**Fig. 2C and Suppl. Fig. 4A,B**). Before cross-  
172 linking, each double-cysteine mutant showed a gel shift after incubation with mPEG5K, thus  
173 demonstrating PEGylation of free cysteines (**Fig. 2C and Suppl. Fig. 4C**), albeit the shift of  
174 K166C-I250C is less prominent (**Fig. 2C**). In contrast, after treatment with o-PDM, all the  
175 double cysteine mutants were protected from PEGylation, thus showing that all mutants were  
176 successfully cross-linked. The cysteine-less control LtaA, did not show a gel shift in any of the  
177 conditions (**Fig. 2C and Suppl. Fig. 4C**), demonstrating that the shifts observed for the mutants  
178 were due to PEGylation of cysteines. These results support that when embedded in the  
179 membrane of proteoliposomes, LtaA can adopt conformations where residues at the lateral  
180 openings lined by TM2-TM11 and TM5-TM8 display similar distances to those reported by the  
181 outward-facing structure and the inward-facing model.

182

183 ***Alternating-access to the central cavity is essential for function***

184 The cross-linking results described above showed that LtaA can cycle through outward-  
185 and inward-facing conformations. However, it is not known whether this is required for  
186 function. This is important because some flippases and scramblases use a 'credit card' mode  
187 of transport, where exposing a side cleft or a cavity to one side of the membrane is sufficient  
188 for catalysis of lipid transport across the membrane [36-38, 41, 49]. To answer whether  
189 alternating between inward- and outward-facing conformations is important for LtaA activity,  
190 we performed copper chloride catalyzed cross-linking of residues located at the lateral

191 openings lined by TM2-TM11 and TM5-TM8, and determined proton-coupled glycolipid  
192 transport activity of cross-linked LtaA variants in proteoliposomes (**Fig. 3**). In this assay, the  
193 addition of the K<sup>+</sup>-selective ionophore valinomycin generates a membrane potential of about  
194 -60 mV, which drives proton influx. Acidification of the lumen of proteoliposomes quenches  
195 the fluorophore 9-amino-6-chloro-2-methoxyacridine (ACMA) causing a decrease in the  
196 fluorescence [26]. The double-cysteine mutants A53C-T366C (TM2-TM11) and K166C-I250C  
197 (TM5-TM8) close the extracellular side openings, whereas the mutants K80C-E339C (TM2-  
198 TM11) and K141C-N276C (TM5-TM8) close the cytoplasmic openings (**Fig. 3A and Suppl. Fig.**  
199 **4A,B**). Our results show that except for K141C-N276C (TM5-TM8), independent cross-linking  
200 of the remaining three lateral openings decreases LtaA activity relative to non-cross-linked  
201 mutants (**Fig. 3A**).

202 In addition, we aimed to completely close the cytoplasmic or extracellular cavities and  
203 test the effect on LtaA activity (**Fig. 3B**). To do this, we constructed the mutant A53C-T366C-  
204 K166C-I250C that after cross-linking would close the extracellular pathway, while the mutant  
205 K80C-E339C-K141C-N276C would close the cytoplasmic pathway (**Fig. 3B and Suppl. Fig. 4A,B**).  
206 Our results show that in contrast to non-cross-linked proteins, both mutants display  
207 background quenching levels, similar to that observed for protein-free liposomes, thus,  
208 indicating strong inhibition of transport activity (**Fig. 3B**). We confirmed cross-linking of each  
209 double- and tetra-cysteine mutant reconstituted in proteoliposomes by gel shift assays after  
210 incubation with mPEG5K (**Fig. 3C,D and Suppl. Fig. 4D**), which showed that after treatment  
211 with copper chloride, all the mutants were protected from PEGylation, whereas before cross-  
212 linking a gel shift was observed. This confirmed that all mutants were successfully cross-linked  
213 in the proteoliposomes samples used in the assay.

214 Taking together, these results reveal that alternating opening to both sides of the  
215 membrane is a requirement for LtaA function. However, not all lateral openings seem to have  
216 the same functional relevance. In particular, our results demonstrate that while both  
217 extracellular lateral openings are similarly important for function, the cytoplasmic lateral  
218 opening lined by TM2 and TM11 has a more significant role, as revealed by the low activity of  
219 the cross-linked variant K80C-E339C. In contrast, cross-linking of the cytoplasmic lateral  
220 opening lined by TM5 and TM8, does not seem to affect LtaA function strongly.

221

222



## 223 **Dynamics of lateral openings**

224 For lipid transporters that adopt a ‘trap-and-flip’ mechanism, substrate binding and  
225 release involve movement of lipids through lateral openings of the translocation channel [18,  
226 21, 40, 43, 44]. We studied the dynamic behavior of the lateral openings in LtaA when the  
227 protein is embedded in a lipid bilayer. To do this, we performed molecular dynamics (MD)  
228 simulations of outward- and inward-facing LtaA in a membrane composed of POPS (65%),  
229 diacylglycerol (20 %), cardiolipin (10 %), and gentiobiosyl-diacylglycerol (5 %), resembling the  
230 membrane of *S. aureus* [50]. During the simulations both states were found to be stable as  
231 judged by RMSD plots (**Suppl. Fig. 5**). The simulations revealed that all the optimized inward-  
232 facing models exhibit a cavity which is open to the cytoplasm and closed to the extracellular  
233 space, whereas the cavity of the outward-facing state is open to the extracellular space and  
234 closed to the cytoplasm (**Fig. 4A**). In agreement with the observed wider opening of the  
235 extracellular lateral openings, the simulations of outward-facing LtaA showed the intrusion of  
236 glycolipid and POPS molecules into the putative translocation pathway (**Fig. 4C**). The glycolipid  
237 was seen to intrude from the TM5-TM8 opening, with one of the aliphatic tails reaching to the  
238 C-terminal hydrophobic pocket, whereas two POPS molecules intrude from the TM2-TM11  
239 opening (**Fig. 4C**). By contrast, simulations of inward-facing LtaA did not show lipids entering  
240 the cytoplasmic cavity.

241 In the MD simulations of the inward-facing conformation, we found that the  
242 cytoplasmic opening between TM2 and TM11 is wider and more dynamic than that between  
243 TM5 and TM8 (**Fig. 4B**). The lateral opening between TM2 and TM11 is thus a likely route for  
244 entry of glycolipids into the substrate binding site of the transporter. By contrast, in the  
245 outward-facing conformation, the two gates have similar widths and dynamics (**Fig. 4B**).  
246 Together with the results from the cysteine trapping analysis, this data suggests that both  
247 extracellular openings are a likely route for glycolipids exit, whereas the cytoplasmic lateral  
248 opening lined by TM2 and TM11 is more relevant for function as it may provide more room  
249 for glycolipids to enter the translocation pathway.

250

## 251 **The hydrophobic pocket is relevant for lipid transport**

252 Inspection of the central cavity in the *in silico* analysis shows that similarly to what was  
253 observed in the outward-facing crystal structure of LtaA [26], the central cavity of the inward-  
254 facing state is amphipathic (**Fig. 5A**). The cavity displays a hydrophilic pocket, enclosed mainly



255 by residues from the N-terminal domain (E32, R35, D68, W127 and W150), which we have  
256 previously shown to be relevant for recognition of the glycolipid headgroup [26], and a  
257 hydrophobic pocket, enclosed mainly by residues from the C-terminal domain (V234, L237,  
258 C263, L296, L300, L309, I316, and Y320) (**Fig. 5A**). The recent structure of the MFSD2A  
259 transporter trapped in an inward-facing conformation, displays a similar amphipathic central  
260 cavity (**Suppl. Fig. 6**) [21]. Computational docking of a glycolipid molecule to inward-facing  
261 LtaA suggests that the gentiobiosyl headgroup is preferentially accommodated in the  
262 hydrophilic pocket, whereas the diacylglycerol aliphatic tails are docked into the hydrophobic  
263 pocket (**Fig. 5B**).

264 A striking feature of the central cavity observed in LtaA and MFSD2A [21], and to our  
265 knowledge, not observed in other MFS structures available to date, is the presence of the  
266 highly hydrophobic pocket at the C-terminal domains of these transporters (**Fig. 5A and Suppl.**  
267 **Fig. 6**). To test the importance of this pocket in LtaA, we have designed mutants that introduce  
268 polar residues, thus making it more hydrophilic. We then evaluated growth of *S. aureus*  
269 NCTC8325  $\Delta$ *ltaA* cells complemented with ectopic copies of the *ltaA* gene carrying these  
270 mutations (**Fig. 5C and Suppl. Fig 7**). The variants V234T/L237N/I297S, C263S/L309Q/I316N,  
271 and Y320R display marked growth defects, whereas the mutant L296D/I316N do not affect  
272 growth. Each mutant was also purified and reconstituted into proteoliposomes, followed by  
273 determination of their flipping activity (**Fig. 5D**). In agreement with the results from *S. aureus*  
274 growth assays, the mutants V234T/L237N/I297S, C263S/L309Q/I316N, and Y320R display low  
275 relative activity compared to LtaA-WT (**Fig. 5D**), whereas L296D/I316N display the highest  
276 activity among all mutants. In contrast, introducing a mutation that scarcely increases the  
277 polarity of the cavity but that changes the size of residues V234 and I316, displayed low  
278 relative flipping activity compared to LtaA-WT, but does not affect growth of *S. aureus*  
279 NCTC8325  $\Delta$ *ltaA* cells (**Fig. 5C,D and Suppl. Fig 7**). Taken together, these results support a  
280 fundamental role of the hydrophobic pocket in glycolipid transport. As suggested by docking  
281 analysis, it is likely that this pocket is involved in binding of the aliphatic tails of the glycolipid  
282 substrate. The striking hydrophobicity of the C-terminal TM helices 7, 8, and 10 in multiple  
283 MFS lipid transporters (**Suppl. Fig. 8**), and the involvement in coordination of the aliphatic  
284 chain of lysophospholipid as revealed by the structure of MFSD2A [21], suggest a shared  
285 mechanistic role of the hydrophobic pocket in lipid-tails binding in MFS lipid transporters.

286

## 287 **The hydrophilic pocket dictates substrate specificity**

288           So far, our results suggest that during transport, LtaA encloses the full glycolipid  
289 substrate in the amphipathic cavity. However, understanding the relevance of the individual  
290 parts of the substrate molecule, headgroup and aliphatic chains, is fundamental for future  
291 design of pharmacologically relevant molecules targeting this and other MFS lipid transporters.  
292 To gain insight into whether LtaA displays higher selectivity towards the headgroup than for  
293 the diacylglycerol moiety, we performed flipping assays with LtaA-WT co-reconstituted in  
294 proteoliposomes together with NBD-labeled Glc<sub>2</sub>-DAG (gentiobiosyl-diacylglycerol) and  
295 increasing concentrations of Gal<sub>2</sub>-DAG (digalactosyl-diacylglycerol) (**Fig. 5E**). Glucose and  
296 galactose differ only in the orientation of the -OH group at the C-4 position. Thus, we  
297 hypothesized that if the headgroup is more relevant for substrate recognition than the  
298 aliphatic chains, then transport of Glc<sub>2</sub>-DAG-NBD will be not be affected, since the difference  
299 between glucose and galactose would preclude Gal<sub>2</sub>-DAG from being a good competitor. On  
300 the other hand, if the diacylglycerol moiety is more relevant for substrate recognition, we  
301 expect Gal<sub>2</sub>-DAG to be a strong competitor, thus, resulting in marked decrease of Glc<sub>2</sub>-DAG-  
302 NBD transport. Our results show that even under a high excess of Gal<sub>2</sub>-DAG, there is no  
303 significant effect on Glc<sub>2</sub>-DAG-NBD transport (**Fig. 5E**). We have previously shown that  
304 gentiobiose ( $\beta$ -D-Glc-(1,6)-D-Glc), a disaccharide with the same composition and  
305 conformation as the glycolipid headgroup (Glc<sub>2</sub>-DAG), inhibits lipid transport [26]. Taken  
306 together, these results suggest that an intact headgroup is highly relevant for substrate  
307 binding and transport, and that even small changes to the headgroup abolishes recognition.  
308 Independent of the presence of the diacylglycerol moiety and its predicted binding to the  
309 hydrophobic pocket, the headgroup seems to dictate whether a glycolipid can be a substrate  
310 for LtaA or not.

311

## 312 **Discussion**

313           Several transporters of the MFS superfamily have been structurally characterized in  
314 one or multiple conformational states [4, 8-17] However, except for the outward-facing  
315 structure of LtaA [26], solved by X-ray crystallography, and the inward-facing structure of  
316 MFSD2A [21], solved by single particle cryo-electron microscopy, there are no additional  
317 structures available of MFS lipid transporters. Despite the differences among their lipid  
318 substrates, the distinct composition of bacterial and eukaryotic membranes, and their

319 opposite vectorial lipid transport directions, LtaA and MFSD2A share multiple architectural  
320 similarities, including a canonical MFS fold of 12 TM helices and an amphipathic central cavity  
321 with asymmetric distribution of hydrophobic and hydrophilic residues (**Suppl. Fig. 6**). A similar  
322 arrangement of central cavity residues has been predicted to be present in the bacterial  
323 lysophospholipid transporter LpIT [18], and are likely to be part of the architecture of other  
324 MFS lipid transporters (**Suppl. Fig. 8**). These characteristics suggest a common mechanism of  
325 substrate recognition and translocation among these proteins. Indeed, LtaA and MFSD2A  
326 display strong selectivity towards the headgroup of their lipid substrates [6, 26]. In the case of  
327 MFSD2A the zwitterionic charge of the phosphatidylcholine headgroup is fundamental for  
328 ligand transport, whereas LtaA displays strong selectivity towards the gentiobiosyl  
329 disaccharide headgroup of the glycolipid. Furthermore, LtaA selects against an isomer of the  
330 disaccharide headgroup as shown by the poor competition displayed by digalactosyl-  
331 diacylglycerol in transport assays (**Fig. 5E**). In contrast, LpIT has been shown to exhibit a more  
332 relaxed specificity towards the lipid headgroup, being able to transport  
333 lysophosphatidylethanolamine and lysophosphatidylglycerol lipids [18].

334 Although MFSD2A and LpIT have been shown to strongly select for lysophospholipids,  
335 they display relaxed selectivity towards the length of the aliphatic chains [6, 18]. MFSD2A  
336 transports docosahexaenoic acid (DHA), an essential omega-3 fatty acid for brain growth and  
337 cognitive function, in the form of lysophosphatidylcholine, but can also transport other lipids  
338 with at least 14-carbons acyl chain [6]. It is noteworthy that *S. aureus* membranes are rich in  
339 diacylglycerols with chains length ranging from C<sub>15</sub> to C<sub>18</sub>, with the most dominant lipid species  
340 having a C<sub>17</sub>:C<sub>15</sub> composition [25]. This variability among diacylglycerols in *S. aureus*, and the  
341 measurable translocation of Glc<sub>2</sub>-DAG-NBD [26], which has a C<sub>10</sub> acyl chain length and an NBD  
342 group linked to one of the diacylglycerol chains, suggest that LtaA displays similar relaxed  
343 specificity towards the length of the lipid part.

344 Our results strongly suggest that in contrast to mechanisms proposed for other lipid  
345 transporters, LtaA transports gentiobiosyl-diacylglycerol by a ‘trap-and-flip’ mechanism,  
346 which follows the classical alternating-access model of transport [51], with the entire  
347 glycolipid entering and leaving the central translocation pathway (**Fig. 5F**). Inward-facing LtaA  
348 binds a glycolipid molecule which enters through the lateral opening lined by TM2 and TM11,  
349 as suggested by the cross-linking analysis and MD simulations. This triggers a conformational  
350 change to an outward-facing state, in which the glycolipid is released into the membrane

351 presumably through any of the two extracellular lateral openings. Protonation of residues in  
352 the hydrophilic pocket allows transition to the inward-facing conformation, followed by  
353 proton release to the cytoplasm.

354         Similar to the asymmetric opening of extracellular and cytoplasmic cavities described  
355 here for LtaA, MFSD2A exhibits a wider opening of its cytoplasmic lateral openings [21]. In this  
356 case, the extracellular opening is predicted to be narrow due to the constriction imposed by  
357 a disulfide bond at the extracellular side of the transporter [21]. Our results suggest that LtaA  
358 exhibit wide open extracellular lateral openings, whereas the cytoplasmic openings are  
359 narrower. The distinct constrictions of the cavities that recruit the lipid substrate, cytoplasmic  
360 cavity in LtaA and extracellular cavity in MFSD2A, might be part of a selectivity filter that  
361 confers substrate specificity.

362         In summary, our results provide insights into the molecular mechanism of glycolipid  
363 transport by LtaA and support a ‘trap-and-flip’ model where asymmetrically open lateral  
364 ‘gates’ and extracellular and cytoplasmic cavities play an essential role. Our data suggests that  
365 the highly selective hydrophilic pocket dictates substrate specificity, but that the hydrophobic  
366 pocket is fundamental for aliphatic chains transport. The mechanistic elements described here  
367 might be shared by other MFS lipid transporters and can be decisive for the design of drugs  
368 targeting these proteins.

369

## 370 **Methods**

371 **LtaA expression and purification.** The gene encoding *S. aureus* LtaA was cloned into a  
372 modified pET-19b vector (Novagen), with an N-terminal His<sub>10</sub> affinity tag. LtaA WT and  
373 mutants were expressed in *E. coli* BL21 Gold (DE3) (Stratagene) cells. Cells were grown in  
374 Terrific Broth (TB) medium supplemented with 1% glucose (w/v) at 37°C. Overexpression was  
375 induced with 0.2 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) for 1h. All following steps  
376 were performed at 4 °C, unless different specified. Cells were harvested by centrifugation,  
377 resuspended in 50mM Tris-HCl, pH 8.0; 500mM NaCl; 5mM β-mercaptoethanol; 0.5mM PMSF  
378 and disrupted in a M-110L microfluidizer (Microfluidics) at 10000 psi chamber pressure.  
379 Membranes were pelleted by ultracentrifugation and solubilized in 50 mM Tris-HCl, pH 8.0;  
380 200mM NaCl; 20mM Imidazole; 15% glycerol (v/v); 5mM β-mercaptoethanol; 1% Lauryl  
381 Maltose Neopentyl Glycol (w/v) (LMNG, Anatrace); 1% N-dodecyl-β-D-maltopyranoside (w/v)  
382 (DDM, Anatrace). After removing debris, the supernatant was loaded onto a pre-equilibrated

383 NiNTA superflow affinity column (Qiagen). The column was washed with 50mM Tris-HCl, pH  
384 8.0; 200mM NaCl; 50mM Imidazole; 10% glycerol (v/v); 5mM  $\beta$ -mercaptoethanol; 0.02%  
385 LMNG and 0.02% DDM and then further washed with the same buffer only containing 0.02%  
386 LMNG. Elution was performed in the same buffer containing 200mM Imidazole. Buffer  
387 exchange to buffer 10 mM Tris-HCl pH 8.0; 150mM NaCl; 0.02% LMNG was performed using  
388 PD-10 columns (GE Healthcare). Analytical size exclusion chromatography was performed on  
389 a Superdex 10/300 GL column (GE Healthcare) in buffer 10 mM Tris-HCl, pH 8.0; 150 mM NaCl;  
390 0.02% LMNG. [52]

391  
392 **Mutagenesis.** LtaA mutants were generated using overlap Extension-PCR, followed by DpnI  
393 digestion for two hours at 37°C, and transformation into *E. coli* DH5 $\alpha$  cells. The mutations  
394 were confirmed by DNA sequencing (Microsynth). All oligos used for mutagenesis are listed in  
395 **Suppl. table 2.**

396  
397 **YpfP expression and purification.** The gene encoding *S. aureus* YpfP was cloned into a  
398 modified pET-19b vector (Novagen) with an N-terminal His<sub>10</sub> affinity tag. YpfP was  
399 overexpressed in BL-21 Gold (DE3) (Stratagene) cells. Cells were grown in TB medium  
400 supplemented with 1% glucose (w/v) at 37 °C until a cell density of OD<sub>600</sub> = 3. Subsequently,  
401 cells were induced with 0.2 mM IPTG for 16h at 24 °C. Cells were harvested by centrifugation  
402 and resuspended in buffer A (50mM Tris-HCl pH 8.0; 200mM NaCl; 3% glycerol; 3mM  $\beta$ -  
403 mercaptoethanol) plus 0.5mM PMSF. Cells were disrupted using a tip sonication. After  
404 differential centrifugation, the supernatant containing YpfP was incubated with NiNTA resin  
405 and left stirring for 1h at 4 °C. Washing was performed with buffer A complemented with 50  
406 mM imidazole pH 8.0, followed by elution with buffer A complemented with 200 mM  
407 imidazole pH8.0. YpfP was desalted in buffer 50 mM Tris-HCl pH 8.0; 200 mM NaCl; 10%  
408 glycerol using PD-10 columns (GE healthcare). If required YpfP was concentrated using a  
409 Vivaspin 20 30MWCO until 2.4 mg/ml, flash frozen in liquid nitrogen and stored at -80 °C until  
410 further use.

411  
412 **Synthesis of NBD-glycolipid and glycolipid.** Synthesis of glycolipid and nitrobenzoxadiazole  
413 (NBD)-labelled glycolipid was performed using a modification of the protocol described by  
414 Jorasch et al [53] and Kiriukhin et al [54]. A final concentration of 2mM UDP-Glucose (Sigma),

415 2mM NBD-decanoyl-2-decanoyl-sn-Glycerol (Cayman), and 1.2mg/ml purified YpfP were  
416 incubated together for 16h at 30°C. The reaction product was separated using thin-layer  
417 chromatography (TLC) with a silica gel matrix (Sigma) in a solvent mixture consisting of  
418 chloroform:methanol:water (65:25:4, vol/vol/vol). Silica containing the NBD-glycolipid was  
419 recovered from plates, and the NBD-glycolipid was extracted from the silica by incubation with  
420 a solvent mixture of chloroform:methanol (50:50, vol/vol), followed by drying of the anchor-  
421 LLD under argon atmosphere, and subsequently resuspension in 20 mM Tris-HCl pH 8.0; 150  
422 mM NaCl. NBD-glycolipid was flash frozen in liquid nitrogen, and stored at -80°C until further  
423 use. Reaction products were previously characterized [26]. Non-labelled glycolipid was  
424 prepared similarly by incubation of 2mM UDP-Glucose, 2 mM 1,2-dimyristoyl-sn-glycerol  
425 (Avanti) and 1.2 mg/ml YpfP for 16h at 30°C.

426  
427 **Formation of LtaA proteoliposomes.** LtaA was reconstituted in unilamellar liposomes  
428 prepared by extrusion through polycarbonate filters (400 nm pore size) from a 3:1 (w/w)  
429 mixture of *E. coli* polar lipids and L- $\alpha$ -phosphatidylcholine (Avanti polar lipids) resuspended in  
430 20 mM Tris-HCl pH 8.0; 150mM NaCl and 2mM  $\beta$ -mercaptoethanol. After saturation with DDM  
431 (Anatrace), liposomes were mixed with purified LtaA in a 50:1 (w/w) lipids/protein ratio. DDM  
432 was removed after incubation with BioBeads (BioRad). Proteoliposomes were centrifugated,  
433 washed and resuspended to a final concentration of 20mg/ml lipids; 7.8 $\mu$ M LtaA. The  
434 proteoliposomes were flash-frozen in liquid nitrogen and stored at -80°C until further use.

435  
436 **In vitro flipping assay.** Before performing flipping assays, proteoliposomes were thawed, their  
437 resuspension buffer was exchanged to 20 mM MES pH 6.5; 150 mM NaCl, and the product of  
438 the NBD-glycolipid synthesis reaction was incorporated by performing freeze/thaw cycles.  
439 Proteoliposomes and protein-free liposomes were diluted to a concentration of 2 mg/ml lipids  
440 followed by extrusion through polycarbonate filters (400 nm pore size). Proteoliposomes were  
441 immediately used for flipping assays. In case of competition assays with  
442 digalactosyldiacylglycerol (DGDG). DGDG powder (Avanti) was resuspended in 20 mM Tris-  
443 HCl; 150 mM NaCl and incorporated into proteoliposomes during freeze/thaw cycles together  
444 with the NBD-glycolipid. Flipping of NBD-glycolipid was assessed by determining the  
445 percentage of NBD-fluorescence that is quenched after addition of a 5 mM sodium dithionite  
446 (Sigma) after 200 seconds of starting fluorescence recording. 100 seconds before finishing



447 data recording, 0.5% Triton X100 was added to permeabilize the liposomes, making all NBD-  
448 glycolipid molecules accessible to dithionite reduction. The fluorescence after Triton X100  
449 addition was used for baseline calculations. Fluorescence was recorded at 20°C using a Jasco  
450 Fluorimeter. The excitation and emission wavelengths were 470 and 535 nm, respectively. For  
451 analysis the fluorescence intensity was normalized to  $F/F_{\max}$ . Relative flipping activities were  
452 calculated as follows: relative activity =  $100 \times ((F/F_{\max})_i - (F/F_{\max})_{\text{liposomes}}) / ((F/F_{\max})_{\text{wt}} -$   
453  $(F/F_{\max})_{\text{liposomes}})$ , where i corresponds to each respective treatment/mutants, liposomes  
454 corresponds to liposomes without protein, wt corresponds to wild type LtaA and  $F/F_{\max}$   
455 values correspond to the normalized fluorescence values at the plateau after addition of  
456 sodium dithionite. Curves were plotted using GraphPad Prism 8. Time courses of the  
457 dithionite-induced fluorescence decay in liposomes were repeated at least 3 times for each  
458 individual experiment.

459

460 **Proton-transport assay.** LtaA proteoliposomes and protein-free liposomes were thawed, and  
461 their internal buffer exchanged to 5 mM HEPES pH7.3; 100mM KCl. Glycolipid was  
462 incorporated during freeze/thaw cycles followed by extrusion through polycarbonate filters  
463 (400 nm pore size). After 90s of sonication, proteoliposomes and protein-free liposomes were  
464 diluted 25-fold in buffer containing 5 mM HEPES pH 7.3; 10 mM KCl; 90 mM NaCl; 0.5  $\mu\text{M}$  9-  
465 amino-6-chloro-2-methoxyacridine (ACMA). Fluorescence was recorded using a Jasco  
466 Fluorimeter with excitation and emission wavelengths of 410 and 480 nm respectively. When  
467 the fluorescence signal was stable,  $\text{H}^+$  influx was initiated by establishing a membrane  
468 potential by the addition of the potassium ionophore valinomycin (5 nM). Time courses of the  
469 proton-transport assay in proteoliposomes were repeated at least 3 times for each individual  
470 experiment. Crosslinking was performed before the measurement by addition of 2 mM  $\text{CuCl}_2$   
471 to the proteoliposomes during the buffer exchange and incorporation of glycolipid steps. After  
472 1h incubation at RT in the dark,  $\text{CuCl}_2$  was removed by centrifugation, and proteoliposomes  
473 were resuspended in buffer 5 mM HEPES, pH7.3; 100mM KCl.

474

475 **LtaA crosslinking and PEGylation.** LtaA mutants incorporated into proteoliposomes were  
476 incubated with 2 mM  $\text{CuCl}_2$  or N,N'-1,2-phenylenedimaleimide (o-PDM) for 1h at RT in the  
477 dark. In case of non-crosslinked samples, proteoliposomes were incubated with a proportional  
478 volume of DMSO or buffer. Crosslinkers were removed by centrifugation and washing with



479 buffer. To PEGylate free cysteines, LtaA mutants were incubated for 3h at RT in the presence  
480 of 0.5 mM mPEG5K-Maleimide (Sigma) and 0.5% SDS. Proteins were separated on 15%  
481 polyacrylamide gels and visualized with QuickBlue Protein stain (Lubio science).

482

483 **Sample preparation for LC-MS analysis.** LtaA mutants were purified as described above, and  
484 concentrated to a concentration of 0.6 mg/ml. Purified LtaA was incubated for 1h at RT in the  
485 dark in the absence or presence of 2 mM o-PDM. Afterwards, 10 mM  $\beta$ -mercaptoethanol was  
486 added to quench the crosslinker. 1-2  $\mu$ g of either crosslinked or non-crosslinked LtaA protein  
487 were dissolved in 20  $\mu$ l digestion buffer (0.02% of LMNG; 1 M urea; 0.1 M  
488 ammoniumbicarbonate; 10 mM tris(2-carboxyethyl) phosphine (TCEP); 15 mM  
489 chloroacetamide, pH = 8.5), reduced and alkylated for 1h at 37 °C. Proteins were digested by  
490 incubation with either sequencing-grade modified trypsin (1/50, w/w; Promega, Madison,  
491 Wisconsin), chymotrypsin sequencing grade (1/50, w/w, Sigma-Aldrich) or lys-C (1/100, w/w,  
492 Wako) overnight at 37°C. Then, the peptides were cleaned using iST cartridges (PreOmics,  
493 Munich) according to the manufacturer instructions. Samples were dried under vacuum and  
494 dissolved in 0.1 % formic acid solution at 0.5 pmol/ $\mu$ l. All samples were prepared in triplicates.

495

496 **Label-free targeted PRM-LC-MS analysis of cysteine-containing peptides.** In a first step,  
497 parallel reaction-monitoring (PRM) assays [55] were generated for all the peptides of LtaA WT  
498 and the peptides of the 5 different LtaA cysteine mutants, for each protease. These peptides  
499 include the reference peptide for normalization, that is shared for all mutants. Therefore, the  
500 specific peptide sequences were loaded into Skyline (version 20.2.0.343 (<https://brendanx-uw1.gs.washington.edu/labkey/project/home/software/Skyline/begin.view>) and transitions  
501 were predicted using the integrated PROSIT algorithm for double and triple charged  
502 precursors. Then, protease and isoform specific isolation mass lists were exported and used  
503 to generate specific targeted LC-MS analyses. This analysis was carried as described previously  
504 [56]. Chromatographic separation of peptides was carried out using an EASY nano-LC 1000  
505 system (Thermo Fisher Scientific), equipped with a heated RP-HPLC column (75  $\mu$ m x 30 cm)  
506 packed in-house with 1.9  $\mu$ m C18 resin (Reprosil-AQ Pur, Dr. Maisch). Aliquots of 1 pmol total  
507 peptides were analyzed per LC-MS/MS run using a linear gradient ranging from 95% solvent A  
508 (0.15% formic acid, 2% acetonitrile) and 5% solvent B (98% acetonitrile, 2% water, 0.15%  
509 formic acid) to 30% solvent B over 90 minutes at a flow rate of 200 nl/min. Mass spectrometry  
510

511 analysis was performed on a Q-Exactive plus mass spectrometer equipped with a  
512 nanoelectrospray ion source (both Thermo Fisher Scientific) using a hybrid DDA (top5)/PRM  
513 LC-MS analysis. In detail, each MS1 scan was followed by high-collision-dissociation (HCD) of  
514 the precursor masses of the imported isolation list and the 5 most abundant precursor ions  
515 with dynamic exclusion for 20 seconds. For each mutant and protease, a specific LC-MS  
516 method was generated. Total cycle time was approximately 1 second. For MS1, 3e6 ions were  
517 accumulated in the Orbitrap cell over a maximum time of 100 ms and scanned at a resolution  
518 of 70,000 FWHM (at 200 m/z). Targeted MS2 scans were acquired at a target setting of 3e6  
519 ions, accumulation time of 100 ms and a resolution of 35,000 FWHM (at 200 m/z) and a mass  
520 isolation window to 0.4 Th. MS1 triggered MS2 scans were acquired at a target setting of 1e5  
521 ions, a resolution of 17,500 FWHM (at 200 m/z) and a mass isolation window of 1.4 Th. Singly  
522 charged ions and ions with unassigned charge state were excluded from triggering MS2 events.  
523 The normalized collision energy was set to 27% and one microscan was acquired for each  
524 spectrum. The acquired raw-files were converted to mgf-file format using MSConvert (v 3.0,  
525 proteowizard) and searched using MASCOT (Matrix Science, Version: 2.4.1) against a decoy  
526 database containing normal and reverse sequences of the predicted SwissProt entries of  
527 *Staphylococcus aureus* (strain NCTC 8325 / PS 47, [www.ebi.ac.uk](http://www.ebi.ac.uk), release date 2020/08/21).  
528 The 5 LtaA mutants and commonly observed contaminants (in total 6,574 sequences) were  
529 generated using the SequenceReverser tool from the MaxQuant software (Version 1.0.13.13).  
530 The search criteria were set as following: full tryptic specificity was required (cleavage after  
531 lysine or arginine residues); 3 missed cleavages were allowed; carbamidomethylation (C) was  
532 set as fixed modification and oxidation (M) as variable modification. The mass tolerance was  
533 set to 10 ppm for precursor ions and 0.02 Da for fragment ions. Then, Scaffold (version  
534 Scaffold\_4.11.1, Proteome Software Inc., Portland, OR) was used to validate MS/MS based  
535 peptide and protein identifications. Peptide identifications were accepted if they could be  
536 established at a probability greater than 97.0% by the Scaffold Local FDR algorithm. Protein  
537 identifications were accepted if they could be established at a probability higher than 99.0%  
538 to achieve an FDR less than 1.0% and contained at least 2 identified peptides. Protein  
539 probabilities were assigned by the Protein Prophet algorithm [57]. Proteins that contained  
540 similar peptides and could not be differentiated based on MS/MS analysis alone were grouped  
541 to satisfy the principles of parsimony. Subsequently, all raw-files were imported into Skyline  
542 for protein/peptide quantification. To control for variation in sample amounts, all intensities

543 were normalized against the 4 cysteine-free reference peptides. Only peptides that could be  
544 confidently identified by database searching were considered for quantification by PRM using  
545 the predicted transitions. Statistical analysis and ratio calculations to compare the relative  
546 abundance of the peptides between non-crosslinked and crosslinked peptides were  
547 performed in Excel. Histograms and P values were generated using Prism 9.

548  
549 **Docking of glycolipid.** A 1,2-dihexadecanoic-3-O-( $\beta$ -D-glucopyranosyl-1 $\rightarrow$ 6-O- $\beta$ -D-  
550 glucopyranosyl-sn-Glycerol molecule was docked to the LtaA inward-facing model with  
551 Autodock Vina [58]. The initial glycolipid coordinates were generated from 2D geometry in  
552 Phenix (eLBOW) [59]. The stereochemistry was corrected in Phenix (REEL)[59]. Docking was  
553 performed over a search space of  $50 \times 44 \times 76 \text{ \AA}^3$  covering the central cavity.

554  
555 ***S. aureus* phenotypic complementation assay.** Generation of pLOW-ltaA and of  
556 *Staphylococcus aureus* NCTC8325  $\Delta$ ltaA genotype was previously described [26]. pLOW vector  
557 was used for construction of ltaA complementary strains. Point mutations were generated by  
558 extension overlap PCR, and then with restriction-ligation cloning using Sall and NotI cloned  
559 into pLOW vector [60]. For cloning purposes *E. coli* IM08B was used[61]. The sequence of the  
560 resulting constructs was confirmed by DNA sequencing (Microsynth). After conformation of  
561 the correct constructs, pLOW vector carrying ltaA WT or point mutations were introduced into  
562 *S. aureus* NCTC8325  $\Delta$ ltaA by electrophoresis with erythromycin selection (5 $\mu$ g/ml). *S. aureus*  
563 cells were grown in 3 ml of Luria-Bertani (LB) medium at 37 °C with 200 rpm until OD<sub>600</sub> of  
564 0.3. For complementary strains containing a pLOW vector, a final concentration of 5  $\mu$ g/ml  
565 was added to the medium. For the serial dilutions, 5  $\mu$ l of the original and its dilutions were  
566 spotted on LB agar plates buffered with sodium phosphate at pH 6.4 complemented with 0.1  
567 mM IPTG. The plates were incubated overnight at 37 °C. Pictures were taken the next morning.

568  
569 **Preparation of *S. aureus* membranes for LC-MS analysis.** *S. aureus* cells were grown in 3 ml  
570 LB medium at 37 °C with 200 rpm until OD<sub>600</sub> of 0.4. For complementary strains containing a  
571 pLOW vector, a final concentration of 5  $\mu$ g/ml and 0.1 mM IPTG were added to the medium.  
572 After harvesting the cells were resuspended in 10 mM Tris pH8.0; 1 mM EDTA; 25  $\mu$ g/ml  
573 lysostaphin, and incubated for 0.5h at 37 °C. Cells were further subjected to sonication,  
574 followed by collection of membranes by ultracentrifugation. The membranes were

575 resuspended in 100 mM Tris-HCl; 5% SDS; 10 mM tris(2-carboxyethyl) phosphine (TCEP).  
576 Samples were sonicated for 10 minutes, followed by shaking for 1h at 37 °C with 500 rpm. To  
577 reduce and alkylate the disulfides a final concentration of 15 mM iodoacetamide was added,  
578 and the samples were incubated for 0.5h in the dark at room temperature. Samples were  
579 loaded on S-trap Micro Spin column (Protifi). After washing, on column peptide digestion was  
580 performed by addition of trypsin in 50 mM triethylammonium bicarbonate (TEAB) buffer, and  
581 incubation of 1h at 47 °C. Digested peptides were collected by passing 50 mM  
582 triethylammonium bicarbonate (TEAB) buffer, 0.2 % formic acid (w/v) in distilled water, and  
583 0.2 % formic acid (w/v) in 50% acetonitrile (v/v) through the column and dried in a SpeedVac  
584 (Labconco). Dried peptides were re-suspended in 0.1% formic acid (w/v) and stored at -20 °C.

585

586 **Targeted PRM LC-MS analysis of LtaA WT and mutants.** As a first step, PRM assays [55] for all  
587 possible peptides of LtaA with a length of 6 to 25 amino acids comprising double- and triple-  
588 charged precursor ions were created. Five peptides were identified to match the length and  
589 charge criteria, leading to ten PRM assays in total. These were used to identify LtaA membrane  
590 fractions of wild-type *S. aureus*. The setup of the  $\mu$ RPLC-MC system was previously  
591 described[56]. Mass spectrometry analysis was conducted using a Q-Exactive mass  
592 spectrometer with a nano-electrospray ion source (both Thermo Fisher Scientific). Each MS1  
593 scan was followed by high-collision-dissociation (HCD) of the ten LtaA precursor ions in PRM  
594 mode using a global isolation mass list. By applying strict identification criteria, three peptide  
595 ions of LtaA LTNYNTRPVK (2+ and 3+ ion) and MQDSSLNNYANHK (2+) were identified, and  
596 these were used for label-free PRM quantification. To control for protein variation between  
597 different samples, the total ion chromatography (only comprising peptide with two or more  
598 charges) was determined for each sample by label-free quantification using Progenesis Q1  
599 (version 2.0, Waters) and then used to normalize the samples. The integrated peak areas of  
600 the three peptide ions that were quantified by PRM were summed up, and used for LtaA  
601 quantification.

602

603 **Modeling of inward-facing conformation.** The inward-facing conformation was modelled  
604 under the assumption of inverted repeats [47]. Sequence alignments between the two  
605 repeats of each domain of LtaA were performed. We structurally aligned R1D1 (residues 16–  
606 105) with R2D1 (residues 109-189), and R1D2 (residues 220-302) with R2D2 (residues 309-

607 393) using the structure alignment program TMalign resulting in two pairs of alignments.  
608 These two pairs of alignments were then used together to build up the final pair-wise  
609 alignment between the LtaA sequence and a template in which the LtaA sequence repeats  
610 were rearranged in the order R1D2-R1D1-R2D2-R2D1. The initial sequence alignment was  
611 then refined by removing gaps in the transmembrane regions and in the secondary structure  
612 elements. Further refinements were made to match the secondary structure as observed in  
613 the outward-open crystal structure. In particular, we aimed to maintain the helical regions in  
614 the template where possible, subject to the pseudo-symmetry between the two MFS  
615 transporter domains. We used this alignment and the X-ray crystal structure of LtaA (PDB  
616 entry 6S7V)[26] to construct the inward-facing model templates using Modeller 9v24. In 100  
617 templates, the side chain packing of the models were re-built using SCWRL4 model. Next, we  
618 selected 7 models with the highest MODELLER score and the best MolProbity [62] profile for  
619 further analysis. Then, we repacked the side chains using SCWRL4.0[63] and as a last step the  
620 models were energetically minimized after placing them in the lipid bilayer using the Gromacs  
621 2019.6 steepest descent algorithm for 5,000 steps [64]. To further validate the quality of the  
622 models, we assessed the stereochemistry. Evaluation of the model using MolProbity showed  
623 that the final minimized models have reasonable qualities (MolProbity score: 2.00-2.3,  
624 Ramachandran favored: 92.1-93.6%, and Ramachandran outliers: 0.8-1.90%) (**Suppl. Table 1**).

625  
626 **Molecular dynamics simulations of inward-facing conformation models.** To study their  
627 dynamics, each of the optimized inward-facing models was placed in a heterogenous lipid  
628 bilayer (POPG (65%), diacylglycerol (20 %), cardiolipin (10 %), and gentiobiosyl-diacylglycerol  
629 (5 %)) and then solvated in TIP3P water with 150 mM NaCl. The all-atom CHARMM36m force  
630 field was used for lipids, ions, and protein [65-67]. All simulations were performed using  
631 GROMACS 2019.6 [64]. The starting systems were energy-minimized for 5,000 steepest  
632 descent steps and equilibrated first for 1 ns of MD simulations in a canonical (NVT) ensemble  
633 and then for 7.5 ns in an isothermal-isobaric (NPT) ensemble under periodic boundary  
634 conditions. The initial restrains on the positions of nonhydrogen protein atoms were 4,000  
635 kJ·mol<sup>-1</sup>·nm<sup>2</sup>. During equilibration, these restraints were gradually released. Particle-mesh  
636 Ewald summation with cubic interpolation and a 0.12-nm grid spacing was used to treat long-  
637 range electrostatic interactions [68]. The time step was initially 1 fs, and was then increased  
638 to 2 fs during the NPT equilibration. The LINCS algorithm was used to fix all bond lengths [69].

639 Constant temperature was set with a Berendsen thermostat [70], combined with a coupling  
640 constant of 1.0 ps. A semi-isotropic Berendsen barostat [70] was used to maintain a pressure  
641 of 1 bar. During production runs, the Berendsen thermostat and barostat were replaced by a  
642 Nosé–Hoover thermostat [71] and a Parrinello–Rahman barostat [72] The unconstrained  
643 production trajectories were analyzed with Visual Molecular Dynamics (VMD) [73] and  
644 MDAAnalysis package [74, 75]. A simulation of each inward-facing model was performed for  
645 150 ns.

646  
647 **Molecular dynamics simulations of outward-facing conformation.** The outward-facing  
648 structure of LtaA (PDB ID 6S7V) was embedded in a lipid bilayer composed of POPG-DAG-CL-  
649 gentiobiosyl-diacylglycerol using CHARMM-GUI [76]. The system was then solvated in TIP3P  
650 water with 150 mM NaCl. The all-atom CHARMM36m force field was used for lipids, ions, and  
651 protein [65-67]. All simulations were performed using GROMACS 2019.6 [64]. Simulations  
652 were performed with similar protocols as described above for inward-facing models. The  
653 simulation of the outward-facing structure was performed for 960 ns.

654

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835

836 **Author Contributions**

837 E.L. performed *in vitro* and *in vivo* biochemical characterization of LtaA and variants. A.R.M.  
838 performed computational analysis. C.P. supervised the biochemical analysis. G.H. supervised  
839 computational analysis. A.S. and E.L. performed mass spectrometry analysis. E.L., A.R.M, and  
840 C.P analyzed the computational, structural and functional data. C.P. conceived and directed  
841 the project. All authors contributed to manuscript writing and revision.

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843 **Author Information**

844 Competing interests: None declared.

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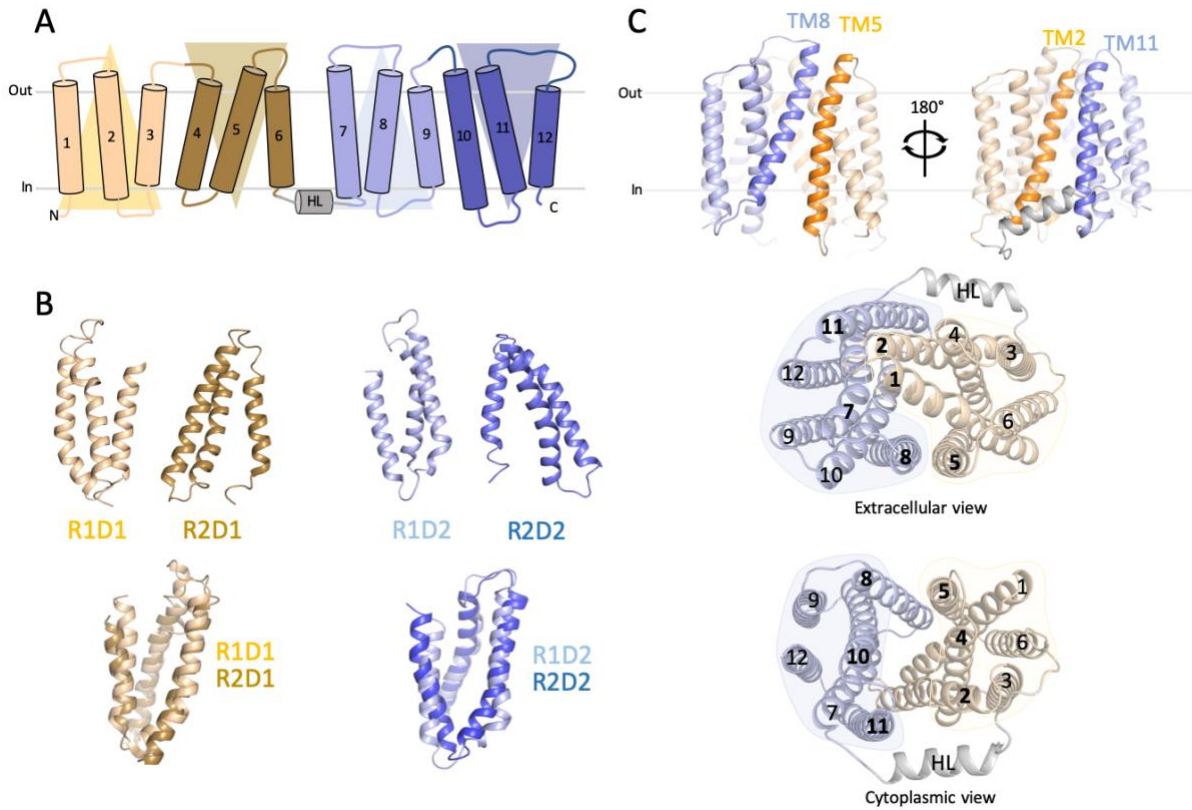
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859 **Figures and Figure legends**



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861 **Figure 1. 'Repeat swap' modeling of inward-facing LtaA.** A. Topology representation of LtaA.

862 Domain-1 (N-terminal) and domain-2 (C-terminal) are shown in light orange and light blue,

863 respectively. B. Individual repeat domains as observed in outward-facing LtaA (PDB ID 6S7V),

864 and superposition of inverted repeats (r.m.s.d. = 2.5 Å and 3.0 Å for aligned Ca atoms of

865 R1D1/R2D1 and R1D2/R2D2, respectively). R1D1 and R2D1 indicate the first and second

866 repeats in the N-terminal domain, respectively, whereas R1D2 and R2D2 indicate the first and

867 second repeat in the C-terminal domain, respectively. Colors are according to panel A. C. Side-

868 views of inward-facing LtaA model showing TM helices that line the lateral openings.

869 Extracellular and cytoplasmic views are also shown.

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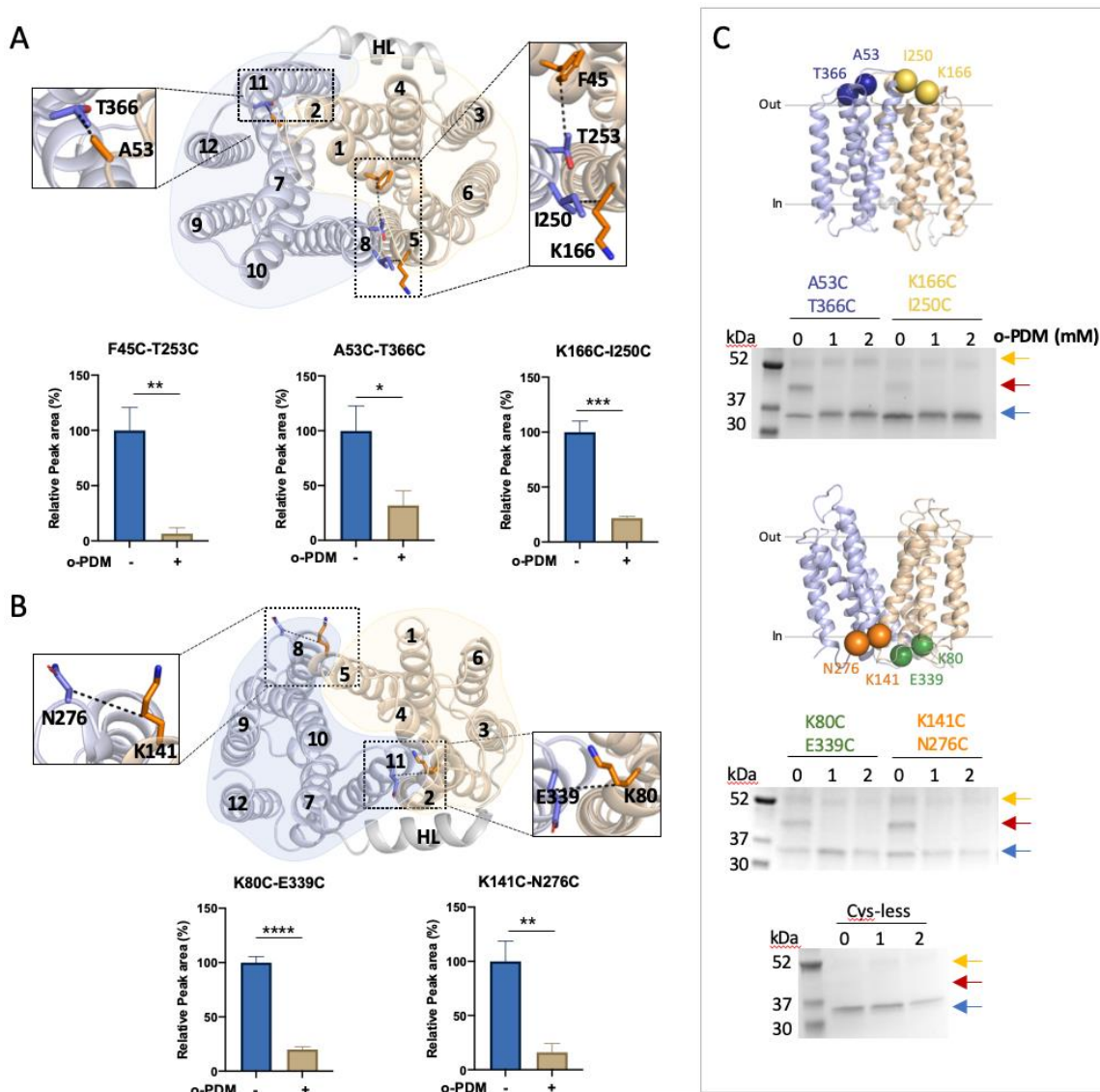
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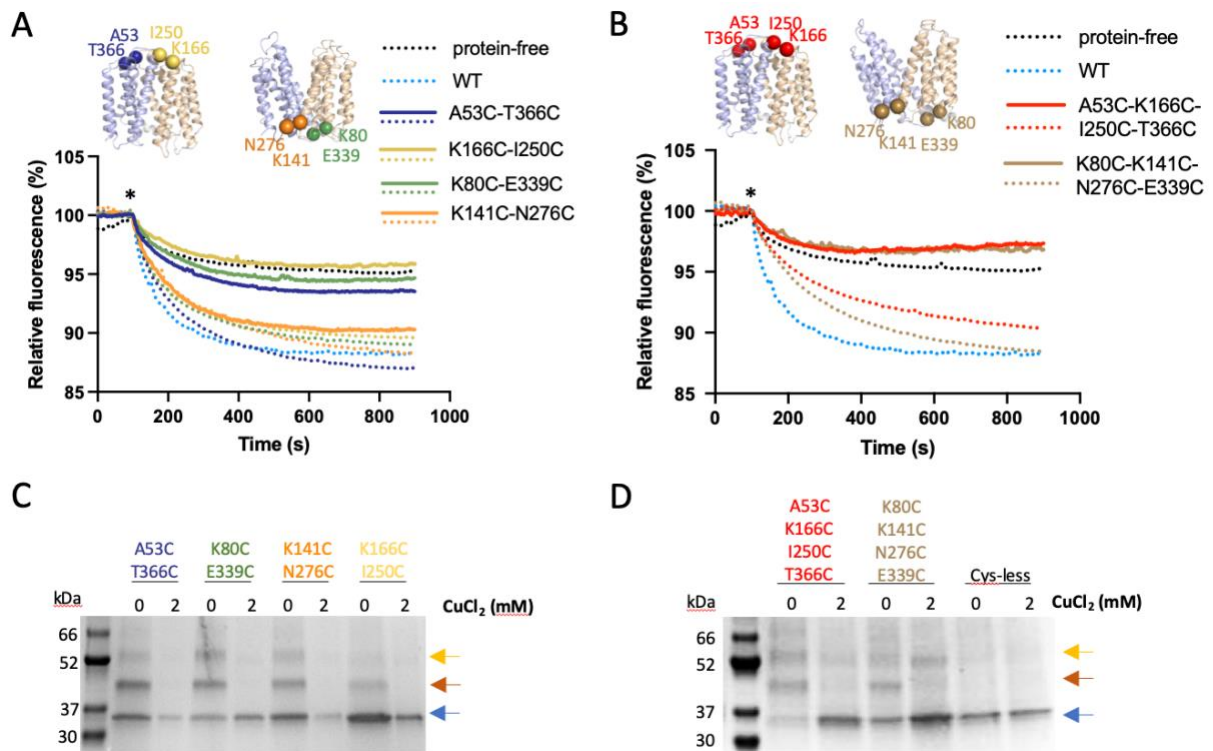
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877 **Figure 2. LtaA adopts inward- and outward-facing states.** Selected residues for cross-linking  
 878 of LtaA in inward-facing conformation (A) and outward-facing conformation (B). N-terminal  
 879 and C-terminal domains are shown in light orange and light blue, respectively. The relative  
 880 abundance of cysteine containing peptides in absence (-) or presence (+) of o-PDM is shown  
 881 in histograms. Collision-induced dissociation (CID) spectrum of cysteine containing peptides  
 882 and elution profiles of peptide fragments are shown in supplementary figure 3. Error bars  
 883 indicate standard deviation (s.d.), n≥3. \*: P≤0.05, \*\*: P≤0.01, \*\*\*: P≤0.001, \*\*\*\*: P≤0.0001. C.  
 884 Cross-linking analysis of LtaA in proteoliposomes. Positions of selected cysteine pairs at the  
 885 extracellular and cytoplasmic regions of LtaA are shown as spheres. SDS-PAGE show band  
 886 shifts of samples treated with mPEG5K after irreversible cross-link with o-PDM. Separated  
 887 species are indicated with arrows. Blue arrow: non-PEGylated LtaA; red arrow: PEGylated  
 888 LtaA; orange arrow: LtaA dimer.





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890 **Figure 3. Cycling through outward- and inward-facing conformations is essential for LtaA**

891 **activity.** **A,B.** Proton-transport activity of LtaA and variants after chemical crosslinking with

892  $\text{CuCl}_2$  (solid lines) or in absence of cross-linking treatment (dotted lines) ( $n \geq 3$ ).

893 Proteoliposomes and protein-free liposomes containing 100 mM KCl were diluted in buffer

894 containing 10 mM KCl, 90 mM NaCl and ACMA.  $\text{H}^+$  influx was initiated by establishing a

895 membrane potential upon addition of the potassium ionophore valinomycin (asterisk). **C.D.**

896 SDS-PAGE shows band shifts of samples treated with mPEG5K after cross-link with  $\text{CuCl}_2$ .

897 Separated species are indicated with arrows. Blue arrow: non-PEGylated LtaA; red arrow:

898 PEGylated LtaA; orange arrow: LtaA dimer.

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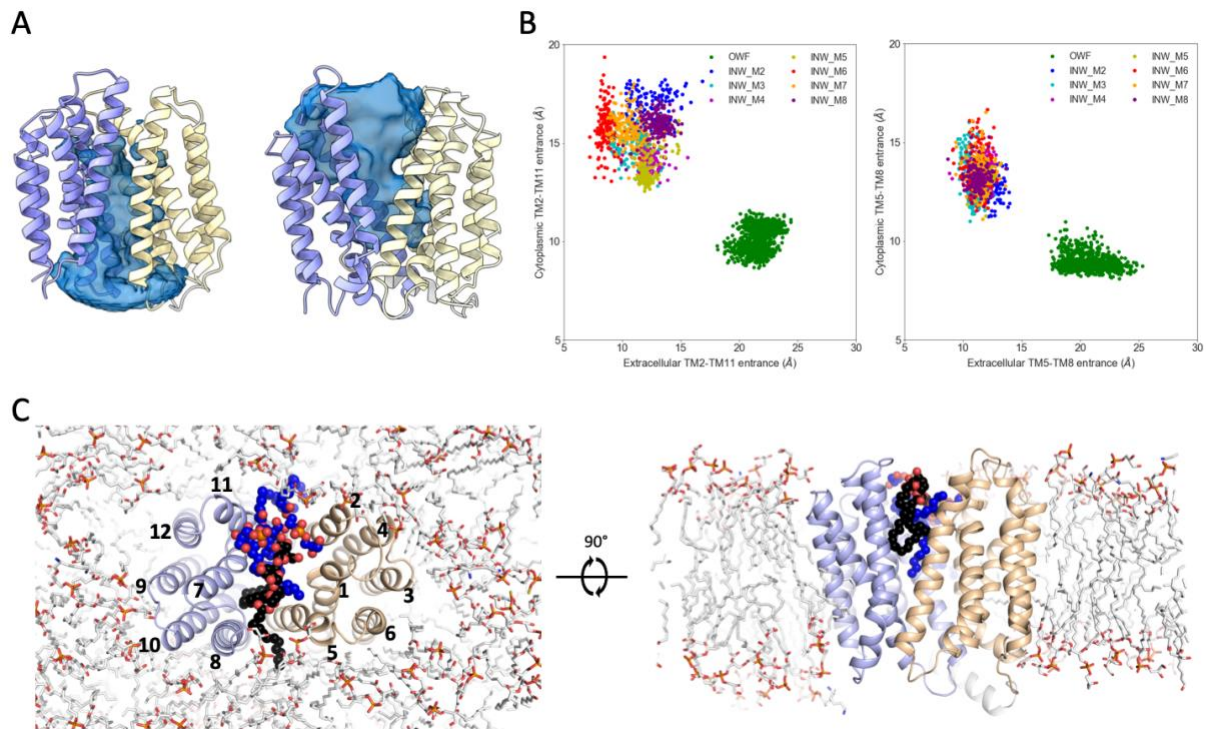
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909 **Figure 4. LtaA displays asymmetric opening of cavities and lateral ‘gates’.** A. Representative

910 view of solvent exposed cavity of inward-facing and outward-facing LtaA as observed during

911 MD simulations. B. Analysis of distances between TM helices lining the cytoplasmic and

912 extracellular lateral openings of outward-facing and inward-facing models. The center of

913 masses of the C $\alpha$  atoms of extracellular residues 52-57 (TM2), 163-167 (TM5), 250-255 (TM8),

914 364-367 (TM11), and of cytoplasmic residues 77-81 (TM2), 139-143 (TM5), 273-276 (TM8),

915 341-344 (TM11), were used for the calculation of inter-TM distances. C. Intrusion of

916 gentiobiosyl-diacylglycerol (black spheres) and POPG (blue spheres) molecules in the

917 extracellular cavity of LtaA during simulations. N-terminal and C-terminal domains are shown

918 in light orange and light blue, respectively.

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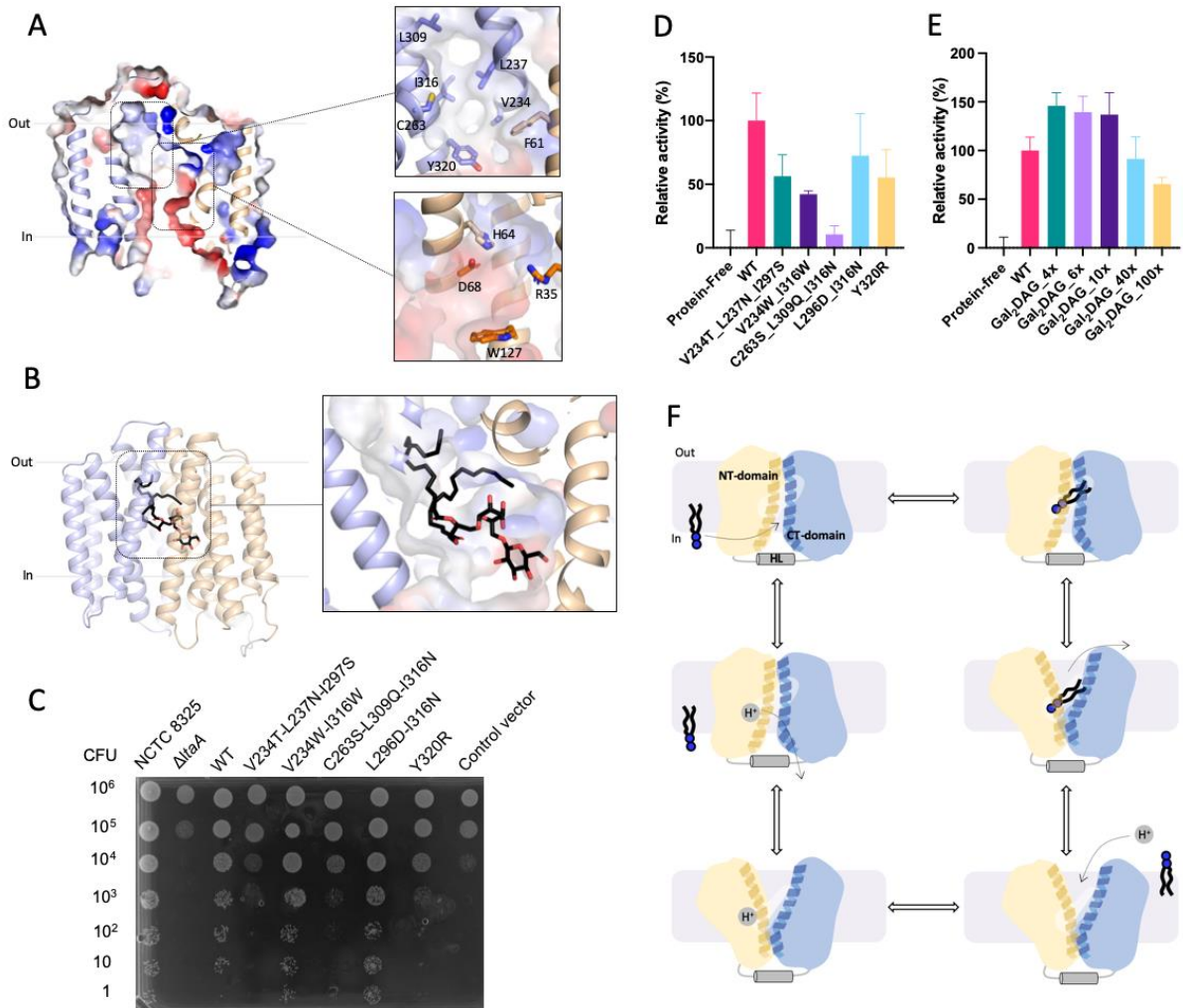
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929 **Figure 5. Hydrophilic and hydrophobic cavities participate in 'trap-and-flip' of lipids. A.**

930 Vacuum electrostatic surface representation of inward-facing model of LtaA. Residues

931 forming the hydrophobic and hydrophilic pockets are shown. **B.** A model of a glycolipid

932 molecule docked into the amphipathic cavity of LtaA. The lipid tail length corresponds to C16

933 chains. N-terminal and C-terminal domains are shown in light orange and light blue,

934 respectively. **C.** *S. aureus* cell growth on LB agar plates containing 0.1 mM IPTG, buffered at

935 pH 6.4. The  $\Delta ltaA$  mutant is complemented with pLOW vector carrying a *ltaA*-WT gene or the

936 annotated point mutations; Control vector indicates the pLOW vector carrying an unrelated

937 gene (dCas9). **D.** Mutagenesis analysis of the hydrophobic pocket. Relative flipping activity of

938 LtaA-WT and variants. Error bars show s.d. of technical replicates,  $n \geq 3$ . **E.** Headgroup

939 selectivity analysis. Relative flipping activity of LtaA in the presence of different concentrations

940 of digalactosyl-diacylglycerol (Gal<sub>2</sub>DAG). Molar excess of Gal<sub>2</sub>DAG over Glc<sub>2</sub>-DAG-NBD is

941 indicated. Error bars show s.d. of technical replicates,  $n \geq 3$ . **F.** Proposed mechanism of LtaA

942 catalyzed glycolipid transport. Schematic of conformational states throughout LtaA transport  
943 cycle.