

1 **Molecular basis for the maintenance of lipid asymmetry in the outer membrane of**
2 ***Escherichia coli***

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19

20 **Abstract**

21 A distinctive feature of the Gram-negative bacterial cell envelope is the asymmetric outer
22 membrane (OM), where lipopolysaccharides (LPS) and phospholipids (PLs) reside in the outer
23 and inner leaflets, respectively. This unique lipid asymmetry renders the OM impermeable to
24 external insults. In *Escherichia coli*, the OmpC-MlaA complex is believed to maintain lipid
25 asymmetry by removing mislocalized PLs from the outer leaflet of the OM. How it performs this
26 function is unknown. Here, we define the molecular architecture of the OmpC-MlaA complex to
27 gain insights into its role in PL transport. We establish that MlaA sits entirely within the bilayer
28 in complex with OmpC and provides a hydrophilic channel possibly for PL translocation across
29 the OM. Furthermore, we show that flexibility in a hairpin loop adjacent to the channel
30 modulates MlaA activity. Finally, we demonstrate that OmpC plays an active role in maintaining
31 OM lipid asymmetry together with MlaA. Our work offers glimpses into how the OmpC-MlaA
32 complex transports PLs across the OM and has important implications for future antimicrobial
33 drug development.

34

35 **Introduction**

36 The outer membrane (OM) of Gram-negative bacteria is an extremely asymmetric
37 bilayer, comprising lipopolysaccharides (LPS) in the outer leaflet and phospholipids (PLs) in the
38 inner leaflet (1, 2). LPS molecules pack tightly together in the presence of divalent cations to
39 form an outer layer with markedly reduced fluidity and permeability (3). Thus, the OM serves as
40 an effective barrier against toxic compounds including detergents and antibiotics. This function
41 is fully dependent on the establishment and maintenance of lipid asymmetry; cells generally
42 become more sensitive to external insults when OM lipid asymmetry is disrupted, which is
43 typically characterized by the accumulation of PLs in the outer leaflet (4, 5). The OM is also
44 essential for viability.

45 The requisite lipid asymmetry of the OM is likely initially established by direct
46 placement of LPS and PLs into the outer and inner leaflets, respectively. LPS assembly into the
47 outer leaflet of the OM is mediated by the well-established Lpt (lipopolysaccharide transport)
48 machinery (6), but proteins that transport and insert PLs into the inner leaflet have not been
49 identified. For entropic reasons, there is a natural tendency for PLs to appear in the outer leaflet
50 of the OM, although how they traverse the bilayer is unclear. This occurs more readily with
51 perturbations in the OM, especially when assembly of other OM components is disrupted (4, 5,
52 7). Since loss of lipid asymmetry compromises the barrier function of the OM, several
53 mechanisms exist to remove PLs aberrantly localized in the outer leaflet of the membrane: (i) the
54 OM phospholipase OmpLA hydrolyzes both acyl chains from outer leaflet PLs (8), (ii) the OM
55 acyltransferase PagP transfers an acyl chain from outer leaflet PLs to LPS (9) or
56 phosphatidylglycerol (PG) (10), and (iii) the OmpC-Mla system, a putative PL trafficking
57 pathway, removes outer leaflet PLs and shuttles them back to the inner membrane (IM) (11, 12).

58 The OmpC-Mla system comprises seven proteins located across the cell envelope.
59 Removing any component results in PL accumulation in the outer leaflet of the OM, and
60 therefore sensitivity to SDS/EDTA (11, 12). The OM lipoprotein MlaA forms a complex with
61 osmoporin OmpC that likely extracts PLs from the outer leaflet of the OM (12). The periplasmic
62 protein MlaC serves as a lipid chaperone and is proposed to transport lipids from the OmpC-
63 MlaA complex to the IM (11, 13, 14). At the IM, MlaF and MlaE constitute an ATP-binding
64 cassette (ABC) family transporter together with two auxiliary proteins, MlaD and MlaB (14, 15);
65 this complex presumably receives PLs from MlaC and inserts them into the membrane. MlaD
66 has been shown to bind PLs, while MlaB is important for both assembly and activity of the
67 transporter (15). Recently, the function of the OmpC-Mla system in retrograde (OM-to-IM) PL
68 transport has been demonstrated in *E. coli* (7).

69 The molecular mechanism by which the OmpC-MlaA complex extracts PLs from the
70 outer leaflet of the OM, presumably in an energy-independent manner, is an interesting problem.
71 Aside from the LptDE machine, which assembles LPS on the surface (4, 5), the OmpC-MlaA
72 complex is the only other system proposed to catalyze the translocation of lipids across the OM.
73 OmpC is a classical trimeric porin that typically only allows passage of hydrophilic solutes
74 across the OM (3, 16), while MlaA is believed to be anchored to the inner leaflet of the
75 membrane; how the two proteins are organized in a complex for the translocation of amphipathic
76 PLs is not known. In this paper, we establish that MlaA is in fact an integral membrane protein
77 that forms a channel adjacent to OmpC trimers in the OM, likely allowing the passage of PLs.
78 We first demonstrated that MlaA binds the OmpC trimer within the OM bilayer by mapping the
79 interaction surfaces using *in vivo* crosslinking. Using a recently predicted structural model of
80 MlaA (17), we obtained molecular views of the OmpC-MlaA complex by molecular dynamics

81 (MD) simulations, and experimentally established the existence of a hydrophilic channel within
82 OM-embedded MlaA. Combining charge mutations in this channel modulated MlaA activity,
83 suggesting functional importance. Furthermore, mutations altering the flexibility of a hairpin
84 loop that could interact with the hydrophilic channel led to predictable in vivo effects on MlaA
85 function. Finally, we identified a key residue on OmpC found at the OmpC-MlaA interacting
86 surface that is important for proper function of the complex. Our findings provide important
87 mechanistic insights into how PLs may be translocated across the OM to ensure proper lipid
88 asymmetry.

89

90 **Results**

91 **The OmpC trimer contacts MlaA directly along its membrane-facing dimeric interfaces.**

92 To develop a detailed architectural understanding of the OmpC-MlaA complex, we
93 carried out in vivo photocrosslinking to map intermolecular interactions within the complex.
94 Guided by the crystal structure of the OmpC trimer (18), we introduced the UV-crosslinking
95 amino acid, *para*-benzoyl-L-phenylalanine (*p*Bpa), at 49 positions in OmpC via amber
96 suppression (19). Initial selection focused on residues that are either solvent-accessible (i.e. loop
97 and lumen) or located near the membrane-water boundaries (i.e. aromatic girdle). Upon UV
98 irradiation, a ~65 kDa crosslinked band that contains both OmpC (~37 kDa) and MlaA (~28
99 kDa) could be detected in cells expressing OmpC variants substituted with *p*Bpa at three
100 positions (L50, Q83, or F267) (Fig. 1A). These residues are found on the periplasmic turns at the
101 dimeric interfaces of the OmpC trimer (Fig. 1C), thus localizing possible binding sites for MlaA.
102 We have previously proposed that OmpC may allow MlaA to traverse the bilayer and gain
103 access to PLs that have accumulated in the outer leaflet of the OM (12). As none of the six
104 selected residues in the OmpC lumen crosslinked to MlaA, we decided to probe for interactions

105 between MlaA and the membrane-facing side walls of OmpC, specifically around the dimeric
106 interfaces of the OmpC trimer. Remarkably, out of the additional 49 positions tested in this
107 region, 10 residues allowed photoactivated crosslinks between OmpC and MlaA when replaced
108 with *p*Bpa (Fig. 1B and Fig. S1). In total, these 13 crosslinking residues clearly demarcate an
109 extensive MlaA-interacting surface on OmpC (Fig. 1C). This explains why OmpC exhibits
110 strong interactions and can be co-purified with MlaA on an affinity column, as we have
111 previously reported (12). Two positions, Y149 and L340, are located right at or near the
112 membrane-water boundary exposed to the extracellular environment, suggesting that MlaA
113 traverses the entire width of the OM. We conclude that MlaA binds along the dimeric interfaces
114 of the OmpC trimer in the membrane.

115

116 **Two specific regions on MlaA contact the membrane-facing dimeric interfaces of the**
117 **OmpC trimer.**

118 We sought to map in greater detail the OmpC-MlaA interacting surface in vitro. To do
119 that, we first overexpressed and purified the OmpC-MlaA complex to homogeneity. We showed
120 that this complex forms a single peak on size exclusion chromatography (SEC) (Fig. 2A). OmpC
121 within this complex exhibits the characteristic heat-modifiable gel shift commonly observed for
122 OM β -barrel proteins (20), consistent with the presence of the folded trimer. Multi-angle light
123 scattering (MALS) analysis revealed that one copy of MlaA interacts with the OmpC trimer (Fig.
124 S2), suggesting that only one of the three dimeric interfaces within the trimer is available for
125 binding (Fig. 1C). We next performed protease digestion experiments to identify specific
126 region(s) on MlaA that may interact stably with OmpC. OM β -barrel proteins such as OmpC are
127 known to be protease-resistant (21). Given that some parts of MlaA contact OmpC within the
128 membrane, we expect these bound regions to also be protected from proteolytic degradation.

129 Treatment of the purified OmpC-MlaA complex with trypsin results in almost complete
130 degradation of MlaA, with the OmpC trimer remaining intact (Fig 2A). Following SEC,
131 however, we found that an ~8 kDa peptide (presumably from MlaA) remains stably bound to the
132 trimer. N-terminal sequencing and tandem mass spectrometry (MS) analyses revealed that this
133 peptide corresponds to MlaA_{D61-K124} (Figs. 2A and 2C, and Fig. S3). These results suggest that
134 MlaA interacts strongly with OmpC in the membrane in part via this specific region.

135 To define how OmpC contacts the MlaA_{D61-K124} peptide, we next attempted to
136 overexpress and purify *pBpa*-containing OmpC variants in complex with MlaA, and determine
137 which of the previously identified 13 OmpC residues interacts with MlaA_{D61-K124} in vitro. We
138 sequentially performed UV crosslinking and trypsin digestion to potentially link MlaA_{D61-K124} to
139 specific residues on OmpC. This approach may also allow trapping of other potential interacting
140 regions of MlaA, which might not have been stably retained on the wild-type complex after
141 trypsin digestion. We successfully detected trypsin-resistant crosslinked products for seven
142 OmpC_{*pBpa*}-MlaA complexes (Fig. 2B); these appeared slightly above OmpC between 37 to 50
143 kDa, indicating that peptides in the range of ~6-10 kDa are crosslinked to OmpC. N-terminal
144 sequencing of six of the seven adducts showed the presence of OmpC, and an MlaA peptide
145 beginning at residue D61 (Figs. 2C and 2D, and Fig. S3C). Given the approximate sizes of the
146 crosslinked adducts, we concluded that all these residues interact with MlaA_{D61-K124}.
147 Interestingly, an additional peptide on MlaA starting at residue F133 was also found to crosslink
148 at two (Y149 and L340) of these six positions on OmpC (Figs. 2B–D, and Fig. S3C). These
149 adducts can be detected by an α -MlaA antibody that recognizes an epitope within V182-Q195 on
150 MlaA (Fig. 2B), suggesting that a peptide from F133 to at least R205 (next trypsin cleavage site)

151 may be crosslinked (Fig. 2C). Thus, in addition to MlaA_{D61-K124}, our crosslinking strategy
152 revealed a second point of contact (MlaA_{F133-R205}) between OmpC and MlaA.

153 At the point of these findings, there was no available molecular structure for MlaA;
154 however, a structural model has been predicted based on residue-residue contacts inferred from
155 co-evolution analysis of metagenomic sequence data (17). Using a rigorously-validated quality
156 score, this method of structure determination has generated reliable models for 614 protein
157 families with currently unknown structures. We experimentally validated the model for MlaA by
158 replacing residue pairs far apart on the primary sequence with cysteines, and showed that only
159 those that are highly co-evolved (and predicted to be residue-residue contacts) allow disulfide
160 bond formation in cells (Fig. S4). We therefore proceeded to use this MlaA model to understand
161 the organization of the OmpC-MlaA complex. Interestingly, the positions of the two OmpC-
162 contacting peptides on the MlaA model are spatially separated in a way consistent with the
163 arrangement of the residues on OmpC that crosslink to each peptide (Fig. 2D). This not only
164 reveals how MlaA may potentially be oriented and organized around the dimeric interface of the
165 OmpC trimer, but also suggests that the entire MlaA molecule may reside in the membrane. In
166 fact, the overall surface of MlaA, other than the putative periplasmic-facing region, is largely
167 hydrophobic (Fig. S5). Furthermore, using all-atomistic MD simulations, we found that the
168 structural fold of MlaA appears to be more stable in a lipid bilayer than in an aqueous
169 environment (Fig. S6). Consistent with this, we note that even without its N-terminal lipid
170 anchor, MlaA is not a soluble protein, and can only be extracted and purified from the OM in the
171 presence of detergent. Collectively, these observations lend strong support to the validity of the
172 predicted MlaA structure.

173

174 **MlaA provides a hydrophilic channel that may allow PL translocation across the OM.**

175 To obtain a physical picture of how OmpC interacts with MlaA within the complex, we
176 used MD simulations to dock MlaA onto the OmpC trimer within a PL bilayer. Using a
177 previously reported protocol (22), we first docked the MlaA model onto the OmpC trimer
178 structure, both as rigid bodies. Interestingly, all initial docked structures contained MlaA binding
179 at one dimeric interface of OmpC. Based on information derived from crosslinking, we selected
180 two most consistent models differing slightly in how MlaA is oriented with respect to OmpC for
181 unrestrained refinements using all-atomistic simulations. Multiple simulations were run for each
182 MlaA orientation in a PL bilayer until overall root-mean-square deviations (RMSD) stabilized;
183 remarkably, the resulting equilibrium models fulfilled all observed experimental crosslinking
184 data. We performed clustering on all available trajectories, and identified the most populated
185 conformations of the OmpC-MlaA complex in our simulations (Fig. 3A and Fig. S7A for one
186 MlaA orientation, and Fig. 3B and Fig. S7B for the other). These conformational models all
187 show MlaA sitting in the bilayer, tucked nicely into the dimeric interface of the OmpC trimer.
188 Evidently, the MlaA_{D61-K124} peptide interacts extensively with OmpC in these models, consistent
189 with why this peptide remains stably bound to OmpC after protease digestion (Fig. 2A). We also
190 mutated several MlaA residues found at the OmpC-MlaA interfaces to *p*Bpa, and performed in
191 vivo crosslinking experiments. We identified one position L109 that allowed strong
192 photoactivatable crosslinking to OmpC when replaced with *p*Bpa (Fig. 3C). This residue lies
193 within the MlaA_{D61-K124} peptide (Figs. 3A and 3B), confirming that this region does in fact
194 contact OmpC in cells.

195 One striking feature present in all the simulated OmpC-MlaA structures is a negatively-
196 charged hydrophilic channel within MlaA that spans the lipid bilayer (Fig. 4A, and Fig. S8).

197 Based on its function in removing PLs from the outer leaflet of the OM, we hypothesize that this
198 channel may allow passage of charged headgroups as PLs translocate across the membrane. To
199 test the existence of this hydrophilic channel in MlaA in cells, we selected 27 residues in and
200 around the putative channel in a representative model (Fig. 4, and Fig. S9), and determined their
201 solvent accessibility using the substituted cysteine accessibility method (SCAM). We first
202 showed that these cysteine mutants are functional (Fig. S9C). Solvent-exposed residues are
203 expected to be reactive with the charged membrane-impermeable thiol-labelling reagent, sodium
204 (2-sulfonatoethyl)methanethiosulfonate (MTSES). Remarkably, we found that residues predicted
205 to be within the putative channel (Figs. 4A and 4B) or at the membrane-water boundaries (Figs.
206 S9A and S9B) are indeed accessible to MTSES. We therefore conclude that MlaA forms a
207 hydrophilic channel in the OM in cells.

208 To ascertain whether this channel is functionally important, we separately mutated 19
209 polar and (negatively) charged residues near or within the channel to alanine or arginine, and
210 tested for MlaA function. However, all of these MlaA mutants are functional (Fig. 5B, and Fig.
211 S10A), indicating that single residue changes are not sufficient to perturb channel properties to
212 affect PL transport. We noticed that four (D160, D161, D164, and D167) of the five negatively-
213 charged channel residues are in close proximity (Fig. 5A). To alter channel properties more
214 drastically, we combined arginine mutations for these aspartates; interestingly, only the
215 D161R/D167R double mutant (Fig. S10B) and D160R/D161R/D164R triple mutant (MlaA_{3D3R})
216 disrupted function; cells expressing these variants are highly sensitive to SDS/EDTA (Fig. 5B,
217 and Fig. S10B). For the MlaA_{3D3R} mutant, this is likely due to the accumulation of PLs in the
218 outer leaflet of the OM (as judged by PagP-mediated acylation of LPS; Fig. 5C). In fact, this
219 mutant exhibits OM defects that are more pronounced than the $\Delta mlaA$ strain (Fig. 5B),

220 suggesting gain of function. Consistent with this idea, the *3D3R* mutation gives rise to the same
221 defects in strains also expressing the wild-type *mIaA* allele, revealing a dominant negative
222 phenotype (Figs. 5B and 5C). We showed that MlaA_{3D3R} is produced at levels comparable to
223 wild-type MlaA on a plasmid (Fig. S11A), and is still able to interact strongly with OmpC (Fig.
224 S11B). Taken together, these results suggest that the MlaA channel plays a functional role in
225 maintenance of lipid asymmetry.

226

227 **Flexibility of a hairpin loop adjacent to the channel on MlaA is required for function.**

228 The gain-of-function/dominant negative phenotype of the *mIaA*_{3D3R} mutant is similar to a
229 previously reported *mIaA** (or *mIaA*_{ΔNF}) mutant (Figs. 5B and 5C) (23), suggesting that these
230 mutations may have similar effects on MlaA structure and/or function. Interestingly, the
231 positions of these mutations on the OmpC-MlaA models flank a hydrophobic hairpin loop
232 (G141-L158) within MlaA (Fig. 6A). Therefore, we hypothesized that the loop could play a
233 functional role in MlaA, and that these mutations may affect interactions with this loop. To
234 examine this possibility, we created three separate mutations at the hairpin structure and tested
235 each variant for MlaA function. Two of these mutations, Y¹⁴⁷VQL→4A (*L1*) and
236 F¹⁵²YGSF→5A (*L2*), are designed to disrupt interactions with other regions of MlaA. The other
237 mutation, P151A, removes a proline that may be critical for the hairpin turn structure. The N-
238 terminus of the loop is connected to the rest of MlaA via an unstructured glycine-rich linker,
239 which we reasoned may influence conformation of the entire hairpin structure. Thus, we
240 constructed two additional mutants, G¹⁴¹VGYG→A¹⁴¹VAYA (*3G3A*) and
241 G¹⁴¹VGYG→P¹⁴¹VPYP (*3G3P*), to reduce possible flexibility in this region. Remarkably, *L1*,
242 *L2*, and *3G3P* mutations resulted in similar extents of SDS/EDTA sensitivity (Fig. 6B), as well

243 as OM outer leaflet PL accumulation (Fig. 6C), when compared to the $\Delta mlaA$ mutation. Given
244 that these mutations also do not affect MlaA levels or interaction with OmpC (Fig. S11), we
245 conclude that they are loss-of-function mutations. The hairpin loop, along with its surrounding
246 structures, forms an important functional region on MlaA.

247 Phenotypes observed for the loop rigidifying mutation (*3G3P*) and gain-of-function
248 mutations (*3D3R* and *mlaA**) suggest that flexibility in the hairpin loop is critical for MlaA
249 function. We hypothesize that the hairpin loop may exist in two distinct conformations. The
250 *3D3R* or *mlaA** mutations could alter interactions with the loop, resulting in it adopting one
251 conformation, and somehow giving rise to gain-of-function/dominant negative phenotypes; in
252 the case of *mlaA**, it was proposed that this mutation caused MlaA to be in a “leaky” or “open”
253 state, and allowed PLs to flip out to the outer leaflet of the OM (23). In contrast, the *3G3P*
254 mutation may lock the hairpin loop in a second conformation, where MlaA is in a “closed” state,
255 thus abolishing function in PL transport. If these were true, we predict that rigidification of the
256 hairpin loop with the *3G3P* mutation would be able to correct gain-of-function/dominant
257 negative phenotypes observed in the *3D3R* and/or *mlaA** mutants. Indeed, the *3G3P/3D3R* and
258 *3G3P/mlaA** combination mutants no longer exhibit gain-of-function/dominant negative
259 phenotypes, but behave like the *3G3P* or null mutants (Fig. 6D). Again, these variants are
260 expressed at comparable levels to the single mutants, and still interact with OmpC (Fig. S11).
261 Taken together, these results indicate clear importance of dynamics in the hairpin loop in
262 controlling the function of MlaA.

263

264 **A specific residue in the dimeric interface of OmpC is important for its function in**
265 **maintaining lipid asymmetry.**

266 Given that MlaA sits in the membrane and provides a channel that putatively allows
267 movement of PLs across the OM, it is not clear why it should bind at one of the dimeric
268 interfaces of OmpC trimers, and what the exact role of OmpC may be. To understand the
269 importance of OmpC-MlaA interaction, we attempted to engineer monomeric OmpC constructs
270 that we predict would no longer interact with MlaA. We installed specific mutations (G19W
271 and/or R92L) in OmpC that were found previously to disrupt the oligomerization state of its
272 homolog OmpF (G19W and R100L correspondingly) in vitro (24) (Fig. 7A). Both the OmpC_{G19W}
273 and OmpC_{R92L} single mutants can interact with MlaA, and still form trimers in vitro, albeit
274 slightly destabilized compared to wild-type OmpC (Fig. S12). Combination of these mutations
275 further weakens the OmpC trimer, with noticeable monomer population at physiological
276 temperature. Intriguingly, both the double mutant and the R92L single mutant accumulated PLs
277 in the outer leaflet of the OM (Fig. 7B), indicating that R92 is important for the role of OmpC in
278 OM lipid asymmetry. Consistent with this idea, we demonstrated that cells expressing the
279 OmpC_{R92A} variant also exhibit perturbations in OM lipid asymmetry. We further showed that all
280 these *ompC* alleles can rescue severe SDS/EDTA sensitivity known for cells lacking OmpC (Fig.
281 7C), suggesting normal porin function; however, cells expressing OmpC_{G19W/R92L} and OmpC_{R92A},
282 unlike WT and OmpC_{R92L}, are still sensitive to SDS at higher concentrations of EDTA. These
283 phenotypes mirror those observed for cells lacking MlaA, suggesting the loss of Mla function in
284 these mutant strains. It appears that the R92 residue is critical for this function, although it is not
285 clear why the single R92L mutation did not result in SDS/EDTA sensitivity. The exact role of
286 R92 is not known, but the phenotypes observed for the single R92A mutation cannot be due to
287 disruption of OmpC trimerization (Fig. S12). We conclude that OmpC has an active role in
288 maintaining OM lipid symmetry together with MlaA.

289 **Discussion**

290

291 Osmoporin OmpC interacts with MlaA to maintain lipid asymmetry in the OM; how this
292 complex is organized to extract PLs from the outer leaflet of the OM is not known. In this study,
293 we have employed photoactivatable crosslinking and MD simulations to gain insights into the
294 molecular architecture of the OmpC-MlaA complex. We have established that MlaA interacts
295 extensively with OmpC at one of the dimeric interfaces of the porin trimer, and resides entirely
296 within the OM lipid bilayer. We have also demonstrated that MlaA forms a hydrophilic channel,
297 likely allowing PLs to translocate across the OM. This overall organization of the OmpC-MlaA
298 complex is quite remarkable, especially how MlaA spans the OM and possibly gains access to
299 outer leaflet PLs. Very few lipoproteins are known to span the OM; some notable examples
300 include the LptDE and Wza translocons, which transports LPS and capsular polysaccharides,
301 respectively. In the LptDE complex, the OM lipoprotein LptE serves as a plug, and stretches
302 across the bilayer through the lumen of the LptD β -barrel (25, 26). In the octameric Wza
303 translocon, each protomer provides a C-terminal α -helix to form a pore that spans the membrane
304 (27). MlaA is unique in that it is essentially an integral membrane protein, capable of forming a
305 channel on its own. In many ways, MlaA behaves like typical OM β -barrel proteins, even though
306 it is predominantly α -helical. Furthermore, being overall a hydrophobic protein also poses a
307 problem for MlaA to transit across the periplasmic space. How MlaA is shielded from the
308 aqueous environment, in addition to the requirement of the Lol system (28), necessitates further
309 investigation.

310 Very recently, crystal structures of MlaA in complex with trimeric porins have in fact
311 been solved (29). Interestingly, the experimentally-determined structures of MlaA closely

312 resembled the initial MlaA model predicted from co-evolution analysis (Fig. S13A). These
313 structures revealed that MlaA interacts with trimeric porins at one or more of their dimeric
314 interfaces (Fig. S13B), in an orientation similar to one of our simulated OmpC-MlaA models
315 (Fig. 3B). They also showed that MlaA contains a channel. Clearly, these findings converged
316 with the main conclusions derived from our biochemical and modelling studies, which is quite
317 remarkable. We note, however, a couple of discrepancies between the solved structures and our
318 biochemical data, suggesting that these static structures may represent only one of several
319 possible stable conformations of MlaA, which may exist as part of the mechanism associated
320 with lipid transport in the native OM environment. First, the distance separating porin residues
321 equivalent to Y149/L340 in OmpC and the MlaA_{F133-R205} peptide (Fig. S13B) is not consistent
322 with the detection of strong photoactivatable crosslinks between these regions in the complex
323 (Fig. 2B). Second, the proposed location of some MlaA residues, specifically M39, F42, and
324 N43, at the interior of the lipid bilayer (Fig. S13B) is not in agreement with high or partial
325 solvent accessibility of these sites in cells, as highlighted in the SCAM data (Fig. S9B). Upon
326 close examination of the structures, we found multiple non-native MlaA-MlaA and MlaA-porin
327 contacts in all the crystal forms (Fig. S13C); these artificial crystal contacts, some with
328 substantial buried surface areas, may have influenced the observed conformation of MlaA. Given
329 that the MlaA-porin structures were also not solved in the context of a native lipid bilayer, we
330 suspect that they do not yet provide the complete picture.

331 Our functional data on the OmpC-MlaA complex provide a glimpse of how PLs may
332 translocate across the OM during maintenance of lipid asymmetry. One key aspect of MlaA
333 function resides in a hairpin loop structure juxtaposed against the hydrophilic channel. Dynamics
334 of this loop appear to control whether MlaA exists in a “closed” or “open” state, and thus access

335 of PLs through the channel. A mutation that likely rigidifies the loop locks MlaA in the non-
336 functional “closed” state (Fig. 6), while mutations that possibly affect interactions with the loop
337 favors the “open” state, and gives rise to gain of function (Fig. 5). Therefore, the hairpin loop
338 may directly gate the channel, providing access for outer leaflet PLs across the OM. This idea
339 has also been suggested based on the solved MlaA structures, where a putative disulfide bond
340 that apparently locks the loop in position renders MlaA non-functional (29).

341 How OmpC participates in maintaining OM lipid asymmetry as part of the complex is not
342 clear, especially given that MlaA alone provides a channel for PL translocation. It is possible that
343 OmpC may play a passive role, and simply be important for stabilizing the structure and
344 orientation of MlaA in the OM. However, we have previously shown that MlaA also interacts
345 with OmpF, yet removing OmpF has minimal effects on OM lipid asymmetry (12); this argues
346 against a mere passive role for OmpC in PL translocation. Furthermore, we have now identified
347 a specific residue R92 on OmpC that is required for maintaining OM lipid asymmetry (Fig. 7).
348 Therefore, we believe that OmpC plays an active role in the process. R92 lies in the dimeric
349 interface of the OmpC trimer, which incidentally is where MlaA binds. Even though this residue
350 has been shown to be important for gating the porin (30), it is not obvious how this gating
351 function may influence the translocation of PLs by MlaA. Instead, we speculate that being close
352 to Y149 and L340, R92 may somehow affect interactions between OmpC and the MlaA_{F133-R205}
353 peptide, possibly influencing MlaA conformation in the OM, and in turn, properties of the
354 hydrophilic channel. The role that R92 plays in OmpC-MlaA function should be further
355 characterized.

356 The OmpC-MlaA complex is proposed to extract PLs from the outer leaflet of the OM
357 and hand them over to MlaC, which resides in the periplasm. Consistent with this idea, *E. coli*

358 MlaC has been crystallized with a bound PL, and shown to interact with a complex of OmpF-
359 MlaA in vitro (14). However, it is not clear how transfer of PLs from OmpC-MlaA to MlaC
360 takes place, even though this must presumably occur in an energy-independent fashion. Since PL
361 movement from the outer to inner leaflets of the OM is entropically disfavored, it is likely that
362 translocation of PLs by the OmpC-MlaA complex would be coupled to transfer to MlaC, i.e.
363 extracted PLs do not go into the inner leaflet. If this were true, it may be possible that MlaC also
364 influences the function of the OmpC-MlaA complex. Specifically, binding of MlaC to the
365 complex may be required for PL extraction from the outer leaflet of the OM. MlaC could alter
366 the structure and/or dynamics of MlaA in the OmpC-MlaA complex, ultimately leading to
367 efficient PL translocation across the OM.

368 Lipid asymmetry is critical for the OM to function as an effective permeability barrier.
369 Thus, understanding mechanistic aspects of how bacterial cells maintain OM lipid asymmetry
370 would guide us in designing strategies to overcome the barrier. Our work on elucidating the
371 architecture and function of the OmpC-MlaA complex has revealed critical insights into the role
372 of a hairpin loop on MlaA in modulating activity, a feature that can be exploited in drug
373 discovery efforts. In particular, small molecules that can potentially influence dynamics of this
374 loop may induce either loss or gain of function, thereby leading to increased sensitivity to
375 existing antibiotics.

376

377 **Experimental procedures**

378

379 **Bacterial strains and plasmids.** All strains and plasmids used are listed in Table S1 and S2,
380 respectively.

381

382 **Growth conditions.** Luria Bertani (LB) broth and agar were prepared as described previously
383 (12). Unless otherwise noted, ampicillin (Amp) (Sigma-Aldrich, MO, USA) was used at a
384 concentration of 200 µg/mL, chloramphenicol (Cam) (Alfa Aesar, Heysham, UK) at 15 µg/mL,
385 kanamycin (Kan) (Sigma-Aldrich) at 25 µg/mL, and spectinomycin (Spec) (Sigma-Aldrich) at 50
386 µg/mL. For crosslinking experiments, *para*-Benzoyl-L-phenylalanine (*p*Bpa; Alfa Aesar) was
387 dissolved in 1 M NaOH at 0.25 M, and used at 0.25 mM unless otherwise mentioned.

388

389 **Plasmid construction.** To construct most plasmids, the desired gene or DNA fragments were
390 amplified by PCR from the DNA template using primers listed in Table S3. Amplified fragments
391 were digested with relevant restriction enzymes (New England Biolabs) and ligated into the
392 same sites of an appropriate plasmids using T4 DNA ligase (New England Biolabs). NovaBlue
393 competent cells were transformed with the ligation products and selected on LB plates
394 containing appropriate antibiotics. All constructs were verified by DNA sequencing (Axil
395 Scientific, Singapore).

396

397 **Construction of chromosomal *ompC* mutants using negative selection.** All chromosomal
398 *ompC* mutations were introduced via a positive-negative selection method described previously
399 (31). To prepare electro-competent cells, strain MC4100 harbouring pKM208 (32) grown

400 overnight at 30 °C was inoculated into 15 mL SOB broth with 1:100 dilution. Cells were grown
401 at the same temperature until OD₆₀₀ reached ~0.3-0.4. 1 mM of IPTG was added and the culture
402 was grown for another 60 min at 30 °C. Cells were then subjected to heat shock at 42 °C for 15
403 min, followed by incubation for 15 min on ice, with intermittent agitation. Subsequently, cells
404 were centrifuged at 5000 x g for 10 min and made competent by washing twice with prechilled
405 sterile water followed by cold 10 % glycerol. Competent cells were pelleted and resuspended in
406 cold 10 % glycerol. For positive selection, 1 µg *kan-PrhaB-tse2* cassette amplified from pSLC-
407 246 (31) using ompC_NS_N5 and ompC_NS_C3 primer pairs was transformed into the
408 competent cells using 1-mm electroporation cuvettes (Biorad) in Eppendorf Eporator[®]
409 (Eppendorf) with an output voltage of 1800 V. Cells were recovered in LB with 2 % glucose at
410 37 °C for at least 4 h, plated onto LB plates supplemented with Kan and 2 % glucose, and
411 incubated at 37 °C for 24 h. For negative selection, 1 µg PCR product of *ompC* wild-type or
412 mutant constructs amplified using ompC_NS_N5_C and ompC_NS_C3_C primer pairs were
413 transformed into competent cells made from positive selection using similar procedures. After
414 transformation, cells were plated onto minimal (M9) plates supplemented with 0.2 % rhamnose,
415 and incubated for 48 h at 37 °C. Surviving colonies were PCR screened and verified by DNA
416 sequencing (Axil Scientific, Singapore).

417

418 **In vivo photoactivatable crosslinking.** We adopted previously described protocol (19) for all in
419 vivo photoactivatable crosslinking experiments. Briefly, amber stop codon (TAG) was introduced
420 at selected positions in either pDSW206*ompC* or pCDF*m1aA-His* plasmids via site directed
421 mutagenesis using primers listed in Table S3. For *OmpC* crosslinking, MC4100 with
422 $\Delta ompC::kan$ background harbouring p*Sup-BpaRS-6TRN* (33) and pDSW206*ompC* were used.

423 For MlaA crosslinking, MC4100 with $\Delta mlaA::kan$ background harbouring *pSup-BpaRS-6TRN*
424 (33) and *pCDFm1aA-His* were used. An overnight 5 mL culture was grown from a single colony
425 in LB broth supplemented with appropriate antibiotics at 37 °C. Overnight cultures were diluted
426 1:100 into 10 mL of the same media containing 0.25 mM *pBpa* and grown until OD_{600} reached
427 ~1.0. Cells were normalized by optical density before pelleting and resuspended in 1 mL ice cold
428 TBS (20 mM Tris pH 8.0, 150 mM NaCl). Samples were either used directly or irradiated with
429 UV light at 365 nm for 20 min at 4 °C or room temperature. All samples were pelleted again and
430 finally resuspended in 200 μ L of 2 X Laemmli buffer, boiled for 10 min, and centrifuged at
431 21,000 x *g* in a microcentrifuge for one min at room temperature; 15 μ L of each sample
432 subjected to SDS-PAGE and immunoblot analyses.

433
434 **Over-expression and purification of OmpC-MlaA-His complexes.** All proteins were
435 overexpressed in and purified from BL21(λ DE3) derivatives. We found that BL21(λ DE3) strains
436 from multiple labs do not actually produce OmpC (gene is somehow missing in these strains);
437 therefore, to obtain OmpC-MlaA complexes, we deleted *ompF* from the chromosome and
438 introduced *ompC* on a plasmid. OmpC-MlaA-His protein complexes were over-expressed and
439 purified from BL21(λ DE3) cells with chromosomal $\Delta ompF::kan$ background co-transformed
440 with either *pDSW206ompC_{pBpa}*, *pSup-BpaRS-6TRN* and *pCDFdmlaA-His* (for in vitro
441 crosslinking experiments), or *pACYC184ompC* and *pET22b(+)**dmlaA-His* (for characterization
442 of the wildtype complex). An overnight 10 mL culture was grown from a single colony in LB
443 broth supplemented with appropriate antibiotics at 37 °C. The cell culture was then used to
444 inoculate a 1-L culture and grown at the same temperature until OD_{600} reached ~ 0.6. For
445 induction, 0.5 mM IPTG (Axil Scientific, Singapore) was added and the culture was grown for

446 another 3 h at 37 °C. Cells were pelleted by centrifugation at 4,700 x g for 20 min and then
447 resuspended in 10-mL TBS containing 1 mM PMSF (Calbiochem) and 30 mM imidazole
448 (Sigma-Aldrich). Cells were lysed with three rounds of sonication on ice (38 % power, 1 second
449 pulse on, 1 second pulse off for 3 min). Cell lysates were incubated overnight with 1 % n-
450 dodecyl β -D-maltoside (DDM, Calbiochem) at 4 °C. Cell debris was removed by centrifugation
451 at 24,000 x g for 30 min at 4 °C. Subsequently, supernatant was incubated with 1 mL Ni-NTA
452 nickel resin (QIAGEN), pre-equilibrated with 20 mL of wash buffer (TBS containing 0.025 %
453 DDM and 80 mM imidazole) in a column for 1 h at 4 °C with rocking. The mixture was allowed
454 to drain by gravity before washing vigorously with 10 x 10 mL of wash buffer and eluted with 10
455 mL of elution buffer (TBS containing 0.025% DDM and 500 mM imidazole). The eluate was
456 concentrated in an Amicon Ultra 100 kDa cut-off ultra-filtration device (Merck Millipore) by
457 centrifugation at 4,000 x g to ~500 μ L. Proteins were further purified by SEC system (AKTA
458 Pure, GE Healthcare, UK) at 4 °C on a prepacked Superdex 200 increase 10/300 GL column,
459 using TBS containing 0.025% DDM as the eluent. Protein samples were used either directly or
460 irradiated with UV at 365 nm for in vivo photoactivable crosslinking experiments.

461

462 **SEC-MALS analysis to determine absolute molar masses of OmpC-MlaA-His complex.**

463 Prior to each SEC-MALS analysis, a preparative SEC was performed for BSA (Sigma-Aldrich)
464 to separate monodisperse monomeric peak and to use as a quality control for the MALS
465 detectors. In each experiment, monomeric BSA was injected before the protein of interest and
466 the settings (calibration constant for TREOS detector, Wyatt Technology) that gave the well-
467 characterized molar mass of BSA (66.4 kDa) were used for the molar mass calculation of the
468 protein of interest. SEC purified OmpC-MlaA-His was concentrated to 5 mg/mL and injected

469 into Superdex 200 Increase 10/300 GL column pre-equilibrated with TBS and 0.025 % DDM.
470 Light scattering (LS) and refractive index (n) data were collected online using miniDAWN
471 TREOS (Wyatt Technology, CA, USA) and Optilab T-rEX (Wyatt Technology, CA, USA),
472 respectively, and analyzed by ASTRA 6.1.5.22 software (Wyatt Technology). Protein-conjugate
473 analysis available in ASTRA software was applied to calculate non-proteinaceous part of the
474 complex. In this analysis, the refractive index increment dn/dc values (where c is sample
475 concentration) of 0.143 mL/g and 0.185 mL/g were used for DDM and protein complex,
476 respectively (34). For BSA, UV extinction coefficient of 0.66 mL/(mg.cm) was used. For the
477 OmpC-MlaA-His complex, that was calculated to be 1.66 mL/(mg.cm), based on its predicted
478 stoichiometric ratio OmpC₃MlaA.

479
480 **Affinity purification experiments.** Affinity purification experiments were conducted using
481 $\Delta mlaA$ strains expressing MlaA-His at low levels from the pET23/42 vector. For each strain, a
482 1.5-L culture (inoculated from an overnight culture at 1:100 dilution) was grown in LB broth at
483 37 °C until OD₆₀₀ of ~0.6. Cells were pelleted by centrifugation at 4700 x g for 20 min and then
484 resuspended in 10-mL TBS containing 1 mM PMSF (Calbiochem) and 50 mM imidazole
485 (Sigma-Aldrich). Cells were lysed with three rounds of sonication on ice (38 % power, 1 second
486 pulse on, 1 second pulse off for 3 min). Cell lysates were incubated overnight with 1 % n-
487 dodecyl β -D-maltoside (DDM, Calbiochem) at 4 °C. Cell debris was removed by centrifugation
488 at 24,000 x g for 30 min at 4 °C. Subsequently, supernatant was incubated with 1 mL Ni-NTA
489 nickel resin (QIAGEN), pre-equilibrated with 20 mL of wash buffer (TBS containing 0.025%
490 DDM and 80 mM imidazole) in a column for 1 h at 4 °C with rocking. The mixture was allowed
491 to drain by gravity before washing vigorously with 10 x 10 mL of wash buffer and eluted with 5

492 mL of elution buffer (TBS containing 0.025% DDM and 500 mM imidazole). The eluate was
493 concentrated in an Amicon Ultra 100 kDa cut-off ultra-filtration device (Merck Millipore) by
494 centrifugation at 4,000 x g to ~100 μ L. The concentrated sample was mixed with equal amounts
495 of 2X Laemmli buffer, boiled at 100 °C for 10 min, and subjected to SDS-PAGE and
496 immunoblot analyses.

497
498 **Trypsin digestion for protein N-terminal sequencing and mass spectrometry analyses.** A 1
499 mg/mL solution of purified OmpC-MlaA-His (OmpC was either wild-type or substituted with
500 *p*Bpa at selected positions) complex was incubated with or without 50 μ g/mL trypsin (Sigma-
501 Aldrich) for 1 h at room temperature. *p*Bpa substituted samples were irradiated with UV at 365
502 nm before trypsin digestion. Samples were then analyzed by SDS-PAGE, followed by SEC. Peak
503 fractions from SEC for each sample were pooled, concentrated using an Amicon Ultra 100 kDa
504 cut-off ultra-filtration device (Merck Millipore), and resuspended in 2 X Laemmli sample buffer
505 before analyses by SDS-PAGE and immunoblot using α -MlaA antibody. For N-terminal
506 sequencing, samples were transferred onto PVDF membrane, followed by Coomassie Blue
507 staining (1–2 s). The desired protein bands were carefully excised with a surgical scalpel. For
508 tandem MS, protein bands were excised from a Coomassie Blue stained Tricine gel. Samples
509 prepared for N-terminal sequencing and tandem MS were kept in sterile 1.5 mL centrifuge tubes
510 before submission for analyses at Tufts University Core Facility, Boston, USA, and Taplin
511 Biological Mass Spectrometry Facility, Harvard Medical School, Boston, USA, respectively.

512
513 **Substituted cysteine accessibility method (SCAM).** 1-mL cells were grown to exponential
514 phase (OD_{600} ~0.6), washed twice with TBS (pH 8.0), and resuspended in 480 μ L of TBS. For

515 the blocking step, four tubes containing 120 μ L of cell suspension were either untreated (positive
516 and negative control tubes added with deionized H₂O) or treated with 5 mM thiol-reactive
517 reagent *N*-ethylmaleimide (NEM, Thermo Scientific) or sodium (2-sulfonatoethyl)
518 methanethiosulfonate (MTSES, Biotium). As MTSES is membrane impermeable, it is expected
519 to react with the free cysteine in MlaA variants only when the residue near or at the membrane-
520 water boundaries, or in a hydrophilic channel. In contrast, NEM is expected to label all MlaA
521 cysteine variants as it is membrane permeable. Reaction with MTSES or NEM blocks the
522 particular cysteine site from subsequently labelling by maleimide-polyethylene glycol (Mal-
523 PEG; 5 kDa, Sigma-Aldrich). After agitation at room temperature for 1 h, cells were washed
524 twice with TBS, pelleted at 16,000 \times *g*, and resuspended in 100 μ L of lysis buffer (10 M urea,
525 1% SDS, 2 mM EDTA in 1 M Tris pH 6.8). Both NEM- and MTSES-blocked samples and the
526 positive control sample were exposed to 1.2 mM Mal-PEG-5k. After agitation for another hour
527 with protection from light, all samples were added with 120 μ L of 2 X Laemmli buffer, boiled
528 for 10 min, and centrifuged at 21,000 \times *g* in a microcentrifuge for one min at room temperature;
529 20 μ L from each sample tubes were subjected to SDS-PAGE and immunoblot analyses.

530

531 **In vivo disulfide bond analysis.** Strain NR1216 ($\Delta dsbA$) harbouring pET23/42*mLaA-His*
532 expressing MlaA-His with site specific cysteine substitutions was grown overnight in LB broth
533 at 37 °C. A 0.5 mL of overnight culture was normalized by optical density, added with
534 trichloroacetic acid (TCA) at final concentration of ~14 % and mixed thoroughly at 4 °C. This
535 step was performed to prevent scrambling of disulfide bond formed in the cysteines substituted
536 MlaA-His. Proteins precipitated for at least 30 min on ice were centrifuged at 16,000 \times *g* for 10
537 min at 4 °C. The pellet was washed with 1 mL of ice-cold acetone and centrifuged again at

538 16,000 × *g* for 10 min at 4 °C. Supernatants were then aspirated and the pellet was air dried at
539 room temperature for at least 20 min. Samples were resuspended thoroughly with 100 μL of
540 either 100 mM Tris.HCl pH 8.0, 1% SDS (for non-reduced samples), or the same buffer
541 supplemented with 100 mM of dithiothreitol (DTT) (for reduced samples), incubated for 20 min
542 at room temperature. The samples were finally mixed with 4 X Laemmli buffer, boiled for 10
543 min and subjected to SDS-PAGE and immunoblotting analyses using α-His antibody.

544 **Docking of MlaA to OmpC.** The ClusPro server (22) was used to dock MlaA (ligand, uniprot
545 ID: P76506, https://gremlin2.bakerlab.org/meta_struct.php?page=p76506) (17) to OmpC
546 (receptor, PDB ID: 2J1N) (18). The default server settings were used in the docking procedure.
547 The minimum distance between 6 residues on OmpC and the corresponding cross-linked peptide
548 regions of MlaA was calculated for all the predicted structures obtained from the server. Two
549 OmpC-MlaA model with the smallest average minimum distance of all residue and peptide pairs
550 were selected as the initial structures for use in the all-atom simulations.

551

552 **Simulation procedures and setup.** All simulations were performed using version 5.1.4 of the
553 GROMACS simulation package (35, 36).

554

555 *All-atom simulations.* In total, 7 all-atom simulations were performed (Table A). The simulations
556 were performed using the CHARMM36 force field parameter set (37). The equations of motion
557 were integrated using the Verlet leapfrog algorithm with a step size of 2 fs. Lengths of hydrogen
558 bonds were constrained with the LINCS algorithm (38). Electrostatic interactions were treated
559 using the smooth Particle Mesh Ewald (PME) method (39), with cutoff for short-range
560 interactions of 1.2 nm. The van der Waals interactions were switched smoothly to zero between

561 1.0 and 1.2 nm. The neighbor list was updated every 20 steps. The Nose-Hoover thermostat (40,
 562 41) with a coupling constant of 1.0 ps was used to maintain a constant system temperature of 313
 563 K. The protein, membrane and solvent (water and ions) were coupled to separate thermostats.
 564 The Parrinello-Rahman barostat (42) with a coupling constant of 5.0 ps was used to maintain a
 565 pressure of 1 bar. Semi-isotropic pressure coupling was used for all the membrane systems,
 566 while isotropic coupling was used for the solvent-only system. Initial velocities were set
 567 according to the Maxwell distribution.

568 Proteins were inserted into a pre-equilibrated, symmetrical 1,2-dimyristoyl-
 569 phosphatidylethanolamine (DMPE) membrane over 5 ns using the membed tool (43) in the
 570 GROMACS simulation package. Subsequent equilibration, with position restraints of 1000 kJ
 571 mol⁻¹ placed on all non-hydrogen protein atoms, was performed for 20 ns to allow the solvent
 572 and lipids to equilibrate around the proteins. The position restraints were removed before
 573 performing the production runs.

574 Table A. Summary of all-atom molecular simulations: system compositions and simulation times

Protein Configuration	Lipids	Water and Ions	Simulation time (# of simulations x ns)
MlaA	N/A	9439 H ₂ O 29 K ⁺ 19 Cl ⁻	1 x 500
MlaA	272 DMPE	11734 H ₂ O 42 K ⁺ 32 Cl ⁻	1 x 500
OmpC trimer MlaA (ClusPro model) in orientation 1	980 DMPE	36113 H ₂ O 98 K ⁺ 98 Cl ⁻	1 x 500 1 x 320 1 x 130
OmpC trimer MlaA (ClusPro model) in orientation 2	980 DMPE	36113 H ₂ O 98 K ⁺ 98 Cl ⁻	1 x 500 1 x 500

575

576 All of the simulations performed are summarized in Table A. For the OmpC trimer docked with
577 MlaA in orientation 1, three separate production simulations with different initial velocities were
578 performed for the OmpC-MlaA complex, resulting in 3 trajectories of 500 ns, 320 ns, and 130 ns
579 in length, respectively. For the OmpC trimer docked with MlaA in orientation 2, two separate
580 production simulations with different initial velocities were performed for the complex, resulting
581 in 2 trajectories of 500 ns in length. Clustering was performed on the MlaA structures obtained
582 from a combined trajectory of all three (MlaA orientation 1) or two (MlaA orientation 2)
583 atomistic simulations – a total of 4750 frames spaced every 0.2 ns. The structures were assigned
584 to clusters using Root Mean Squared Distance (RMSD) with a 0.1 nm cut-off. Four and two
585 clusters, respectively, were observed for MlaA in the two orientations to contain greater than 100
586 frames. The central structure of these four clusters was used to generate the representative
587 OmpC-MlaA models (Fig. S7). `trj_cavity` was used to identify the location the pore cavity (44),
588 and `Hole` was used to create the pore profile (45).

589

590 **Temperature titration for chromosomal *ompC* mutants.** Purified wild-type and mutant
591 OmpC-MlaA-His complexes were aliquoted into 1.5 mL centrifuge tubes and incubated in water
592 bath set at different temperatures for 10 min. 20 μ l of each sample were transferred into separate
593 tubes and mixed immediately with equal volume of 2 X Laemmli buffer and subjected to SDS-
594 PAGE in 12 % Tris.HCl gels, followed by Coomassie Blue staining (Sigma-Aldrich).

595

596 **LPS labeling and lipid A isolation.** Mild acid hydrolysis of [32 P]-labeled cultures was used to
597 isolate lipid A according to a procedure described previously (4, 12, 52) with some
598 modifications. 5 mL cultures were grown (inoculated with overnight cultures at 1:100 dilution)

599 in LB broth at 37 °C until OD₆₀₀ reached ~0.5–0.7 (exponential) or ~2–4 (stationary). Cultures
600 were uniformly labeled with 1 μCi mL⁻¹ [³²P]-disodium phosphate (Perkin-Elmer) from the start
601 of inoculation. One MC4100 wild-type culture labeled with [³²P] was treated with
602 25 mM EDTA, pH 8.0 for 10 min prior to harvesting. Cells (5 mL and 1 mL for exponential and
603 stationary phase cultures respectively) were harvested by centrifugation at 4700 × g for 10 min
604 and washed twice with 1 mL PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄,
605 1.8 mM KH₂PO₄, pH 7.4) at 5000 × g for 10 min. Each cell pellet was resuspended in 0.32 mL
606 PBS, and converted into single phase Bligh/Dyer mixture (chloroform/methanol/water:1/2/0.8)
607 by adding 0.8 mL methanol and 0.4 mL chloroform. The single phase Bligh/Dyer mixture was
608 incubated at room temperature for 20 min, followed by centrifugation at 21,000 × g for 30 min.
609 Each pellet obtained was washed once with 1 mL freshly made single phase Bligh/Dyer mixture
610 and centrifuged as above. The pellet was later resuspended in 0.45 mL 12.5 mM sodium acetate
611 containing 1 % SDS, pH 4.5. The mixture was sonicated for 15 min before incubation at 100 °C
612 for 40 min. The mixture was converted to a two-phase Bligh/Dyer mixture
613 (chloroform/methanol/water: 2/2/1.8) by adding 0.50 mL methanol and 0.50 mL chloroform. The
614 lower phase of each mixture was collected after phase partitioning by centrifugation at
615 21 000 × g for 30 min. The collected lower phase was washed once with 1 mL of the upper phase
616 derived from the freshly made two-phase Bligh/Dyer mixture and centrifuged as above. The final
617 lower phase was collected after phase partitioning by centrifugation and dried under N₂ gas. The
618 dried radiolabeled lipid A samples were redissolved in 100 μL of chloroform/methanol mixture
619 (4/1), and 20 μL of the samples were used for scintillation counting (MicroBeta2®, Perkin-
620 Elmer). Equal amounts of radiolabeled lipids (cpm/lane) were spotted onto the TLC plate (Silica
621 Gel 60 F254, Merck Millipore) and were separated using the solvent system consisting of

622 chloroform/pyridine/96 % formic acid/water (50/50/14.6/4.6) (5). The TLC plate was then dried
623 and exposed to phosphor storage screens (GE Healthcare). Phosphor-screens were visualized in a
624 phosphor-imager (Storm 860, GE Healthcare), and the spots were analyzed by ImageQuant TL
625 analysis software (version 7.0, GE Healthcare). Spots were quantified and averaged based on
626 three independent experiments of lipid A isolation.

627
628 **OM permeability studies.** OM sensitivity against SDS/EDTA was judged by colony-forming-
629 unit (CFU) analyses on LB agar plates containing indicated concentrations of SDS/EDTA.
630 Briefly, 5 mL cultures were grown (inoculated with overnight cultures at 1:100 dilution) in LB
631 broth at 37 °C until OD₆₀₀ reached ~0.4-0.6. Cells were normalized by optical density, first
632 diluted to OD₆₀₀ = 0.1 (~10⁸ cells), and then serially diluted (ten-fold) in LB broth using 96-well
633 microtiter plates. 2.5 µL of the diluted cultures were manually spotted onto the plates, dried, and
634 incubated overnight at 37 °C. Plate images were visualized by *G:Box Chemi-XT4* (Genesys
635 version 1.4.3.0, Syngene).

636
637 **SDS-PAGE, immunoblotting and staining.** All samples subjected to SDS-PAGE were mixed
638 1:1 with 2X Laemmli buffer. Except for temperature titration experiments, the samples were
639 subsequently either kept at room temperature or subjected to boiling at 100 °C for 10 min. Equal
640 volumes of the samples were loaded onto the gels. As indicated in the figure legends, SDS-
641 PAGE was performed using either 12% or 15% Tris.HCl gels (53) or 15% Tricine gel (54) at 200
642 V for 50 min. After SDS-PAGE, gels were visualized by either Coomassie Blue staining, or
643 subjected to immunoblot analysis. Immunoblot analysis was performed by transferring protein
644 bands from the gels onto polyvinylidene fluoride (PVDF) membranes (Immun-Blot 0.2 µm, Bio-

645 Rad, CA, USA) using semi-dry electroblotting system (Trans-Blot Turbo Transfer System, Bio-
646 Rad). Membranes were blocked for 1 h at room temperature by 1 X casein blocking buffer
647 (Sigma-Aldrich), washed and incubated with either primary antibodies (monoclonal α -MlaA (12)
648 (1:3000) and α -OmpC (31) (1:1500)) or monoclonal α -His antibody (pentahistidine) conjugated
649 to the horseradish peroxidase (HRP) (Qiagen, Hilden, Germany) at 1:5000 dilution for 1 – 3 h at
650 room temperature. Secondary antibody ECL™ anti-mouse IgG-HRP was used at 1:5000
651 dilution. Luminata Forte Western HRP Substrate (Merck Millipore) was used to develop the
652 membranes, and chemiluminescence signals were visualized by G:Box Chemi-Xt4 (Genesys
653 version 1.4.3.0, Syngene).

654

656 **Acknowledgements.** We thank Swaine Chen and Varnica Khetrpal (Genome Institute of
657 Singapore, A*STAR) for providing α -OmpC antibody, and reagents for negative selection and
658 assistance in this technique. We also thank Michael Berne (Analytical Core Facility, Tufts
659 Medical School) and Ross Tomaino (Taplin Mass Spectrometry Facility, Harvard Medical
660 School) for performing Edman sequencing and MS/MS, respectively. We gratefully
661 acknowledge computing resources provided by the National Supercomputing Center Singapore
662 (<http://www.nsc.sg>). Finally, we thank Swaine Chen for critical discussions and comments on
663 the manuscript. J.Y. was supported by the National University of Singapore Graduate School for
664 Integrative Sciences and Engineering scholarship. J.K.M. and P.J.B. acknowledge support from
665 the Singapore Ministry of Education Academic Research Fund Tier 3 grant (MOE2012-T3-1-
666 008). All experimental work was supported by the National University of Singapore Start-up
667 funding, the Singapore Ministry of Education Academic Research Fund Tier 1 and Tier 2
668 (MOE2013-T2-1-148) grants (to S.-S.C.).

669
670 **Conflict of Interest.** The authors declare that they have no conflicts of interest with the contents
671 of this article.

672
673 **Author contributions:** J.Y., K.W.T., D.A.H., P.J.B. and S.-S.C designed research; J.Y., K.W.T.,
674 Z.-S.C. performed all wet lab experiments described in this work; D.A.H. and J.K.M. performed
675 all MD simulations; J.Y., K.W.T., D.A.H., P.J.B. and S.-S.C. analyzed and discussed data; J.Y.,
676 K.W.T., and S.-S.C. wrote the paper.

677

678

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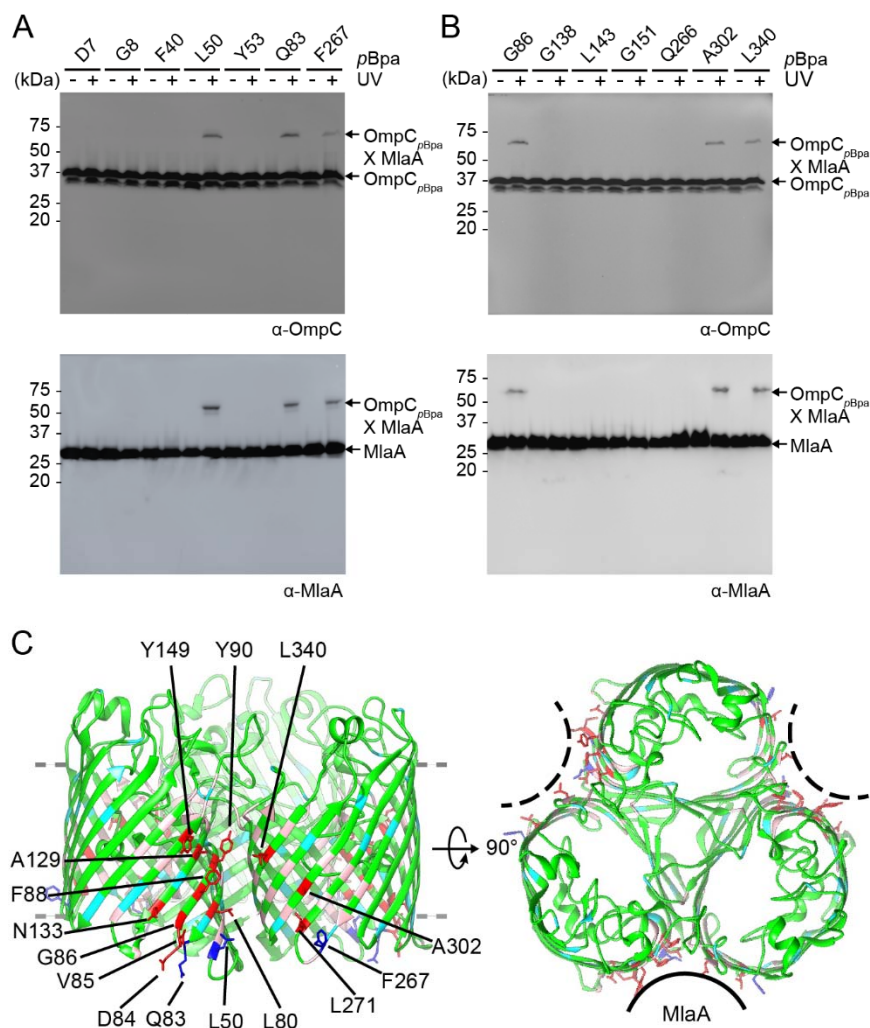
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811

812 **Figures**

813



814

815 **Figure 1.** MlaA binds at the dimeric interfaces of the OmpC trimer in vivo. (A, B)

816 Representative immunoblots showing UV-dependent formation of crosslinks between OmpC and

817 MlaA in $\Delta ompC$ cells expressing OmpC substituted with *pBpa* at indicated positions, selected in

818 a (A) global, or (B) localized search. (C) Side (left) and top (right) views of cartoon

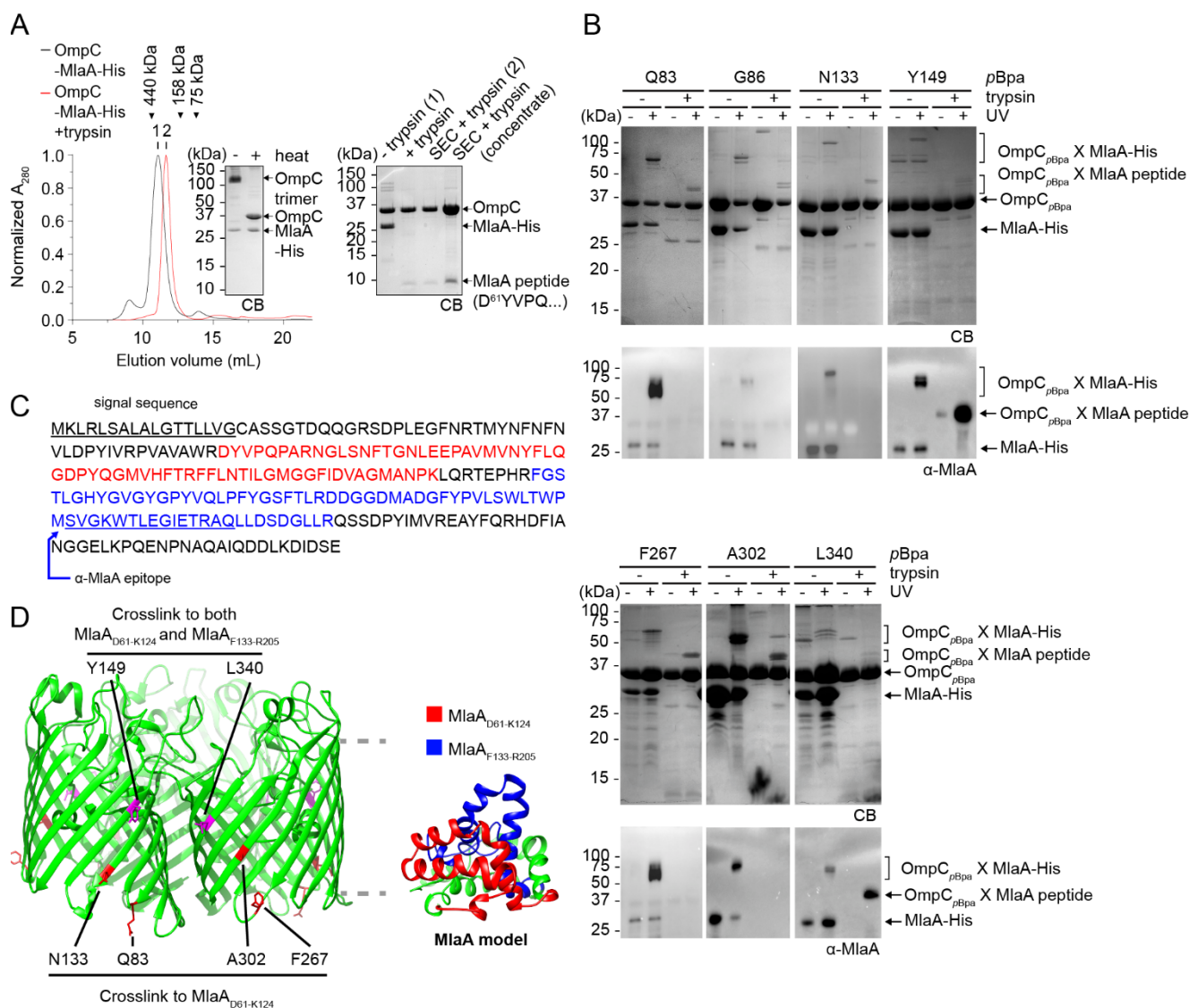
819 representations of the crystal structure of *E. coli* OmpC (PDB ID: 2J1N) (18) with positions that

820 crosslink to MlaA highlighted. Residues selected in the global search for MlaA interaction are

821 colored *cyan* (no crosslinks) and *blue* (sticks; crosslinks detected), while those selected in the

822 localized search are colored *light pink* (no crosslinks) and *red* (sticks; crosslinks detected). The
823 OM boundary is indicated as gray dashed lines. MlaA binding sites are indicated as solid or
824 dashed curves on the top-view representation.

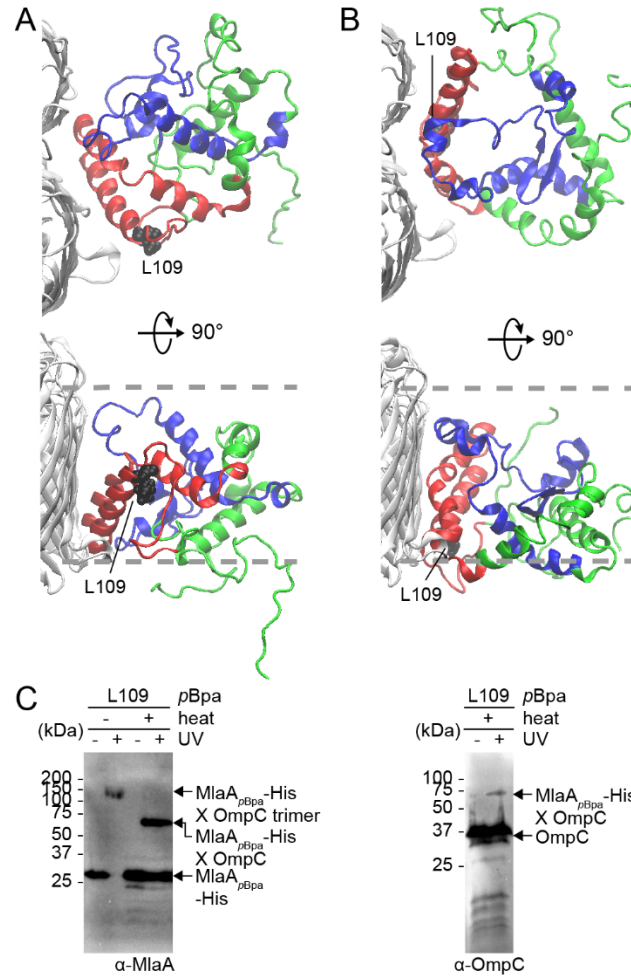
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827 **Figure 2.** OmpC contacts two specific regions on MlaA. (A) SEC profiles and SDS-PAGE
828 analyses of purified OmpC-MlaA-His complex before (*black*) or after (*red*) treatment with
829 trypsin. Peak fractions from SEC were subjected to denaturing SDS-PAGE (15% Tris.HCl gel),
830 followed by Coomassie Blue (CB) staining (*right*). Non-trypsin treated samples were also
831 analysed by seminitive SDS-PAGE (*left*). Edman degradation and tandem MS analyses revealed
832 that the MlaA peptide that remains bound to OmpC following trypsin treatment begins at D61
833 (Fig. S3). (B) SDS-PAGE (15% Tris.HCl gel) and immunoblot analyses of purified OmpC _{ρ Bpa}-

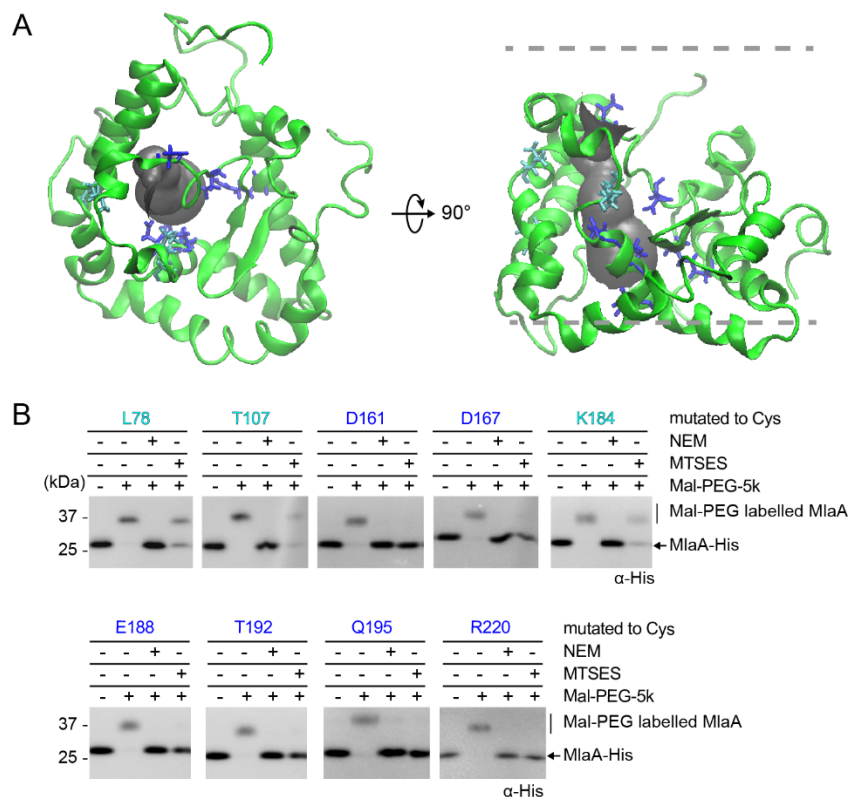
834 MlaA-His complexes following sequential UV irradiation and trypsin digestion. The resulting
835 OmpC_{pBpa}-MlaA_{peptide} crosslinked products were N-terminally sequenced (see Fig. S3). (C)
836 Amino acid sequence of MlaA with the two peptides found crosslinked to OmpC_{pBpa} highlighted
837 (*red*: MlaA_{D61-K124}, *blue*: MlaA_{F133-R205}). The signal sequence and α -MlaA binding epitope are
838 underlined and annotated. (D) Cartoon representations of the crystal structure of *E. coli* OmpC
839 with positions that crosslink to specific MlaA peptides indicated (*left*), and a structural model of
840 MlaA (17) with peptides crosslinked by OmpC_{pBpa} highlighted (*right*). The OM boundary is
841 indicated as gray dashed lines.
842



843

844 **Figure 3.** Molecular models of the OmpC-MlaA complex depict how MlaA may interact with
845 OmpC in the OM bilayer. (A) and (B) Representative MlaA structures bound to OmpC in two
846 possible orientations selected from all-atomistic MD simulation trajectories. MlaA_{D61-K124} and
847 MlaA_{F133-R205} peptides are highlighted in *red* and *blue*, respectively, as in Fig. 2D. L109 is
848 labelled and depicted as *black* spheres on MlaA. The OM boundaries are indicated as *gray*
849 dashed lines. (C) Immunoblots showing UV-dependent formation of a crosslink between MlaA
850 and OmpC in $\Delta mlaA$ cells expressing MlaA_{L109}*pBpa*-His. As expected, the crosslinked product
851 also exhibits heat-modifiable gel shift, indicative of the presence of OmpC.

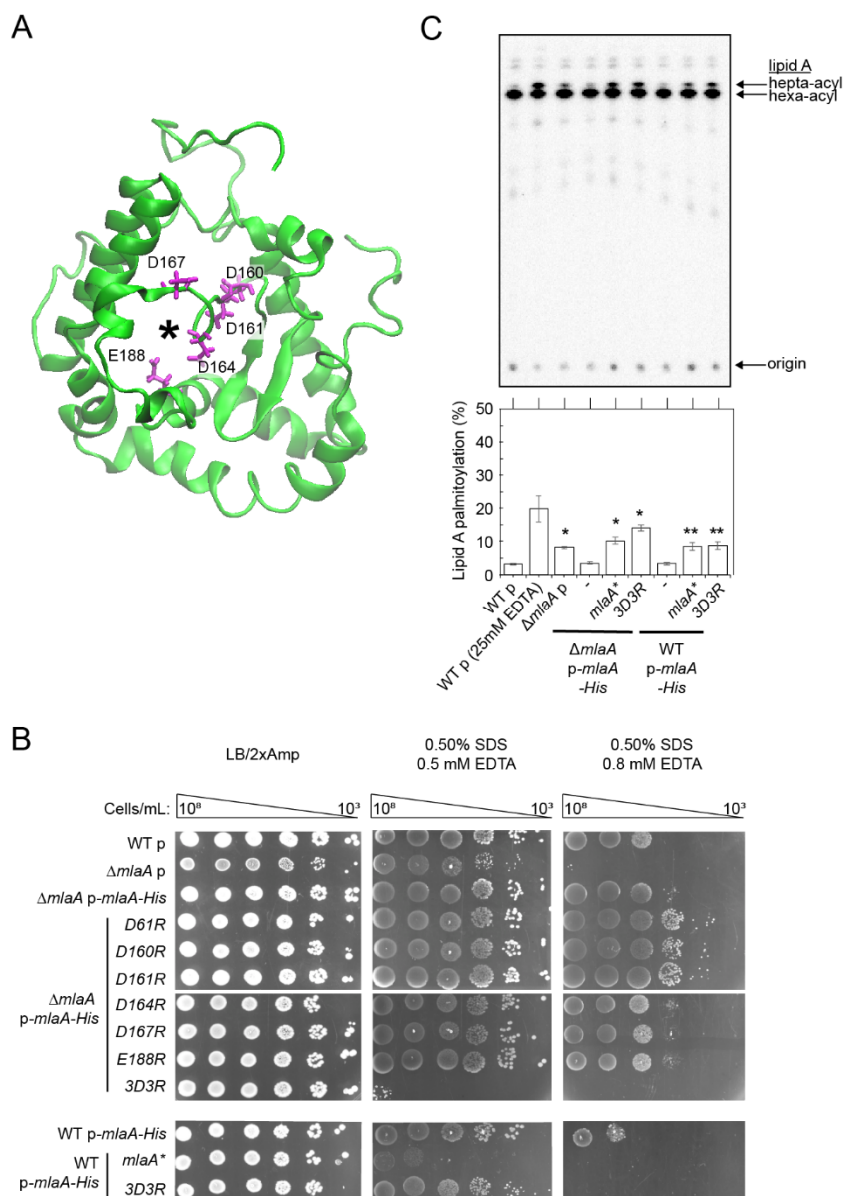
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853

854 **Figure 4.** MlaA forms a hydrophilic channel in the OM. (A) A representative structure of MlaA
855 from all-atomistic MD simulations with its putative channel depicted in *gray*. Residues in the
856 channel that are fully or partially solvent accessible, based on SCAM in (B), are highlighted in
857 *blue* and *cyan*, respectively. (B) Immunoblots showing maleimide-polyethylene glycol (Mal-
858 PEG) alkylation of MlaA variants containing channel-facing residues substituted with cysteine
859 (as depicted in (A)) following labelling by membrane permeable *N*-ethylmaleimide (NEM) or
860 impermeable (MTSES) reagents. Mal-PEG alkylated MlaA_{Cys}-His variants show a ~5 kDa mass
861 shift. Positions fully or partially blocked by MTSES, which reflects the level of solvent
862 accessibility, are highlighted in *blue* or *cyan*, respectively.

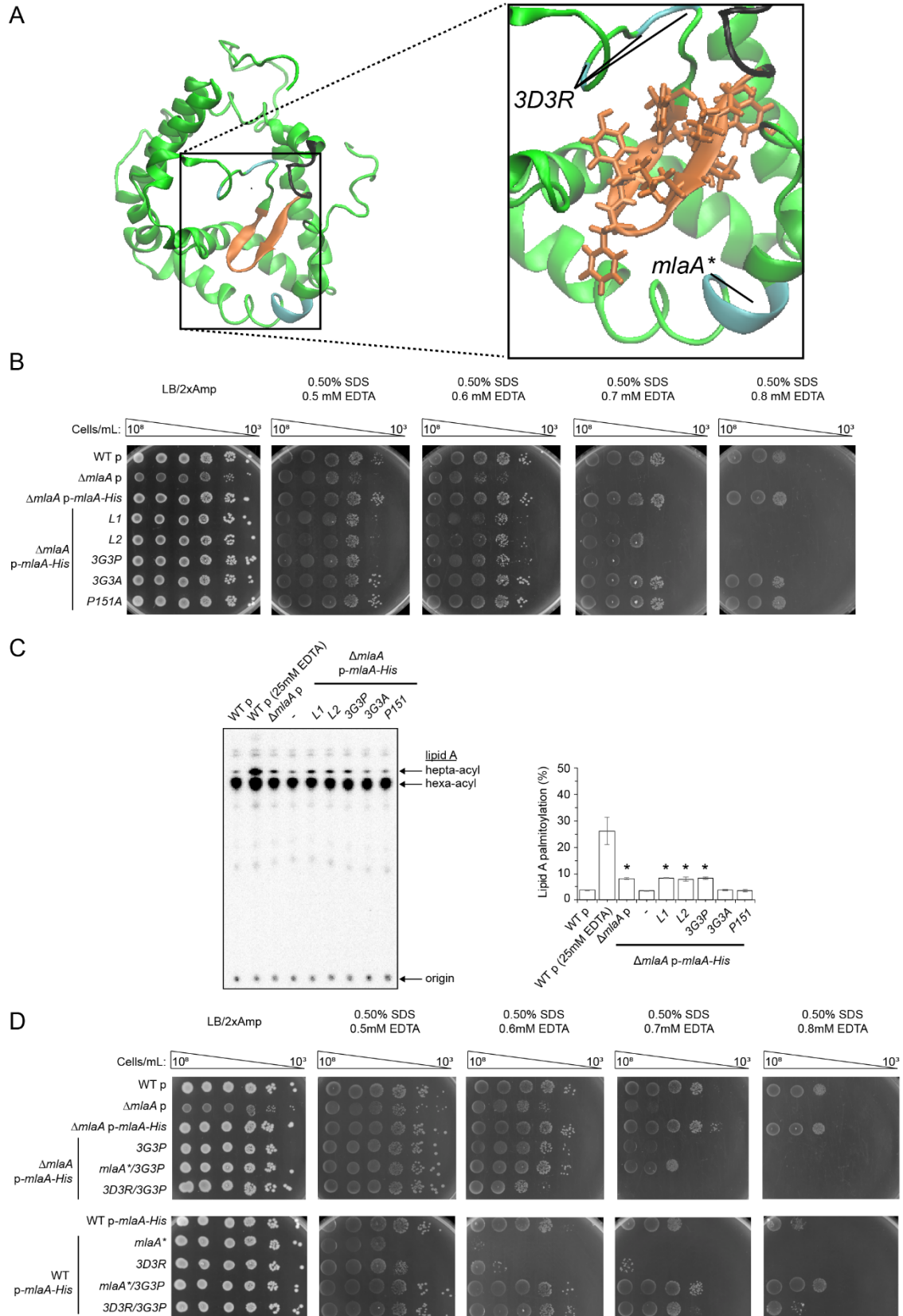
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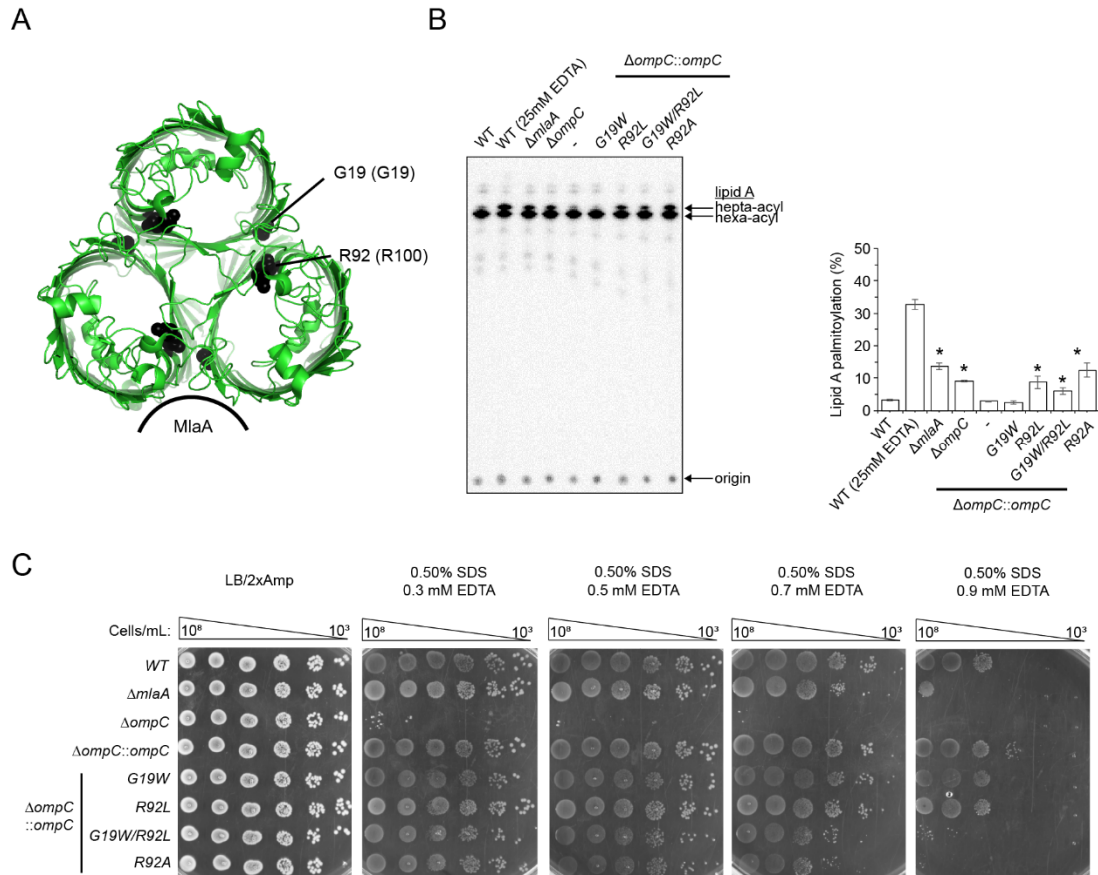
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865 **Figure 5.** A triple charge-inversion mutation in the hydrophilic channel within MlaA results in
 866 gain-of-function phenotypes. (A) A representative structure of MlaA from all-atomistic MD
 867 simulations (as in Fig. 3, top view) with position of the channel depicted by an *asterisk*.
 868 Negatively charged residues mutated to arginine are highlighted (*magenta*, in sticks). (B)
 869 Analysis of SDS/EDTA sensitivity of wild-type (WT) and $\Delta mlaA$ strains producing indicated
 870 MlaA variants at low levels from the pET23/42 vector (p) (12). Serial dilutions of respective
 871 cultures were spotted on LB agar plates containing Amp, supplemented with or without 0.50%

872 SDS and 0.5/0.8 mM EDTA, as indicated, and incubated overnight at 37 °C. (C) Representative
873 thin layer chromatography (TLC)/autoradiographic analysis of [³²P]-labeled lipid A extracted
874 from exponential phase cultures of strains described in (B). As a positive control for lipid A
875 palmitoylation, WT cells were treated with 25 mM EDTA for 10 min prior to extraction. Equal
876 amounts of radioactive material were spotted for each sample. Average percentages of
877 palmitoylation of lipid A and the standard deviations were quantified from triplicate experiments
878 and plotted below. Student's t-tests: *, $p < 0.005$ compared to WT with empty vector; **, $p <$
879 0.001 compared to WT *p-mlaA-His*.



881 **Figure 6.** Flexibility in a hairpin loop structure on MlaA adjacent to the hydrophilic channel is
882 critical for function. (A) A representative structure of MlaA from all-atomistic MD simulations
883 (as in Fig. 3B) with the hairpin loop adjacent to the hydrophilic channel highlighted. In the
884 expanded representation, the *3D3R* and *m1aA** mutations, the hairpin loop, and the glycine rich
885 region N-terminal to the loop are colored in *cyan*, *orange* and *black*, respectively. Residues on
886 the hairpin loop chosen for mutation are represented in sticks. (B) Analysis of SDS/EDTA
887 sensitivity of wild-type (WT) and $\Delta m1aA$ strains producing indicated MlaA loop variants from
888 the pET23/42 vector (p). (C) Representative TLC/autoradiographic analysis of [32 P]-labeled lipid
889 A extracted from exponential phase cultures of strains described in (B). Equal amounts of
890 radioactive material were spotted for each sample. Average percentages of palmitoylation of
891 lipid A and the standard deviations were quantified from triplicate experiments and plotted on
892 the right. Student's t-tests: *, $p < 0.0005$ compared to WT with empty vector. (D) Analysis of
893 SDS/EDTA sensitivity of wild-type (WT) and $\Delta m1aA$ strains producing indicated MlaA variants
894 from the pET23/42 vector (p).



895

896 **Figure 7.** A specific mutation in the dimeric interface of the OmpC trimer results in perturbation
 897 in OM lipid asymmetry. (A) Cartoon representation of the crystal structure of OmpC trimer
 898 illustrating the positions of G19 and R92 region. (B) Analysis of SDS/EDTA sensitivity of wild-
 899 type (WT) and $\Delta ompC$ strains producing indicated OmpC variants from the chromosomal locus.
 900 (C) Representative TLC/autoradiographic analysis of [32 P]-labeled lipid A extracted from
 901 stationary phase cultures of strains described in (B). Equal amounts of radioactive material were
 902 spotted for each sample. Average percentages of palmitoylation of lipid A and the standard
 903 deviations were quantified from triplicate experiments and plotted on the right. Student's t-tests:
 904 *, $p < 0.005$ compared to $\Delta ompC::ompC$.