Lipid interactions are important for the Tol-Pal complex in maintaining outer membrane lipid homeostasis

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Abstract:

Gram-negative bacteria are intrinsically resistant to many antibiotics in part due to the asymmetric architecture and barrier function of their outer membrane (OM). To establish proper lipid asymmetry, cells need to ensure an intricate balance of constituent OM components, especially lipids. In this regard, the conserved, trans-envelope Tol-Pal complex plays a primary role in maintaining OM lipid homeostasis, thus OM integrity, possibly via retrograde phospholipid transport. However, mechanistic details for this process are unknown, owing to the lack of evidence for direct lipid binding. In this study, we discover that the periplasmic protein TolB, a key component of the Tol-Pal system, associates directly with membranes in vitro, via specific interactions with cardiolipin (CL). Using coarse-grained molecular dynamics simulations, we identify a CL-binding site on TolB; a single amino acid mutation at this site abolishes in vitro membrane interaction, consequently impairing cellular Tol-Pal function in maintaining OM homeostasis in *Escherichia coli*. Curiously, we find that the functional requirement for TolB-CL interactions can be bypassed in cells lacking CL, suggesting compensatory effects through other lipids. Our findings reveal a previously unappreciated lipid-binding role for TolB, and provide novel insights into how the Tol-Pal complex may facilitate phospholipid transport across the cell envelope. Our work will inform future strategies towards developing new antibiotics against Gram-negative bacteria.

37 **Significance:**

38 The integrity of a biological membrane enables the definition of cellular boundaries, and of life.
39 In Gram-negative bacteria, the outer membrane presents a formidable barrier against
40 antibiotics, making it a key contributor to intrinsic antimicrobial resistance. While the Tol-Pal
41 complex is known to ensure outer membrane stability and lipid homeostasis, its precise
42 mechanism remains elusive. This study reveals a direct lipid-binding role for TolB, a
43 periplasmic component of the complex, and identifies cardiolipin as a critical interacting partner.
44 Our work establishes a novel dimension of Tol-Pal function in outer membrane lipid
45 homeostasis and transport, opening new avenues for targeting envelope maintenance
46 systems in Gram-negative pathogens.

47 **Introduction:**

48 Gram-negative infections pose a significant threat to global health due to existing/intrinsic and
49 emerging/acquired resistance to antibiotics.[1] A large part of such antibiotic resistance stems
50 from the formidable barrier conferred by the complex cell envelope, particularly the outer
51 membrane (OM), which encapsulates the peptidoglycan layer and the inner membrane (IM).[2]
52 Consequently, a great many efforts have been focused on understanding Gram-negative OM
53 biogenesis and homeostasis, culminating in well-established mechanisms for the assembly of
54 major OM components, including β-barrel proteins,[3] lipoproteins,[4] and lipopolysaccharides
55 (LPS).[5] Even so, little is known about the bidirectional transport of bulk phospholipids (PLs),
56 the most basic building block of any bilayer, across the cell envelope.[6-8] In this regard, AsmA
57 superfamily proteins and the Tol-Pal complex have only recently been implicated in
58 anterograde (IM-to-OM) and retrograde (OM-to-IM) PL transport, respectively.[9-13]

59 The Tol-Pal complex is critically important for OM stability and lipid homeostasis.[10, 11] In the
60 absence of the Tol-Pal complex, cells accumulate excess PLs in the OM; such lipid imbalance
61 gives rise to OM instability and permeability defects, characterized by hypervesiculation, and
62 severe sensitivity to antibiotics and detergents.[14-17] In fact, *tol-pal* deletion cells exhibit slower
63 retrograde PL transport, suggesting critical involvement in the process.[11] The Tol-Pal complex
64 is also involved in cell division; constituent components are enriched at mid-cell during division,
65 and mutants display defects in OM constriction, and/or septal cell wall separation (under
66 hypoosmotic conditions).[18, 19] However, it has now been established that septal localization of
67 the Tol-Pal complex is not required for OM integrity, underscoring its primary role in OM lipid
68 homeostasis and transport.[10]

69 The Tol-Pal complex comprises the TolQRA and TolB-Pal sub-complexes at the IM and the
70 OM, respectively.[20] Pal is an OM lipoprotein that either binds the cell wall,[21-23] thus tethering
71 the OM, or interacts with the periplasmic protein TolB.[21, 24] The TolQRA sub-complex
72 harnesses the proton motive force (pmf) to transduce a force on the TolB-Pal sub-complex,
73 through TolA-TolB interaction across the cell envelope.[20] While such force transduction is
74 believed to be important for localization of Pal to the division site, and is hijacked by colicins
75 for cellular entry,[20] how the same mechanism contributes to the main function of the Tol-Pal
76 complex in OM lipid homeostasis is unclear.[10, 11, 18, 25] It has been proposed that the Tol-Pal
77 complex directly mediates retrograde transport of bulk PLs.[11] Yet, there is no evidence of lipid
78 binding by any of the Tol-Pal components.

79 In this study, we establish that the function of TolB requires direct interaction with membranes,
80 with specificity for a particular PL species, cardiolipin (CL). While trying to characterize TolB-

81 Pal interaction at the membrane in vitro, we serendipitously discover that TolB alone binds
82 directly to liposomes. In addition, we find that this membrane association depends on the
83 presence of CL, both in vitro and in silico. Furthermore, molecular dynamics (MD) simulations
84 revealed a putative CL-binding site on TolB; we demonstrate that a specific mutation at this
85 site disrupts TolB interactions with CL-containing membranes, as well as its cellular function.
86 Our work underscores the functional relevance of TolB-membrane association, and provides
87 novel insights into how the Tol-Pal complex may mediate PL transport.

88

89 **Results:**

90 **TolB interacts with membranes.**

91 We initially set out to study how TolB interacts with Pal in a native environment. The strong
92 interaction between TolB and Pal was previously observed using purified full-length TolB and
93 truncated Pal proteins that are entirely soluble in aqueous solution.[24] However, full-length Pal
94 is a lipoprotein anchored to the inner leaflet of the OM via its N-terminal triacyl modification,
95 and it is not known how the TolB-Pal interaction may be affected at/near a lipid bilayer.
96 Therefore, we reconstituted Pal into liposomes, and examine its interaction with TolB using
97 isothermal calorimetry (ITC). In our proteoliposomes derived from *E. coli* polar lipids, ~80% of
98 Pal is anchored on the surface, accessible to TolB added in the external solution (Fig S1). We
99 first showed that control titrations of TolB into buffer or buffer into Pal proteoliposomes gave
100 negligible background heat changes. Interestingly, we also did not observe significant signals
101 between TolB and Pal proteoliposomes, in contrast to strong heat changes detected between
102 TolB and soluble Pal under identical conditions (Fig 1A).[24] This unexpected result suggests
103 either that TolB cannot bind Pal at the membrane, or that other interactions, possibly with the
104 bilayer, mask TolB-Pal binding.

105 We hypothesized that TolB may interact with membranes, therefore titrated TolB into empty
106 liposomes made from *E. coli* polar lipids, comprising phosphatidylethanolamine (PE):
107 phosphatidylglycerol (PG):CL in a ratio of ~67:23:10. Remarkably, we detected endothermic
108 heat changes between TolB and liposomes. As a control, the soluble domain of the OM
109 lipoprotein LolB (sLolB) exhibited no interaction with empty liposomes (Fig 1B).[4] Furthermore,
110 we found that TolB, but not sLolB, affected the sedimentation of liposomes in an in vitro assay
111 (Fig S2). Taken together, our results demonstrate that TolB interacts directly with the lipid
112 bilayer.

113

114 **TolB exhibits specificity for CL binding.**

115 To understand the nature of interaction between TolB and the lipid bilayer, we also examined
116 TolB binding to empty liposomes derived from synthetic lipids representing major species in *E.*
117 *coli* (PE:PG 3:1). Interestingly, we did not observe any heat changes when TolB was titrated
118 into these synthetic lipid liposomes (Fig 1B). TolB also did not impact these liposomes during
119 sedimentation in vitro (Fig S2), consistent with the lack of interaction. A notable distinction
120 between liposomes derived from synthetic lipids or *E. coli* polar lipids is the presence of CL in
121 the latter, suggesting that TolB interacts with membranes in a CL-dependent manner.

122 To explore the possibility of TolB-CL interaction, we conducted interaction studies in silico
123 using coarse-grained (CG) molecular dynamics (MD) simulations. We positioned TolB ~8 nm
124 from membranes approximating our in vitro setup, namely PE:PG:CL at 67:23:10 (+CL), or
125 PE:PG at 75:25 (-CL). By running 5 x 10 μs simulations in each condition, we were able to
126 follow prospective interactions of TolB with either membrane. In the absence of CL, the
127 distance between TolB and the bilayer fluctuated constantly, indicating brief and unstable
128 interactions (Fig 2A). In stark contrast, when CL is present, TolB rapidly binds to the bilayer
129 and stays bound throughout the simulations (Fig 2A). Averaging the overall time that TolB
130 remains bound to the membrane (in all binding poses) over the 5 runs revealed an extremely
131 high membrane binding likelihood in the presence of CL, ca. 91% vs 39% when CL was absent.
132 These data corroborate our experimental observation that TolB interacts with membranes in a
133 CL-dependent manner.

134

135 **Disrupting the CL binding ability of TolB abolishes its function in OM lipid homeostasis.**

136 We employed PyLipID, a software tool designed for predicting lipid binding sites within proteins,
137 to identify possible CL binding sites on TolB based on our CG-MD data.[26, 27] PyLipID enables
138 the calculation of residence times and binding probabilities between proteins and lipid
139 molecules from MD simulations. Our analysis revealed that TolB indeed harbors a CL-binding
140 site, which remained occupied with CL for ~81% of the total simulation time, clustering on
141 average ~4.4 CL molecules via their head groups (Fig 2B). Considering binding through this
142 site alone, membrane binding likelihood of TolB effectively reduced to zero in the absence of
143 CL (Fig 2C). TolB contains a C-terminal six-bladed β-propeller domain linked to a short peptide,
144 termed the 'TolA box', at the N terminus, via a α/β globular domain.[28] The TolA box and the β-
145 propeller domain interact with TolA and Pal, respectively.[24, 28, 29] The putative CL-binding site
146 comprises residues from 4 adjacent loop regions in the β-propeller domain of TolB, collectively
147 presenting 4 positively charged side chains (Arg^{223}, Arg^{244}, Lys^{264}, Arg^{287}) for interaction with

148 the phosphate moieties in the CL head groups (Fig 2D).[27] These Arg/Lys residues, along with
149 neighbouring side chains, all exhibit ~60% median CL occupancy (Fig 2B).

150 To investigate the importance of the putative CL-binding site, we substituted each Arg/Lys
151 position with either alanine or glutamate, and assessed whether these mutations perturbed
152 TolB function. The alanine variants behaved similarly to wildtype TolB (Fig 3A). Remarkably,
153 we found that R244E rendered this TolB charge-swapped variant incapable of rescuing
154 sensitivity to SDS-EDTA and vancomycin in the Δ*tolB* strain (Fig 3A, 4A), as well as the
155 increased cell width phenotype associated with OM lipid dyshomeostasis (Fig 4B).[10]
156 Importantly, this mutation did not impact TolB stability (Fig S3A), its ability to facilitate colicin A
157 entry (Fig S3B), nor its cellular interaction with Pal (Fig S4), indicating a true and specific loss
158 of function in maintaining OM integrity. In fact, we demonstrated that $TolB^{R244E}$ failed to restore
159 OM defects in a strain expressing a chimeric version of the Tol-Pal complex, whose only
160 function is in OM lipid homeostasis along the cell periphery (Fig 3B).[10] These findings indicate
161 that the functional impairment caused by the R244E mutation is most likely directly linked to
162 the disruption of TolB-CL binding.

163 R244 occupies a central position within the predicted CL-binding site. Therefore, we
164 hypothesize that changing this positively charged residue to a negative one (R244E) could
165 introduce repulsive interactions with the negatively charged head groups of CL, and disrupt
166 binding. Consistent with this idea, introduction of R244E in silico significantly reduced the
167 binding likelihood of TolB to CL-containing membranes, to ~49% chance (Fig 3C). We further
168 demonstrated that $TolB^{R244E}$ no longer exhibits significant heat changes when titrated into
169 liposomes derived from *E. coli* polar lipids (Fig 3D), indicating loss of its ability to bind CL.
170 $TolB^{R244E}$ also does not affect liposome sedimentation (Fig S5). Taken together, we conclude
171 that CL binding via R244 is critical for TolB function as part of the Tol-Pal complex to maintain
172 OM lipid homeostasis in wildtype cells.

173

174 **The requirement for TolB-CL binding for OM lipid homeostasis is bypassed in strains**
175 **lacking CL.**

176 Since CL binding is important for TolB function, one might expect CL-deficient strains to exhibit
177 phenotypes associated with an impaired Tol-Pal complex. However, we found that Δ*clsABC*
178 mutations (Δ*cls*) do not cause apparent OM permeability defects (Fig S6). These observations
179 suggest that the presence of CL may not be strictly essential for Tol-Pal function. To further
180 test this idea, we examined whether the CL binding ability of TolB is still required in the Δ*cls*
181 mutant. $TolB^{R244E}$ was produced at comparable levels to wildtype TolB in Δ*tolB* strains (Fig S7).
182 To our surprise, $TolB^{R244E}$ was able to rescue sensitivity of Δ*cls* Δ*tolB* strain to SDS-EDTA and

183 vancomycin, to a large extent (Fig 4A). This mutation is even able to rescue the width defect
184 in Δ*tolB* cells in the absence of CL (Fig 4B), overall demonstrating that TolB^{R244E} remains
185 functional in OM lipid homeostasis in cells lacking CL. Collectively, our results suggest that
186 although CL-binding is important for TolB activity in wild-type cells, this requirement is
187 bypassed when CL is absent, potentially due to compensatory changes in the membrane
188 environment.

189

190 **TolB-CL binding is important for proper cell division**

191 Despite having a primary function in maintaining OM lipid homeostasis, the Tol-Pal complex
192 is recruited to mid-cell to facilitate OM invagination and/or septal cell wall separation. These
193 roles during cell division are thought to involve Pal-mediated OM-cell wall tethering,
194 modulation of cell wall enzymes, and/or possible OM lipid remodelling.[10, 18, 19] We examined
195 the impact of TolB^{R244E} on cell division phenotypes, and found that cells expressing this variant
196 alone exhibited impaired growth and cell chaining under low osmolarity conditions (Fig S8).
197 Using TolB-sfGFP constructs expressed at comparable levels (Fig S9), we showed that R244E
198 did not affect enrichment of TolB to the mid cell, under normal osmolarity conditions (Fig 5A).
199 Furthermore, Pal-mCherry still localized to the cell septum in the presence of TolB^{R244E}, albeit
200 less strongly than in strains expressing wildtype TolB (Fig 5C). Interestingly, removing CL (Δ*cls*)
201 also enabled TolB^{R244E} to partially rescue growth defects, though not cell chaining, under low
202 osmolarity conditions (Fig S7). While strains lacking CL overall exhibited increased Pal
203 localization to mid cell, it was still weaker for TolB^{R244E}-expressing cells (Fig 5B and D).
204 Regardless of whether CL was available, however, Pal recruitment to the division site was still
205 significant when TolB^{R244E} was present, as opposed to no enrichment in the absence of TolB
206 (Fig 5C and D), to facilitate OM-cell wall tethering. We conclude that loss of TolB-CL binding
207 may impact cell division instead via its effect on OM lipid homeostasis.

208

209 **Discussion:**

210 How the Tol-Pal complex maintains OM lipid homeostasis has remained elusive. While it has
211 been implicated in retrograde transport of bulk PLs, there has been a lack of evidence for any
212 protein-lipid interaction within the Tol-Pal complex.[10, 11] In this study, we have uncovered the
213 ability of the periplasmic protein, TolB, to bind lipid membranes in a CL-specific manner. We
214 have shown that disruption of a putative CL-binding site on TolB abolished binding of CL-
215 containing membranes, and impaired overall cellular function in OM lipid homeostasis.
216 Interestingly, CL-binding became dispensable for Tol-Pal function in cells lacking CL,

217 suggesting possible compensatory effects of other membrane lipids. Overall, our work
218 provides critical insights into the direct involvement of the Tol-Pal complex in lipid transport.

219 The discovery of TolB-CL interaction facilitates new ideas on how the Tol-Pal complex may
220 maintain OM lipid homeostasis and transport. Due to its cone-shaped structure, CL is generally
221 believed to be a non-bilayer lipid, which may preferentially localize to membrane regions of
222 high (negative) curvature.[30] This property is often attributed for why CL is enriched at the cell
223 poles,[31] albeit along with PG, a cylindrical bilayer lipid. In this vein, it is possible that CL would
224 be found in other highly curved or strained membrane regions around the cell periphery. In
225 cells lacking the Tol-Pal complex, excess PLs accumulate in the OM,[11] which may induce local
226 "ruffling" of the membrane, particularly in the inner leaflet. We speculate that CL may localize
227 to such bulges in the OM, presumably of high curvature, therefore marking sites of excess
228 lipids for TolB recognition. This process could be aided by Pal binding to TolB, positioning TolB
229 closer to the inner leaflet of the OM. Binding of TolB to CL at these sites may facilitate
230 subsequent interactions with TolA, which exerts a pulling force via the TolQR motor-stator
231 complex at the IM. Therefore, CL may serve as a spatial cue for TolB recruitment, enabling
232 the Tol-Pal complex to possibly mediate lipid transport, and maintain OM homeostasis. In the
233 absence of CL, and perhaps only so, we reason that other non-bilayer lipids, e.g. PA or PE,
234 could play compensatory role(s), rendering TolB-CL interactions non-essential. Further
235 (biophysical) characterization of TolB-membrane binding will be critical. Given the essential
236 role of TolB in OM homeostasis, its membrane-binding activity could be exploited as a potential
237 drug target.

238 The primary function of the Tol-Pal complex is to maintain OM lipid homeostasis, possibly via
239 retrograde PL transport.[10, 11] In this context, given its ability to bind membranes, TolB is quite
240 likely the main effector. Interestingly, the identified binding site to CL lies in the C-terminal β-
241 propeller domain of TolB, lining the region(s) where TolB interacts with colicins or with Pal.[24,
242 32] We propose that TolB binding to the membrane bulges may allow its N-terminal "TolA box"
243 to become accessible (to TolA), similar to the effect of binding to colicins, instead of Pal. The
244 pulling force thus transduced by the TolQRA complex on TolB and its associated OM leaflet
245 could bring this assembly closer to the IM, eventually leading to hemifusion, and possibly
246 retrograde diffusion of PLs across a membrane bridge.[13] Here, the role of Pal as a cell wall
247 anchor may define permissive sites across the peptidoglycan mesh, and facilitate such trans-
248 envelope membrane dynamics while preserving overall cell envelope integrity. This
249 mechanistic working model, while speculative, provides a useful framework for our continued
250 understanding of the Tol-Pal complex and its function in OM lipid homeostasis and transport
251 in Gram-negative bacteria.

Material and Methods:

Strains, plasmids, and growth conditions

The strains, plasmids and primers used were summarized in Table S1-4. All strains were grown in lysogeny broth (LB – 1% (w/v) tryptone and 0.5% (w/v) yeast extract with or without 1% (w/v) NaCl) liquid culture at 37 ºC or on solid LB agar plates (1.5% (w/v) Bacto-Agar added to LB media) supplemented with ampicillin (100 µg/mL), spectinomycin (20 µg/mL), chloramphenicol (30 µg/mL), 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG). The gene deletion alleles were constructed using λ Red recombination and transduced into strains using P1 transduction.[33] Plasmids expressing TolB-sfGFP, Pal-mCherry, TolB-Pal-mCherry were constructed using Gibson assembly.[34]

Isothermal Calorimetry (ITC)

TolB, sPal and Pal proteoliposomes in PBS were used for ITC. All proteins used in the experiment were in the same buffer (20 mM phosphate, 300 mM NaCl) to ensure no heat release from the mixture of different buffers. Prior to performing ITC, samples were centrifuged (10,000 x *g*, 4 ºC, 5mins) to remove any precipitate. ITC was carried using NanoITC Microcalorimeter (TA Instruments). 50 µL of TolB at concentration of 140 µM (7 nmol) was injected into 20µM (166 µL, 3.32 nmol) of sPal or Pal proteoliposomes over 48 injections with an interval of 220 seconds. Experiments were carried out at 25 ºC. 4.15 nmol of Pal proteins were used to generate Pal proteoliposomes, with ~80% surface-exposed (~3.32 nmol).

Coarse-grained molecular dynamics simulations

Membrane binding of TolB was assayed using coarse-grained (CG) simulations. The monomeric TolB crystal structure was used as an input [Protein Data Bank ID code 2W8B; chain A.[24] Protein atoms were converted to the CG Martini 3 force field[35] using the martinize2 method.[36] Additional bonds of 500 kJ mol^{-1} nm^{-2} were applied between all protein backbone beads within 1 nm. Proteins were oriented randomly using the Gromacs *editconf* tool, with a different starting orientation for each repeat. Proteins were placed 8 nm from a membrane (centre-of-mass distance), which were built using the insane protocol.[37] Simulation boxes were 11 x 11 x 21 nm. All systems were solvated with Martini waters and Na^+ and Cl^- ions to a neutral charge and 150 mM. Systems were minimized using the steepest descents method, followed by 1 ns equilibration with 5 fs time steps, then by 100 ns equilibration with 20 fs time steps, before 10 x 10 µs production simulations using 20 fs time steps, all in the NPT ensemble at 323 K with the V-rescale thermostat (τ_t = 1.0 ps) and semi-isotropic Parrinello-Rahman pressure coupling at 1 bar (τ_p = 12.0 ps). The Reaction-Field method was used to model long-range electrostatic interactions. Bond lengths were constrained to their equilibrium values

286 using the LINCS algorithm. Simulations were run in Gromacs 2020.3.[38, 39] Plots were made
287 using Prism 10. MD snapshots were made using VMD.[40] Data were analysed using Gromacs
288 tools and the PyLipID package.[26]

289 **Efficiency of plating (EOP)**

290 Strains were grown to mid-log (OD_{600} ~0.6) and normalized to an OD_{600} of 0.1. 10-fold dilution
291 was performed to obtain a range of concentration of strains. 5µL of these diluted cultures were
292 spotted on LB agar plates containing 0.5% sodium dodecyl sulfate (SDS)/ 0.5 mM
293 Ethylenediaminetetraacetic acid (EDTA), 60 µg/mL vancomycin or without NaCl, unless
294 otherwise stated. The LB agar plates were then incubated at 37 °C (for SDS EDTA and
295 vancomycin plates) or at 42 °C (for plates without NaCl) for approximately 18hrs. All results
296 shown are representative of at least three independent replicates.

297 **Microscopy**

298 Overnight cultures of the strains were grown in LB and diluted (1:2000) in fresh media. The
299 sub-cultures were grown to mid-log (~ 3 hrs) and concentrated 10-fold by centrifugation (3,000
300 x *g*, 5 mins). 10 µL of the sample was spotted on an agarose pad (1% agarose, in LB medium)
301 and viewed under a confocal microscope (Olympus FV3000 Confocal Microscope). Images
302 were analysed using ImageJ/FIJI (version 1.53c) and the MicrobeJ (version 5.13I) plugin.[41, 42]

303

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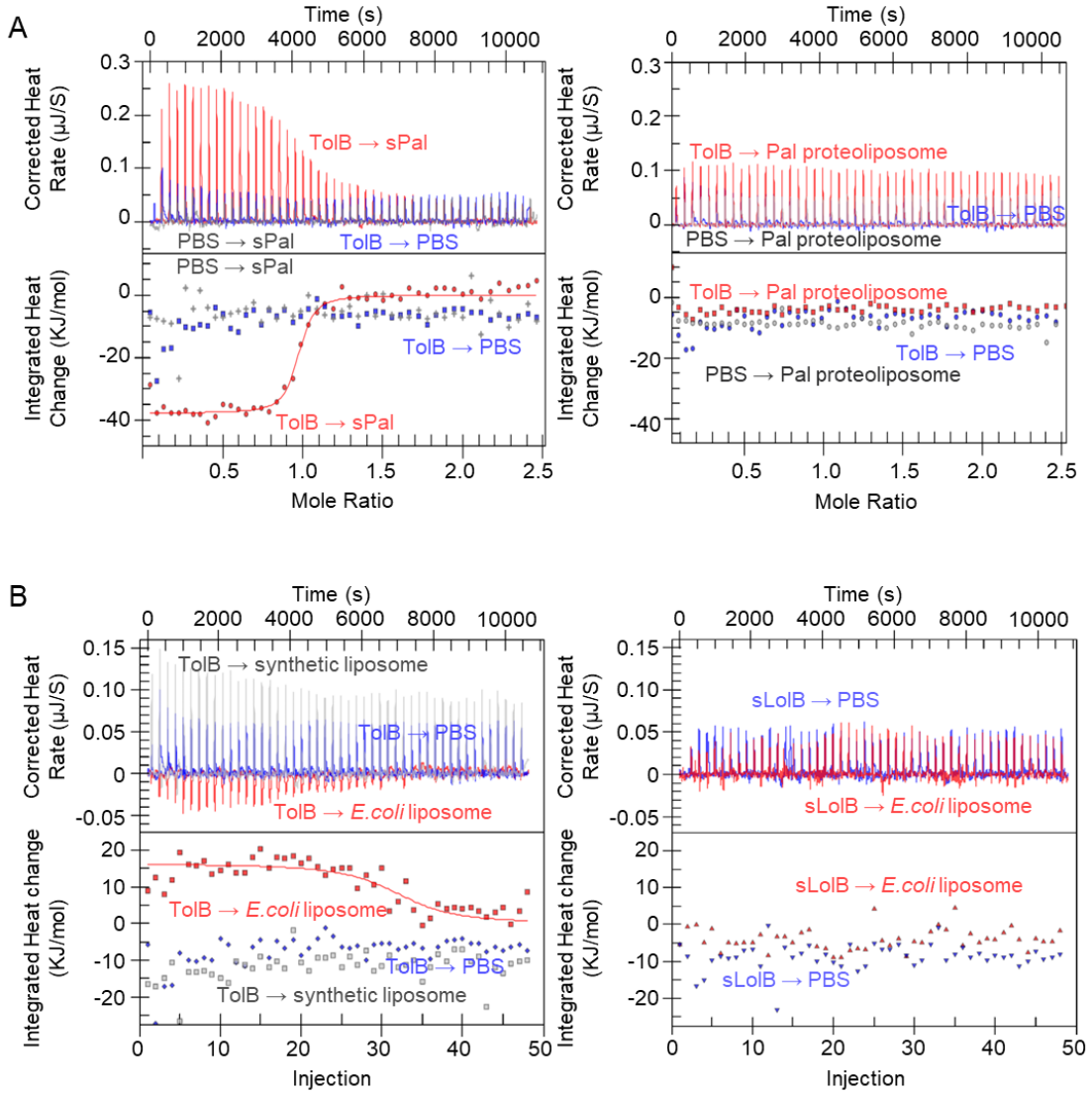
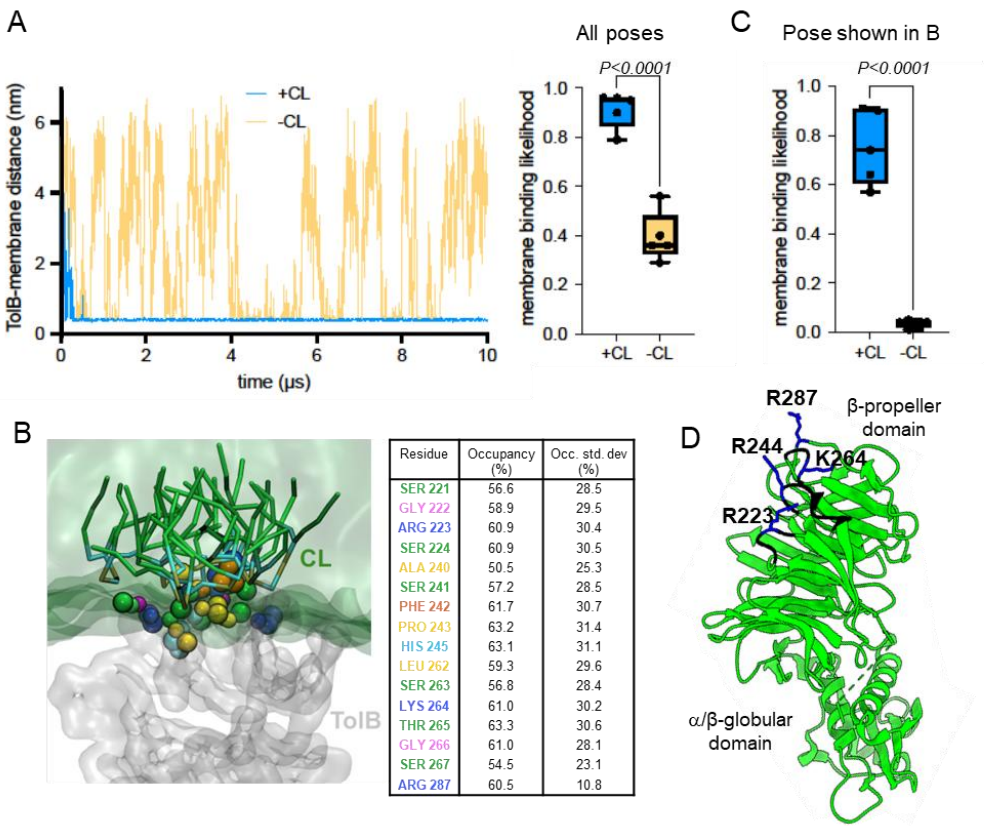
448 **Figures:**



449

450 **Fig 1: TolB interacts with lipid bilayers in vitro.** (A) Representative ITC data for TolB (140
451 µM, 7 nmol) titrated into (*left*) soluble Pal (sPal, 20 µM, 3.32 nmol) or (*right*) Pal
452 proteoliposomes (25 µM, 3.32 nmol surface-exposed) in phosphate-buffered saline (PBS),
453 with indicated control titrations. (B) Representative ITC data for (*left*) TolB or (*right*) LolB (140
454 µM, 7 nmol) titrated into empty liposomes (5 mg lipids) composed of *E. coli* polar lipid extract
455 (PE:PG:CL ~67:23:10) or synthetic lipids (PE:PG 3:1), with indicated control titrations. Each
456 titration was performed at 25 ^{o}C, and repeated more than 3 times.



Residue	Occupancy (%)	Occ. std. dev (%)
SER 221	56.6	28.5
GLY 222	58.9	29.5
ARG 223	60.9	30.4
SER 224	60.9	30.5
ALA 240	50.5	25.3
SER 241	57.2	28.5
PHE 242	61.7	30.7
PRO 243	63.2	31.4
HIS 245	63.1	31.1
LEU 262	59.3	29.6
SER 263	56.8	28.4
LYS 264	61.0	30.2
THR 265	63.3	30.6
GLY 266	61.0	28.1
SER 267	54.5	23.1
ARG 287	60.5	10.8

Fig 2: TolB interacts with CL in silico via a putative binding site. (A) Analysis of TolB-membrane distance over time in a representative 10-μs coarse-grain MD simulation, in the presence or absence of CL. Membrane binding likelihood, averaged over 5 repeat runs, considering all binding poses, is shown on the right. Two-tailed Student's t-test was used to test for statistical significance. (B) The most representative pose of how TolB (*grey*) interacts with CL in coarse-grain simulations, showing residues (colored spheres) in contact with CL molecules (*green/cyan/tan* sticks), and their percentage CL occupancies (in table). (C) Membrane binding likelihood, just considering the binding pose in (B). (D) Contact residues are mapped onto a cartoon representation of TolB structure. Basic or other residues are colored *blue* (with side chain) or *black* (backbone only), respectively.

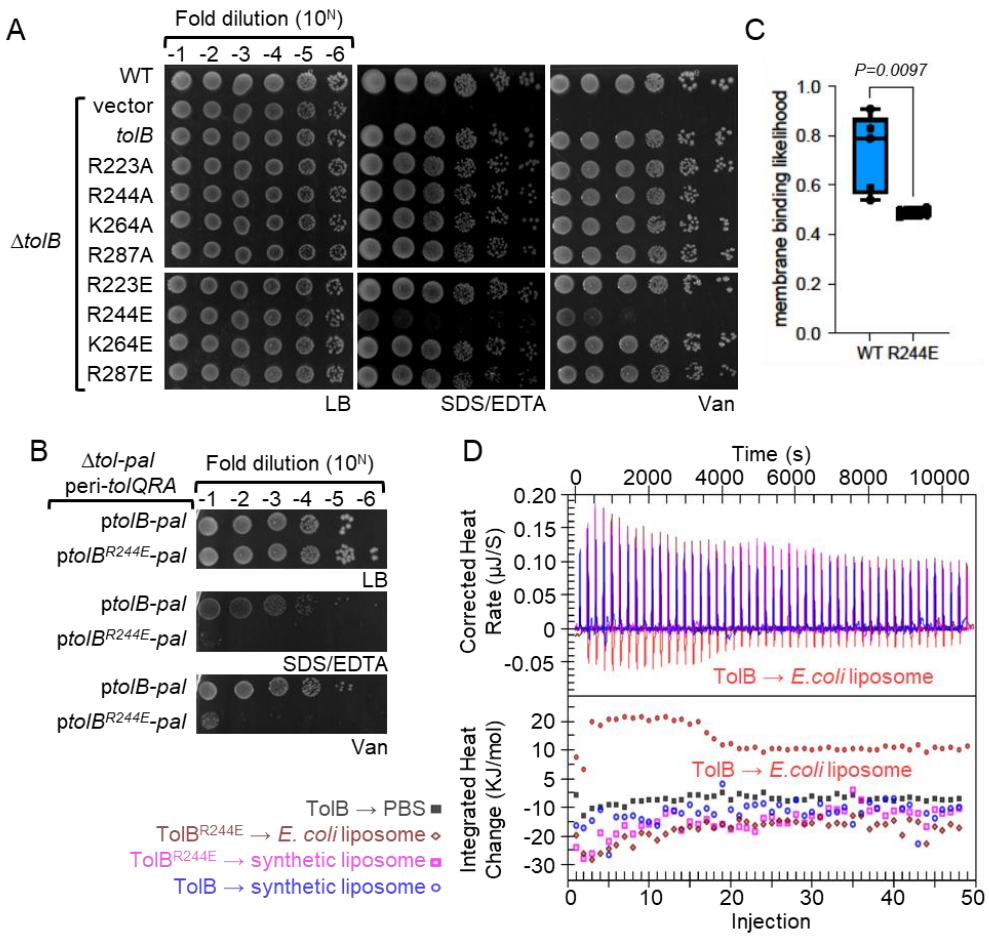


Fig 3: Mutation within putative CL-binding site resulted in loss of TolB function. (A) Efficiency of plating (EOP) of MG1655 wild type (WT), and Δ*tolB* strains, containing either pET22/42 empty vector or the plasmid expressing TolB and its mutants, on LB agar plates supplemented with SDS/EDTA (0.5% SDS/0.5 mM EDTA) or vancomycin (60 μg/mL) at 37°C. (B) EOP of MG1655 Δ*tol-pal* strain expressing a peripheral version of the TolQRA complex that remains randomly distributed along the cell periphery, and is known to restore OM defects,[10] containing either pBAD43-*tolB-pal* or pBAD43-*tolB*^{R244E}*-pal*, on LB agar plates supplemented with SDS/EDTA (0.3% SDS/0.3 mM EDTA) or vancomycin (30 μg/mL) at 37 °C. Peripheral TolQRA is engineered using a chimeric TonB-TolA that complexes with ExbBD (homolog of TolQR). (C) Membrane binding likelihood, averaged over 5 x 10 μs MD runs, of wildtype TolB and TolB^{R244E} binding to CL-containing membrane. (D) Representative ITC data of TolB and TolB^{R244E} (140 μM, 7 nmol) titrated into *E. coli* or synthetic liposomes (5 mg lipids) at 25 °C, with indicated control titrations.

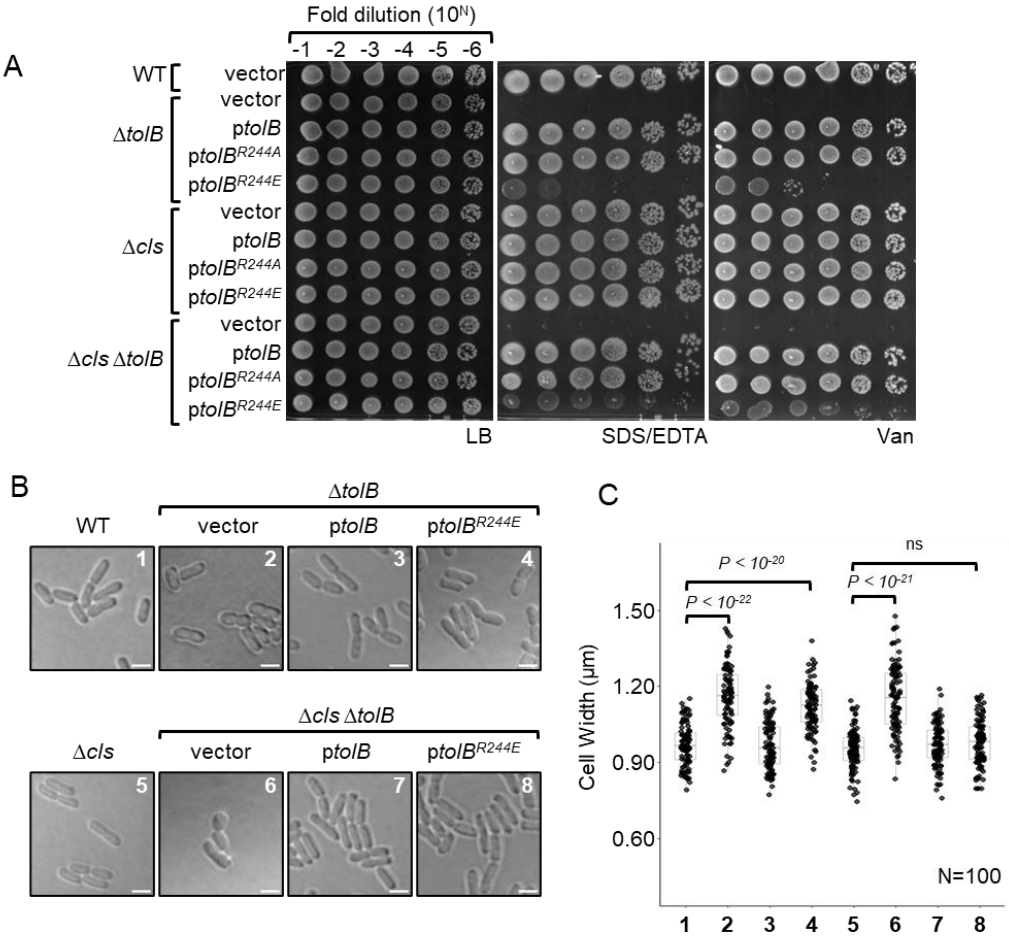


Fig 4: TolB^{R244E} regains partial function in strains without CL. (A) EOP of MG1655 wild type (WT), Δ*tolB*, Δ*cls*, and Δ*cls* Δ*tolB* strains, containing either pET22/42 empty vector or the plasmid expressing TolB and its mutants, on LB agar plates supplemented with SDS/EDTA (0.5% SDS/0.5 mM EDTA) or vancomycin (60 µg/mL) at 37 °C. (B) Differential interference contrast (DIC) microscope images of indicated strains from (A) grown in LB at 37 °C. Scale bar represent 2 µm. Cropped images are representative of at least three different uncropped field-of-view. (C) Quantification of the cell width of 100 individual cells of same strains (numbered) shown in (B). Box plot defines the median (center) and the 25th and 75th percentile (lower and upper hinge); the whisker represents the range excluding the outliers (outlier defined by sample with value further than 1.5 interquartile-range from the corresponding hinge). A two-sided Wilcoxon ranked sum test was used to test for statistical significance.

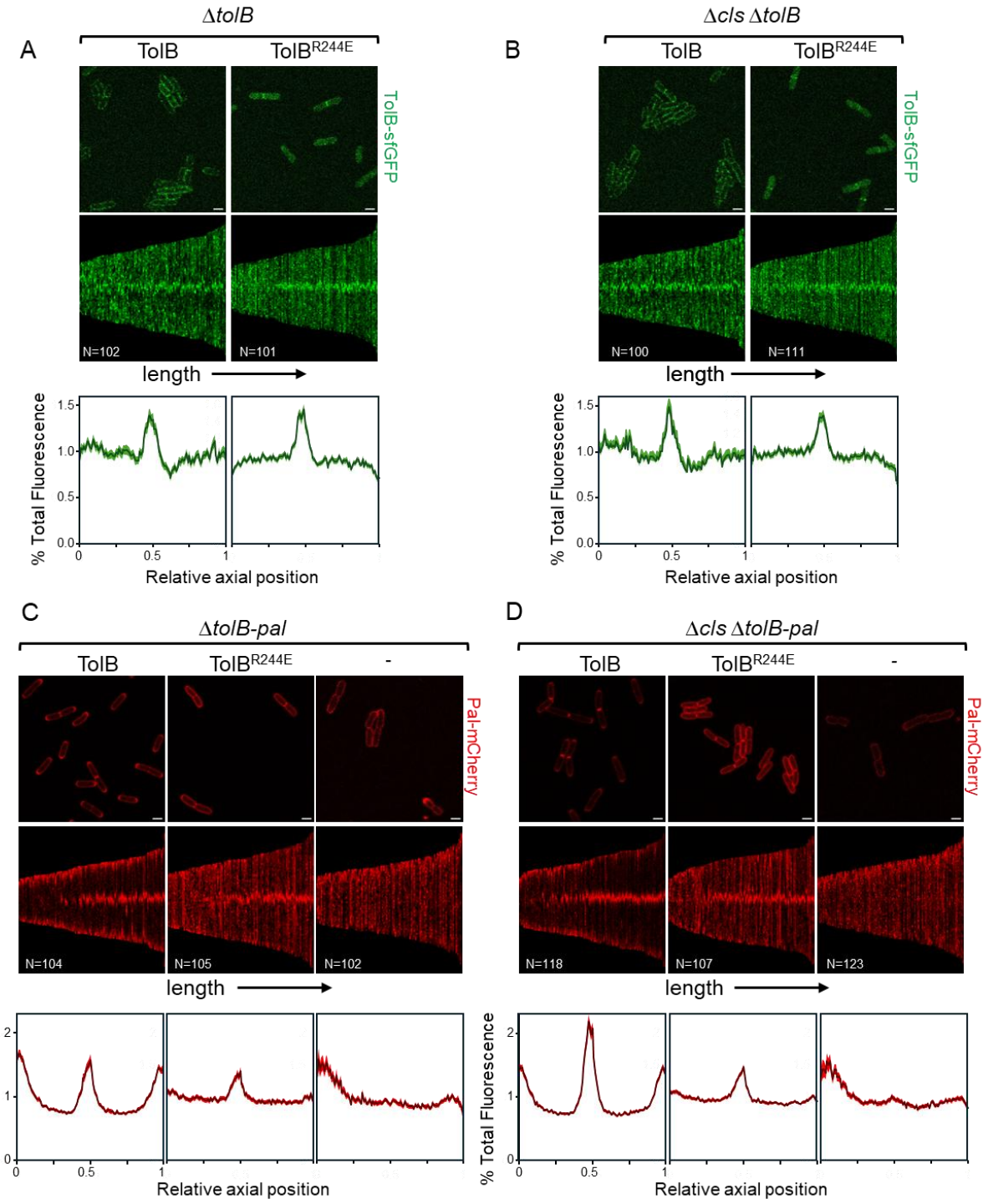


Fig 5: TolB and Pal still localize to cell septa in the presence of *tolB*^{R244E} mutation, and/or in the absence of CL. (*top*) Fluorescence microscopy images of (A) MG1655 Δ*tolB::kan* and (B) Δ*cls* Δ*tolB::kan* strains, expressing TolB-sfGFP or TolB^{R244E}-sfGFP from pET22/42, and of (C) MG1655 Δ*tolB-pal::kan* and (D) Δ*cls* Δ*tolB-pal::kan* strains, expressing Pal-mCherry, TolB/Pal-mCherry or TolB^{R244E}/Pal-mCherry from pBAD43. Scale bar represents 2 µm. (*middle*) Demographic representation of the fluorescence signals in the indicated strains measured along the long axis of individual cells, sorted according to cell length and aligned to mid-cell.

502 (*bottom*) Average percentage distribution of the fluorescence signals along the long axis of all
503 cells measured, where the relative axial position of 0.5 corresponds to mid-cell, and 0 to 1
504 correspond to the poles.