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TITLE – Evidence for phospholipid export from the bacterial inner membrane by the Mla ABC transport system.

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26 **Abstract**

27 The Mla pathway is believed to be involved in maintaining the asymmetrical Gram-
28 negative outer membrane via retrograde phospholipid transport. The pathway is composed of
29 3 components: the outer membrane MlaA-OmpC/F complex, a soluble periplasmic protein,
30 MlaC, and the inner membrane ATPase, MlaFEDB complex. Here we solve the crystal
31 structure of MlaC in its phospholipid free closed apo conformation, revealing a pivoting β -
32 sheet mechanism which functions to open and close the phospholipid-binding pocket. Using
33 the apo form of MlaC we provide evidence that the inner membrane MlaFEDB machinery
34 exports phospholipids to MlaC in the periplasm. Furthermore we confirm that the
35 phospholipid export process occurs through the MlaD component of the MlaFEDB complex
36 and that this process is independent of ATP. Our data provides evidence of an apparatus for
37 lipid export away from the inner membrane and suggests that the Mla pathway may have a
38 role in anterograde phospholipid transport.

39

40 **Introduction**

41 Gram-negative bacteria have a complex envelope consisting of an inner (IM) and
42 outer membrane (OM), separated by a thin peptidoglycan layer and aqueous periplasm¹. The
43 OM has a unique structure and contains two types of lipid, phospholipids (PL) and
44 lipopolysaccharide (LPS), which are asymmetrically distributed. PL is found primarily in the
45 inner leaflet and LPS is found in the outer leaflet. The structure of this asymmetric membrane
46 serves as a formidable barrier with asymmetry key in maintaining barrier function²⁻⁴.

47 Research from various groups over the last two decades has provided considerable
48 mechanistic understanding of the processes involved in the formation and trafficking of
49 components to the OM, including the β -barrel assembly machine (Bam) complex for OM
50 proteins⁵⁻⁸, the Lpt pathway for LPS transport⁹⁻¹² and the Lol pathway for lipoprotein

51 trafficking to the OM ^{13,14}. In the case of PLs, it is known there is ATP independent
52 bidirectional movement of PLs between the IM and OM ^{15,16,17}. However, the mechanism by
53 which PLs are trafficked between the membranes remains largely unresolved.

54 The first evidence for a proteinaceous system responsible for PL trafficking between
55 the IM and OM came in the form of the Maintenance of Lipid Asymmetry (Mla) pathway, a
56 six protein (MlaA-F) system with components in the IM, periplasm, and OM ¹. It was
57 described as having a role in maintaining lipid asymmetry in the OM by removing and
58 trafficking PLs found in the outer leaflet of the OM to the IM. Through genetic knockouts,
59 structural and modelling-based studies, the individual components have been identified and
60 characterised (Supplementary Figure 1) ¹⁸⁻²⁴. Furthermore, several studies have indicated that
61 they are important for infection ²⁵⁻²⁷. The components include an OM lipoprotein termed
62 MlaA, which has been found to be associated with the major porins OmpF and OmpC, and is
63 believed to extract PLs from the OM, a soluble periplasmic component known as MlaC,
64 thought to ferry PLs between the membranes; and an inner membrane ATPase composed of
65 MlaF, MlaE, MlaD and MlaB in the a 2:2:6:2 stoichiometry that is assumed to accept and
66 insert PLs into the IM. However, little information regarding the mechanism of this proposed
67 PL trafficking pathway has been confirmed. In particular, questions still remain regarding the
68 directionality of PL transport, and why such a complex transport mechanism is present when
69 other simpler systems, such as the OM phospholipase A₂, PldA ²⁸ and the lipopolysaccharide
70 palmitoyl transferase, PagP ²⁹, are available in the outer membrane to maintain asymmetry?

71 In this study we sought to address these questions focusing on the interaction between
72 the periplasmic MlaC and the MlaFEDB complex. Here we provide evidence that the
73 MlaFEDB complex can function in an anterograde direction, extracting PLs from the IM and
74 passing them to MlaC via MlaD, and that it does so in an ATP independent manner.

75

Results

Detergent treatment of MlaC can produce a phospholipid free variant

In order to investigate the directionality of PL transport within the Mla system we required the isolation of both PL bound and PL free species of MlaC. Using *E. coli* pBE1203²¹ we overexpressed and purified MlaC by published methods²¹ (Supplementary Figure 2). Thin layer chromatography (TLC) analysis of MlaC extracts revealed it co-purified with PL (Supplementary Figure 3A), with the ratios of PG to PE varying depending on purification (Compare Figures 2A, 2D, 3C, 5D, 5E & Supplementary Figure 3A). Furthermore, unlike previous observations we also noted the presence of cardiolipin (Supplementary Figure 3A). Throughout we term this species, MlaC-PL, to represent phospholipid bound MlaC. In order to produce protein free of bound PL (MlaC-apo), we washed the protein extensively using buffer containing the detergent β -octyl glucoside. The generation of this lipid free variant was then confirmed through a combination of TLC analyses, native mass spectrometry³⁰, and MALDI-TOF mass spectrometry (Supplementary Figure 3). Circular dichroism spectroscopy and Analytical ultracentrifugation indicated that the secondary structure and oligomeric state of MlaC-apo was indistinguishable from MlaC-PL (Supplementary Figure 4A-C). This was confirmed by NMR spectroscopy, using $^1\text{H}^{15}\text{N}$ heteronuclear single quantum coherence spectroscopy we were able to observe both the ^{15}N -MlaC-PL and ^{15}N -MlaC-apo spectra (Supplementary Figure 4D). Both showed the presence of folded species, however clear differences in peak positions were noted throughout suggesting a large conformational change occurred during PL addition/removal.

The phospholipid binding cavity of MlaC is controlled via a β -sheet hinged lid

To determine the structural changes that occur in MlaC upon loss of PL, we determined the crystal structure of MlaC-apo at a resolution of 2.25 Å (Supplementary Table

1). Within the asymmetric unit two molecules of MlaC were observed, one in a conformation similar to the published lipid-bound MlaC structure (5uwa) but lacking density associated with PL, which we have termed MlaC-open (see Supplementary Discussion for further details) and another in a previously undescribed closed conformation (MlaC-apo) (Figure 1A & B).

Unlike MlaC-open, MlaC-apo shows the absence of a pocket (Figure 1C & D). Comparison between the apo and open conformations indicates that the majority of the protein undergoes only minor conformational changes (0.66Å over α helices 1-5 & 7; residues 23-112,183-208) between the open and apo conformations. However, large changes are observed across the β -sheet, in particular a ~ 2.5 Å translation of the entire β -sheet and a pivot of approximately 15° (Figure 1E). To accommodate this movement a further pivot is manifest at Gly182 within helix $\alpha 6$ resulting in an angular shift of part of this helix by 17.9° (Defined across residues 169-182) (Figure 1F). These movements together allow the residues at the mouth of the cavity to come together, which results in its closing (Figure 1G & H).

To corroborate the MlaC-apo structure, molecular dynamics simulations were performed. Simulations utilising the PL bound crystal structure with the PL removed (5uwa) resulted in movements of the protein towards the observed MlaC-apo structure (Supplementary Figure 5 and Supplementary Table 2). Additional simulations performed on the MlaC-apo structure demonstrate the stability this conformation and highlight its similarity to the structures observed in simulations with the bound PLs removed (Supplementary Figure 6). A further series of PL removed simulations performed on MlaC structures determined from *Ralstonia solanacearum* (2qgu) and *Pseudomonas putida* (4fcz) demonstrate a very similar closure of the PL binding pocket upon PL removal (Supplementary Figure 7).

MlaC cannot bind free phospholipid

We next investigated whether MlaC-apo is able to bind passively to free PL. To this end, we incubated hexa-histidine-tagged MlaC-apo with buffer containing *E. coli* PL SUVs and removed the unbound PLs by purifying MlaC using Ni-NTA resin. Analysis of the Ni-NTA eluate by TLC indicated that MlaC-apo did not bind free PL (Figure 2A). To more accurately confirm this result we probed PL binding using NMR. By comparing spectra taken of both ^{15}N -MlaC-PL and ^{15}N -MlaC-apo we were able to monitor any changes in binding on incubation of MlaC-apo with excess *E. coli* polar lipids (Figure 2B & Supplementary Figure 8). Following co-incubation for 18 h at room temperature minimal PL loading occurred, as evidenced by chemical shifts predominantly remaining in the apo position. These data suggest that loading is either an extremely slow process or requires interaction with another Mla component. Consistent with these results molecular dynamics simulations of MlaC-apo performed to advance the conformational sampling demonstrates that once the structure is in its apo conformation it does not readily sample the open conformation (Supplementary Figure 9).

MlaC accepts phospholipid from MlaD

Malinverni & Silhavy (2009) suggest that MlaC passes PLs to the MlaFEDB complex¹ (Supplementary Figure 1). Because the MlaD component of this complex has been shown to interact with MlaC²¹ we sought to investigate PL movement between these two components. Thus we overexpressed and purified a construct of MlaD consisting of its periplasmic domain lacking its N-terminal membrane anchor (residues 29-183) with a C-terminal hexa-histidine tag (MlaD $^{\Delta\text{tm}}$) (Supplementary Figure 10). Like MlaC, MlaD $^{\Delta\text{tm}}$ copurified with PLs (MlaD $^{\Delta\text{tm}}$ -PL) (Figure 2D). We generated PL free MlaD $^{\Delta\text{tm}}$ (MlaD $^{\Delta\text{tm}}$ -apo) using the same method we described for MlaC-apo. Through SPR we were able to probe the interaction between these different species of MlaC and MlaD (MlaC-apo, MlaC-PL, MlaD $^{\Delta\text{tm}}$ -apo

MlaD^{Δtm}-PL), we show that both forms of MlaC were able to interact with both species of MlaD^{Δtm} and surprisingly all showed similar equilibrium dissociation constants of around 12μM (K_D) (Supplementary Table 3 & Supplementary Figure 11). Next we probed PL transfer between MlaC and MlaD^{Δtm} by co-incubation of apo and PL forms. After co-incubation each protein species was separated by size exclusion chromatography and their PL content analysed by TLC (Figure 2C). Unexpectedly, no lipid transfer was observed between MlaC-PL and MlaD^{Δtm}-apo. In contrast, lipid transfer from MlaD^{Δtm} to MlaC was clearly observed when MlaC-apo was co-incubated MlaD^{Δtm}-PL (Figure 2D) as evidenced by PL association with MlaC and diminished levels of PL associated with MlaD^{Δtm}. The ratios of PL bound to MlaC-apo were consistent with those of MlaD^{Δtm} prior to incubation, suggesting MlaC showed no PL specificity following transfer.

To confirm the transfer event yielded a structure consistent with MlaC-PL we again utilised NMR. Analysis of ¹⁵N-MlaC-apo following incubation with MlaD^{Δtm}-PL and subsequent purification showed that MlaC adopted the MlaC-PL conformation (Figure 2B & Supplementary Figure 8A). However incubation of ¹⁵N-MlaC-PL with MlaD^{Δtm}-apo showed no change in chemical shift confirming the TLC results. Owing to the passivity of these interactions (i.e. in the absence of an energy source) the clear unidirectional movement of PL to MlaC suggests that it has a far higher binding affinity for PL than MlaD^{Δtm}, consistent with the recent results of Ercan *et al.* (2018)³¹.

MlaFEDB transfers phospholipid from the inner membrane to MlaC

Given that MlaC has a higher affinity for PL than MlaD^{Δtm}, we hypothesised that the full MlaFEDB complex may increase the affinity of MlaD for PL or promote uptake of PL from MlaC. MlaFEDB has been established as an ATP-binding cassette (ABC) family transporter¹⁹. Using a similar construct to those previously published^{19,21} but with a hexa-

histidine affinity tag present at the N-terminal of MlaF, we purified the complex in detergent micelles (Figure 3A) and were able to show intrinsic ATPase activity (Figure 3B) in line with others¹⁹. By binding MlaFEDB to metal chelate resin, we were able to stably reconstitute the complex back into PL. This allowed us to perform interaction studies by flowing MlaC over the complex (in the presence of ATP), which could then be analysed for the presence of bound PLs (Figure 3C & D). The results corroborated the observations for MlaD^{Δtm} and again showed the movement of PLs from MlaFEDB to MlaC-apo. Consistent with the MlaD-MlaC interaction, no obvious differences in the PL ratio were observed bound to MlaC-apo compared to MlaFEDB following PL transfer. Whilst movement of PL in the retrograde direction, from MlaC-PL to MlaFEDB, was not observed. To confirm that binding to the resin resulted in no adverse effects on ATPase functionality we measured ATPase activity whilst bound to resin beads (Supplementary Figure 12). Only a small decrease was noted, suggesting tethering had minimal effects on function.

To further address and confirm transfer directionality and provide a method to investigate rate changes we utilised the technique of quartz crystal microbalance with dissipation monitoring (QCM-D). Using the approach of Giess *et al.* (2004) a protein tethered PL bilayer was generated³². This required adsorption of MlaFEDB to a gold surface functionalized with Cu²⁺-NTA, followed by reconstitution of a PL bilayer around the complex (Supplementary Figure 13). Copper was chosen over Nickel to provide a stronger interaction with the His-tag, again this did not overly affect activity (Supplementary Figure 12).

Using the same construct of MlaFEDB with a hexa-histidine tag on the N-terminal of MlaF, which we had already shown was active, allowed for correct orientation on the surface, around which a supported POPC bilayer was formed (Figure 4A1-6 & Supplementary Table 4). Because the Mla pathway does not appear to show head group preference²¹, POPC was

chosen due to its well-characterised thermotropic properties and demonstrated ability to form supported lipid bilayers on protein functionalised surfaces³³. Although not a constituent of *E. coli* membrane, MlaFEDB functionality was not impaired by the presence of POPC (Supplementary Figure 14A & B). We also confirmed that MlaC is not able to passively take up free POPC (Supplementary Figure 14C). We then sought to see if the addition of MlaC-apo or MlaC-PL, in the presence of ATP, would either lead to deposition or removal of PLs from the surface through MlaFEDB, after first confirming that no interaction takes place with the surface in the absence of MlaFEDB (Figure 4B). Addition of MlaC-PL resulted in no persistent changes in mass (Figure 4A-7 & 4A-8, Black; Supplementary Figure 15 A-C; Supplementary Table 4) suggesting no addition of PL to the bilayer occurred. However, on addition of MlaC-apo clear increases in frequency were observed (Figure 4A-7 & 4A-8, Red; Supplementary Figure 16 A-D; Supplementary Table 4), consistent with mass loss from the surface, and taken with the TLC is consistent with the removal of PL from the bilayer via MlaFEDB.

To confirm that the mass loss observed from the surface was due to PL and not protein we employed the use of attenuated total reflection (ATR) fourier transform infrared (FTIR) spectroscopy (Figure 4C-F). Briefly, FTIR spectroscopy provides an analysis of the chemical bonds present within a sample by their characteristic bond vibrational frequencies. Deposition of active MlaFEDB proteoliposomes onto a silicon oxide surface of a single-crystal Si ATR substrate resulted in vesicle rupture and the formation of a supported bilayer, which we confirmed by both QCM-D and neutron reflectometry (Supplementary Figure 17). A clear distinction between PL and protein could be achieved by the presence of the unique protein amide-I C=O vibration at $\sim 1650\text{ cm}^{-1}$ and the corresponding PL molecular vibrations of C=O ester, CH₃, CH₂ asymmetric and CH₂ symmetric stretching vibrations at 1731, 2855, 2924 and 2958 cm^{-1} , respectively (Figure 4C). On passage of MlaC-apo over the supported

bilayer surface, clear decreases in vibrations associated with PL were observed, with no concomitant drop in protein signal (Figure 4D), such loss was not observed in the presence of MlaC-PL (Figure 4E), nor for MlaC-apo in the presence of POPC alone (Figure 4F) confirming that protein disassociation did not contribute to the mass loss observed from the surface in QCM-D measurements. By measuring changes in peak area, over the 4 hour interval approximately 36% of PL was removed from the surface, but it must be noted that in this system MlaFEDB orientation was not defined, it could adopt one of two orientations, either with MlaD exposed on the surface or with MlaF/B exposed, thus likely leading to an underestimation of the percentage of PL loss.

To identify whether MlaFEDB functions in an enzymatic fashion, e.g. can undergo multiple rounds of PL extraction, we utilised QCM-D with continuous passage of MlaC-apo over the surface (Figure 5A). Under the assumption that mass changes in response to the adsorption of DDM-solubilised MlaFEDB is solely down to MlaFEDB, this gives an estimate of the MlaFEDB surface concentration of $5.83 \text{ pmol}\cdot\text{cm}^{-2}$. Similarly, if we assume that the decrease of adsorbed mass in response to MlaC-apo in the presence of ATP is solely due to POPC removal, this yields a PL loss of $0.39 \text{ nmol}\cdot\text{cm}^{-2}$. These estimates, in turn, provide a molar ratio of 66.63 moles of POPC removed per mole of MlaFEDB complex, strongly suggesting that MlaFEDB functions in an enzymatic fashion. However, this ratio is likely an overestimation, as the acoustic mass sensed by QCM-D also contains contributions from coupled solvent, and in the case of the MlaFEDB surface concentration estimation, DDM micelles, which are not accounted for in our analysis.

ATPase activity increases in the presence of MlaC-apo

Thong *et al.* (2016) suggested that MlaC might regulate MlaFEDB ATPase activity via direct interactions at the PL bilayer²⁰. To establish the role of the PL environment and

probe the ability of MlaC to influence MlaFEDB ATPase activity, MlaFEDB was introduced into *E. coli* polar lipid liposomes and subsequently incubated with either MlaC-apo or MlaC-PL, the ATP hydrolytic activity of MlaFEDB was then assessed through an enzyme-coupled ATPase assay³⁴. Following the introduction of MlaFEDB into polar lipid liposomes, ATPase activity was dramatically diminished with the rate at 500 μ M ATP decreasing from 0.05 μ M ATP s⁻¹ (MlaFEDB in detergent micelles) to 0.009 μ M ATP s⁻¹ (Figure 5F). Following incubation with MlaC-PL, this rate remained unaffected. However, upon addition of MlaC-apo, the ATPase activity of MlaFEDB was significantly increased to an average of 0.024 μ M ATP s⁻¹ at 500 μ M ATP; whilst this represents a significant increase in ATP hydrolysis, it remains a lower rate than is exhibited by MlaFEDB in detergent micelles (Figure 5F). Together these results suggest that PL environment is essential in regulating MlaFEDB ATPase activity, and that when constrained in a bilayer MlaFEDB requires the interaction of MlaC-apo to drive ATP hydrolysis, further highlighting the importance of the MlaC PL bound state in defining PL movement.

ATP hydrolysis has no obvious effect on phospholipid transfer

QCM-D, FTIR and TLC allow for a more in-depth analysis of the role ATP hydrolysis plays in PL transport. Using QCM-D and measuring frequency changes during interaction with MlaC (apo or PL) in the presence or absence of ATP we were able to probe changes in rate. Strangely no obvious differences in the rate of PL loss from the surface were noted (Figure 5A & B, Supplementary Figures 15-19). The mass changes observed on addition of either MlaC-apo or MlaC-PL remained comparable irrespective of the presence or absence of ATP suggesting that the removal of PL from the surface was ATP independent (Supplementary Table 4). We have already shown that ATPase activity is still present following binding to Cu²⁺ NTA beads and reconstitution into a PL bilayer suggesting that

surface tethering should not impact function (Supplementary Figure 12). However to confirm this in the absence of 6xHis tethering, we used FTIR (Figure 5C). Similar results were noted, the rate of PL movement from MlaFEDB to MlaC-apo occurred at the same rate irrespective of the presence of ATP and again no PL movement was noted from MlaC-PL to MlaFEDB either in the presence or absence of ATP. Furthermore, using our TLC transport assay, neither the absence of ATP nor the presence of the non-hydrolysable ATP analogue, ATP- γ S, impacted on the observed transport of PLs from MlaFEDB to MlaC-apo. For both conditions (Figure 5D & E), similar PL transport was noted between MlaFEDB and MlaC-apo whilst no PL movement was noted in the retrograde direction i.e. from MlaC-PL to MlaFEDB. These results combined with the observation that ATPase activity is upregulated in the presence of MlaC-apo suggest that the ATPase activity of MlaFEDB is not directly related to the PL export process but has an alternate function within the Mla pathway.

Discussion

Understanding the molecular mechanisms of PL transport between the IM to the OM represents one of the major challenges surrounding bacterial PL homeostasis³⁵. Whilst it has been well established that a host of PLs can move bidirectionally between the inner and outer membranes¹⁵, it remains unclear as to how these processes occur. Genetic studies have led to the Mla pathway being considered to maintain OM lipid asymmetry by removing PLs from the outer leaflet of the OM then shuttling them across the periplasmic space via MlaC to the inner membrane^{20,21} where MlaFEDB, through utilising ATP, was hypothesised to insert those PLs into the IM^{1,19}. Here we provide evidence to the contrary and suggest that MlaFEDB transports PLs from the IM, transferring them to MlaC in an ATP independent fashion. This is feasible considering how dominant mutations in MlaA result in both increased levels of PLs at the OM and a concurrent deterioration of the IM³⁶. In addition,

Malinverni & Silhavy (2009) were unable to follow the Mla dependent movement of PLs out of the OM¹. More recently, Kamische *et al.* (2019) showed accumulation of newly synthesised PLs at the inner membrane in *mla* mutants of *Acinetobacter baumannii*²⁴. Together, this is suggestive of a system inclined toward anterograde PL transport. Whether this represents the sole function of the pathway remains to be seen, with further studies required to fully elucidate the directionality of PL transport.

These findings allow us to think of MlaC as a potential PL-shuttle, funnelling PLs toward the OM MlaA complex. This shuttling activity of MlaC is reliant upon the reversible transition between PL bound and PL free states, here we demonstrate the ability of MlaC to form this PL free condition. Furthermore, through crystallographic studies we have determined its structure to a resolution of 2.25 Å, and show that during the transition between PL bound and PL free states, MlaC experiences global structural changes, involving a unique β -sheet pivot mechanism (see Supplementary Information for further discussion).

Through a combination of QCM-D, FTIR and TLC we have demonstrated that the movement of PLs from MlaFEDB to MlaC occurs independently of ATP hydrolysis, with transport occurring at comparable rates regardless of the presence or absence of 500 μ M ATP. This is feasible considering the transport of PLs from the IM to the OM would involve movement down a concentration gradient. Furthermore, Sutterlin *et al.* (2016) noted that even in response to ATP synthase inhibition, dominant MlaA mutations continue to promote PL flow away from the IM, again suggesting passivity in this mechanism³⁷.

Whilst it has been suggested that ATP hydrolysis may facilitate PL transfer from MlaC to MlaFEDB²¹, our evidence suggests otherwise and as such the role of the MlaFEDB ATPase remains ambiguous. Therefore, elucidating how ATPase activity is coupled to PL transport represents an important area for future study. Considering the function of MlaFEDB at the IM, it is possible that its ATPase activity may be linked to potential flippase ability,

with MlaC binding promoting MlaFEDB dependent ATP hydrolysis and a subsequent flipping of PLs between inner and outer leaflets, which may be significant in maintaining the integrity of the IM. In fact, a similar ABC transporter, MsbA, has been shown to function in a comparable manner at the IM, where it acts to flip LPS and PLs from the inner to the outer leaflet in an ATP dependent fashion^{37,38}. In addition, we have shown MlaFEDB dependent ATP hydrolysis to be highly regulated in terms of PL environment and putative MlaC interactions, with ATPase activity dramatically increasing exclusively in the presence of MlaC-apo. This is unsurprising considering the ATPase activity of other well-characterized ABC transporters is intrinsically linked to the binding of their corresponding substrate-binding proteins^{39,40}. Moreover, this would avoid redundant ATP hydrolysis, allowing for potential coupling of MlaFEDB flippase ATPase activity to the process of MlaC PL loading. This theory is supported by recent work from Kamischke and colleagues who demonstrated that ATPase null mutants promoted an accumulation of PLs at the IM²⁴.

The model we present here, in which MlaFEDB is involved in PL transport out of the IM (Figure 6), opens up the notion of MlaC acting as a soluble, periplasmic PL store. Whilst we have focused on MlaFEDB driven PL transport, it is possible that in situations in which both MlaFEDB and MlaA are present this process would not occur in such a unidirectional fashion. As such, *in vivo* MlaC may function as a PL sensor, with its overall lipid state defining the directionality of transport. Therefore, if MlaC is involved in bidirectional PL transport, the inability of MlaC-PL to deliver PLs into a bilayer via MlaFEDB would signal towards an as yet unknown mechanism for MlaC PL recycling, which given the number of MCE protein complexes involved in as of yet poorly understood PL transport processes, represents a viable concept²¹.

In summary, this study provides compelling evidence for PL export from the inner membrane to the periplasmic space and suggests that the Mla pathway may be involved in

anterograde transport to the bacterial outer membrane and that the export process appears to occur in an ATP independent manner.

Methods

Expression and purification of MlaC and MlaD

DNA corresponding to MlaC was previously cloned into a custom plasmid (pBE1203) with an N-terminal hexa-histidine tag followed by a TEV protease cleavage site²¹. MlaD DNA corresponding to its periplasmic domain (residues 23-183) was chemically synthesised (Genscript) and cloned into pET vector, pET26b (Merckmillipore) to contain a C-terminal hexa-histidine tag, which we refer to as MlaD^{Δtm} throughout. Both MlaC and MlaD^{Δtm} plasmids were then used to transform *E. coli* strain BL21(DE3). Expression was performed by growing overnight cultures in lysogeny broth at 37°C to an OD₆₀₀ = 0.6 whereupon protein expression was induced by the addition of IPTG to a final concentration of 1 mM. Protein expression was allowed to continue at 15°C overnight. Cells were harvested by centrifugation (6000 x g, 10 min) and pellets resuspended in 50 mM Tris pH 8, 500 mM NaCl, 10 mM imidazole. Cells were lysed by three passes through an Emulsiflex C3 disruptor (Avestin), then centrifuged (75,000 x g, 45 min) to pellet cell debris. The clarified lysate was passed over a 5 mL Ni-NTA column (GE Healthcare), washed with 5 column volumes of 50 mM Tris pH 8, 500 mM NaCl, 50 mM imidazole and bound protein eluted with 50 mM Tris pH 8, 500 mM NaCl, 500 mM Imidazole. Fractions containing MlaC were further purified on a Superdex 75 gel filtration column (GE Healthcare) equilibrated in 50 mM Tris pH 8, 300 mM NaCl, whilst MlaD^{Δtm} containing fractions were purified on a Superdex 200 size exclusion column (GE Healthcare) equilibrated in 50 mM Tris pH 8, 300 mM NaCl.

Expression and purification of MlaFEDB

MlaFEDB was expressed and purified according to the method of Ekiert *et al.* (2017). Briefly, Rosetta 2 (DE3) cells (Novagen) were transformed with plasmid pBE1196²¹. This plasmid contains the complete *mlaFEDCB* operon (from MlaF start codon to MlaB stop codon) with the addition of a hexa-histidine tag on the C-terminal of MlaF. For expression, overnight cultures were grown at 37°C with shaking to an OD₆₀₀ of 0.9 then induced by the addition of arabinose to a final concentration of 0.1% (w/v) for an additional 4 h shaking at 37°C. Cells were harvested by centrifugation (6000 x g, 10 min) and pellets resuspended in 50 mM Tris pH 8, 500 mM NaCl, 10 mM imidazole. Cells were lysed by three passes through an Emulsiflex C3 disruptor (Avestin) before centrifugation (20,000 x g, 30 min) to remove cell debris. The lysate was then further centrifuged (100,000 x g, 30 min) to pellet membranes. Membrane pellets were resuspended and solubilised in 50 mM Tris pH 8, 500 mM NaCl, 10 mM imidazole, 25 mM n-Dodecyl-β-D-Maltopyranoside (DDM) then incubated for 1 h at 4°C with mixing. Insoluble material was pelleted by centrifugation (100,000 x g, 30 min). The supernatant was passed over a 5 mL Ni-NTA column (HIS-Trap – GE Healthcare), washed with 50 mM Tris pH 8, 500 mM NaCl 50 mM imidazole, 0.5 mM DDM and bound protein eluted with 50 mM Tris pH 8, 500 mM NaCl, 500 mM Imidazole, 0.5 mM DDM. Fractions containing MlaFEDB were pooled and concentrated before additional purification on a Superdex 200 size exclusion column (GE Healthcare) equilibrated in 50 mM Tris pH 8, 300 mM NaCl, 0.5 mM DDM.

Lipid removal

Purified protein was bound to a Ni-NTA affinity column (GE Healthcare) and washed with three washes of 25 mL 50 mM Tris pH 8, 500 mM NaCl, 10 mM imidazole, 25 mM β-octyl glucoside with 1 h static incubation period for each wash, followed by a final wash of 50 mL 50 mM Tris pH 8, 500 mM NaCl, 10 mM imidazole and protein was eluted using 50

mM Tris pH 8, 500 mM NaCl, 250 mM imidazole. Fractions containing protein were pooled, concentrated and then dialysed against 50 mM Tris pH 8, 150 mM NaCl.

Crystallisation and structure determination of MlaC

Size exclusion fractions containing purified MlaC were concentrated to 20-40 mg·mL⁻¹. Vapour diffusion trials were performed via the sitting drop method using the JCSG+ crystal screen (Molecular dimensions). Crystals grew from drops consisting of 2 µl protein plus 2 µl of a reservoir solution consisting of 2.0 M ammonium sulphate, 0.2 M sodium chloride, 0.1 M sodium cacodylate pH 6.5. These were then seeded 1:100, and additional vapour diffusion trials performed using the Morpheus crystal screen (Molecular Dimensions). Crystals grew from drops consisting of 100 nl 1:100 seed solution, 100 nl MlaC (30 mg·mL⁻¹), 200 nl reservoir solution consisting of 0.1 M Tris/Bicine pH 8.5, 0.03 M sodium fluoride, 0.03 M sodium bromide, 0.03 M sodium iodide, 20% (v/v) glycerol and 10% (w/v) PEG4000. No additional cryo-protectant was needed. Native diffraction data was collected at Diamond light Source beamline I04, indexed to spacegroup P3₁21 and processed using XIA2⁴¹. The structure was phased by molecular replacement using PHASER⁴² with a search model comprised of the published PL-bound MlaC structure (5uwa). The resulting model was manually rebuilt/adjusted in Coot⁴³ and refined using PHENIX⁴⁴ and PDB-REDO⁴⁵. The final refined model consists of 2 copies of MlaC within the asymmetric unit, with a final R/Rfree of 19.7/23.6.

Thin layer chromatography

To analyse PL movement within the Mla system a Ni-NTA affinity column protocol coupled with thin layer chromatography (TLC) was employed. To measure whether MlaC could bind PL directly from the environment, without the need for MlaD or MlaFEDB

426 facilitation, we adjusted both MlaC-PL (natively extracted MlaC shown to be bound to PL)
427 and MlaC-apo to 2 mg·mL⁻¹ and bound it to a Ni-NTA affinity column equilibrated in 50 mM
428 Tris pH 8, 150 mM NaCl. Small unilamellar vesicles of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-
429 phosphocholine (POPC) or *E. coli* polar lipid extract (Avanti) (4 mg·mL⁻¹ in 50 mM Tris pH
430 8, 150 mM NaCl) were then flowed through the column for 30 min. The column was
431 subsequently washed for 45 min in 50 mM Tris pH 8, 150 mM NaCl, removing any unbound
432 PLs. MlaC was then eluted from the column in 50 mM Tris pH 8, 150 mM NaCl, 250 mM
433 imidazole and collected prior to PL extraction.

434 To investigate the transport of PLs between MlaC and MlaD^{Δtm} equimolar
435 concentrations of each were co-incubated together for 30 min then separated via passage
436 through a Superdex S75 column and fractions containing either MlaC or MlaD^{Δtm} collected,
437 analysed by SDS-PAGE to confirm separation and pooled. Samples were adjusted to 0.5
438 mg·mL⁻¹ in 2 mL and added to 2 mL of methanol and 1 mL chloroform (2:2:1 v/v/v).
439 Samples were then vortexed continuously for 5 min, incubated for 30 min at 50 °C, and
440 vortexed again for 5 min. The mixture was centrifuged (2000 x g, 10 min), and the lower
441 phase extracted and evaporated. Dried PLs were resuspended in 100 μL chloroform and 5 μL
442 loaded onto a Silica TLC plate (Sigma) and run with a 6.5:2.5:1 (chloroform:methanol:acetic
443 acid) solvent. The TLC plate was dried for 30 min, stained with 10 % (w/v)
444 phosphomolybdic acid (PMA) in ethanol, and heated until staining occurred.

445 To investigate the transport of PLs between MlaC and MlaFEDB, 2 mg of purified
446 MlaFEDB was bound to a 1 mL Ni-NTA affinity column equilibrated in 50 mM Tris pH 8,
447 150 mM NaCl, 0.5 mM DDM. DDM was then exchanged for 25 mM β-octylglucoside (β-
448 OG) in 50 mM Tris pH 8, 150 mM NaCl. The MlaFEDB bound Ni-NTA affinity column was
449 then incubated for 30 min with 4 mg·mL⁻¹ of either POPC or *E. coli* polar lipid small
450 unilamellar vesicles (SUVs) resuspended in 50 mM Tris pH 8, 150 mM NaCl, 25 mM β-OG.

MlaFEDB was then reconstituted into a PL bilayer environment by flowing POPC or *E. coli* polar lipid small unilamellar vesicles (4 mg·mL⁻¹ in 50 mM Tris pH 8, 150 mM NaCl) through the column for 30 min. To remove any unbound PLs MlaFEDB was then thoroughly washed for 45 min in 50 mM Tris pH 8, 150 mM NaCl, with the flow through collected prior to lipid extraction. Either 2 mg MlaC-apo or MlaC-PL in 50 mM Tris pH 8, 150 mM NaCl was incubated with the bound MlaFEDB on the Ni-NTA affinity column for 30 min. MlaC was then washed off the column in the same buffer and collected for lipid extraction. Bound MlaFEDB was then eluted in 50 mM Tris pH 8, 150 mM NaCl, 250 mM imidazole and collected for lipid extraction.

Samples were adjusted to 0.5 mg·mL⁻¹ and 2 mL and added to 2 mL methanol and 1 mL chloroform (2:2:1 v/v). Samples were then vortexed continuously for 5 min, incubated for 30 min at 50°C, and vortexed again for 5 min. The mixture was centrifuged (2000 x g, 10 min), and the lower phase extracted and evaporated. Dried lipids were resuspended in 100 µL chloroform and 5 µL loaded onto a Silica TLC plate (Sigma) and run with a 6.5:2.5:1 (chloroform:methanol:acetic acid) solvent. The TLC plate was dried for 30 min, stained with 10% (w/v) PMA in ethanol, and heated until staining occurred.

MlaFEDB ATPase activity

An NADH enzyme-linked assay ³⁴ adapted for a microplate reader ⁴⁶ was used to determine the rate of ATP hydrolysis by MlaFEDB in both detergent micelles and polar lipid liposomes. All assays were performed in triplicate and at room temperature. For MlaFEDB in detergent micelles, assays were performed in 50 mM Tris pH 8.0, 150 mM NaCl, 5 mM MgCl₂, 0.5 mM DDM; whilst for MlaFEDB in liposomes an assay buffer of 50 mM Tris pH 8.0, 150 mM NaCl, 5 mM MgCl₂ was used. Using a reaction volume of 75 µl, in 96-well plates (Sigma-Aldrich), 200 mM NADH (Sigma-Aldrich), 20 U·mL⁻¹ lactic dehydrogenase

(Sigma-Aldrich), 100 U·mL⁻¹ pyruvate kinase (Sigma-Aldrich), 0.5 mM phosphoenolpyruvate (Sigma-Aldrich) and different ATP (Sigma-Aldrich) concentrations were added to the appropriate assay buffer. The change in absorbance at 340 nm was then measured using an Anthos Zenyth 340rt (Biochrom) absorbance photometer equipped with ADAP software. Reactions were followed from 10-60 min (allowing for all ATP to be hydrolysed) with measurements taken every 15 s. ATP hydrolysis rates were then determined using maximal 340 nm absorbance readings (with 0 μM ATP), and minimal 340 nm absorbance readings (with 0 μM NADH) from these readings the concentration of NADH at each time-point was calculated, allowing for a linear fit of the reduction in NADH absorbance and conversion to ATP hydrolysis.

Circular Dichroism

MlaC was diluted to 10 μg·mL⁻¹ in 50 mM sodium phosphate pH 7, 300 mM NaCl and circular dichroism performed on a Jasco J-1500 CD spectrometer with a path length of 1 cm at 20°C. A thermal melt was performed with spectra recorded every 5°C up to a temperature of 90°C.

Analytical ultracentrifugation

Twin channel AUC cells were prepared with 400 μl of protein sample at a concentration of 0.3 mg·mL⁻¹ to 1.4 mg·mL⁻¹ in one channel and 420 μl of relevant buffer blank in the other. The cells were loaded into an An50Ti rotor in a XL-I analytical ultracentrifuge (Beckman Coulter) and the samples centrifuged at 129,000 x g and at a temperature of 20°C until the sample sedimented. The protein samples within the cell were monitored by measuring the absorbance at 280 nm. The data were analysed using the program Sedfit⁴⁷ and fit using a continuous c(S) distribution model.

Nuclear magnetic resonance

NMR experiments were performed at 25°C on an Oxford Instruments 900 MHz magnet equipped with a Bruker Avance III console and TCI 5mm z-PFG cryogenic probe. Experiments were conducted using ¹⁵N-labelled MlaC at concentrations between 0.1-0.3 mM in 50 mM sodium phosphate buffer pH 7, 50 mM NaCl and 0.02% (w/v) NaN₃ in 90% H₂O/10% D₂O. ¹⁵N labelled MlaC was purified as above but with the exception of substituting lysogeny broth for M9 minimal media containing ¹⁵NH₄Cl as the sole nitrogen source. ¹H, ¹⁵N heteronuclear single quantum coherence (HSQC) experiments⁴⁸⁻⁵⁰ were recorded with a spectral width of 7978 Hz and 1024 points in the ¹H dimension and 1772 Hz and 256 points in the ¹⁵N dimension in the presence and absence of 2 mg·mL⁻¹ *E. coli* polar lipid extract (Avanti). All spectra were processed with NMRPipe⁵¹ and analysed using SPARKY⁵².

Molecular dynamics simulations

MlaC-apo simulations were conducted using both the MlaC-apo structure determined herein and previously published PL bound MlaC structures from *E. coli* (5uwa), *R. solanacearum* (2qgu) and *P. putida* (4fcz). For all structures, missing terminal residues were added back to the structures prior to simulation using MODELLER, version 9.15⁵³. Prior to simulation the structures were solvated within rhombic dodecahedral boxes, using 100 mM NaCl, while also ensuring system neutrality, and minimised using 1000 steps of the steepest descents method.

Apo simulations initiated from the three different lipid bound MlaC structures were performed using three different force fields: the all-atom AMBER99SBNMR-ILDN⁵⁴ and CHARMM36⁵⁵ force fields, plus the united-atom GROMOS54A7 force field⁵⁶. Appropriate

simulation settings were chosen for each force field. AMBER99SBNMR-ILDN simulations used a 1.0 nm cut-off for both Coulombic and van der Waals interactions. These simulations also used the standard TIP3P water model⁵⁷. For the CHARMM36 force field, a 1.2 nm cut-off for the Coulombic interactions was applied, while the van der Waals interactions were switched off between 1.0 and 1.2 nm. No long-range dispersion correction was applied. CHARMM36 simulations were performed using the CHARMM TIP3P water model^{58,59}. GROMOS 54A7 simulations used a 1.4 nm cut-off for both Coulombic and van der Waals interactions and the SPC water model⁶⁰. Unlike the all-atom force field simulations, which used a simulation time step of 2 fs, the GROMOS 54A7 force field simulations used virtual interaction sites for the hydrogen atoms allowing a 4 fs time step to be applied⁶¹. For all these simulations: long-range interactions were treated using the Particle Mesh Ewald method⁶²; a simulation temperature of 310 K and a pressure of 1 bar were maintained using Nose-Hoover^{63,64} and Parrinello-Rahman^{65,66} schemes with coupling constants of 2.0 and 5.0 ps, respectively; for each protein and force field combination, five independent simulations utilising different starting velocities were performed for 500 ns each, following an initial 10 ns period in which the positions of the protein atoms were restrained. Starting velocities for the independent simulations were randomly generated according to a Maxwell distribution at 310 K. These simulations were all performed using GROMACS, version 5.0.6⁶⁷.

Simulations initiated from the MlaC-apo structure were performed using the AMBER99SBNMR-ILDN force field and followed the same settings as described above. Two standard simulations were performed for 500 ns, each using different starting velocities. Simulations were also performed using both elevated temperature and scaled down interactions, as per the higher replica levels of temperature and Hamiltonian replica exchange methods^{68,69}, in an attempt to accelerate the sampling within these simulations. The elevated temperature simulations were performed at a temperature of 400 K, while the interactions

were scaled down by a factor of 0.7. We note here that applying a higher temperature of 500 K or a scaling factor of 0.5 led to the protein unfolding during test simulations. As per the standard MlaC-apo simulations, two independent simulations were performed for each setting using different starting velocities. Scaling of the interactions was performed using the partial_tempering script of the PLUMED code ⁷⁰. Apart from the higher temperatures and scaled interactions, these simulations were performed using the same methods described for the standard simulations. Simulations of the MlaC-apo structure were all performed using GROMACS version 5.1.4 ⁶⁷. Analysis of the simulations was performed using the GROMACS tools, the trj_cavity program ⁷¹ and VMD ⁷².

Native mass spectrometry

Native mass spectrometry experiments were performed on a QExactive HF (ThermoFisher Scientific) coupled with a Nanomate Triversa (Advion). MlaC-apo and MlaC-PL were initially concentrated to 15 μ M and then buffer exchanged into 100 mM ammonium acetate pH 7 using Amicon centrifugal filters with 10 kDa MWCO. Measurements were performed in positive ion mode using a voltage of 1.75 kV. The capillary was at 250 °C and in-source dissociation and HCD energy set to zero to preserve any non-covalent complexes present. CsI was used for calibration and all mass spectra were acquired over a mass range 1500-3500 m/z. The spectra were processed using XCalibur v.4 (ThermoFisher Scientific).

MALDI-TOF mass spectrometry

1 μ L of MlaC-apo at 0.35 mM in a buffer of 50mM Tris-HCl was loaded onto a metal target plate with an equal volume of 2',4',6'-trihydroxyacetophenone matrix. This was repeated with the addition of various concentrations of β -OG ranging from equimolar (0.35mM) to a final concentration of 43.75 μ M. Spectra of MlaC-apo were acquired in

positive ion reflection mode using an ultrafleXtreme MALDI-TOF (Bruker, Massachusetts, US). The spectra provided are the sum of 10,000 laser shots.

Surface Plasmon Resonance

Equilibrium Dissociation constants (K_D) for the interactions between MlaC-apo/PL and MlaD^{Δtm}-apo/PL were determined through surface plasmon resonance (SPR). MlaC-apo, MlaC-PL, MlaD^{Δtm}-apo and MlaD^{Δtm}-PL were all dialysed into a buffer of 0.01 M HEPES pH 7.4, 0.15 M NaCl (HBS). SPR was conducted using a Biacore 3000 (GE Healthcare, Illinois, US) with proteins immobilized through nickel-chelation affinity onto a Sensor Chip NTA (GE Healthcare, Illinois, US) to an RU of ~2000. The chip was first washed with a buffer of 0.01 M HEPES pH 7.4, 0.15M NaCl, 0.005% v/v surfactant P20 (HBS-P). Flow cells were regenerated using 5 mL of 350 mM EDTA in HBS-P adjusted to pH 8.3. Binding of both MlaD-apo and MlaD-PL was performed sequentially to separate flow cells, whilst a third flow cell was used as control. Experiments were performed at a flow rate of 5 $\mu\text{L}\cdot\text{min}^{-1}$ with 5 μL injections of MlaC at various concentrations. Due to the nature of the interaction and potential exchange of PLs, MlaD was regenerated on the surface between each experimental condition. Processing of the data for K_D was performed using BIAevaluation (GE Healthcare, Illinois, US) and PRISM software (Graphpad, San Diego, US).

Functionalization of gold surfaces

QCM-D sensors with gold surface coating were cleaned by UV-ozone treatment for 10 min then immersed in a 5:1:1 solution of H₂O:H₂O₂ (25%):NH₄OH (30%) heated to 75 °C for 5 min. Sensors were thoroughly rinsed in ultra-pure water, dried and subject to a final UV-ozone treatment for 10 min. The gold surfaces were then functionalized based on the method of Giess to introduce a nitrilotriacetic acid functionalized surface³². Briefly, surfaces

were immersed in a solution of 2 mg·mL⁻¹ dithiobis (*N*-succinimidyl propionate) in dry DMSO for 30 min, then rinsed with dry DMSO, ultra-pure water, and ethanol and dried in a stream of nitrogen. The surfaces were then immersed for 2 h in a solution of 150 mM *N*-(5-amino-1-carboxypentyl) iminodiacetic acid (ANTA) buffered to pH 9.8 with 0.5 M K₂CO₃, washed with ultra-pure water and then incubated for 30 min in 40 mM CuSO₄ in 50 mM sodium acetate buffer. As a final step, the surfaces were washed with ultra-pure water and dried in a nitrogen stream prior to use. Evidence for correct surface deposition was given by QCMD, showing a characteristic frequency change of ~8 Hz on Cu²⁺ incubation (Supplementary Figure 20).

Quartz crystal microbalance with dissipation monitoring (QCM-D)

QCM-D is a surface sensitive technique utilising the piezoelectric effect for real-time measurements of surface interactions. The change in the resonant frequency (Δf) is inversely proportional to the amount of mass deposited at the surface (Δm), as given by the Sauerbrey equation ⁷³:

$$\Delta f = -\frac{2f_0^2}{A\sqrt{\rho_q\mu_q}}\Delta m$$

Where f_0 is the resonant frequency, A is the piezoelectrically excited area of the crystal, ρ_q and μ_q are the density and shear modulus of the quartz crystal, respectively. QCM-D was performed using a Q-sense Analyzer QCM-D system (Gothenburg, Sweden). Sensors were mounted into a temperature controlled flow cell at 20°C attached to a calibrated peristaltic pump and filled with ultra-pure H₂O. A flow rate of 0.1 mL·min⁻¹ was used throughout unless otherwise stated. Frequency and dissipation changes (Δf and ΔD) were monitored using multiple harmonics (n=3, 5, 7, 9, 11, 13) of the resonant frequency, however for simplicity data are reported using the 3rd harmonic only. A baseline was acquired for 5

min in H₂O to allow for temperature equilibration before the measurement was started. Buffer was exchanged for DDM buffer (50 mM Tris pH 8, 150 mM NaCl, 0.5 mM DDM) for 10 min. MlaFEDB with a hexa-histidine tag at the N-terminal of MlaF was diluted to 0.1 mg·mL⁻¹ in the same buffer and injected into the cell to bind to the Cu²⁺ ion complexed surface. Once Δf_3 (change in frequency of the 3rd harmonic) reached -50 Hz ($\Delta D = 1.6$ ppm), an arbitrary value chosen for consistency and to limit surface saturation by MlaFEDB and to provide adequate space for PL coverage, excess MlaFEDB was removed by flowing DDM buffer over the surface for 7 min. DDM was exchanged for β -octylglucoside (β -OG) (25 mM in 50 mM Tris pH 8, 150 mM NaCl) for 15 min to ensure thorough detergent exchange. Mixed POPC and β -OG micelles were prepared by resuspension of a desiccated POPC film in 25 mM β -OG, 50 mM Tris pH 8, 150 mM NaCl to a concentration of 2 mg·mL⁻¹ and subsequent sonication for 30 minutes to ensure homogeneity, as confirmed by dynamic light scattering (DLS). POPC/ β -OG mixed micelles were injected into the QCM-D flow cells for 15 min to allow for detergent to exchange for PLs. POPC small unilamellar vesicles (SUVs) were prepared by resuspension of a desiccated POPC film in 50 mM Tris pH 8, 150 mM NaCl to a concentration of 2 mg·mL⁻¹ and subsequent sonication for 30 minutes until an optically transparent solution was obtained. The formation of SUVs of ~ 50 nm diameter was confirmed by DLS. POPC SUVs were then flowed for 10 min to deposit a supported bilayer.

Addition of detergent solubilised MlaFEDB resulted in a decrease in frequency corresponding to an increase in mass coupled to the surface (Figure 5B-1 & Supplementary Table 4). The surface was then washed (Figure 5B-2), exchanged into a β -octyl glucoside detergent buffer that more suited vesicle deposition (Figure 5B-3) and still maintained MlaFEDB activity (Supplementary Figure 21). A supported bilayer was formed by first an addition of mixed detergent/POPC (Figure 5B-4) and finally POPC alone leading to a characteristic initial adsorption and subsequent rupture of vesicles indicative of bilayer

deposition at the surface ⁷⁴ (Figure 5B-5). A final wash was then performed to remove excess liposomes (Figure 5B-6 & Supplementary Table 4).

The surfaces were thoroughly washed for 20 min in 50 mM Tris pH 8, 150 mM NaCl to ensure excess PL had been removed. MlaC was diluted to 0.1 mg·mL⁻¹ in 50 mM Tris pH 8, 150 mM NaCl in the presence or absence of 0.5 mM ATP, 5 mM MgCl₂ for ATP dependence experiments. MlaC was injected into the cell at a flow rate of 0.1 ml·min⁻¹. Excess MlaC was removed by washing the surfaces in 50 mM Tris pH 8, 150 mM NaCl. This allowed Δf in response to MlaC to be obtained.

Due to the large dissipation shifts observed with QCM-D, the MlaFEDB-containing films cannot be considered rigid. Therefore, we have made the assumption that these films are laterally homogeneous, as supported by neutron reflectometry, and performed viscoelastic modelling in order to estimate the mass changes at the surface in response to each analyte (Supplementary Table 4). Viscoelastic modelling was performed using Dfind software (Biolin Scientific, Gothenburg, Sweden). Frequency and dissipation values of the 3rd, 5th, 7th, 9th, 11th and 13th harmonics were modelled using the included 'Broadfit' viscoelastic modelling algorithm, with data collected for 50 mM Tris pH 8, 150 mM NaCl in the absence of protein, detergent or phospholipids used as reference values. In line with Reviakine *et al.* (2011) the assumption that the interfacial material is laterally homogeneous with a density similar to that of the bulk solvent, at 1000 g/L was made ⁷⁵. The density of the bulk solvent was held constant at 1006 g/L, taking into account the density of water at 293 K and buffer components.

Attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR)

ATR-FTIR spectra were collected using a Thermo-Nicolet iS50 instrument fitted with an ATR flow cell accessory (Specac) attached to a syringe pump and a water cooling loop

connected to a temperature controlled water bath, a cryo-cooled mercury cadmium telluride detector and a dry-air purge operating at a flow rate of 40 L·min⁻¹ in order to minimise absorbance from residual water vapour in the beam path. All spectra were obtained at 20 °C, with a resolution of 4 cm⁻¹ and 128 interferograms collected for each spectra. Fourier self-deconvolution was performed automatically by the data acquisition software (OMNIC 9 - Thermo Fisher Scientific) with a constant bandwidth applied across all spectra.

Single crystal silicon ATR substrates were cleaned by immersion in 2% (w/v) SDS for 30 min before rinsing extensively with ultrapure water and drying under a stream of nitrogen. Substrates were then UV-ozone cleaned for 10 min, washed with ultrapure water and then UV-ozone cleaned a final time for 10 min. The substrate was mounted in the flow cell dry and the volume filled with buffer A (50 mM Tris, 150 mM NaCl, pD 8.0 in D₂O) for background collection. For experiments investigating the effect of ATP, all buffers were supplemented with 0.5 mM ATP, 5 mM MgCl₂. Proteoliposomes for deposition were prepared by mixing POPC and MlaFEDB in a 3:1 mass ratio in the buffer B (50 mM Tris, 150 mM NaCl, 25 mM β-OG, pD 8.0 in D₂O) to a final concentration of 0.3 mg·mL⁻¹ MlaFEDB and 0.1 mg·mL⁻¹ POPC. This mixture was then diluted 10-fold in buffer A to lower the detergent concentration below the critical micelle concentration (~23mM), and incubated at 6 °C for 30 min. This resulted in spontaneous self-assembly of proteoliposomes of approximately 100 nm diameter, as confirmed by dynamic light scattering. 2 mL POPC/MlaFEDB proteoliposomes were then manually injected over the Si substrate and incubated for 15 min to allow for MlaFEDB-containing POPC bilayer deposition. The proteoliposome suspension was diluted a further 10-fold in buffer A and 2 mL injected into the flow cell to allow for β-OG to exchange out of the deposited bilayer. Finally, the POPC/MlaFEDB bilayer was washed with 5 mL buffer A at a flow rate of 0.5 mL·min⁻¹ and spectra collected.

MlaC was introduced into the cell at a concentration of $10 \mu\text{g}\cdot\text{mL}^{-1}$ in buffer A under a constant flow of $0.1 \text{ mL}\cdot\text{min}^{-1}$ for 4 h. Spectra were continuously collected every 80 s throughout. After four hours, the flow cell was washed with 5 mL buffer A at a flow rate of $0.5 \text{ mL}\cdot\text{min}^{-1}$ and a final spectrum collected.

All spectra were corrected for removal of water vapour by scaling and subtracting spectra collected prior to deposition of the POPC/MlaFEDB bilayer from the bilayer spectra. No further processing was performed. Peak integrations were performed over the aliphatic C-H stretch region from 2990 cm^{-1} – 2810 cm^{-1} . This includes contributions from the symmetric and asymmetric C-H₂ stretching vibrations and the C-H₃ stretching vibrations arising from the PL tails as a comparison of the extent of PL removal from the bilayer.

Neutron Reflectometry

Specular neutron reflectometry (NR) measurements were carried out using the white beam INTER reflectometer ⁷⁶ at the Rutherford Appleton Laboratory (Oxfordshire, UK). Data were collected at a resolution (dQ/Q) of 3.5%, yielding a total illuminated length of 60 mm. Solid/liquid flow cells connected to a liquid chromatography pump were employed allowing for easy exchange of the solution isotopic contrast within the (3 ml volume) sample cell. For each isotopic contrast change a total of 15 ml of 50 mM Tris pH 8, 150 mM NaCl solution was pumped through the cell (5 cell volumes) at a speed of 1.5 ml/min. A Piranha acid cleaned silicon substrate was assembled into the flow cell. 100% D₂O buffer solutions were then flowed into the cell and NR from the solid liquid interface was examined. Next a suspension of POPC:MlaFEDB ($0.2 \text{ mg}\cdot\text{mL}^{-1}$) vesicles in the D₂O buffer solution (prepared in an identical fashion to FTIR deposition) was injected into the cell and incubated for 40 minutes while the vesicles ruptured onto the solid/liquid interface. The vesicle solution was then pumped out of the cell, washed with 50 mM Tris pH 8, 150mM NaCl, 100% D₂O) and

interfacial structure was again examined using NR. Due to the complex nature of the interfacial membrane structure a number of data sets were collected under varying solution isotope mixtures, namely; 100% D₂O, 80% D₂O: 20% H₂O, Silicon matched water (38% D₂O) and 100% H₂O.

Neutron Reflectometry data analysis

NR data were analysed using the in-house software, RasCal (version 2014 Beta, A. Hughes, ISIS Spallation Neutron Source, Rutherford Appleton Laboratory) which employs optical matrix formalism (described in detail by Born and Wolf (1970)⁷⁷) to fit layer models of the interfacial structure to the experimental NR data. In this approach, the interface is described as a series of slabs, each of which is characterised by its scattering length density (SLD, ρ), thickness and roughness, where the roughness parameter is applied as a Gaussian smearing across the interface. The reflectivity for the model starting point is then calculated and compared with the experimental data. A least squares minimisation is used to adjust the fit parameters to reduce the differences between the model reflectivity and the data. In all cases the simplest possible model (i.e. least number of layers), which adequately described the data, was selected.

The clean silicon/water interface was examined in a single solution isotopic condition and the membrane coated interface was examined under four solution isotope contrast conditions. The reflectivity profiles from the bilayer containing sample were constrained to fit to a single profile of layer thickness and roughness. The fitted layer structure at the interface between the solid support and the bulk solution was found to consist of layers of silicon dioxide, a thin water layer, the inner bilayer head-group region, the bilayer tail region, the outer bilayer head-group region and a layer of protein on the surface of the bilayer. Once the membrane isotopic contrast data series was found to simultaneously fit this structure NR

data of clean silicon/water interface collected before the addition of the membrane was added to the simultaneously fitted data group to additionally resolve the fitting of the substrate roughness and the silicon oxide layers.

The data were fitted using a model of the interfacial structure where it was assumed that the scattering length density of the head group and lipid tail regions were composed of the combined SLD contributions of the lipid, protein and water multiplied by their volume fraction within a given layer, i.e:

$$\rho_{\text{fitted}} = (\rho_{\text{lipid}} \cdot \phi(\text{lipid})) + (\rho_{\text{protein}} \cdot \phi(\text{protein})) + (\rho_{\text{solution}} \cdot \phi_{\text{solution}})$$

Where ρ_{fitted} is the fitted SLD value, ρ_{protein} , ρ_{lipid} and ρ_{solution} are known SLD values for the sample components (see Supplementary Table 5) and ϕ is the component volume fraction⁷⁸. Simultaneous data analysis was performed by varying the volume fraction contributions of each of these components until a satisfactory model to data fit was achieved for all experimental data sets. This approach has been used previously to resolve the structure of interfacial membrane complexes^{79,80}.

The SLD values for each layer in each isotopic contrast were allowed to vary depending on the expected layer identity but were constrained such that the volume fractions of the constituents of each layer did not exceed 100%. The parameter fit values and the scattering length density profiles these describe were then used to determine the bilayer structure, surface coverage (i.e. volume fraction of bilayer defects across the surface⁸¹) and interfacial roughness. The coverage of protein in the peripheral protein layer was determined by comparing the fitted SLD values for this layer to the known SLD values of the protein and solution as described previously for protein adsorbed lipid bilayers⁷⁹. Error estimation of the fitted parameters was carried out using Rascal's in-built Bayesian analysis routines

^{82,83}, with the log-likelihood function described in terms of the overall chi-squared ⁸². In addition to the model parameters, the backgrounds, scale factors and instrument resolutions were also fitted. Marginalised posteriors were obtained using a Delayed Rejection Adaptive Metropolis algorithm ^{84,85}, and the best fit parameters taken as the distribution maxima, and the uncertainties obtained from the shortest 95% confidence intervals of each distribution (See Supplementary Table 6).

Data availability

The MlaC-apo X-ray structure has been deposited in the PDB with the following accession number: 6GKI. In addition, the data that support the findings of this study are available from the corresponding author upon request.

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Author Contributions

GWH, SCLH, CSL, PS, CH, TJP, PJW, ACL, DGW, MJ, VS, ITC, CH, GLI, JAB, YY, MJ, DH, IRH, LAC, ALL, TJK all participated in the conception and design of the work. GWH, SCLH, CSL, PS, AHM, TJP, PJW, ACL, DGW, RJP, LAC, ALL, TJK all participated in the data acquisition, analysis or interpretation of the work. GWH, SCLH, CSL, PS, TJP, PJW, ACL, DGW, LAC, ALL and TJK were involved in writing and editing the manuscript. All authors approved the final version submitted for publication.

Competing Interests

The authors declare that they have no competing interests with the contents of this article.

Figure Legends

Figure 1 – The MlaC-apo structure. A) Cartoon representation of the two molecules of MlaC observed in the asymmetric unit. The MlaC-open conformation is shown coloured light blue (helix) and pink (sheet) and the closed MlaC-apo conformation coloured dark blue (helix) and green (sheet). B) The individual open and apo conformations of MlaC highlighting the numbering of secondary structural elements. C) Stripped away structure showing the MlaC PL binding pocket observed in the MlaC open conformation overlaid with the position of the PL observed in crystal structure 5uwa highlighting the position of Y105 bisecting the pocket. PL binding pocket calculated using 3vee⁸⁶. D) Cut-away view of the MlaC surface, slice through shown in grey. Both the MlaC open (left) and apo (right)

conformations are displayed and coloured according to hydrophobicity (Blue hydrophobic, white – neutral, red – hydrophilic), thus showing the closing of the PL binding pocket. E) Observed shift of the β -sheet between the open and apo conformations with a lateral movement of 2.5 Å and 15° shift in angle occurring. F) Corresponding shift of part of helix $\alpha 6$ pivoting from Gly182 resulting in an angle change of 17.9°. Zoom in view of the residues surrounding the binding pocket in the open (G) and apo (H) conformations showing little conformational change in the residues involved.

Figure 2 – MlaC requires MlaD for phospholipid loading. A) TLC of MlaC purified from the cell showing the presence of bound PL (MlaC-PL); following washing with detergent leading to PL removal and the formation of MlaC-apo; and MlaC-apo following incubation with *E. coli* phospholipid SUVs (MlaC-apo-pPL) showing no addition of PL to MlaC. Representative image of 3 independent experiments. B) Excerpts from $^1\text{H}^{15}\text{N}$ -HSQC spectra of 0.2mM ^{15}N -labelled MlaC showing the H-N resonance positions for A85, G74 and G170 in the presence of PL (MlaC-PL), the absence of PL (MlaC-apo), following 18 h incubation of MlaC-apo with 2mg·mL⁻¹ *E. coli* phospholipid SUVs (MlaC-apo pPL), and following 30 min incubation of MlaC-apo with MlaD^{Δtm}-PL and subsequent SEC purification (MlaC-apo post MlaD^{Δtm}-PL). Representative of 2 independent experiments. C) Size exclusion chromatogram of the purification of MlaC and MlaD^{Δtm} following co-incubation to allow PL transfer to occur. Inset shows the un-boiled SDS-PAGE of the components following separation with MlaD^{Δtm} shown forming characteristic oligomers^{19,21}. Size exclusion chromatography and SDS-PAGE was performed at least on 3 occasions with representative images provided. D) TLC showing the movement of PL between MlaC and MlaD^{Δtm}. Positions of phosphatidylglycerol (PG), phosphatidylethanolamine (PE) and cardiolipin (Cl) are shown. Ec PL corresponds to *E. coli* polar phospholipids. Below is shown an extract of an

SDS-PAGE showing the separation of MlaC and MlaD^{Δtm} for each sample. Transfer studies were performed independently 3 times, with representative data shown.

Figure 3 – MlaFEDB is active and exports inner membrane phospholipids to MlaC. A) Size exclusion chromatogram of detergent solubilised MlaFEDB yielding a single complex. Inset showing SDS-PAGE demonstrating that MlaF, MlaE, MlaD and MlaB form a complex, with characteristic oligomers of MlaD shown in the unboiled (UB) sample which are lost in the boiled (B) sample. Experiments were performed on at least 3 independent occasions, yielding consistent results. B) Enzyme coupled ATPase assay of MlaFEDB (0.1μM) performed in detergent micelles (0.05% DDM). Average ATP hydrolysis rates were plotted against ATP concentrations and fitted against an expanded Michaelis-Menten equation that includes a term for the Hill coefficient (n). $V_{max} = 0.05 \mu\text{M ATP s}^{-1}$, $k_{cat} = 0.5 \pm 0.1 \text{ s}^{-1}$, $K_m = 200.0 \pm 44.6 \mu\text{M}$, $n = 1.81 \pm 0.35$. Error bars indicate standard deviation in data, n=3 independent experiments. C) TLC showing the movement of PL between MlaFEDB and MlaC in the presence of 500 μM ATP. B – Buffer, Ec PL – *E. coli* polar phospholipids; FEDB PL – MlaFEDB reconstituted into *E. coli* polar phospholipid liposomes, W – Wash following binding of MlaFEDB in *E. coli* polar phospholipid liposomes to Ni-NTA column. Representative images of at least 3 independent lipid transfer assays are shown. D) SDS-PAGE of the samples shown in C) confirming separation of the various species following incubation. Separation was confirmed on 3 separate occasions.

Figure 4 – QCMD and FTIR further suggests MlaFEDB exports inner membrane phospholipids to MlaC. A) QCM-D measurements performed in the presence of 500μM ATP showing the frequency (solid line) and dissipation (broken line) responses, indicative of mass changes to [1] the attachment of MlaFEDB, [2] washing, [3] exchange of DDM for β-

OG, [4] exchange of β -OG with a mixture of β -OG and POPC, [5] POPC vesicle deposition and popping, [6] washing off excess POPC, [7] addition of MlaC either apo (red) or PL bound (black), [8] repeat injection of MlaC, [9] buffer wash. QCM-D measurements were performed on at least 3 separate occasions. B) QCM-D measurements showing the frequency (solid line) and dissipation (broken line) responses, indicative of mass changes to [1] POPC fusion and rupture, [2] washing with water, [3] washing with buffer, [4] addition of MlaC either apo (red) or PL bound (black), [5] wash with buffer. Measurements were performed on 3 separate occasions, yielding consistent results. C) ATR-FTIR spectra of a Si-adsorbed supported POPC bilayer containing reconstituted MlaFEDB. Spectra were collected before interaction with MlaC-apo (red spectra) and then at selected time points during interaction with MlaC-apo (orange through indigo spectra). Dashed vertical lines highlight absorbances corresponding to stretching vibrations of symmetric and asymmetric C-H₂ and C-H₃ groups, present primarily within the POPC fatty acyl tails, the ester C=O stretch, from the glycerol-ester bond of the PL tails, and the amide I band from the HNC=O bonds in the protein backbone. The strong negative absorbance band at 2500 cm⁻¹ is due to fewer O-D bonds from the D₂O buffer within the penetration depth of the IR effervescent wave after bilayer deposition relative to background spectra collected on bare Si. D, E) Excerpts from ATR-FTIR spectra showing the absorbance of aliphatic C-H_x bonds (left panel) and carbonyl bonds (right panel) during the interaction of MlaFEDB/POPC bilayers with D) MlaC-apo or E) MlaC-PL. F) Excerpts of ATR-FTIR spectra of a POPC bilayer in the presence of MlaC-apo. All spectra displayed are taken at timepoints as indicated in Figure 4C and collected in the presence of 500 μ M ATP throughout.

Figure 5 – MlaFEDB driven PL export occurs in an ATPase independent manner, however ATP hydrolysis is linked to MlaC-apo binding. A) Side-by-side comparison of

QCM-D profiles of MlaC-apo interaction with MlaFEDB in the presence (Red) and absence (Black) of ATP with numbering according to Figure 4A. Measurements were performed on at least 3 separate occasions. B) QCM-D measurements showing the frequency and dissipation response observed as in Figure 4A but in the absence of ATP. C) Changes to ATR-FTIR integral values for aliphatic C-H_x peaks (2900 – 2810 cm⁻¹) of Si-adsorbed MlaFEDB/POPC supported bilayers upon introduction of MlaC (apo/lipid) in the presence or absence of ATP. D) TLC showing the movement of PL between MlaFEDB and MlaC in the absence of ATP labelled according to Figure 3C. Representative image of 3 independent experiments. E) TLC showing the movement of PL between MlaFEDB and MlaC in the presence of 500 μM ATPγS, labelled according to Figure 3C. Representative image of 3 separate assays. F) Enzyme coupled ATPase assay of MlaFEDB *E. coli* polar phospholipid proteoliposomes in the presence and absence of 500μM ATP, MlaC-apo and MlaC-PL. Shown for comparison is the equivalent ATPase rate for DDM solubilised MlaFEDB. Error bars indicate standard deviation of the mean in data, n=3 independent experiments.

Figure 6 – Schematic summarising questions for the mechanisms of the Mla system arising from this study. Experimental evidence strongly suggests that MlaFEDB is capable of, and shows a preference for, transferring PL to MlaC. Further work is needed to fully elucidate the functions of each of the Mla components, which likely reach beyond that of the classically theorised retrograde PL trafficking.

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