Structural and functional characterization of the LetAB complex reveals a new family of transporters

OR

Structural basis of phospholipid lipid transport across the bacterial envelope by LetAB

OR

Structural and functional characterization of the LetAB complex reveals a non-canonical mechanism of lipid transport

OR

LetA utilizes a non-canonical transport mechanism to drive lipids across the bacterial envelope

Cristina C. Santarossa[1], Yupeng Li[2], Sara Yousef[1], Hale S. Hasdemir[2], Carlos C. Rodriguez[2], Max Haase[3], Minkyung Baek[7], Nicolas Coudray[1,4,], John Pavek[5], Kimber Focke[5], Annika Silverberger[5], Johannes Yeh[6], Michael M. Marty[5], David Baker[7], Emad Tajkhorshid[2], Damian Ekiert[1]* and Gira Bhabha[1]*

[1]Department of Biology, Johns Hopkins University, Baltimore, MD, USA
[2]Theoretical and Computational Biophysics Group, Department of Biochemistry, and Center for Biophysics and Quantitative Biology, NIH Resource for Macromolecular Modeling and Visualization, Beckman Institute for Advanced Science and Technology, University of Illinois Urbana-Champaign, Urbana, IL, USA
[3]Max Planck Institute of Molecular Physiology, Dortmund, Germany
[4]Applied Bioinformatics Laboratories, NYU School of Medicine, New York, USA
[5]Department of Chemistry and Biochemistry, University of Arizona, Tucson, AZ, USA
[6]Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, USA
[7]Institute for Protein Design, University of Washington, Seattle, Washington, USA

*For correspondence: gira.bhabha@be-labs.org and damian.ekiert@be-labs.org

ABSTRACT

Membrane transport proteins import and export a diverse array of cargos across cellular membranes, ranging from small sugars to entire proteins. A small number of structurally distinct membrane transporter families have been described to date, and are presumed to account for the vast majority of transport processes in cells. Here we characterize LetAB, a membrane transporter from *E. coli*, which we find is structurally and evolutionarily unrelated to previously described families, and functions in lipid transport mediated by MCE proteins. The founding member of this proposed transporter family, LetA, functions as a pump at one end of a ~225 Å long tunnel formed by its binding partner, LetB. Together, they create a pathway for lipid transport between the inner membrane and outer membranes. We determined the structure of the LetAB complex, which shows that LetA adopts a fold evolutionarily related to non-transporter proteins from eukaryotes, such as TARPs and Claudins. Deep mutational scanning of LetA sheds light on functional regions, revealing a potential proton shuttling pathway to energize transport, and a periplasmic pocket poised to bind substrate. MD simulations suggest a pathway for lipid transport. Our cryo-EM structure, together with MD simulations, the use of AlphaFold to predict alternate conformations, and functional studies in cells, allow us to propose a model for how this new family of membrane transporters may drive the transport of hydrophobic molecules across the bacterial cell envelope.

INTRODUCTION

Membrane transport is critical for cell growth, division and homeostasis. Transporters mediate either the active or passive transport of a wide variety of substrates across cellular membranes, including nutrients, ions, and drugs[1–3]. Active transporters utilize the energy from ATP hydrolysis or energy stored in electrochemical gradients across the membrane to translocate substrates against their concentration gradient, while passive transporters facilitate diffusion of molecules across membranes along their concentration gradient. To date, several transporter folds have been identified[4–8], including the F-type/V-type ATPases, P-type ATPases, and members of the ATP-binding cassette (ABC) and Solute Carrier families. However, many membrane proteins hypothesized to be transporters have not been well characterized, even in well-studied model organisms such as *Escherichia coli*. Some of these hypothetical transporters may be evolutionarily related to known transporter families, but have diverged beyond recognition at the sequence level. Alternatively, these unstudied protein families may represent new kinds of transporters that await experimental characterization.

The mammalian cell entry (MCE) family of proteins has been implicated in lipid transport across the cell envelope in double-membraned bacteria. Members of this family play an important role in maintaining the cell envelope of both Gram-negative bacteria[9,10] and *Mycobacteria*[11] by providing sheltered passage of hydrophobic molecules across the aqueous periplasm. To perform this challenging task, MCE proteins adopt a range of architectures to form pathways between membranes[12,13]. MCE proteins are usually associated with other membrane proteins, which are thought to drive lipid translocation through the pathways formed by the MCE protein. The *E. coli* Maintenance of Lipid Asymmetry (Mla)[14,15] and *Mycobacterium smegmatis* Mce1[13] systems are the best characterized and both utilize ABC transporters as pumps to energize substrate translocation. However, many other MCE gene clusters do not encode components of an ABC transporter and it is unknown how lipids are translocated, or what the energy source may be for this process in systems that do not encode ATPases.

LetB (Lipophilic Envelope-spanning Tunnel B) is a large MCE protein that forms a hydrophobic tunnel, long enough to span the gap between the IM and outer membrane in *E. coli*[16] (Figure 1A). The prevailing model in the field is that LetB transports lipids through the central tunnel, between the two membranes[16]. The mechanism by which lipids enter the LetB tunnel remains unknown. LetB is encoded in an operon together with hypothetical integral IM protein LetA (Figure 1A), which has no detectable sequence homology to ABC transporters or other membrane protein families. In Gram-negative bacteria, proteins homologous of LetA are encoded adjacent to MCE proteins, including the Paraquat Inducible (Pqi) system in *E. coli*, suggesting that these proteins may have evolved to function together in lipid transport. LetA is a multipass transmembrane protein that is predicted to reside in the bacterial IM, and is well-poised to act as a pump for substrate translocation through the LetB tunnel. Given its apparent divergence from known transporter folds and absence of canonical ATP binding motifs, LetA has the potential to define a new class of membrane transport proteins.

Here, we show that LetA is functionally linked to LetB, and that the two proteins physically interact. We report the structure of the LetAB complex, and find that LetA is distantly related to other well-known eukaryotic membrane proteins without any known transporter activity, including the TARPs[17] and claudins[18]. Our structure, coupled with deep mutational scanning, MD simulations, and AlphaFold predictions of alternate states, suggest a model for how LetA may drive phospholipid transport to maintain outer membrane integrity in *E. coli*, and provide our first insights into a previously uncharacterized family of putative active transporters in bacteria.

RESULTS

LetA and LetB are functionally linked

Deletion of *letA* and *letB* together (Δ*letAB*) in *E. coli* was previously shown to cause mild sensitivity to the bile salt, cholate, and to the zwitterionic surfactant, lauryl sulfobetaine (LSB)[16,19] are exacerbated when *pqiAB*, a second *E. coli* MCE system, is also deleted (Supplementary Figure 1A)[16,19]. To assess the relative contributions of *letA* and *letB* to cholate and LSB sensitivity, we deleted *letA* and *letB* individually in a ΔpqiAB background. Strains lacking *letA* or *letB* exhibit similar growth defects to each other and to Δ*letAB* mutants, which can be rescued by complementation with a plasmid carrying WT *letAB* (Figure 1B). These results suggest that LetA and LetB function in the same pathway.

To examine if LetA and LetB physically interact, we co-expressed both proteins in *E. coli* and pulled down His-tagged LetA, and found that untagged LetB is pulled down in a LetA-dependent manner (Supplementary Figure 1B). Further purification by size exclusion chromatography reveals that LetA and LetB co-fractionate, forming a large complex with an apparent molecular weight of ~670 kDa (Supplementary Figures 1C-D), similar to the expected size of a LetB hexamer (~570 kDa) plus 1-2 copies of LetA and a detergent micelle. Negative stain EM of purified LetAB reveals particles with seven characteristic bands of density resembling LetB[16], and an additional globular density at one end (Figure 1C), which we hypothesized corresponds to LetA in a detergent micelle. Overall, our data show that LetA and LetB interact to form a stable complex.

LetA is required for lipid entry into LetB tunnel

Previous studies have shown that the soluble, periplasmic domain of LetB binds phospholipids[12], and crosslinking experiments in *E. coli* lysates suggest that the binding sites for phospholipid substrates is in the LetB central tunnel[16]. It is unclear, however, if phospholipids spontaneously enter the tunnel of the full-length, membrane-embedded LetAB complex *in vivo*. To address this question, we used an *in vivo* crosslinking assay. We grew *E. coli* in the presence of ^{32}P orthophosphate, to label bulk phospholipids and other phosphate-containing molecules[16], and over-expressed LetAB with the unnatural photocrosslinking amino acid *p*-benzoyl-L-phenylalanine (BPA) incorporated at specific sites in LetB[16,20]. We then irradiated live cells with UV light to allow *in vivo* crosslinking of molecules in proximity to the site of the BPA probe. Following purification of LetAB complexes, we analyzed the crosslinking of ^{32}P-labeled molecules to LetB by electrophoresis and phosphorimaging. BPA was positioned inside the LetB tunnel (F468) or on the periplasm-facing exterior surface (E854, negative control) (Figure 1D), locations validated in previous work[16]. We detect strong ^{32}P incorporation into LetB when BPA was positioned inside the tunnel, but minimal ^{32}P incorporation with BPA positioned outside the tunnel, as expected based on previous work (Figure 1E)[16]. To test if lipid access to the LetB tunnel is dependent on LetA, we assessed lipid crosslinking in the LetB tunnel with or without co-expression of LetA. Efficient crosslinking was observed when LetA and LetB were co-expressed, but crosslinking was greatly reduced when LetA was not co-expressed (Figure 1F). LetB expression and membrane localization were similar, with or without LetA co-expression (Supplementary Figure 1E). Taken together, these results suggest that LetA is necessary for phospholipid entry into the tunnel of full-length LetB *in vivo*. The simplest interpretation of this result is that LetA is an exporter that loads lipids into LetB.

Overall structure of LetAB complex

To understand the physical basis for how LetA and LetB function together, we determined the structure of the LetAB complex using single particle cryo-EM. Cryo-EM samples were prepared both in the presence and absence of crosslinking. The crosslinked and uncrosslinked datasets yielded maps with similar average resolutions across the LetAB complex (2.5-4.6 Å: Figure 2A, Supplementary Figure 2, Table S1), and the overall backbone conformation is similar in both structures (root-mean square displacement of 2.097 Å, 5217 c-α positions). We will primarily focus our discussion on the crosslinked LetAB structure, except where noted, as the transmembrane (TM) region is better resolved (Supplementary Figures 2C, 2H).

The LetAB complex is an elongated assembly (Figures 2A-B), ~290 Å long and ~90 Å wide, and consists of six copies of LetB and one copy of LetA. The LetB periplasmic domain hexamerizes to form the tunnel, consistent with previous structures[16,19], and dominates the assembly, accounting for ~225 Å of the total length and 92% of the mass. The seven MCE domains in the primary sequence of LetB associate with neighboring LetB protomers to form seven rings, which stack to create a central hydrophobic tunnel that runs through the entire length of the LetB periplasmic domain (Supplementary Figures 1F-G). Each LetB protomer also contains a single N-terminal TM helix, resulting in a total of six TM helices that anchor the assembly in the IM. A single copy of LetA associates with LetB in the IM, making interactions with MCE ring 1 and the TM helices of LetB, and resulting in the formation of an asymmetric complex (Figures 2C-D). Consistent with its membrane localization, LetA contains a ~30 Å hydrophobic belt around its circumference, which would likely interface with lipids in the IM (Figure 2E). Notably, density for only four of the six LetB TM helices are apparent in the EM map, and these four helices interact with LetA. The remaining two TM helices are not resolved (Figure 2C) and may not stably interact with LetA.

The lining of the LetB tunnel is formed by pore-lining loops that emerge from each MCE domain[16]. Previous cryo-EM structures have shown that the pore-lining loops from rings 1, 5, 6 and 7 can adopt multiple conformational states to control the diameter of the central tunnel, thereby potentially regulating the passage of substrates. LetA is positioned right underneath the pore of MCE ring 1. In the absence of LetA, MCE ring 1 of LetB is predominantly in the closed state, in which the pore through the ring is not wide enough to allow passage of a phospholipid (Figure 2F)[16]. Interestingly, in our structure of the LetAB complex, MCE ring 1 of LetB adopts an open state, suggesting that binding to LetA modulates the conformation of the LetB tunnel (Figure 2F).

Overall structure of LetA

LetA is a single polypeptide that consists of two related modules, which we term “LetA modules”. Each LetA module consists of a cytoplasmic Zinc Ribbon (ZnR) domain followed by a transmembrane domain (TMD) (Figures 3A-C). The TMD of each LetA module contains four transmembrane helices, one interfacial helix in the membrane, and a three-stranded β-sheet extending into the periplasm (Figures 3A-D). The two LetA modules, which share only ~25% sequence identity, associate in a head-to-head manner in the structure, resulting in an intramolecular dimer with 2-fold pseudo-symmetry (Figure 3E). The two TMDs form an inverted V-shape, creating a large, hydrophilic cleft that faces the cytoplasm (Figure 3F). In this cleft, we observed the presence of a ^{361}GRWSM-Ψ-D-Ψ-F^{369} motif (where Ψ denotes an aliphatic amino acid: L, I, V, or M) that is well conserved across a diverse set of LetA-like proteins (Supplementary Figures 3A-C), suggesting that this site may be important for LetA function. In addition to this cleft, LetA contains an amphipathic periplasmic pocket 174 Å^3 in volume (Figure 3G), which is formed primarily by residues of TMD^C,

along with TM3 of TMD^N. This pocket sits directly below the entrance to the LetB tunnel, with the LetA periplasmic β-sheets serving as a hydrophobic bridge to connect the pocket to the pore lining loops of LetB ring 1 (Figure 3H). In contrast, an equivalent pocket is not present in TMD^N. The cleft and periplasmic pocket could potentially serve as substrate binding sites, and function as part of the substrate translocation pathway.

On the cytoplasmic side, ZnR^N and ZnR^C interact to form a structural unit. Each ZnR consists of two stacked β-hairpins (Figure 3I)[21] and contains a tetracysteine motif involved in metal binding (CXXC-X_n-CXXC, where n ranges from 11 to 18, Supplementary Figure 3A). ZnR^C connects TMD^N to TMD^C, and interacts non-covalently with ZnR^N to form a ZnR dimer on the cytoplasmic side of LetA, close to the pseudo-2-fold axis, perhaps thereby stabilizing the association between the N-terminal and C-terminal halves of LetA. In prokaryotes, members of the ZnR family can bind various transition metals depending on the ZnR domain's functional role[22], most commonly zinc[23] or iron[24]. To assess if LetA is a metal-binding protein and to profile its metal-binding specificity, we performed inductively coupled plasma mass spectrometry (ICP-MS) on purified LetA protein, which suggests that LetA specifically binds ~1.9 zinc atoms per protein molecule (n=2, range: 1.7 - 2), indicating that both ZnR domains likely coordinate zinc under our experimental conditions. It is possible that under different environmental conditions and different metabolic states of the cell, the ZnR domains could coordinate a different metal.

LetA modules are widespread in Proteobacteria[9]. While two LetA modules are found within a single gene in *E. coli* LetA, in some species, such as *Pseudomonas aeruginosa*, each module is encoded by a separate gene (PA4690a and PA4690), and presumably the resulting proteins associate to form an intermolecular dimer in contrast to the intramolecular dimer of *E. coli* LetA. To assess whether the N-terminal and C-terminal halves of *E. coli* LetA can assemble and function in the absence of a covalent linkage, as hypothesized for *P. aeruginosa* PA4690a and PA4690, we co-expressed the N- and C-terminal modules of *E. coli* LetA from separate genes (split-LetA). This split-LetA construct fully rescued the cholate and LSB sensitivity of the *ΔpqiAB ΔletAB* strain (Supplementary Figure 3D), suggesting cellular function comparable to WT LetA. Additionally, pull down experiments show that the two halves of split-LetA interact with each other, and with LetB (Supplementary Figure 3E). Thus, it is likely that LetA proteins in bacteria can either be encoded as a single polypeptide with two LetA modules, as in *E. coli*, or as two separate gene products, each containing a single module, as in *P. aeruginosa*.

LetA is unrelated to known transporters but distantly related to TARPs and Claudins

To gain further insights into LetA and its evolutionary relationship to other protein families, we examined if LetA has homology to proteins of known structure and function. Using both sequence- and structure-based searches, we were unable to identify any previously determined structures in the PDB with a domain organization similar to either the whole LetA protein or a LetA module. We were also unable to identify any structural similarity to any other known transporter family, suggesting that LetA is quite different from previously characterized proteins, and may represent a new kind of membrane transport protein.

Surprisingly, a structure-based search of the PDB using FoldSeek revealed that an individual LetA TMD is structurally related to eukaryotic integral membrane proteins, and are most similar to the Transmembrane AMPA receptor Regulatory Protein (TARP) and claudin families (Figures 3J-K). TARPs play a role in regulation of ion channel function[25], and Claudins are important for cell-cell adhesion[18]; neither TARPs or Claudins are transporters themselves. The TMDs from LetA, TARPs,

and claudins have the same 4-pass transmembrane topology and a small β-sheet on the extracytoplasmic side of the membrane (Figures 3J-K). The region that differs most between LetA, TARPs and claudins is the periplasmic β-strands; TARPs and claudins contain five β-strands, while in LetA, two of the β-strands have been structurally replaced with an interfacial helix (IF). LetA shares a few key features with TARPs. First, TM2 is broken into two segments in both LetA and TARPs, which we refer to as TM2a and TM2b in LetA and has been referred to as the extracellular helix domain and TM2 in TARPs[16]. Second, while LetA, TARPs, and Claudins have very low sequence identity (~6-16%), Type 1 TARPs share a conserved hydrophobic motif near the extracytoplasmic end of $TM4^{C}$ (^{393}FYFG^{396}). This sequence is clearly present in approximately the same location in LetA (Supplementary Figure 3G)[26]. The way the FYFG motif interacts with neighboring TM helices is somewhat different between LetA and TARPs (Supplementary Figure 3H), and we note that only F395 is conserved in a larger set of LetA-like proteins (Supplementary Figure 3A). As the function of the motif is not well understood, it is hard to predict whether it serves a similar role in TARPs and LetA. Taken together, the LetA module may share a common ancestor with TARPs and claudins, but is more closely related to TARPS and that evolution has shaped each of these derived protein families for a substantially different role in membrane biology.

A Foldseek search of the AlphaFold database revealed potential uncharacterized structural homologs of full-length LetA that are present in some eukaryotes (Supplementary Figure 3I). The AlphaFold predictions suggest that certain parasites and marine protists encode proteins resembling LetA, but lacking clear ZnR domains. Parasites and marine protists are not known to contain MCE proteins. However, LetA-like proteins identified in kinetoplastids and dinoflagellates appear to be fused to an extracytoplasmic β-jellyroll domain with a hydrophobic groove, reminiscent of the bridge-like lipid transport domains of VPS13, AsmA, and the LPS exporter (Supplementary Figure 3I)[27,28]. Thus, these distantly related relatives of LetA may potentially mediate the transport of lipids or other hydrophobic molecules in some eukaryotes.

Deep mutational scanning reveals functionally important regions in LetA

To gain unbiased insight into functionally important residues in LetA, we used deep mutational scanning (DMS). We generated a library of single amino acid substitution mutants, in which each position in LetA was mutated to the 19 other possible amino acids, as well as a stop codon, expected to contain 8,540 unique mutations. We transformed the library into our *ΔpqiAB ΔletAB* strain and used selection based on resistance to cholate and LSB to probe the relative fitness of each LetA mutant in the library (Supplementary Figures 4-5). Heatmaps illustrating the impact of each mutation on LetA fitness show similar patterns with cholate and LSB (Supplementary Figures 5A-B). As expected, mutation of the start codon, or introducing a stop codon at most positions resulted in reduced fitness. To identify positions likely to be important for LetA function, we calculated a tolerance score for each residue (See Methods)[29,30], ranging from 0 to 1, where 0 means no mutations are tolerated and 1 means all mutations are tolerated (Figure 4A, Supplementary Figures 5A-B). The majority of residues (~90%) tolerate mutations well (TS ≥0.7), including a ~25 residue cytoplasmic extension at the N-terminus of LetA (Figure 4A). LetA constructs truncating this region are expressed and largely rescue growth of the Δ*pqiAB* Δ*letAB* strain in the presence of cholate or LSB (Supplementary Figures 4D-E). However, a subset of positions in LetA were less tolerant of mutation (TS < 0.7), including 53 positions for cholate and 37 positions for LSB (FigureA 4A, Supplementary Figures 5A-B). The majority of these putative functionally important residues cluster in three discrete

regions of the LetA structure: 1) the periplasmic pocket in TMD^C, 2) a polar network in TMD^C, and 3) the ZnR domains (Figure 4B).

Periplasmic pocket

The periplasmic pocket (Figure 4C) is open to the periplasm, and lies just below the pore through ring 1 of LetB. Thus, this pocket is well positioned to serve as a binding site for lipids moving between IM and the LetB tunnel. From the cholate and LSB datasets combined, approximately half of all residues with low tolerance scores clustered to this region (28 residues), suggesting that the periplasmic pocket is of functional importance (Figure 4D). Of the 28 residues, 19 have a TS <0.7 in both the cholate and LSB datasets, while the remainder follow similar fitness patterns in LSB and cholate, albeit with slightly higher tolerance scores in the cholate dataset. The majority of these residues are hydrophobic, and are less tolerant to mutations to polar residues, suggesting that maintaining the hydrophobic character of this pocket is critical, consistent with a role in binding to lipids or other hydrophobic molecules. Most of the 28 residues are buried within the pocket or cluster to TMD^C strands β1 and β3, which may allow β1 and β3 to act as a hydrophobic "slide" for lipid translocation between the periplasmic pocket of LetA and the pore of LetB ring 1 (Figures 3H,4C). Taken together, DMS and the structure reveals a functionally important, hydrophobic, outward-open pocket that could potentially function as a substrate-binding site involved in lipid translocation between LetA and LetB.

Polar network in TMD^C

From our DMS analysis, our attention was drawn to residues K178, D181, S321, K328, S364, D367, and T402, which have low tolerance scores. These polar residues reside within the transmembrane region of LetA, which is unusual for transmembrane domains[31](Figures 4E-F). Mapping these residues onto the structure shows that they form a polar network running across the membrane, from the periplasmic pocket to the cytoplasm via the central cleft (Figure 4E). Multiple sequence alignment of a panel of LetA-like proteins show that they are well conserved (Supplementary Figure 3A). Mutating each of the seven polar network residues to alanine resulted in stable protein complexes capable of binding to LetB, suggesting that these mutations do not drastically disrupt LetA folding or stability, but rather likely have a specific role in the transport mechanism (Supplementary Figure 5C). Studies of similar polar networks in other transmembrane proteins have shown that these residues can be important for energy transduction by forming proton transfer pathways[1,32], often via a combination of ionizable residues and intervening, bridging water molecules. Consistent with a potential role in proton shuttling, several of these residues are predicted to have significantly perturbed pKa's: K178 and K328 have predicted pKa's of 8.72 and 8.35, respectively, which are lower than the normal pKa of lysine (≥10)[33,34] while D367 has a predicted pKa (6.71) that is higher than the normal pKa of aspartate (4.0)[35]. This leads to the hypothesis that the polar network in LetA may be a proton transfer pathway, and LetA may be driven by proton motive force. To assess whether the residues in the polar network are capable of shuttling protons, we performed equilibrium molecular dynamics (MD) simulations to examine their solvent accessibility. Water density is clearly observed in the vicinity of the polar network, bridging the periplasmic and cytoplasmic spaces (Supplementary Figure 5D). Notably, only the core of TMD^C is accessible to water, whereas the core of TMD^N, which lacks a polar network, remains inaccessible. The polar residues within the core of TMD^C, spanning from D181 to T402, are connected by a network of water molecules, forming a continuous pathway through which protons could potentially be shuttled (Figure 4E, Supplementary Figure 5E) to energize conformational changes of LetA for lipid transport.

ZnR domains

The cysteines of ZnR domains are expected to be important for function, as they typically maintain the structural fold through tetrahedral coordination of the metal ion[36]. Consistent with this expectation, we found that the metal-coordinating cysteines cannot tolerate mutations (Figure 4G, Supplementary Figures 5A-B,). Mutating the cysteines to alanine failed to rescue the cholate and LSB sensitivity of the *ΔpqiAB ΔletAB* strain (Supplementary Figure 5F).

Surprisingly, aside from the metal-coordinating cysteines, no other residues in the ZnR domains were sensitive to mutation (TS >0.7) (Figure 4A). This led us to hypothesize that the overall sequence of the ZnR domains has little impact on LetA function as long as the domains can adopt a ZnR fold, and the metal-coordinating cysteines can bind Zn. To test this hypothesis, we replaced the ZnR domains of LetA with the ZnR domains from another protein with substantially different sequences. We generated a ZnR-swap mutant, in which we replaced both ZnR^N and ZnR^C with those of PqiA, a second LetA-like protein from *E. coli*, which shares 21% and 48% sequence identity with the LetA ZnRs, respectively (Supplementary Figure 5G). The ZnR-swap mutant showed cholate and LSB resistance similar to the WT, suggesting that substantial sequence divergence can be tolerated outside of the Zn coordinating cysteines (Figure 4H, Supplementary Figure 5H). Given the surprisingly high tolerance for sequence divergence, we hypothesized that the low fitness of metal-coordinating cysteines could be explained simply by the effect of these mutations on protein folding, and questioned whether the ZnRs were required for function at all. Western blotting of the Cys mutants shows reduced levels of LetA protein, consistent with the hypothesis that the mutants fold poorly or are less stable (Supplementary Figure 5I).

To test whether the ZnRs are required for function at all, we generated two mutants, in which either ZnR^N or ZnR^C were deleted, and tested these in complementation assays. LetAΔZnR^N fails to rescue growth, while LetAΔZnR^C partially rescues growth (Figure 4I) despite reduced expression in cells compared to the WT (Supplementary Figure 5J). In pull down assays, LetAΔZnR^N protein is unable to pull down LetB, suggesting that this mutation may interfere with LetA folding (Supplementary Figure 5K). Notably, two residues in $TM4^C$, D410 and R412, are intolerant to mutations, and form a salt bridge network with ZnR^N, suggesting ZnR^N and TMD^C may be a structurally stable unit. In contrast, the LetAΔZnR^C mutant pulls down LetB, suggesting that the protein is folded and at least partially functional (Supplementary Figure 5K), and consequently, that ZnR^C is not strictly necessary for function. Consistent with this idea, we have observed LetA-like sequences lacking ZnR^C, such as in Shewanella (Uniprot A0A4Y6I6U8). Overall, our ZnR deletion mutants suggest that the resulting LetA protein is less fit than the WT, but can function without ZnR^C. Taken together, this leads to a model in which the ZnR domains are likely playing a role in modulating the structure or conformational ensemble sampled by LetA, rather than making an essential contribution to transport such as substrate binding, or the recruitment of other essential protein subunits.

Lipid binding and specificity

In the central cleft in LetA, additional density is apparent in the uncrosslinked EM map, consistent with the size and shape of a diacyl lipid, which we call Lipid 1 (Figure 5A, Supplementary Figure 6A). Site-specific crosslinking suggests that the Lipid 1 site is occupied by a phospholipid *in vivo* (Supplementary Figures 6B-C). MD simulations show that a lipid remains stably bound at this site, whether or not it is included in the starting model (Supplementary Figures 6D-E). LetA residues

contacting this lipid are generally insensitive to mutation by DMS. Collectively, these findings suggest that Lipid 1 may function as a structural lipid rather than a substrate for transport.

Intriguingly, in our MD simulations, we observed spontaneous upward movement of another lipid (Lipid 2) in the cleft, which is on the opposite side relative to the Lipid 1 site. Lipid 2 originated from the cytoplasmic leaflet of the IM, and moved along the cleft to a position approximately halfway across the membrane towards the periplasmic pocket. The lipid then remains stably bound at this position (Figure 5A, Supplementary Figure 6F, Movies S1-2), without any further movement towards the periplasm. We hypothesize that Lipid 2 may represent a substrate and suggest a transport mechanism, involving 1) spontaneous movement of a lipid from the cytoplasmic leaflet to the site observed near the middle of the membrane, 2) translocation to the periplasmic pocket, perhaps following a conformational change, and 3) transfer from the periplasmic pocket of LetA to the LetB tunnel. To explore the possible trajectory of Lipid 2 between its stable position in the LetA central cleft to the periplasmic pocket, we performed steered MD simulations. We pulled on either one or both tails of Lipid 2, or its head group, from the bottom of the periplasmic pocket to the top. We found that to accommodate Lipid 2, the IF^C and $TM2a^C$ helices open laterally as a unit, similar to a “flap”, revealing an amphipathic groove (Figure 5B). Pulling on one tail resulted in the tails being splayed apart, shielded from the solvent by hydrophobic residues, and the headgroup is inside the pocket interacting with polar residues (Figures 5B-C, Movies S3-4). The configuration of the splayed Lipid 2 is reminiscent of lipids identified in the cryo-EM structure of another MCE system in *E. coli*, MlaFEDB [14]. While MlaFEDB is energized by an ABC transporter (MlaF and MlaE), which is unrelated to LetA, MlaD is an MCE protein that is homologous to LetB. The splayed configuration of the lipid being transported may be advantageous in both cases, such that one tail is transported first, followed by the other, avoiding energetically unfavorable interactions and minimizing water intrusion. Notably, pulling one tail of the lipid required the least accumulated work amongst the three pulling strategies (Supplementary Figure 6G). The steered MD simulations lead us to hypothesize that Lipid 2 can be transported from the LetA central cavity to the periplasmic pocket, facilitated by conformational changes.

Following steered MD simulations, we conducted 300 ns equilibrium simulations for each scenario, to observe if and how Lipid 2 relaxes into the periplasmic pocket (Supplementary Figure 6D). We found that Lipid 2 is highly flexible in the periplasmic pocket, and samples an ensemble of configurations (Supplementary Figures 6H-I). However, a common theme emerged: 1) the negatively charged phosphate headgroup of the lipids tended to dock against R63 from LetB, which is on the bottom of ring 1, facing towards the periplasmic pocket of LetA (Figure 5D), and 2) the fatty acyl tails sample the surrounding hydrophobic surfaces of LetA, primarily either downwards into the periplasmic pocket or upwards towards the pore of LetB (Figure 5C, Movies S3-4). This sampling along the hydrophobic surfaces in this region supports our hypothesis that the periplasmic β-strands of LetA provide a pathway for lipid movement from LetA to LetB (Figures 3H, 4C). To test the functional importance of LetB R63, we generated three mutants, R63A, R63N, and R63D. These mutants are sensitive to both cholate and LSB, despite showing expression levels similar to WT (Figures 5E-F), suggesting that R63 is indeed a critical residue for LetAB function.

Our MD simulations do not speak to lipid specificity for the substrate. All lipids used in the simulation (PE, PG and cardiolipin) are observed to occupy the Lipid 2 position. To further explore the lipid binding specificity of LetAB, we performed lipidomics experiments on purified LetAB. We found that both PE and PG were enriched in purified LetAB relative to the *E. coli* membrane as a whole, while cardiolipin was depleted (Supplementary Figures 6J-K). Taken together, our data suggest the

presence of two lipids, Lipid 1, which is likely a structural lipid stably bound in the LetA cleft, and Lipid 2, which likely represents the substrate.

Conformational changes that may facilitate transport in LetA

If LetA is indeed a transporter, it must cycle through a series of conformational states to enable lipid translocation across the bilayer. Experimentally, we have only been able to capture one state of LetA thus far. To explore potential additional states sampled by LetA, we turned to protein structure prediction. We used AlphaFold2[37–39] with reduced multiple sequence alignment depth, which was recently pioneered as an approach to predict alternative states of transporters[38,40]. We generated 160 predictions (see Methods), which can be grouped into five major clusters based upon the state of the LetA TMDs (see Methods, Figure 6A, Supplementary Figures 7A-B, Supplementary Table S4). The models in Cluster 1 are similar to our cryo-EM structure. In the remaining four clusters, we observe two major types of motion, which occur to varying degrees and in different combinations. First, in Clusters 2-5, TMD^C rotates relative to TMD^N, over a range of ~20-44 degrees, around an axis roughly perpendicular to the plane of the membrane (Figure 6B, Movie S5). Second, in Clusters 4 and 5, one TM3 segment slides past the other at the interface between the TMDs (Figures 6B-C, Movie S5). The combination of TMD rotation and TM3 sliding motions result in the opening of an amphipathic groove between the the TM3 helices on one side and a flap formed by the IF^C and $TM2a^C$ helices on the other, that connects the location of Lipid 2 from our MD simulations to the periplasmic pocket (Figure 6D). The groove is composed of polar and hydrophobic residues that are sensitive to mutations. This predicted state suggests a possible pathway for lipid movement that is consistent with our steered MD simulations.

To assess whether LetA indeed samples alternative states similar to those predicted by AlphaFold2, we focused on Model 149, which represents some of the largest conformational changes in our predicted states, and we used an *in vivo* cysteine-based crosslinking assay. We introduced pairs of cysteine mutations at positions in LetA, for which the distance between the cysteine pair is expected to change between the cryo-EM state and Model 149. Utilizing a bi-functional maleimide reagent with a very short linker between reactive groups (BMOE; ~8 Å), the mutations we engineered are predicted to be selective for crosslinking in either Model 149 or the cryo-EM structure, but not in both. The LetA L94C/I383C double mutant is predicted to be crosslinked in the cryo-EM state only, while LetA Q180C/R380C double mutant is predicted to be crosslinked in Model 149 only (Figure 6E). We used our split-LetA construct with non-essential cysteines removed as the background for these mutations (Cys-light LetA; see Methods), as successful crosslinking is expected to covalently link the two LetA Modules, resulting in a large mobility shift when analyzed by SDS-PAGE. The Cys-light LetA and L94C/I383C and Q180C/R380C derivatives are functional in cells (Supplementary Figure 7C). BMOE treatment of the L94C/I383C pair led to a ~16 fold increase in the crosslinking efficiency relative to that of the DMSO control (Figures 6F), suggesting that the cryo-EM state is indeed sampled in cells. BMOE treatment of the Q180C/R380C pair led to a ~10 fold increase in the crosslinking efficiency relative to that of the DMSO control (Figures 6F), suggesting that the Model 149 conformation is also sampled in cells. The L94C, I383C, Q180C, and R380C single mutant controls exhibit background levels of crosslinking, suggesting that the crosslinking in the double mutants is specific. Together, these results support the existence of an “open-groove” LetA state that is consistent with AlphaFold2 predictions, which may play a role in facilitating transport.

Discussion

Model for LetAB mediated lipid transport. LetB forms a tunnel across the bacterial cell envelope, and has previously been shown to bind lipids, leading to a model in which the hydrophobic tunnel provides a sheltered passageway for lipid transport between the IM and outer membrane. How lipids may be loaded into LetB was unknown, and LetA, a gene in the same operon as LetB, was hypothesized to play a role in the lipid transport mechanism. Combining cryo-EM, DMS, MD simulations and AlphaFold predictions followed by experimental validation of alternate states, we propose a model for LetAB-mediated lipid transport (Figure 7). 1) In the absence of LetA, the LetB tunnel can sample both open and closed conformations *in vitro*, but Ring 1, which is juxtaposed with the IM, is predominantly in the closed conformation, preventing the passage of lipids through the tunnel. LetA binding to LetB results in the opening of LetB Ring 1, mediated by a conformational change in the Ring 1 pore-lining loops. Under physiological conditions, it is possible that LetA is constitutively bound to LetB, resulting in a tunnel that is predominantly open, or it may be that LetA binding regulates the opening of the LetB tunnel. The opening of the Ring 1 pore facilitates lipid loading into the LetB tunnel. Consistent with this, our data show that the LetB tunnel in cells is only loaded with lipids in the presence of LetA. How does LetA extract a lipid from the IM to load into LetB? 2) LetA may cause local distortion of the membrane, enabling the spontaneous movement of phospholipids from the inner leaflet of the IM into the LetA central cleft at the middle of the membrane. MD simulations suggest that after this point, a conformational change in the protein is required for translocation along the transport pathway. DMS data and our cryo-EM structure revealed a polar network of functionally important residues running through LetA. MD simulations suggest how these residues and associated water molecules are poised to shuttle protons across the IM, further bolstering the model that LetA is driven by the proton motive force. 3) LetA undergoes a conformational change, likely driven by proton shuttling, revealing an amphipathic groove. This conformational change was predicted by AlphaFold2, and validated in cells using crosslinking. 4) The lipid traverses the amphipathic groove in a splayed out configuration, in which each fatty acyl tail interacts with functionally important hydrophobic and polar residues. 5) The lipid arrives in the Let A periplasmic pocket, where it is largely flexible, and constrained by interactions of the polar head group with R63 of LetB Ring 1. The lipid tails interact with surrounding hydrophobic residues from LetA, and when pointed towards the letB tunnel, the lipid slides across the periplasmic β1 and β3 strands of LetA to enter the LetB tunnel. 6) The lipid is extruded from the periplasmic pocket into the LetB tunnel, LetA returns to the lower energy, cryo-EM state. The mechanism of transport through the LetB tunnel likely relies on previously described conformational changes, which, in a peristaltic fashion, modulate the diameter of the tunnel[16]. The simplest interpretation of our data is that LetAB is an exporter. This is based on the facts that, in vivo, full-length LetB is only loaded with lipids in the presence of LetA, and that our MD simulations show spontaneous pulling of a lipid by LetA from the inner leaflet of the IM, the first step in a proposed translocation pathway to LetB. However, an alternate explanation is that there is an unidentified component of the system in the outer membrane, which serves to load LetB in a LetA dependent manner, leading to import of a lipid. Recently, the AsmA family of bridge-like proteins has been proposed to mediate bulk phospholipid transport between the IM and outer membrane, possibly by allowing passive equilibration between the membranes[41]. Future work will be needed to unravel the relative roles of LetAB and the AsmA family in mediating phospholipid export in *E. coli*.

LetA in the context of other transporters. LetA is structurally unrelated to known transporters, but some parallels can be drawn to known transport mechanisms. In particular, LetA encompasses some

elements of the mechanisms used by scramblases and extractors. Scramblases are thought to allow passive equilibration between the two leaflets of cellular membranes, and often function via a "credit card swipe" mechanism, in which a polar groove in a protein spanning the transmembrane region allows lipid head groups to "swipe" across the membrane while the lipid tails remain in the membrane environment. Extractors actively remove lipids and other hydrophobic molecules from the IM and pump them towards the outer membrane and/or out of the cell. The first steps of our proposed transport model, in which the lipid moved from the inner leaflet of the IM to the periplasmic pocket of LetA is similar to a credit card swipe model. However, in contrast to the mechanism used by scramblases which primarily involve interactions with the lipid headgroup, in LetA, the entire lipid may need to slide through the amphipathic groove. Unlike the scramblases, which are generally energy independent and bidirectional, we propose that LetA harnesses energy from the proton motive force to drive unidirectional lipid transport. The later steps of the LetA transport model, in which the lipid moves from the periplasmic pocket to the LetB tunnel may be more similar to extractors like the LPS exporter, which extracts LPS from the outer leaflet of the IM, and extrudes it out of the membrane into a periplasm-spanning bridge to mediate LPS transport to the outer membrane. Analogous to the LPS transport process, a phospholipid that has reached the periplasmic pocket of LetA may be extruded into the tunnel of LetB. Thus, the LetA transport mechanism may combine elements of both scramblases and extractors in order to translocate phospholipids unidirectionally, from the inner leaflet of the IM to the OM.

The structure of LetA revealed an unexpected relationship to eukaryotic proteins, the TARPs and claudins, which have no known transporter functions. LetA and TARPs may share mechanisms for modulating their respective binding partners (Supplementary Figures 7D-E). TARPs dock against the ion channel component of the AMPA receptor and regulate its electrical properties[42,43]. The β-strands of TARPs project toward the AMPAR ligand-binding domains and transiently engage them to modulate gating[42,43]. This is reminiscent of the manner in which the β-strands of LetA interact with LetB to prop open the LetB tunnel. The TARP extracellular helix, which is similar to LetA TM2a, directly interacts with the ligand-binding domain, and plays a key role in the regulation of AMPAR function[17,44]. Notably, LetA TMD^{C} and Type 1 TARPs share a FYFG motif (Supplementary Figures 3G-H), which may play a role in locking the extracellular domain on top of the TMD. In TARPγ8, this motif is part of a pocket that can be targeted using small molecules to negatively regulate AMPAR, suggesting that this motif is part of the gating mechanism that regulates the ion channel[45]. Different from TARPs, LetA appears to be the first example in which TARP/Claudin-like domains are dimerized and associated with ZnR domains. Despite sharing a common fold with LetA and TARPs, claudins self-associate to form tight junctions, where the β-sheets enable the polymerization to form the tight cell-cell adhesion barrier (Supplementary Figure 7F)[42,43]. We propose an evolutionary link between LetA, TARPs and claudins, which share structural homology in their core TCL domains, but include variations on this theme, which repurposes these protein families to carry out entirely different cellular functions.

Figure Legends

Figure 1. LetA and LetB interact and are in the same cellular pathway. (A) Schematic of the *letAB* operon (top). Cross-section of the LetB (purple, PDB 6V0C) tunnel oriented in the context of the IM and outer membrane (bottom). A schematic of LetA (blue) is shown in the IM; it is unclear if LetA

interacts with LetB. (B) Cellular assay to assess the function of *letA*, *letB*, or *letAB* deletion mutants. 10-fold serial dilutions of strains were spotted on LB agar with or without the indicated detergent. (C) 2D class averages from negative stain EM data for LetAB and LetB (PDB 6V0C). (D) Schematic of the LetB tunnel. LetB residues mutated for incorporation of the photo crosslinking amino acid, BPA, are shown as red (inside the tunnel) or blue (outside the tunnel) spheres. (E) SDS-PAGE analysis of purified LetAB, LetB and its BPA mutants, either crosslinked or uncrosslinked and stained by Coomassie (LetB) or phosphor-imaged (^{32}P signal). (F) SDS-PAGE analysis of purified LetAB and its BPA mutants, either crosslinked or uncrosslinked and stained by Coomassie (LetB) or phosphor-imaged (^{32}P signal).

Figure 2. Cryo-EM structure of the LetAB complex. (A) Composite map of crosslinked LetAB. LetA (blue), LetB (purple) and the detergent micelle (white) are highlighted. (B) Surface representation of the LetAB structure oriented in the context of the IM and outer membrane. LetB monomers are depicted in different colors. The LetAB complex is ~290 Å long, where LetA (blue) is embedded in the IM. Phospholipids (orange) and lipopolysaccharides (purple) are shown. (C) Structure of LetA (blue surface) in complex with LetB ring 1 (cartoon). LetB monomers are colored according to Figure 3B. (D) Top and bottom views of the LetAB complex shown as a surface representation. (E) Electrostatic potential molecular surface of the LetAB complex (Map 1). LetA is shown in an inset. (F) Surface representation of LetB ring 1 (purple) in the open or closed (PDB 6V0C) state.

Figure 3. **Structure of LetA.** (A) Schematic of the LetA domain organization. (B) Structure of LetA colored by domain. Zinc atoms are shown as spheres (black). (C) Topology diagram of LetA. (D) Cartoon representation of TMD^N of LetA. The components of the LetA TMD are highlighted according to those in (C) and the β-strands are indicated. (E) TMD^N (blue) is rotated ~160°, which superimposes with TMD^C (light blue). (F) Electrostatic potential molecular surface of LetA. The central cleft is indicated. (G) Side view cross-section of the periplasmic pocket in LetA, which is colored by electrostatic potential. (H) Cartoon representation of LetA and the pore-lining loops of LetB, which are colored according to Chain. Residues in the LetA periplasmic β-strands and LetB pore-lining loops are shown, and the LetA periplasmic pocket is indicated. (I) Cartoon representation of the ZnR domains; metal coordinating cysteines are indicated and the zinc atoms are shown as spheres (black). (J) Cartoon representations of LetA TMD^N, TARPγ2, and claudin-4. The components of the TMD^N are colored according to Figure 3C. The components of TARPγ2 and claudin-4 that are structurally similar to those in LetA TMD are colored the same. (G) Topology diagrams of LetA TMD^N, TARPγ2 and claudin-4.

Figure 4. **Assessing the function of LetA and its structural components.** (A) AlphaFold prediction of LetA (AF-P0AD03-F1-v4), which shows the N- and C-terminal loops, where residues are colored by tolerance scores; the radius of each residue also reflects tolerance, with a larger radius corresponding to low tolerance to mutations. Residues most sensitive to mutation appear as the deepest shade of red and with the largest radius. (B) Cartoon representation of LetA colored by the three clusters identified by DMS. (C) Cartoon representation of LetA (gray). Inset highlights the residues (yellow spheres) in the periplasmic pocket that are sensitive to mutations. (D) Horizontal strips from the heat map shown in Supplementary Figure 5A corresponding to residues in the periplasmic pocket. x-axis: residue position in WT LetA; y-axis: all possible amino acid substitutions. Each square represents the fitness cost of an individual mutation relative to WT. Mutations that

decrease fitness relative to the WT are shown in shades of *magenta*, while mutations that increase fitness are in shades of *green*. Squares containing an "X" indicate mutants that have no sequence coverage in both replicates in either the unselected or selected sample, or both. The colored square above each strip represents the tolerance score. (E) Cartoon representation of LetA (gray). Polar network residues (cyan) are shown (nitrogen: blue, oxygen:red, hydrogen: white). Water molecules are represented using a van der Waals depiction, with oxygen in red and hydrogen in white. Hydrogen bonds between the residues and water molecules are illustrated as black dotted lines. (F,G) Same as Figure 4D, except horizontal strips correspond to residues in either the polar network (F) or ZnR^C domain (G). (H,I) Cellular assay to assess the function of either PqiA ZnR swap mutants (H) or ZnR deletion mutants (I). WT LetB is expressed via the same plasmid. 10-fold serial dilutions of strains were spotted on LB agar with or without the indicated detergent.

Figure 5. **LetA can interact with phospholipids.** (A) Cartoon representation of LetA (blue) and LetB ring 1 (purple) with Lipid 1 (orange) in the central cleft (top). The cartoon is rotated 180° to show Lipid 2 (green) in the central cleft (bottom). (B) Three snapshots of LetA (gray) from the SMD simulation where the lipid is pulled by one tail. LetA can accommodate Lipid 2 (green) by opening a groove (purple), allowing the lipid to enter the perisplasmic pocket (blue). Phosphorus atoms of the bulk lipids are represented as white spheres. (C) Cartoon representation of LetA (gray) and Chain C of LetB (light blue). The inset highlights the residues that are interacting with the lipid (green), and are colored based on their DMS cluster: periplasmic pocket (yellow spheres) and polar network (cyan spheres). Residues that are not sensitive to mutations are shown as gray spheres. Phosphorus atoms of the bulk lipids are represented as white spheres. (D) Cartoon representation of LetA (gray) and Chain C of LetB (light blue). Inset highlights the interaction between the lipid (green) and R63 of LetB (Chain C). Phosphorus atoms of the bulk lipids are represented as white spheres. (E) Cellular assay to assess the function of LetB R63 mutants. WT LetA is expressed from the same plasmid. 10-fold serial dilutions of strains were spotted on LB agar with or without the indicated detergent. (F) Anti-LetB western blot to compare cellular levels of WT LetB and R63 mutants. Membrane-enriched fractions were prepared from all strains used in the complementation experiments and tested for expression of LetB using a rabbit polyclonal antibody against LetB. As a loading control, a rabbit polyclonal antibody against OmpF was used.

Figure 6. **Predicted alternate conformation of LetA.** (A) AlphaFold prediction of a LetA alternate state (Model 149, purple) aligned to the cryo-EM structure of LetA (gray) at TMD^N. ZnR domains are hidden for clarity. (B) Visualization of C-α distances between the cryo-EM structure (white cartoon) and Model 149. Models are aligned on TMD^N. Shorter, gray lines indicate minimal displacement and longer, red lines indicate larger displacement. Black arrows indicate direction of domain movement in Model 149 relative to the cryo-EM structure. (C) Cartoon representation of the cryo-EM structure of LetA and Model 149. The TM3 helices are highlighted (red). (D) Electrostatic potential molecular surface of LetA in the cryo-EM or alternate state (Model 149). Model 149 shows an exposed amphipathic groove that is not present in the cryo-EM structure. (E) Cartoon representations of the cryo-EM LetA structure and Model 149 (G), where residues L94, I383, Q180, and R380 are shown as spheres. The Cβ distances are shown in Å. (F) Genetic complementation of Δ*pqiAB* Δ*letAB E. coli* cells with plasmids expressing WT LetA or ΔCysSplitLetA and its mutants. WT LetB is expressed via the same plasmid. 10-fold serial dilutions of strains were spotted on LB agar with or without the indicated detergent. (G) Anti-LetA (antibody 72) Western blot of split-LetA and its mutants treated with

either DMSO (3%) or BMOE (1 mM). For each BMOE-treated sample, the fold change relative to its uncrosslinked sample is shown.

Figure 7. **Model for LetAB function in lipid transport**. (1) LetB ring 1 is open when bound to LetA. Closed (left, PDB 6V0C) and open LetB states (right) are shown. (2) A phospholipid binds to the central cleft of LetA. (3) LetA adopts an alternate state, possibly driven by the proton motive force, revealing an amphipathic groove. (4) Lipid transverses the groove and (5) arrives in the periplasmic pocket, where R63 of LetB dictates lipid orientation. (6) LetA reverts to the cryo-EM conformation, driving the phospholipid towards the LetB tunnel.

Supplementary Figure 1. **Examining the function of LetA, related Figure 1.** (A) Cellular assay to assess the function of WT, *ΔletAB*, *ΔpqiAB*, *ΔpqiABΔletAB E. coli* cells. 10-fold serial dilutions of strains were spotted on LB agar with or without cholate. (B) Pull-down assay to determine if His-LetA can interact with LetB. Anti-LetA (clone 72) and anti-LetB antibodies were used to detect the input and pull-down via Western blotting. (C) Gel filtration trace for LetAB (black). Gel filtration standards (BioRad) are shown (gray). The LetAB complex elutes at the same volume as the 670 kDa protein standard. (D) Coomassie gel of purified LetAB from the peak fraction shown in (C). (E) Membrane expression of LetAB and LetB each containing BPA at position F468. (F) Composite model of crosslinked LetAB showing a tunnel running through the protein assembly. The tunnel is depicted as a smooth surface colored by the hydrophobicity of pore-facing residues, calculated using CHAP[46]. Cross-sectional view of the MCE 1 domain (gray) where the pore-lining loop (red) is indicated. (G) Radius of the LetAB and LetB (PDB 6V0C) tunnels, measured using CHAP[46].

Supplementary Figure 2. **Cryo-EM data collection and processing for the crosslinked and uncrosslinked datasets, related to Figure 2.** (A) Schematic showing LetB (purple) in complex with LetA (blue, transmembrane). The seven rings of LetB are indicated. The composition of each signal-subtracted map (crosslinked dataset) is shown. (B) Local resolution of Map 1a, 1b, and 1c, as estimated by CryoSPARC. (C) Local resolution map of crosslinked LetA, as estimated by CryoSPARC. (D) Cryo-EM data processing workflow for the crosslinked LetAB dataset; black boxes indicate the classes that were chosen for further processing. See Methods for details. (E) Schematic showing LetB (purple) in complex with LetA (blue, TM). The seven rings of LetB are indicated. The composition of each signal-subtracted map (uncrosslinked dataset) is shown. (F) Local resolution of Map 2a, 2b, and 2c, as estimated by CryoSPARC. (G) Composite map of uncrosslinked LetAB. LetA (blue), LetB (purple) and the detergent micelle (white) are indicated. (H) Local resolution map of crosslinked LetA, as estimated by CryoSPARC. (I) Cryo-EM data processing workflow for the uncrosslinked LetAB dataset; black boxes indicate the classes that were chosen for further processing. See Methods for details.

Supplementary Figure 3. **Conserved motifs and evolutionary relationships with eukaryotic proteins, related to Figure 3.** (A) 20 sequences of LetA and PqiA proteins from Alpha-, Beta, and Gammaproteobacteria aligned using MUSCLE[47]; Uniprot IDs are provided. Positions that are at least 95% conserved are highlighted in blue. Regions of interest are indicated. (B) Cartoon representation of LetA, where residues part of the ^{361}GRWSM-Ψ-D-Ψ-F^{369} motif are shown. (C) Sequence logo of the ^{361}GRWSM-Ψ-D-Ψ-F^{369} motif generated by WebLogo 3[48]. Residues are colored based on hydrophilic (blue), neutral (green), or hydrophobic (black) properties. (D) Cellular assay to assess the function of

the split-LetA construct. 10-fold serial dilutions of strains were spotted on LB agar with or without the indicated detergent. (E) Pull-down assay to determine if the N-terminal LetA module (His-tagged) can interact with the C-terminal LetA module (Strep-tagged) using the split-LetA construct. Anti-His and anti-Strep antibodies were used to detect the input and pull-down via Western blotting. (F) Sequence alignment of the FYFG motifs from LetA and human TARP proteins. Residue boundaries are indicated. (G) Cartoon representations of LetA TMD^C, TARPγ2 and zoomed in views of the FYFG motif. The components of the LetA TMD^C are colored according to Figure 3C. The components of LetA TMD^C and TARPγ2 are colored the same. (H) Cartoon representation of LetA (blue) and LetB ring 1 (purple), highlighting the FYFG motif and its interaction with a TM helix of LetB (left). TARP (blue) in complex with AMPA receptor (purple), highlighting the FYFG motif and its interaction with the periphery of the ion channel. (I) AlphaFold models of LetA-like proteins from the indicated species. Uniprot IDs are provided. Models are colored by the predicted local distance difference test (pLDDT) scores.

Supplementary Figure 4. DMS workflow and identification of functionally important residues, related to Figure 4. (A) Schematic of the DMS experiment and data analysis. See Methods for details. Example horizontal strips, where mutations that decrease fitness relative to the WT are shown in shades of *magenta*, while mutations that increase fitness are in shades of *green*. The square above each strip represents the TS. (B) Relative fitness values of replicate 1 on x-axis and replicate 2 on y-axis give an R^2 of 0.897 for cholate and an R^2 of 0.787 for LSB, validating the reproducibility of the experiments. (C) Histograms showing the frequency of the average fitness cost values for the cholate and LSB datasets. (D) Cellular assay to assess the function of LetA N-terminal truncation constructs. WT LetB is expressed via the same plasmid. 10-fold serial dilutions of strains were spotted on LB agar with or without the indicated detergent. (E) Anti-LetA (clone 45) western blot to compare cellular levels of WT LetA and N-terminal deletion mutants. OmpF levels were probed using an anti-OmpF antibody as a loading control.

Supplementary Figure 5. Results of the DMS experiment performed in the presence of cholate or LSB, related to Figure 4. (A,B) Heat map summarizing results of deep mutational scanning of LetA (average of two biological replicates), where either cholate (A) or LSB (B) was used for selection. x-axis: the sequence of WT LetA from N-terminus to C-terminus; y-axis: all possible amino acid substitutions, including the STOP codon. Each square represents the fitness cost of an individual mutation relative to WT. Mutations that decrease fitness relative to the WT are shown in shades of *magenta*, while mutations that increase fitness are in shades of *green*, with *white* representing neutral mutations, as shown in the key. The tolerance score at each position is shown in the horizontal strip above the LetA sequence. Boxes marked with “X” indicate no coverage in the library. (B) Plot showing the number of residues with the indicated tolerance score. x-axis: tolerance score; y-axis: number of residues. (C) Anti-LetA (clone 45) and anti-LetB western blot to assess if LetA polar network mutants pull down LetB. LetA and WT LetB are expressed from the same plasmid. (D) Water density map for LetA is shown. The map was generated using water oxygens within 3.5 Å of LetA and averaged over all the frames. The proposed proton shuttle residues are depicted in stick representation (orange). LetA is shown in cartoon representation (white), while LetB is omitted for clarity. The averaged water density is illustrated as a blue semi-transparent surface. (E) Hydrogen-bond network between proposed proton shuttle residues and water. The most frequently occurring water bridges in each system, where each edge represents the water bridge that most consistently appears between the

two corresponding nodes throughout the simulation. Each node represents a specific residue, while the edges represent water bridges, with the thickness of the edges corresponding to the relative occupancy of the water bridge throughout the simulation. The color coding of the edges indicates the number of water molecules involved in forming the bridge: 1-W (green) represents a single water molecule bridge, 2-W (blue) indicates a bridge formed through two water molecules, and 3-W (orange) shows a water bridge through three water molecules. The percentage occupancy of each water bridge is annotated alongside the edges. (F) Cellular assay to examine the function of LetA ZnR cysteine mutants. 10-fold serial dilutions of strains were spotted on LB agar with or without the indicated detergent. (G) Anti-LetA western (clone 72) blot to compare cellular levels of WT LetA and ZnR cysteine mutants. OmpF levels were probed using an anti-OmpF antibody as a loading control. (H) Sequence alignment of the LetA and PqiA ZnR domains. (I) Anti-LetA western (clone 72) blot to compare cellular levels of WT LetA and PqiA ZnR swap mutant. OmpF levels were probed using an anti-OmpF antibody as a loading control. (J) Anti-LetA western (clone 72) blot to compare cellular levels of WT LetA and ZnR deletion mutants. OmpF levels were probed using an anti-OmpF antibody as a loading control. (K) Pull-down assay to determine if His-LetA ZnR deletion mutants can interact with LetB. Anti-LetA (clone 72) and anti-LetB antibodies were used to detect the input and pull-down via Western blotting.

Supplementary Figure 6. MD simulations to examine the mechanism of lipid translocation through LetA, related to Figure 5. (A) Cartoon representation of LetA (gray) with EM density (orange) corresponding to Lipid 1 (yellow). (B) Cartoon representation of LetA (gray) with EM density (orange) corresponding to Lipid 1 (yellow). Residues W162 and W267 are shown as spheres (red). Figures 6A-B were prepared in ChimeraX using the zone function with an applied radius of 3.5 Å. (C) SDS-PAGE analysis of purified LetAB and its BPA mutants, either crosslinked or uncrosslinked and stained by Coomassie (LetB) or phosphor-imaged (^{32}P signal). (D) Diagram showing the MD simulations performed and the Movies associated with each step. (E) Time series of the z-position of the phosphorus atom of Lipid 1 in each replica. Lipid 1 was either included (+Lipid 1) or excluded (-Lipid 1) at the start of the simulation. In these simulations, a phospholipid binds to the cleft whether a lipid was modeled to begin with or not. The starting z-position of a phosphorus atom is marked by orange, blue, and green circles for replicas 1, 2, and 3, respectively. The average phosphorous atom position from the lipids around 10 Å of LetA is shown as a black line. (F) Time series of the z-position of the phosphorus atom from the most elevated lipid in the central cavity. Replicas without Lipid 1 (-Lipid 1) are shown in dark colored orange, blue, and green lines for replicas 1, 2, and 3, respectively. Replicas with Lipid 1 (+Lipid 1) are shown in light colored orange, blue, and green lines for replicas 1, 2, and 3, respectively. The lipid type of the most elevated lipid in each replica is indicated in the figure legend. The starting z-position of the phosphorus atom is marked by a circle in the same color as the corresponding line for each replica. The average phosphorus atom position of lipids within 10 Å of LetA is depicted as a black line. (G) Accumulated work profiles for different pulling strategies and lipid types (PMPE and PYPG). The first and second circles on each line indicate that the pulled lipid reached the bottom and middle of the periplasmic pocket, respectively. At the end of the line, the lipid reached the top of the periplasmic pocket. (H) Characterization of the steered lipid conformations. The conformational dynamics of the steered lipids are illustrated in 2D scatter plots showing the orientation angle versus the z position of the phosphate group. Time progression is indicated by a color gradient from dark blue to yellow, with the initial frame highlighted by a red-edged dark blue circle and the final frame by a green-edged yellow circle. The orientation

angle is defined by the angle between the vector pointing from the center-of-mass (COM) of the tail ends to the COM of phosphorus, and the z axis, as depicted in the schematic on the right. (I) Root-mean-square deviation (RMSD) profiles of steered lipids. RMSD plots for each lipid type (PMPE and PYPG) and pulling method (head group, one tail, and two tails) show lipid movement and flexibility within the periplasmic pocket during equilibrium simulations after SMD simulations. (J) Bar plot showing the fold change differences between the purified LetAB and the membrane fraction. Dotted line (red) indicates no enrichment or depletion of the indicated lipid class. (K) Bar plot showing the relative abundance of phospholipids from the membrane fraction of *E. coli* cells overexpressing LetAB and purified LetAB. The summed intensity is used to estimate abundance.

Supplementary Figure 7. Examining the alternate conformation of LetA and how LetA-like TMDs can form different assemblies, related to Figure 6. (A) Heatmap of RMSD values for each AlphaFold2 prediction relative to all other models. Hierarchical clustering analysis was performed, showing five different clusters. x-axis: model number; y-axis: RMSD (Å). (B) Cartoon representation of Model 149 colored by pLDDT score. ZnR domains are hidden for clarity. (C) Cellular assay to examine the function of split-LetA mutants. 10-fold serial dilutions of strains were spotted on LB agar with or without the indicated detergent. (D) LetA (cartoon) and LetB ring 1 (gray surface). AMPAR (gray surface) in complex with TARP. The ligand-binding domains are shown in green. Interaction between TARP and the periphery of the ion channel is shown in the inset. Hypothetical assembly of claudins. One claudin is shown as a cartoon representation, whereas the other five claudins are shown as schematic representations. LetA, TARP and claudins are colored according to Figures 3J-K.

Acknowledgements

We gratefully acknowledge the following funding sources: NIH R35GM128777, NIH 1K99GM157496-01, Charles H. Revson Foundation, and the Burroughs Wellcome Fund. Cryo-EM grids were prepared and screened at NYU Langone Health's Cryo-Electron Microscopy Laboratory and we thank A. Paquette and W. Rice for assistance with cryo-EM grid screening and microscope operation. Cryo-EM data were collected at both the Pacific Northwest Center for Cryo-EM and the New York Structural Biology Center. For data processing, we used computing resources maintained by the High Performance Computing (HPC) Facility at NYU and the Advanced Research Computing at Hopkins (ARCH) core facility.

Methods

Expression and purification of LetAB

Plasmid pBEL1284, which encodes for N-terminal 6xHis2xQH-TEV tagged LetA and untagged LetB, was transformed into OverExpress C43 (DE3) cells (Lucigen, 60446-1) (Key Resources Table). For protein expression, overnight cultures (LB + 100 µg/mL carbenicillin + 1% glucose) were diluted in LB (Difco) supplemented with carbenicillin (100 µg/mL), grown at 37°C with shaking to an OD600 of ~0.9, and then induced by addition of arabinose to a final concentration of 0.2%. Cultures were further incubated at 37°C with shaking for 4 hours, and then harvested by centrifugation. The pellets were resuspended in lysis buffer (50 mM Tris pH 8.0, 300 mM NaCl, 10% glycerol), flash frozen in liquid nitrogen and stored at -80°C. Cells were lysed by two passes through an Emulsiflex-C3 cell disruptor (Avestin), then centrifuged at 15,000g for 30 minutes at 4°C to pellet cell debris. The clarified lysate was subjected to ultracentrifugation at 37,000 rpm (182,460 g) for 45 minutes at 4°C in a Fiberlite F37L-8 x 100 Fixed-Angle Rotor (Thermo Fisher Scientific, 096-087056). The supernatant was discarded and the membrane fraction was solubilized in 50 mM Tris pH 8.0, 300 mM NaCl, 10% glycerol, 25 mM n-Dodecyl-B-D-maltoside (DDM) by rocking overnight at 4°C. Insoluble debris were pelleted by ultracentrifugation at 37,000 rpm for 45 minutes at 4°C. Solubilized membranes were then passed twice through a column containing Ni Sepharose Excel resin (Cytiva). Eluted proteins were concentrated using an Amicon Ultra-0.5 Centrifugal Filter Unit concentrator (MWCO 100 kDa, UFC510096) before separation on the Superdex 200 Increase 10/300 column (Cytiva) equilibrated with gel filtration buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.5 mM DDM and 10% glycerol). Fractions containing LetAB were pooled, concentrated and applied to grids for negative stain EM or cryo-EM. One liter of culture typically yields 30-40 µg of the LetAB complex.

Negative stain EM

To prepare grids for negative-stain EM analysis, a fresh sample of LetAB was applied to a carbon-coated 400 mesh copper grid (Ted Pella, 01754-F), freshly glow discharged for 30 seconds. and blotted off. Immediately after blotting, 3 µL of a 2% uranyl formate solution was applied for staining and blotted off on filter paper (Whatman 1) from the opposite side. Application and blotting of stain was repeated five times. The sample was allowed to air dry before imaging. A negative stain grid of LetB$^{\Delta TM}$ was prepared previously [16] using a similar procedure and stored. New images from this sample were acquired for this study. Data were collected on the Talos L120C TEM (FEI) equipped with the 4k x 4k OneView camera (Gatan) at a nominal magnification of 73,000x corresponding to a pixel size of 2.03 Å/px on the sample and a defocus range of −1 to −2 µm defocus. Negative-stain dataset size was determined to be sufficient by the ability to see features in the 2D classes of picked particles. For both LetAB and LetB$^{\Delta TM}$ datasets, micrographs were imported into cryoSPARC (v.3.3.1) and approximately 200 particles were picked manually, followed by automated template-based picking. Particles were extracted with a 320 pixel box size. Several rounds of 2D classification were performed using default parameters, except that “Force max over poses/shifts” and “Do CTF correction” were both set to False.

Cryo-EM sample preparation and data collection

Grids containing crosslinked LetAB were screened at the NYU cryo-EM core facility on the Talos Arctica (Thermo Fisher Scientific) equipped with a K3 camera (Gatan). The grids were selected for data collection on the basis of ice quality and particle distribution. The selected cryo-EM grid was

imaged on two separate sessions at the Pacific Northwest Center for Cryo-EM (PNCC) on Krios-1, a Titan Krios G3 electron microscope (Thermo Fisher Scientific) equipped with a K3 direct electron detector with a BioContinuum energy filter (Gatan). Super-resolution movies were collected at 300 kV using SerialEM at a nominal magnification of 81,000kx, corresponding to a super-resolution pixel size of 0.5144 Å (or a nominal pixel size of 1.029 Å after binning by 2). 12,029 movies were collected over a defocus range of -0.8 to -2.1 µm and each movie consisted of 50 frames with a total dose of 50 e-/Å^2. Further data collection parameters are shown in Table S1. No statistical methods were used to predetermine sample size for cryo-EM. The cryo-EM dataset size was determined to be sufficient by the ability to reach resolutions beyond 4 Å in 3D construction.

The uncrosslinked LetAB complex was prepared as described in "Expression and purification of LetAB". Grids containing uncrosslinked LetAB were screened at the NYU cryo-EM laboratory on the Talos Arctica (Thermo Fisher Scientific) system equipped with a K3 camera (Gatan). The grid with the best ice quality and particle distribution was imaged at the New York Structural Biology Center (NYSBC) on Krios-1, a Titan Krios G3 electron microscope (Thermo Fisher Scientific) equipped with K3 direct electron detector with a BioContinuum energy filter (Gatan). Super-resolution movies were collected at 300 kV using Leginon at a nominal magnification of 81,000x, corresponding to a super-resolution pixel size of 0.535 Å (or a nominal pixel size of 1.083 Å after binning by 2). Movies were collected over a defocus range of -2 to -5 µm and each movie consisted of 40 frames with a total dose of 51 e-/Å^2. A total of 12,464 movies were collected, consisting of 5372 movies at 0° tilt and 7,083 movies at -30° tilt. Further data collection parameters are shown in Table S1.

To generate the crosslinked LetAB sample, 1% glutaraldehyde was added to purified LetAB at a final concentration of 0.025%. The sample was incubated on ice for one hour and then quenched by the addition of 75 mM Tris-HCl pH 8.0. The sample was incubated for 15 minutes on ice before filtering using an Ultrafree centrifugal filter (catalog #UFC30GVNB) and loading onto a Superdex 200 Increase 10/300 column (Cytiva) to remove aggregated LetAB. Fractions containing the LetAB complex were concentrated to 1 mg/mL using the Amicon Ultra-0.5 centrifugal filter unit concentrator (MWCO 100 kDa, UFC510096). Continuous carbon grids (Quantifoil R 2/2 on Cu 300 mesh grids + 2 nm Carbon, Quantifoil Micro Tools, C2-C16nCu30-01) were glow-discharged for 5 s in an easiGlow Glow Discharge Cleaning System (Ted Pella). 3.5 µL of freshly prepared sample was added to the glow-discharged grid. Grids were prepared using a Vitrobot Mark IV (Thermo Fisher Scientific). Grids were blotted with a blot force of 0 for 3 s at 4°C with 100% chamber humidity and then plunge-frozen into liquid ethane. Grids were clipped for data acquisition.

Cryo-EM data processing and model building for crosslinked LetAB

Data processing workflow for the crosslinked LetAB sample is shown in Table S2. A combination of cryoSPARC (versions 3.2.0-4.3.0) and RELION (version 3.1.0) were used for data processing. Dose-fractionated movies were gain-normalized, drift-corrected, summed and dose-weighted, and binned by 2 using the cryoSPARC Patch Motion module. CTF estimation for each summed image was carried out using cryoSPARC Patch CTF estimation. To generate 2D templates for auto-picking, 1003 particles were manually picked, extracted (box size = 576 px) and subjected to 2D classification. The classes with top, tilted and side views of LetAB were selected as templates for auto-picking, which yielded 3,582,295 particles after extraction (box size = 576 px). The particles were subjected to 2D classification (200 classes) with Force Max over poses/shifts set to False. Well-aligned 2D classes

were selected (1,793,362 particles) and a 3D reconstruction was generated using ab-initio reconstruction. The 3D reconstruction was used as a template for 3D refinement in Relion, which revealed well-resolved density for LetB rings 1-4, poor and noisy density for LetB rings 5-7, and no density for LetA, likely due to rings 1-4 dominating the particle alignment. To improve resolution for LetA, local refinement was performed using a mask around LetB rings 1-4 and the TM region, followed by particle subtraction in Relion where the signal for the TM region and rings 1-2 were kept and re-centered to the middle of the box (256 px). The subtracted particles were imported into cryoSPARC, where they underwent reference-free 3D classification using the ab-initio module (2 classes) to remove misaligned and 'junk' particles, resulting in one "good" class with 1,171,725 particles. The particles were further cleaned using 2D classification and then subjected to non-uniform refinement (942,263 particles). The aligned particles were imported into Relion and sorted using 3D classification without alignment (8 classes), which revealed one class containing density for the TM region with high resolution features. The particles (158,666) were then subjected to local refinement in Relion, yielding a map (Map 1a) with a nominal resolution of 3.4 Å (Supplementary Figure 2C).

To obtain high resolution maps of rings 2-4 and rings 5-7, the 1,793,362 particles from the initial 2D classification step were Fourier cropped to a box size of 128 pixels. The particles were sorted using heterogeneous refinement (5 classes) in cryoSPARC, which revealed only one class where LetB is straight rather than curved. The particles from this class (448,403) were re-extracted (box size = 512 px), aligned using non-uniform refinement, and imported into Relion. The aligned particles underwent local refinement, followed by particle subtraction to yield signal for either rings 2-4 or rings 5-7. During particle subtraction, the subtracted images were re-centered to the middle of the box, which was cropped to either 360 px (rings 2-4) or 256 px (rings 5-7). The two particle subtracted stacks were imported into cryoSPARC, where the particles were subjected to reference-free 3D classification using ab-initio reconstruction (3 classes) to remove misaligned and 'junk' particles. The particles from the selected class were aligned using non-uniform refinement, and then imported into Relion for 3D classification without alignment (8 classes). The classes with the highest resolution features were selected, their particles combined before being imported into cryoSPARC for non-uniform refinement to improve the densities for both the MCE core domains and the pore-lining loops[16], resulting in Map 1b (rings 2-4) and Map 1c (rings 5-7). All refinement steps were performed without symmetry applied (C1).

To build a model for LetAB, RoseTTAFold[49] was used to predict the 3D structure of LetA. The model was fit as a rigid body into the LetA density in Map 1a, followed by rigid body fitting of TMD^N (aa 66-218), TMD^C (aa 261-418), ZnR^N (aa 24-65) and ZnR^C (aa 219-261). Residues 1-26 and 419-427 were deleted due to the absence of density for them. As our ICP-MS data suggests LetA binds zinc, we used Coot (v.0.8.9.2)[50] to add zinc ligands to the densities found in between the predicted metal-coordinating cysteines. To build the model for LetB rings 1-2, PDB 6V0J was used, as it best matches the density in Map 1a. The model was first fit as a rigid body into the density corresponding to LetB, followed by rigid-body fitting of each MCE domain. The PLLs of ring 1 exhibit C3 symmetry. Densities for four out of six TM helices of LetB were observed, and those helices were manually built using Coot. Residues (25-45) of a LetB TM helix (Chain B) were stubbed due to the lack of side chain density.

For Maps 1b and 1c, PDB 6V0F and PDB 6V0E best fit into the density, respectively, after rigid body docking. Each MCE domain was rigid-body fitted into the density. The PLLs of rings 5 and 6 exhibit C3 symmetry. Extra densities that do not correspond to the protein are present near the PLLs

between rings 5-6 and rings 6-7, but the resolution is too low to determine the identity of the ligand. Therefore, ligands were not modeled into these densities. It is possible that these densities represent DDM artifacts. Each model was real-space-refined into its respective map using Phenix [51] with global minimization, Ramachandran, secondary structure and ligand restraints. Using UCSF Chimera [52], Maps 1a and 1c were fitted into Map 1b and resampled such that the maps overlaid with one another. These maps were then used to stitch together the models. The ring 2 model from Map 1b (instead of the one from Map 1a) was used to generate the composite model since this map had complete density for ring 2. The resulting composite model was used as a template to generate a composite density map (Map 1) using the PHENIX Combine Focused Maps module. The model was real-space-refined into Map 1 using PHENIX with global minimization, Ramachandran, secondary structure and ligand restraints. The composite model reveals that LetB rings 3, 5 and 6 are in a single, closed conformation, whereas the conformation of rings 2, 4, and 7 could not be reliably assessed due to weak density for the PLLs (Supplementary Figure 1G).

For validation, statistics regarding the final models (Table S1) were derived from the real_space_refine algorithm of PHENIX and MolProbity[53], EMRINGER[54], and CaBLAM[55] from the PHENIX package[56] were used for model validation. Model correlations to our EM maps were estimated with CC calculations and map-model FSC plot from the PHENIX package.

Cryo-EM data processing and model building for uncrosslinked LetAB

Data processing workflow for the uncrosslinked LetAB sample is shown in Supplementary Figure 2. A combination of cryoSPARC (versions 3.3.1-4.3.0) and RELION (version 4.0-beta) were used for data processing. Particle picking was performed in Relion on the motion-corrected micrographs generated by NYSBC using MotionCor2. 2D templates were generated for auto-picking on manually picked particles. The particles (3,014,365 at 0° tilt; 3,960,481 at -30° tilt) were imported into cryoSPARC and re-extracted (600 px, Fourier cropped to 100 px) from Patch CTF corrected micrographs generated within CryoSparc (gain-normalized, drift-corrected, summed, binned 2x and dose-weighted using the cryoSPARC Patch Motion module). The particles underwent several rounds of 2D classification (200 classes) with Force Max over poses/shifts set to False. Well-aligned 2D classes were selected, resulting in 1,582,691 particles at 0° tilt and 1,918,023 particles at 30° tilt. The particles were combined, sorted by 2D classification, and the selected particles were re-extracted (512 px). The particles (2,658,362) were then aligned by non-uniform refinement (C6 symmetry applied), and imported into Relion, where they were subjected to local refinement with C6 symmetry relaxation applied. The signal for LetB rings 2-7 was removed using the Particle Subtraction module. The subtracted images were re-centered so that the signal for LetA+rings 1-2 was in the middle of the box, which was cropped to 256 px. The subtracted particles were imported into cryoSPARC and sorted by several rounds of 2D classification (200 classes) to remove misaligned and ‘junk” particles. The particles were further sorted using the ab-initio reconstruction (5 classes, 3 rounds) to yield 1,131,012 “clean” particles, which were then aligned using non-uniform refinement. The particles were imported into Relion for 3D classification without alignment and with a mask around LetA, which revealed a class showing high-resolution features. The particles were imported into Relion for non-uniform refinement. To continue filtering out low-resolution particles, the particles were sorted by 3D classification without alignment in Relion, followed by non-uniform refinement in cryoSPARC, two additional times. After non-uniform refinement, the particles underwent local refinement in cryoSPARC to yield a map with a nominal resolution of 3.4 Å (Map 2a, Supplementary Figure 2H).

To obtain high resolution maps of rings 2-4 and rings 5-7, the 2,658,362 particles from the initial 2D classification step were sorted using heterogeneous refinement (5 classes), which revealed only one class where LetB is straight rather than curved. The particles from this class (738,470) were aligned using non-uniform refinement with symmetry applied, and imported into Relion. The aligned particles underwent local refinement, followed by particle subtraction to yield signal for either rings 2-4 or rings 5-7. During particle subtraction, the subtracted images were re-centered to the middle of the box, which was cropped to either 360 px (rings 2-4) or 256 px (rings 5-7). The two particle subtracted stacks were imported into cryoSPARC, where the particles were sorted using ab-initio reconstruction (3 classes) to remove misaligned and 'junk' particles. The "good" classes were selected and the particles were aligned using non-uniform refinement, and then imported into Relion for 3D classification without alignment (8 classes). The classes with the highest resolution features were selected, their particles combined and imported into cryoSPARC for non-uniform refinement to improve the densities for both the MCE core domains and the pore-lining loops [16], resulting in Map 2b (rings 2-4) and Map 2c (rings 5-7).

The crosslinked LetAB model was used to build the model for uncrosslinked LetAB. LetA was rigid-body fitted into the LetA density in Map 2a. A phospholipid (SMILES string = CCCCCCCCCCCC(=O)OCC(COP(=O)(O)OCCN)OC(=O)CCCCCCCCCCC) was manually built into the extra density observed in the central cavity of LetA. Since the resolution of the extra density is too low to unambiguously identify the ligand, the phospholipid is labeled as an unknown ligand (UNL). The ring 1 model from Map 1a was rigid-body docked into Map 2a. The individual MCE domains were then rigid-body fitted into the map. The model was real-space-refined into Map 2a map using PHENIX with global minimization, Ramachandran, secondary structure and ligand restraints.

To build a model for rings 2-4 and rings 5-7, the models from Map 1b and Map 1c were rigid-body fitted into Map 2b and Map 2c, respectively. Each MCE domain was rigid-body fitted into the map. LetB residues 614-619 were deleted from the model due to poor density in the map. Similar to the crosslinked LetAB model, the PLLs of rings 1, 5 and 6 exhibit C3 symmetry. Extra densities are present near the PLLs between rings 5-6 and rings 6-7. Since the resolution is too low to determine the identity of the ligand, the extra densities were left unmodeled. Each model was real-space-refined into its respective map using PHENIX with global minimization, Ramachandran, secondary structure and ligand restraints. Using UCSF Chimera, Maps 2a and 2c were fitted into Map 2b and resampled such that the maps overlaid with one another. These maps were then used to stitch together the models. The ring 2 model from Map 2b was used to generate the composite model since this map had complete density for ring 2. The resulting composite model was used as a template to generate a composite density map (Map 2) using the Phenix Combine Focused Maps module. The model was refined using Phenix.real_space_refine into Map 2 with global minimization, Ramachandran, secondary structure and ligand restraints.

For validation, statistics regarding the final models (Table S1) were derived from the real_space_refine algorithm of PHENIX and MolProbity[53], EMRINGER[54], and CaBLAM[55] from the PHENIX package[56] were used for model validation. Model correlations to our EM maps were estimated with CC calculations and map-model FSC plot from the PHENIX package.

We used this model to assess the conformation of the LetB rings. LetB rings 3, 5 and 6 are in a single, closed conformation, whereas the conformation of rings 2, 4, and 7 could not be reliably assessed due to weak density for the PLLs (Supplementary Figure 1G). However, since the different segments of LetB were processed separately (Supplementary Figure 2I), it is unclear if the conformations of rings 1, 3, 5, and 6 are correlated.

Sequence alignment

LetA and PqiA proteins are widespread across Proteobacteria. Using the *E. coli* LetA sequence, we performed a protein blast to search for LetA and PqiA proteins across the orders within each of the five classes of Proteobacteria. Only sequences that contained both LetA modules were considered. We then performed a tblastn search using the core nucleotide database and the specified organism to determine if the gene is in an operon with *letB* or *pqiB*, which would indicate if the query sequence is a *letA* or *pqiA* gene, respectively. LetB and PqiB can be identified based on sequence length and AlphaFold2 prediction; LetB has six or seven MCE domains while pqiB has three. Through this method, we identified 20 sequences, where nine are LetA and 11 are PqiA proteins. The LetA sequences are from Gammaproteobacteria, whereas PqiA are from Alpha- and Betaproteobacteria. The sequences were aligned using MUSCLE[57] (MUltiple Sequence Comparison by Log-Expectation) with a ClustalW[58] output format and annotated using Jalview (version 2.11.3.3)[59].

Deep Mutational Scanning

A library containing all the possible single amino acid mutants in LetA (*n* = 8,540) was synthesized by Twist Bioscience (San Francisco, CA). Apart from the engineered mutations, these plasmid variants were identical to pBEL2071, which was created to separate ("de-overlap") the LetA and LetB open reading frames so they could be mutated independently of each other, as LetA and LetB overlap by 32 base pairs in the *E. coli* genome. As DNA fragments encoding the entire LetA gene are too large to sequence end-to-end using standard Illumina sequencing (1284 bp), we divided our LetA mutant library into four sub-libraries that each contain all the mutations generated in a ~325 bp segment of the LetA gene (codons 1–104, 105–208, 209-320 or 321-427). Each of the four sub-libraries was kept separate and handled independently throughout the deep mutational scanning experiment, such that the mutated segment from each sub-library could be profiled using a standard Illumina 2x250 protocol to quantify the frequency of each mutant in the population. A Δ*letAB* Δ*pqiAB* strain, bBEL384, was transformed by electroporation with each sub-library and grown overnight at 37°C in LB containing 200 µg/mL carbenicillin. We obtained ~2 x 10^6 CFU for each sub-library. For the replicate experiment, ~1 x 10^7 CFU was obtained. The cultures were diluted 1:20 into fresh LB media containing 100 µg/mL carbenicillin and 50 µg/mL kanamycin and shaken (200 rpm) at 37°C until OD_{600}= ~1. The cultures were plated on LB (BD Difco, catalog #DF0445–07-6) + 100 µg/mL carbenicillin ("no selection"), LB + 100 µg/mL carbenicillin + 0.105% LSB ("selection"), or LB + 100 µg/mL carbenicillin + 8% cholate ("selection"). After overnight incubation on the no selection and selection plates, colonies from each condition were separately scraped and pooled, plasmids were extracted, and amplicons were generated by PCR. The amplicons from each sub-library were then pooled in equimolar amounts to generate the no selection and selection samples. The NEBNext Ultra II Library Prep kit (New England Biologs #E7645) was used to generate the library for Illumina MiSeq 2 X 250 paired-end sequencing. Two independent biological replicates of the deep mutational scanning experiment were performed starting from the same sub-libraries.

Paired-end sequencing data were mapped to a reference WT LetA sequence using the bowtie2 algorithm (v2.4.1), filtered with samtools (v1.9; flags -f 2 -q 42), and overlapping paired ends were merged into a single sequence with pandaseq (v2.11). Lastly, primer sequences used for amplicon amplification were removed using cutadapt (v1.9.1). Processed and merged reads were then analyzed using custom Python scripts to count the frequency of the LetA variants[60]. Briefly, DNA

sequences were filtered by length, removing any sequence larger or smaller than the length of the expected library. Next, sequences were correctly oriented to the proper reading frame and translated to the corresponding protein sequence. Finally, the frequency of each amino acid variant at every position was counted and the counts were normalized to the sequencing depth as read counts per million. These normalized counts were then used for calculation of the relative fitness value (ΔE_i^x), which is defined as the log frequency of observing each amino acid x at each position i in the selected versus the non-selected population, relative to the wild-type amino acid (30). The equation for this calculation is as follows:

$$\Delta E_i^x = log(\frac{f_i^{x,sel}}{f_i^{x,unsel}}) - log(\frac{f_i^{WT,sel}}{f_i^{WT,unsel}}) \quad \text{Equation 1}$$

For cholate, we found that the square of the Pearson correlation coefficient (r^2) between two biological replicates to be $r^2 = 0.897$ (Supplementary Figure 4B). For LSB, the square of the Pearson correlation coefficient is $r^2 = 0.786$. These coefficients indicate replicates are in a good agreement with one another. We were able to extract meaningful fitness information for 8,478/8,540 variants for cholate and 8,504/8,540 variants for LSB. Meaningful fitness information for a mutation could not be extracted if counts were not present in either the unselected or selected dataset. For example, mutations at position 51 had no sequence coverage. The relative fitness values exhibited a bimodal distribution, where the two modes represent the neutral and deleterious mutant groups (Supplementary Figure 4C). We established a cutoff to identify mutations with relative fitness values that are substantially different from the median (0) by calculating the modified Z-score (M_i)[61] for each mutation using Equations 2 and 3, where x_i is a single data value, $\tilde{x}$ is the median of the dataset, and MAD is the median absolute deviation of the dataset:

$$M_i = \frac{0.6745(x_i - \tilde{x})}{MAD} \quad \text{Equation 2}$$

$$MAD = median\{|x_i - \tilde{x}|\} \quad \text{Equation 3}$$

Since modified Z-scores with an absolute value of greater than 3.5 are potential outliers[61], mutations with a Z-score of <-3.5 and >3.5 were considered to be deleterious or advantageous, respectively, to LetA function. For each residue, we calculated a tolerance score based on the number and types of amino acid substitutions that are tolerated. The tolerance scores were calculated by using a modified version of the Zvelebil similarity score[29,30], which is based on counting key differences between amino acids. For each key difference (i.e. ‘small’, ‘aliphatic’, ‘proline’, ‘negative’, ‘positive’, ‘polar’, ‘hydrophobic’ and ‘aromatic), a score of 0.1 is given, such that mutations to dissimilar amino acids (e.g. alanine to arginine) contribute more to the score. If the mutation is tolerated based on our modified Z-score cut-off, the score for that particular mutation is added to a starting score of 0.1. For each sequence position, the scores for tolerant mutations were summed, then divided by the maximum score possible. A score of 1.0 therefore indicates full tolerance in that position, a score of 0.1 denotes no tolerance, and in-between scores suggest different levels of tolerance for that amino acid type.

In the cholate dataset, three of the 53 residues (A128, F141, A272) do not cluster in the three main groups (ZnRs, polar network, and outward-open pocket). In the LSB dataset, two (I131, F141) of the

37 residues are located outside of the three groups. F141 is considered functionally important in both datasets, but it is unclear what its role is, as this residue appears isolated in the membrane. Residues A272 and I131 interact with residues in the LetB TM helices, potentially stabilizing interactions between LetA and LetB in the membrane. As residue A128 precedes the helical “break” of TM3 in TMD^N, this position may only tolerate small hydrophobic residues to maintain the structural integrity of LetA.

Complementation Assays

letA, *letB*, and *letAB* knockout strains were constructed in *E. coli* K-12 BW25113 by P1 transduction from corresponding strains of the Keio collection (Key Resources Table)[62], followed by excision of the antibiotic resistance cassettes using pCP20[63]. To test the impact of *letA*, *letB*, and *letAB* deletion mutants on cell viability, overnight cultures grown in LB were diluted 1:50 into fresh LB without antibiotics. The knockout strains carrying pET17b-*letAB* (Addgene #175804) or its mutants were grown in the presence of 100 µg/mL carbenicillin. Cultures were grown for ≈1.5 hours at 200 rpm and 37°C until reaching an OD600 of ~1.0, then normalized to a final OD600 of 1.0 with fresh LB. From these normalized cultures, 10-fold serial dilutions in LB were prepared in a 96-well plate, and 1 µl of each dilution was spotted onto plates containing LB agar, or LB agar supplemented with either LSB or sodium cholate. The LB agar was prepared from the following components: 10g Tryptone (Gibco, catalog #211705), 10g NaCl (Sigma-Aldrich, catalog #S3014), 5g Yeast extract (Gibco, catalog #212750), 15g Agar (BD Difco, catalog #214530) per one liter of deionized water. Plates were incubated ~18-20 hours at 37°C and then imaged using a ChemiDoc XRS+ System (Bio-Rad). Stock solutions of LSB (5% w/v) and sodium cholate (40% w/v; Thermo Fisher, catalog #A17074.18) were prepared in deionized water and stored at −80°C. At least three independent transformants were used to perform replicates for each phenotypic assay.

Small-scale pull-down assays

Plasmid pBEL1284 was transformed into OverExpress™ C43 (DE3) cells (Lucigen). The *letA* or *letB* regions were mutated using Gibson assembly. Whole plasmid sequencing was performed by Plasmidsaurus using Oxford Nanopore Technology with custom analysis and annotation. Overnight cultures (LB + 100 µg/mL carbenicillin + 1% glucose) were diluted into 20 mL LB (Difco) supplemented with carbenicillin (100 µg/mL), grown at 37°C with shaking to an OD600 of ~0.9, and then induced by addition of arabinose to a final concentration of 0.2%. Cultures were further incubated at 37°C with shaking for 4 hours, and then harvested by centrifugation. The pellets were resuspended in 1 mL of lysozyme resuspension buffer (50 mM Tris pH 8.0, 300 mM NaCl, 1 mg/mL lysozyme, 25 U/mL benzonase, 1 mM TCEP) and were incubated for 1 hr at 4°C. The cells were lysed with eight cycles of a freeze-thaw method, where samples are immersed in liquid nitrogen until fully frozen and then thawed in a 37°C heat block. The lysate containing crude membrane fractions was pelleted at 20,000 g for 15 min, and resuspended in 250 µL of membrane resuspension buffer (50 mM Tris pH 8.0, 300 mM NaCl, 10% glycerol and 25 mM DDM, 1 mM TCEP), and shaken for 1 hr. The sample volume was then increased to 1 mL with 10 mM imidazole wash buffer (50 mM Tris pH 8.0, 300 mM NaCl, 10 mM imidazole) and insoluble material was pelleted at 20,000 g for 15 min. Each supernatant was then mixed with 25 µL of nickel Ni Sepharose Excel resin (Cytiva) for 30 min. The beads were pelleted at 500 g for 1 min and the supernatant removed. The beads were then washed four times with 40 mM imidazole wash buffer (50 mM Tris pH 8.0, 300 mM NaCl, 40 mM imidazole, 10% glycerol, 0.5 mM DDM, 1 mM TCEP) and finally resuspended in 50 µL of elution

buffer (50 mM Tris pH 8.0, 300 mM NaCl, 300 mM imidazole, 10% glycerol, 0.5 mM DDM, 1 mM TCEP). The beads were removed by passing through an Ultrafree centrifugal filter (10,000 g for 1 min) at 4°C. The samples were then mixed with 5x SDS-PAGE loading buffer, and analyzed by SDS-PAGE and stained using InstantBlue Protein Stain (Abcam, Catalog #ab119211). At least two replicates of the experiment were performed, from independently purified proteins.

To obtain membrane fractions without cell debris, lysed samples were centrifuged at 16,000 g for 10 minutes at 4°C. The supernatant was collected and the membrane fraction was isolated by ultracentrifugation in a TLA 120.2 rotor (100,000 rpm, 15 min at 4°C). The supernatant was removed, the pellet resuspended in 500 µL of membrane resuspension buffer, and shaken for 1 hour at 4°C. Added 500 µL of 20 mM imidazole wash buffer (50 mM Tris pH 8.0, 300 mM NaCl, 20 mM imidazole, 10% glycerol, 0.5 mM DDM, 1 mM TCEP), and centrifuged at 20,000 x g for 15 min at 4°C to remove insoluble debris. The samples were then purified using affinity resin as described above.

Generation of LetA monoclonal antibodies

Plasmid pBEL2214 was transformed into Rosetta (DE3) cells (Novagen) for protein expression and overnight cultures were grown in LB supplemented with carbenicillin (100 µg/mL), chloramphenicol (38 µg/mL) and 1% glucose at 37°C. The overnight cultures were diluted 1:50 in fresh LB media supplemented with carbenicillin (100 µg/mL) and chloramphenicol (38 µg/mL). Upon reaching an OD600 of ~0.9, protein expression was induced with the addition of L-arabinose to a final concentration of 0.2%. Cells were cultured for an additional 4 hours at 37°C with shaking, and then harvested by centrifugation. The pellets were resuspended in lysis buffer (50 mM Tris pH 8.0, 300 mM NaCl, 10% glycerol) flash frozen in liquid nitrogen and stored at -80°C. Cells were lysed by three passes through an Emulsiflex-C3 cell disruptor (Avestin), then centrifuged at 15,000g for 30 minutes at 4°C to pellet cell debris. The clarified lysate was subjected to ultracentrifugation at 37,000 rpm (182,460 g) for 45 minutes at 4°C in a Fiberlite F37L-8 x 100 Fixed-Angle Rotor (Thermo Fisher Scientific, 096-087056), and the membrane fraction was solubilized in 50 mM Tris pH 8.0, 300 mM NaCl, 10% glycerol, 25 mM DDM by rocking overnight at 4°C. Insoluble debris were pelleted by ultracentrifugation at 37,000 rpm for 45 minutes at 4°C. Solubilized membranes were then passed twice through a column containing Ni Sepharose resin (Cytiva). Eluted proteins were exchanged into low salt buffer (20 mM HEPES, pH 7.0, 25 mM NaCl, 0.5 mM DDM, 10% glycerol) using an Amicon Ultra-0.5 Centrifugal Filter Unit concentrator (MWCO 30 kDa, UFC503008) before injection into a Mono S 5/50 GL column (Cytiva). The column was eluted using a salt gradient from 25 mM to 1.5 M NaCl over 40 column volumes. The eluted proteins containing LetA were concentrated using an Amicon Ultra-0.5 Centrifugal Filter Unit concentrator (MWCO 30 kDa, UFC503008) before separation in a Superdex 200 Increase 10/300 column (Cytiva) equilibrated in gel filtration buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.5 mM DDM, 10% glycerol).

To generate LetA rat monoclonal antibodies, three six-week old Sprague Dawley rat (Taconics) were immunized with 1 mg of purified LetA protein (100 mg per animal per boost for 5 boosts). Immune response was monitored by ELISA to measure the serum anti-LetA IgG titer from blood samples. After a 60-day immunization course, the rat with the strongest anti-LetA immune response was terminated and 10^8 splenocytes were collected for making hybridomas by fusing with rat myeloma cell line YB2/0, following the standard method[64]. All procedures were approved by the Cold Spring Harbor Laboratory Institutional Animal Care and Use Committee (IACUC).

To select monoclonal antibodies, supernatants collected from individual hybridoma culture media were screened by ELISA to identify hybridoma clones positive for LetA. Positive hybridoma colonies were then isolated and seeded to establish pure hybridoma clones from single cell colonies. For ELISA, purified LetA or negative control protein streptavidin diluted in LetA storage buffer (20 mM Tris, pH8, 150mM NaCl, 10% glycerol, 0.5 mM DDM) were coated (50 ng/well) on ELISA plates (Thermo Fisher Scientific, 464718) following the manufacturer's instructions. Prior to adding the hybridoma supernatant, the coated plate was blocked with LetA storage buffer (20 mM Tris, pH8, 150mM NaCl, 10% glycerol, 0.5 mM DDM) containing 0.5% BSA at 4 °C for six hours. After blocking, hybridoma supernatants diluted in LetA buffer (1:1 dilution) were added to the ELISA plate and incubated at room temperature for 1 hour, followed by three times of extensive wash with LetA buffer. The secondary antibody (112-035-003, Jackson ImmunoResearch for anti-rat IgG HRP) was then added and incubated at room temperature for 30 min, followed by three times of extensive wash with LetA buffer. Chromogenic binding signal was developed by using 3,3',5,5'-Tetramethylbenzidine (TMB) ultra as the HRP substrate (Thermo Fisher Scientific, 34028) following the manufacturer's instructions. Data was collected by measuring the absorbance at 450 nm with a plate reader (Cytation 5, Agilent). The ELISA assay revealed 23 antibody clones to be strong binders of LetA, and two were found to detect LetA in cell lysates via Western blotting (clones 45 and 72). Clone 45 recognizes an epitope in the ZnR domains, and can also recognize PqiA. The epitope recognized by Clone 72 is in the N-terminal extension of LetA, and does not appear to cross-react with PqiA.

Western blotting

To test for protein expression in the strains used for the complementation assays, 10 mL cultures of *E. coli* strains Δ*pqiAB*, Δ*letAB*, Δ*pqiAB* Δ*letAB*, and Δ*pqiAB* Δ*letAB* containing each complementation plasmid was grown to an OD600 of ~0.8. For plasmid containing strains, the cultures were supplemented with carbenicillin (100 µg/mL). The cells were pelleted at 4000 g for 10 mins and resuspended in 1 mL of freeze thaw lysis buffer (PBS pH 7.4, 1 mg/ml lysozyme and 1 µL/ml of benzonase (Millipore), and incubated on ice for 1 hour. The cells were lysed with eight cycles of a freeze-thaw method, where samples are immersed in liquid nitrogen until fully frozen and then thawed in a 37°C heat block. After lysis, the cells were pelleted at 20,000 g for 15 minutes and the pellets were resuspended in 100 µl of SDS-PAGE loading buffer. Each sample (10 µL) was separated on an SDS-PAGE gel and transferred to a nitrocellulose membrane. The membranes were blocked in Phosphate Buffered Saline Tween20 (PBST, 1X PBS + 0.1% Tween20) containing 5% milk for 1 hour at room temperature. Membranes were cut to yield the region of interest to increase the sensitivity of the anti-LetA antibodies. The membranes were then incubated with primary antibody in PBST + 5% milk, either rat monoclonal anti-LetA clone 45 or clone 72 at a final concentration of 0.5 µg/mL, and rabbit polyclonal anti-OmpF (Thermo Scientific, Catalog #PA5121442) at a dilution of 1:2000 overnight at 4°C. The membranes were then washed 3 times with PBST and incubated with Goat anti-rat IgG IRDye® 680RD (LI-COR Biosciences, Catalog #925-68071) and Goat anti-rabbit IgG IRDye® 800CW (LI-COR Biosciences, Catalog #925-32211) polyclonal antibodies in PBST + 5% milk for 1 hour. The membranes were then washed 3 times with PBST and imaged on a LI-COR Odyssey Classic.

ICP-MS

Plasmid pBEL1284 was modified to encode only LetA with a C-terminal 2xQH-7xHis tag to yield pBEL2214. The plasmid was transformed into Rosetta (DE3) cells (Novagen) for protein expression

and overnight cultures were grown in LB supplemented with carbenicillin (100 µg/mL), chloramphenicol (38 µg/mL) and 1% glucose at 37°C. The overnight cultures were diluted 1:50 in fresh LB media supplemented with carbenicillin (100 µg/mL) and chloramphenicol (38 µg/mL). Upon reaching an OD600 of ~0.6, the media was supplemented with 1X metals (50 µM $FeCl_3$, 20 µM $CaCl_2$, 10 µM $MnCl_2$, 10 µM $ZnSO_2$, 2 µM $CoCl_2$, 2 µM $CuCl_2$, 2 µM $NiCl_2$, 2 µM Na_2MoO_4, 2 µM Na_2SeO_3, 2 µM H_3BO_3) and protein expression was induced with the addition of L-arabinose to a final concentration of 0.2%. Cells were cultured for an additional 4 hours at 37°C with shaking, and then harvested by centrifugation. The pellets were resuspended in lysis buffer (50 mM Tris pH 8.0, 300 mM NaCl, 10% glycerol, 1 mM TCEP), flash frozen in liquid nitrogen and stored at -80°C. Cells were lysed by three passes through an Emulsiflex-C3 cell disruptor (Avestin), then centrifuged at 15,000g for 30 minutes at 4°C to pellet cell debris. The clarified lysate was subjected to ultracentrifugation at 37,000 rpm (182,460 g) for 45 minutes at 4°C in a Fiberlite F37L-8 x 100 Fixed-Angle Rotor (Thermo Fisher Scientific, 096-087056), and the membrane fraction was solubilized in 50 mM Tris pH 8.0, 300 mM NaCl, 10% glycerol, 25 mM DDM, 1 mM TCEP by rocking overnight at 4°C. Insoluble debris were pelleted by ultracentrifugation at 37,000 rpm for 45 minutes at 4°C. Solubilized membranes were then passed twice through a column containing Ni Sepharose Excel resin (Cytiva). Eluted proteins were concentrated using the Amicon Ultra-0.5 Centrifugal Filter Unit concentrator (MWCO 30 kDa, UFC503008) before separation on the Superdex 200 Increase 10/300 column (Cytiva) equilibrated in gel filtration buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.5 mM DDM, 10% glycerol, 1 mM TCEP). LetA concentrations were quantified by gel densitometry using bovine serum albumin standards.

Samples for ICP-MS analysis were prepared by adding 0.1 mL trace-metal grade nitric acid to 1.9 mL of protein sample as provided. Samples were analyzed using a Perkin Elmer NexION 350D inductively coupled plasma mass spectrometer. All liquid samples were infused into the nebulizer via peristaltic pump at 0.3 mL/min. For full scan elemental analysis, the instrument "TotalQuant" method was employed, using factory response factors. For quantitative analysis of zinc, calibrators were prepared by dilution of certified single element standard (Perkin Elmer) with 5% nitric acid, and these were used to generate a standard response curve. Except for zinc, none of the 79 other elements tested, such as iron, nickel and cobalt, were enriched in the protein sample relative to the buffer control. These results suggest that the ZnR domains of LetA bind zinc, though we note that the metal binding properties of similar ZnR proteins can be sensitive to the experimental conditions[65].

BPA cross-linking assays

OverExpress™ C43 (DE3) cells were transformed with plasmids to express LetAB (either WT or mutant forms derived from pBEL1284) or LetB (either WT or mutant forms derived from pBEL2782). The cells were co-transformed with pEVOL-pBpF (Addgene #31190) to encode a tRNA synthetase/tRNA pair for the *in vivo* incorporation of p-benzoyl-l-phenylalanine (BPA, Bachem, Catalog #F-2800.0005) in *E. coli* proteins at the amber stop codon, TAG ([14,20]. Bacterial colonies were inoculated in LB broth supplemented with carbenicillin (100 µg/mL) and chloramphenicol (38 µg/mL) and grown overnight at 37°C. The following day, bacteria were pelleted and resuspended in 32P Labeling Medium (a low phosphate minimal media we optimized starting from LS-5052 [66]: 1 mM Na2HP04, 1 mM KH2PO4, 50 mM NH4Cl, 5 mM Na2SO4, 2 mM MgSO4, 20 mM Na2-Succinate, 0.2x trace metals and 0.2% glucose) supplemented with carbenicillin (100 µg/mL) and chloramphenicol (38 µg/mL) and inoculated 1:33 in 20 mL of the same medium. Bacteria were grown to OD600 = ~0.6-0.7 and a final concentration of 0.2% L-arabinose and 0.5 mM BPA, alongside 500

µCi 32 P orthophosphoric acid (PerkinElmer, Catalog #NEX053010MC) were added and left to induce overnight at room temperature.

The following day, the cells were harvested by centrifugation (4,500 g for 10 min) and resuspended in 1 mL of phosphate buffer saline (PBS, pH 7.4), and the 'crosslinked' samples underwent crosslinking by treatment with 365 nM UV in a Spectrolinker for 30 min. Both the crosslinked and uncrosslinked cells were pelleted (6,000 g for 2 min) and resuspended in 1 mL of lysozyme resuspension buffer (50 mM Tris pH 8.0, 300 mM NaCl, 1 mg/mL lysozyme, 25 U/mL benzonase) and were incubated for 1 hr at 4°C. The cells then underwent eight cycles of freeze-thaw lysis by alternating between liquid nitrogen and a 37°C heat block. The lysate was pelleted at 20,000 g for 15 min, and the pellets were resuspended in 250 µL of membrane resuspension buffer (50 mM Tris pH 8.0, 300 mM NaCl, 10% glycerol and 25 mM DDM), and shaken for 1 hr. The sample volume was then increased to 1 mL with 10 mM imidazole wash buffer (50 mM Tris pH 8.0, 300 mM NaCl, 10 mM imidazole) and insoluble material was pelleted at 20,000 g for 15 min. Each supernatant was then mixed with 25 µL of nickel Ni Sepharose Excel resin (Cytiva) for 30 min. The beads were pelleted at 500 g for 1 min and the supernatant collected. The beads were then washed four times with 40 mM wash buffer (50 mM Tris pH 8.0, 300 mM NaCl, 40 mM imidazole, 10% glycerol, 0.5 mM DDM) and finally resuspended in 50 µL of elution buffer (50 mM Tris pH 8.0, 300 mM NaCl, 300 mM imidazole, 10% glycerol, 0.5 mM DDM). The samples were then mixed with 5x SDS-PAGE loading buffer, and the beads spun down at 12,000 g for 2 min. Eluted protein was analyzed by SDS-PAGE and stained using InstantBlue Protein Stain (Abcam, Catalog #ab119211). Relative loading of the LetA or LetB monomer band on the gel was estimated integrating the density of the corresponding bands in the InstantBlue-stained gel in ImageJ (Rueden et al., 2017), and this was used to normalize the amount of protein loaded on a second gel, to enable more accurate comparisons between samples. The normalized gel was stained with InstantBlue and 32P signal was detected using a phosphor screen and scanned on a Typhoon scanner (Amersham). At least two replicates of the experiment were performed, starting with protein expression.

Disulfide-crosslinking assays

To perform these assays, we generated variants of our split-LetA construct. As one cysteine from each pair is within TMD^N and the second Cys from each pair is within TMD^C, we introduced these cysteine pairs into a variant of our split-LetA construct with non-essential cysteines removed (C124S, C266S, C343S; ΔCysSplitLetA), to facilitate the detection of the crosslink of interest. A crosslinking event is predicted to lead to covalent linkage between TMD^N and TMD^C, resulting in a dimer with a large molecular weight shift relative to either domain alone. The metal-coordinating cysteines of the ZnR domains cannot be mutated without affecting LetA function, but are likely protected from maleimide crosslinkers by the bound zinc ion. Given that our DMS data suggests that Q180 and R380 can tolerate mutations to cysteines, we selected these residues to probe the alternate state.

OverExpress™ C43 (DE3) cells (Lucigen) containing pBEL2802 or its mutants were grown overnight at 37°C in LB medium supplemented with carbenicillin (100 µg/mL) and 1% glucose. Overnight cultures were diluted 1:50 to 20 mL of fresh LB media containing carbenicillin (100 µg/mL). Cells were grown to an OD600 of ~0.8, pelleted by centrifugation (4,500 g for 10 mins at 4°C), and resuspended in 1.5 mL PBS pH 7.4. From this stock, 500 µL cell suspension was pipetted into two separate Eppendorf tubes, and either treated with 3% dimethyl sulfoxide (DMSO; solvent used for dissolving cross-linkers) or with 1 mM BMOE (Thermo Scientific Pierce, Catalog #PI22323). To cap

unreacted cysteines, both the DMSO- and BMOE-treated samples were incubated with 2 mM N-ethylmaleimide (NEM, Thermo Scientific Pierce, Catalog #PI23030) and incubated for 10 min at RT while rotating in the dark. To quench unreacted BMOE, the cells were incubated with 10 mM L-cysteine (Sigma, Catalog #168149) for 10 min at RT while rotating. The cells were pelleted by centrifugation (6,000 g for 2 min), flash frozen in liquid nitrogen, and stored -80°C. To lyse the cells, the pellets were resuspended in 1 mL of lysozyme resuspension buffer (50 mM Tris pH 8.0, 300 mM NaCl, 1 mg/mL lysozyme, 25 U/mL benzonase, 1 mM DTT) and incubated for 1 hr at 4°C. The cells then underwent eight cycles of freeze-thaw lysis by alternating between liquid nitrogen and a 37°C heat block. The lysate was pelleted at 20,000 g for 15 min, and resuspended in 250 µL of membrane resuspension buffer (50 mM Tris pH 8.0, 300 mM NaCl, 10% glycerol and 25 mM DDM, 1 mM DTT), and shaken for 1 hr. Insoluble debris was removed by centrifugation at 20,000 ag for 15 min at 4°C. For each sample, 20 µL of the supernatant was mixed with 20 µL 2X SDS-PAGE loading dye supplemented with fresh 50 mM DTT. The samples were heated to 50°C for 15 min, and then 10 µL of the sample was loaded to an SDS-PAGE gel. The LetA bands were probed by Western blotting using the monoclonal anti-LetA antibody (clone #72).

AlphaFold2 predictions

To identify alternate conformations of LetA, we utilized AlphaFold2_multimer_version3 via Colabfold[67]. The sequence of LetA was retrieved from the MG1655 reference genome in the NCBI database. Program outputs yielded five ranked models, each with 32 samples or “seeds”, resulting in 160 predictions. Finding ambiguity in the coevolutionary signal was achieved by reducing the depth of the input multiple sequence alignments (16:32), enabling “dropout”, and setting “recycling”, which is the number of times the structure is fed into the neural network, to 0[67,68]. While many predictions showed ZnR^N and ZnR^C interacting with each other, 37 models showed different degrees of separation between the two ZnRs. In addition, five predictions exhibited severe clashes in ZnR^C. These observations made it difficult to interpret the cytoplasmic region, which also includes the unstructured N- and C-terminal regions that are not observed in our cryo-EM density, and we therefore focused on the TMD region. The RMSD heatmap was built as follows: First, each of the 161 PDBs (LetA cryo-EM structure and 160 models generated by AlphaFold) was aligned with all others PDBs using "align" function from PyMol, restricting the alignment to the carbon atoms and number of cycles to 0. Then, a matrix with the 161 PDBS in x and y was filled with the RMSD. Finally, the dendrogram was computed using the fastcluster python package (using the ward method and euclidean metric). The representative models were selected by calculating the average RMSD for each model in a cluster, and selecting the model with the lowest value.

System preparation for MD simulations

The LetAB complex used in the MD simulations was constructed by integrating the cryo-EM resolved structure with the AF2 multimer predicted model using Chimera and Coot. Missing residues in the C-terminal region of LetA (residues 419-427) were reconstructed using AF2, while the N-terminal disordered region (residues 1-26) was excluded due to its low pLDDT score. Similarly, for LetB, the N-terminal absent residues (residues 1-13) were omitted for the same reason. We retained the TM helices and the first MCE ring of LetB (residues <= 160) to preserve the native environment surrounding LetA, while the remaining portions of LetB were excluded to minimize the system size. Additionally, the two absent TM helices (residues 14-45) of LetB were modeled using AF2. The N-termini of LetA and LetB were capped with an acetylated N-terminus (ACE), while the C-termini of

LetA and LetB were capped with a standard C-terminus (CTER) and a methylamidated C-terminus (CT3), respectively. Protonation states of titratable residues were determined using Propka3[69,70]. The orientation of the protein complex relative to the membrane was established using the Positioning of Proteins in Membranes (PPM) 3.0 web server[71], and the resultant oriented protein complex was embedded into a native Gram-negative bacterial inner membrane (IM) using Membrane Builder module in CHARMM-GUI[72,73]. The composition of each membrane leaflet consists of 1-palmitoyl-2-(cis-9,10-methylene-hexadecanoyl)-phosphatidylethanolamine (PMPE, 16:0/cy17:0), 1-palmitoyl-2-oleoyl-phosphatidylethanolamine (POPE, 16:0/18:1(9Z)), 1-pentadecanoyl-2-(cis-9,10-methylene-hexadecanoyl)-phosphatidylethanolamine (QMPE, 15:0/cy17:0), 1-oleoyl-2-(9Z-hexadecenoyl)-phosphatidylethanolamine (OYPE, 18:1(9Z)/16:1(9Z)), 1-palmitoyl-2-(cis-9,10-methylene-hexadecanoyl)-phosphatidylglycerol (PMPG, 16:0/cy17:0), 1-palmitoyl-2-(9Z-hexadecenoyl)-phosphatidylglycerol (PYPG, 16:0/16:1(9Z)), and 1,1′-palmitoyl-2,2′-(11Z-vacenoyl)-cardiolipin (PVCL2, 1'-[16:0/18:1(11Z)],3'-[16:0/18:1(11Z)]) in ratios of 46, 13, 12, 8, 10, 9, and 2, respectively[74,75]. The cryo-EM resolved lipid was elongated to PMPE, as it represents the most abundant phospholipid in the Gram-negative bacterial IM. To assess the functional role of the cryo-EM resolved lipid, we constructed systems both with and without the modeled lipid (henceforth referred to as "+Lipid cofactor" and "-lipid cofactor", respectively). For each scenario, lipid positions within the membrane were randomly shuffled using the Membrane Mixer plugin[76] in VMD to mitigate any biases from initial lipid placement, resulting in three replicas for each condition, totaling six replicas overall. Finally, the resulting protein-membrane systems were solvated and neutralized with 0.15 M NaCl.

MD simulation protocols

All the MD simulations were executed using the NAMD[77,78] program. CHARMM36m[79,80] and CHARMM36[81] force fields were employed for the proteins and lipids, respectively. TIP3P model was used for water molecules[82]. Temperature was maintained at 310 K via a Langevin thermostat with a damping coefficient of $\gamma = 1$ ps^{-1}, and pressure was held at 1 bar through the Nosé-Hoover piston[83,84]. The Particle-mesh Ewald (PME)[85] method was utilized for calculating long-range electrostatic interactions within periodic boundary conditions at every time step. Non-bonded interactions were calculated with a cutoff of 12 Å, and a switching distance set at 10 Å. To accommodate volumetric changes in the system, a flexible cell was employed, allowing independent fluctuations in three dimensions while preserving a constant x/y ratio for the membrane. The SHAKE[86] and SETTLE[87] algorithms were implemented to constrain bonds involving hydrogen atoms. For the initial equilibration and production runs, a 4-fs timestep was utilized, facilitated by hydrogen mass repartition (HMR)[88,89] to accelerate the simulations. A 2-fs timestep was applied without HMR for non-equilibrium simulations involving collective variables (colvars), as well as any subsequent equilibration and production simulations.

To equilibrate the membrane-protein systems, each system underwent an initial 10,000-step energy minimization using the steepest descent algorithm, followed by equilibrations with gradually reduced harmonic constraints[90]. Initially, only the phospholipid tails were allowed to move without any constraints for 1 ns under NVT, followed by a 10 ns phase where all components excluding the protein were unconstrained under NPT, and subsequently an additional 10 ns allowing all components except the protein backbone to move freely under NPT. A force constant of 10

kcal/mol/Å^2 was applied to the constrained atoms. Finally, all constraints were removed, and each system was subjected to a 2-μs production run.

Exploring the pathway of phospholipid transport by steered MD (SMD) simulations

To elucidate the potential pathways for phospholipid transport and evaluate the feasibility of accommodating a phospholipid within the periplasmic pocket in TMD^C, a series of SMD simulations were conducted. These simulations employed colvars[91] to direct the upward movement of the elevated phospholipids identified in prior MD simulations. Forces were applied to the center of mass (COM) of three specific regions of the elevated lipid: the headgroup, the terminal six carbons of the tail closest to the bottom of the periplasmic pocket, and the terminal six carbons of both tails. Each pulling scenario utilized a stepwise strategy with distanceZ colvars to avoid unintended pathways, ensuring that the pulled atom group traversed the periplasmic pocket in TMD^C. The lipid was initially steered towards the bottom of the periplasmic pocket in TMD^C, followed by movement towards the middle of the pocket, and ultimately to the top of the pocket.Initial configurations for these SMD simulations were derived from the final frame of the 2-μs production run of replica 2 for the system with the lipid cofactor and replica 3 for the system without the lipid cofactor, as they represented the most elevated lipids in each condition. This results in six distinct SMD setups (2 initial configurations × 3 pulling scenarios). To ensure optimal interactions between the pulled lipid and its surrounding environment, the pulling velocity was set to 0.2 Å/ns with a force constant of 10.0 kcal/mol/Å^2. The duration of each simulation is provided in Supplementary Table 2. Throughout the SMD simulations, the centerToReference and rotateToReference options were enabled to align LetA with its initial conformation before calculating distances and forces at each timestep, which avoided the effects of protein translation and rotation on force calculations. Additionally, the z-center of LetA was harmonically constrained using the harmonicWalls function in colvars with a force constant of 10.0 kcal/mol/Å^2 and the lower and upper wall thresholds set at -2 Å and 2 Å, respectively. This constraint prevented the global upward movement of LetA induced by the applied forces on the elevated lipid, which could otherwise distort the local membrane structure.

Following the completion of SMD simulations, the systems underwent an additional 10 ns equilibration phase, during which the protein backbone and the pulled lipid were harmonically constrained with a force constant of 10 kcal/mol/Å^2. All restraints were subsequently removed, and a 300 ns production run was conducted for each system.

Water bridge network analysis

To investigate the potential role of polar residues (D181, K178, S321, K328, S364, D367, and T402) in TMD^c as a proton shuttle pathway, hydrogen bonds were analyzed on a frame-by-frame basis across simulation replicas, which include hydrogen bonds formed directly between the residues, between each residue and adjacent water molecules, and among the water molecules themselves. Hydrogen bonds were defined using the geometric criteria: a donor (D)-hydrogen (H) distance cutoff of 1.2 Å, a donor-acceptor (A) distance cutoff of 3.0 Å, and a minimum D-H-A angle of 120°, ensuring the inclusion of only well-structured hydrogen bonds.

The occupancy of hydrogen bonds is defined as the fraction of total simulation frames in which a given hydrogen bond is observed. Water bridges were classified by their order: direct residue–residue hydrogen bonds with no intervening water molecules were designated as zero-water (0-W) bridges,

while those involving one, two, or three intervening water molecules (1-W, 2-W, and 3-W bridges) were identified by systematically linking residue-to-water and water-to-water hydrogen bonds, thereby constructing higher-order networks. To visually represent the water bridge networks with the highest occupancy, principal component analysis (PCA)[92] was applied to project the spatial arrangement of residues into two dimensions. Each residue was depicted as a node, with edges connecting the nodes to represent the highest-occupancy water bridge between two residues, and the thickness of the edges indicating the relative occupancy of the corresponding water bridge.

Sample Preparation for LC-MS Lipidomics

Three replicates for each protein were analyzed, with each replicate containing a purified protein in detergent, a detergent buffer negative control, and an isolated *E. coli* membrane positive control. The lipids from each sample were extracted via Folch extraction[93]. Briefly, varying amounts of sample, chloroform, methanol, EquiSPLASH LIPIDOMIX (Avanti Polar Lipids) internal standards, and water were combined as described in Supplementary Table S3. The bottom layer was extracted, dried using N_2 gas, and resuspended in 100 µL of LC-MS grade methanol. Each sample was analyzed using data-dependent acquisition (DDA) LC-MS/MS. After DDA data collection, 30 µL of each sample was diluted with 50 µL LC-MS grade methanol to ensure sufficient volume for triplicate MS1-only injections for quantitation.

Collection of Data-Dependent LC-MS/MS Lipidomics Data

Lipids were separated prior to MS analysis using a 21-minute trap-and-elute method as previously described[94,95]. A Waters XBridge Direct Connect HP C_8 column (10 µm, 2.1 x 30 mm) was used as the trap column, and a Waters Premier Acquity UPLC CSH C_{18} column (1.7 µm, 2.1 x 100 mm) was used for analytical separations. Gradients for the C_8 (trap) and C_{18} (analytical) columns were controlled by the Alpha and Beta pumps respectively and are provided in Table 2. The column selection valve position was changed at 0.50 minutes, allowing the Beta pump to flow through both the C_8 and C_{18} columns. At 17.50 minutes the valve reverted to the initial position to allow for washing and re-equilibration of the C_8 and C_{18} columns by the Alpha and Beta pumps, respectively. A 10 µL sample injection volume was used, and the column compartment was held at 60 °C.

Samples were ionized via ESI in negative mode and introduced into a Synapt XS mass spectrometer operated in sensitivity mode. The capillary and sampling cone voltages were set to 2.45 kV and 49 V, respectively. The source offset was 80 V, the source temperature was 120°C, and the desolvation temperature was 250°C. A top-5 DDA method was applied, with an accumulated TIC threshold of 100000 and a maximum acquisition time of 0.25 seconds. MS1 and MS2 spectra were collected from 50 – 2000 Th in continuum mode at a resolution of 10,000 with a scan speed of 0.1 seconds, and fragmentation was performed using collision-induced dissociation (CID) with a collision energy ramp from 20–40 V in the trap cell. Dynamic exclusion was used with an exclusion time of 15 seconds and an exclusion width of 0.5 Da. A fixed exclusion range of 50–450 Th was used to minimize selection of non-lipid precursors. Blank injections of isopropyl alcohol (IPA) were performed every three samples using the same instrumental methods.

Collection of MS1-Only LC-MS Lipidomics Data

After using DDA methods to identify the lipids, we used MS1-only scans to perform quantitation with accurate mass and retention time alignment, as described previously[94,95]. 10 µL of each diluted sample was loaded and separated as described above. MS1 scan parameters were identical to DDA

MS1 scans. Triplicate injections of each sample were performed in a randomized order. 10 µL IPA injections were performed after each sample run to mitigate potential carryover between samples.

Lipid Library Construction using DDA Lipidomics Data

All DDA files were centroided using MSConvert, and mass calibration was performed using an in-house Python script using the known masses and retention times of the EquiSPLASH lipids. Lipid identification based on the calibrated DDA data files was performed using MS-DIAL (version 5.3)[96]. A minimum peak height of 1000 and mass slice width of 0.1 Da were used for peak detection. Only CL, PE, and PG lipids were searched, as these are the most prevalent lipids in *E. coli*. MS1 and MS2 accurate mass tolerances of 0.025 Da were used, and both [M-H]- and [M+CH_3COO^-]- adducts were allowed. For alignment, a retention time tolerance of 0.5 minutes and mass tolerance of 0.015 Da were used. A set of high-quality lipid identifications was then manually checked to produce a lipid library to be used for MS1-based lipid quantification.

Processing of MS1-Only Lipidomics Data

MS1-only files were centroided and calibrated in the same way as DDA files, with the addition of retention time calibration using the known retention times of EquiSPLASH-spiked lipids. Calibrated MS1 files were loaded into Skyline (v23.1.0) and searched against the DDA-constructed lipid library[97]. An ion match tolerance of 0.05 Th and mass accuracy of 10 ppm were used. To ensure accurate quantification, each extracted-ion-chromatogram integration was manually checked and adjusted as necessary. Raw peak areas were then standardized to sample volume and total identified lipid area.

Finally, we compared the enrichment of specific lipid classes between the isolated protein samples and the starting membranes. First, within each individual sample, the total intensity of each lipid class (CL, PE, and PG) was calculated by summing the standardized areas of each individual lipid in each class. The average total peak area for each class was then calculated using the summed areas from each replicate. To compare the lipid composition in the protein samples relative to the *E. coli* membranes, the average fold change was then calculated for each class between the protein and membrane samples. Standard deviations were propagated through the averaging, and a 95% confidence interval for the fold-change was calculated. A two-sample t-test comparing the means of each lipid class area between the protein and membrane samples was performed, and p-values were corrected for multiple testing using the Benjamini-Hochberg method. A significance level of 0.05 was used to determine statistically significant differences.

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