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1 Structural basis for maintenance of bacterial outer membrane lipid
2 asymmetry

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The Gram-negative bacterial outer membrane (OM) is a unique bilayer that forms an efficient permeation barrier to protect the cell from noxious compounds^{1,2}. The defining characteristic of the OM is lipid asymmetry, with phospholipids comprising the inner leaflet and lipopolysaccharide (LPS) the outer leaflet¹⁻³. This asymmetry is maintained by the Mla pathway, a six-component system that is widespread in Gram-negative bacteria and is thought to mediate retrograde transport of misplaced phospholipids from the outer leaflet of the OM to the cytoplasmic membrane⁴. The OM lipoprotein MlaA performs the first step in this process via an unknown mechanism that does not require external energy input. Here we show, using X-ray crystallography, molecular dynamics simulations and *in vitro* and *in vivo* functional assays that MlaA is a monomeric α -helical OM protein that functions as a phospholipid translocation channel, forming a ~20 Å thick donut embedded in the inner leaflet of the OM with a central, amphipathic pore. This architecture prevents access of inner leaflet phospholipids to the pore, but allows outer leaflet phospholipids to bind to a pronounced ridge surrounding the channel, followed by diffusion towards the periplasmic space. Enterobacterial MlaA proteins form stable complexes with OmpF/C^{5,6}, but the porins do not appear to play an active role in phospholipid transport. MlaA represents a lipid transport protein that selectively removes outer leaflet phospholipids to help maintain the essential barrier function of the bacterial OM.

Three systems are known to maintain OM lipid asymmetry; the OM phospholipase A₂ PldA⁷, the LPS palmitoyl transferase PagP⁸, and the Mla (maintenance of outer membrane lipid asymmetry) system. Only the Mla system maintains asymmetry directly via phospholipid extraction, while PldA and PagP both generate lysophospholipids in the outer leaflet that still require removal. The Mla system is conserved in Gram-negative bacteria and plant chloroplasts^{9,10}, consisting of the inner membrane (IM) ABC transporter MlaBDEF, the periplasmic protein MlaC and the OM lipoprotein MlaA^{4,6,11}. Phenotypes from defects in the Mla system are mild under laboratory conditions and require small-molecule OM stressors like SDS/EDTA or antibiotics^{4-6,11}. However, MlaA has been identified as a virulence factor in various bacteria¹²⁻¹⁵. In addition, the Mla system was recently shown to regulate outer membrane vesicle (OMV) formation¹⁶. The deleterious effects of Mla knockouts likely result from phospholipid accumulation in the OM outer leaflet, forming bilayer patches that are portals for entry of lipophilic small molecules¹⁷. So far, structural information on Mla

components is available only for MlaC and MlaD⁵. MlaC likely accepts a phospholipid from MlaA and shuttles it to the ABC transporter for ATP-dependent plasma membrane insertion⁵. MlaA is the most enigmatic Mla component, particularly since its identity as a periplasmically-exposed lipoprotein appears hard to reconcile with its proposed activity on outer leaflet phospholipids.

To elucidate the OM-centered events of the Mla pathway we overexpressed several full-length bacterial MlaA orthologs in *E. coli* (Supplementary Fig. 1). MlaA orthologs from *Klebsiella pneumoniae* (KpMlaA) and *Serratia marcescens* (SmMlaA) could be purified in high yields. Strikingly, both proteins are present in complexes with the trimeric general diffusion porin OmpF (Supplementary Fig. 2a), confirming earlier observations for the *E. coli* complex^{5,6} and indicating that the MlaA-porin interaction is conserved in Enterobacteria. The X-ray crystal structures reveal that KpMlaA forms a ~190 kDa, 3:3 complex with OmpF whereas only one SmMlaA protomer is present per OmpF trimer (Figs. 1a and b and Supplementary Table 1). The difference in stoichiometry is most likely due to a lower stability of the SmMlaA-OmpF complex. Since the estimated number of MlaA molecules per cell is only a few percent of that of OmpF ($\sim 10^5/\text{cell}$)¹⁸, the 3:3 complex observed for KpMlaA is likely due to MlaA overexpression. The physiologically prevalent complex probably contains one MlaA monomer per OmpF trimer (1:3). MlaA co-purifies with OmpF and not OmpC probably due to the low-osmolarity growth medium, favoring OmpF expression. Given a recent suggestion that OmpC-MlaA and not OmpF-MlaA is the active species⁶ we also determined the structure of KpMlaA with *K. pneumoniae* OmpC (OmpK36). We find that KpMlaA-OmpK36 is virtually identical to KpMlaA-OmpF (Supplementary Fig. 2b), calling into question the claim that only OmpC-containing complexes are active⁶. MlaA is bound in the groove between two OmpF/C monomers, and interacts with OmpF/C mainly via van der Waals interactions between hydrophobic residues that would be lipid-exposed in the isolated proteins (Supplementary Fig. 2c). OmpF/C as present in the complexes with MlaA are identical to OmpF/C in isolation (C α r.m.s.d 0.3 Å), demonstrating that the MlaA interaction causes only minimal changes in the porins (Supplementary Fig. 2d).

KpMlaA and SmMlaA share 83% sequence identity and have very similar structures (C α r.m.s.d 0.8 Å; Fig. 1a-c), which as analysed by DALI¹⁹ do not resemble that of any other membrane protein (Supplementary Table 2). The N- and C-terminal ~10-15 residues and the N-terminal lipid anchor are not visible in the electron density. In the following we will discuss

the KpMlaA structure since it has the lowest R/R_{free} values (Supplementary Table 1). Moreover, partially ordered molecules of LPS are present in the conserved porin LPS binding site²⁰ in one of the KpMlaA-OmpF structures, providing a physiologically more relevant picture of the OM outer leaflet (Supplementary Fig. 3). MlaA has a ring-shaped structure and is predominantly α -helical (Figs. 1b and c). The stable association with OmpF facilitates analysis of the orientation of MlaA relative to the OM and reveals that, surprisingly, MlaA is an integral membrane protein (Fig. 1a and Supplementary Fig. 3). Analysis of the electrostatics shows that the entire MlaA surface, apart from the periplasmic face, is hydrophobic (Figs. 1 e-g). Most of the helices are amphipathic and oriented parallel to the OM except helix 6 (H6), which is perpendicular to the membrane plane and spans the lipid bilayer (Figs. 1a and c). The presence of a central channel, ~ 5 Å wide at its narrowest point, gives MlaA the appearance of a donut (Fig. 1d). This channel is lined mostly with hydrophilic residues from H2, the long intra-membrane loop between H4 and H5 ("pore loop"), H5 and the transmembrane H6 (Fig. 1c). The presence of the polar central channel explains why *e.g.* H6 is not a classical hydrophobic transmembrane helix. The extracellular end of the channel is formed by a pronounced, semi-circular ridge comprised of H5, the top of H6 and the loop connecting them (Figs. 1c and d). The top of the ridge including Trp160 and Trp163 is embedded in the interface of the outer leaflet (Fig. 1a and Supplementary Fig. 3).

Atomistic and coarse-grained molecular dynamics (MD) simulations (Supplementary Table 3 and Methods) show that water molecules rapidly fill the central MlaA channel (Figs. 2a and b; Supplementary Movies 1 and 2), indicating that the channel can accommodate polar molecules. Asymmetric systems with mixtures of the minimal biologically active endotoxin molecule Re-LPS (KDO₂-lipid A) and POPE (1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine) in the outer leaflet and POPE in the inner leaflet yielded limited information due to slow movement of the LPS molecules, which do not interact with MlaA within 2 μ s coarse-grained simulations (Fig. 2c) and block access of phospholipids to the MlaA pore. Since phospholipids and LPS do not mix *in vitro*²¹, we decided to simulate symmetrical POPE bilayers, mimicking an OM phospholipid patch¹⁷ (Supplementary Fig. 4a and b). These simulations consistently showed specific interactions of outer leaflet phospholipids with the ridge of MlaA. Following binding, the PE head group moves ~ 15 Å down the channel towards the centre of the bilayer, a movement coupled with tilting of the acyl chains by ~ 45 -90 degrees relative to the membrane normal (Fig. 2a-c; Supplementary Fig. 5a; Supplementary Movies 1 and 2). In both atomistic

and coarse grained simulations, the head group interacts with several negatively charged residues in the channel, including Glu146, Asp149 and Asp152 in helix H5 (Supplementary Figs. 5c and d). Several monovalent cations (Na^+ , K^+) are consistently present at this site and interact with the acidic residues, possibly to facilitate passage of the phosphate moiety. Importantly, POPC (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine) does not interact with MlaA (Supplementary Fig. 5b), indicating head group specificity that may reflect the absence of PC lipids in most enteric bacteria.

PE phospholipids near the channel constriction in the centre of the bilayer are stably bound on the timescale of the simulations, raising the question how the phospholipids move further towards the periplasmic space. The size and polarity of the channel pore in the crystal structure is sufficient to accommodate the phospholipid head group, but does not allow passage of the acyl chains. We propose that a relatively minor shift in the position or conformation of the pore loop tip or H6 (Fig. 1) elongates the pore to accommodate the acyl chains (Supplementary Fig. 6). The pore loop may function as a gate, perhaps opened by MlaC binding, to allow access of the entire phospholipid molecule. The principal residues restricting passage of the phospholipid acyl chains are Tyr138 at the pore loop tip, which side chain interacts with that of Trp170 in H6. Both residues are not well conserved (Fig. 3a) and their substitution by small residues in some orthologues would alleviate the requirement for conformational changes (Supplementary Fig. 6). The opened pore would be amphipathic to provide a matching environment for the substrate (Supplementary Fig. 6d).

A ConSurf analysis²² of MlaA revealed that the pore-lining and MlaA-OmpF/C interface residues are generally poorly conserved (Fig. 3a and Supplementary Fig. 1). Most of the invariant residues are involved in polar helix-helix interactions that likely stabilise the MlaA fold (*e.g.* Asp33 and Arg178; numbering of the mature protein, starting with Cys1). Other invariant residues such as Gln204 are exposed to the periplasmic space and are presumably important for MlaC interaction. The ConSurf output and the computational data were used for selection of mutant *KpmlaA* alleles, which were cloned in a low-copy plasmid and tested for their ability to complement the growth defect of the $\Delta mlaA$ *E. coli* strain in the presence of doxycycline²³ (Fig. 3b) and chlorpromazine²³ (Supplementary Fig. 7). We did not attempt to disrupt the MlaA-OmpF/C interaction because too many residues are involved (Supplementary Fig. 2). We did test the interesting MlaA* variant (lacking residues Asn26-Phe27) that disrupts

phospholipid homeostasis via phospholipid accumulation on the cell surface, leading to cell death²⁴.

The *E. coli* MlaA knockout shows clear growth defects for both doxycycline and chlorpromazine, small molecules with very different structures (Supplementary Fig. 7f). Complementation with wild type KpMlaA almost completely restores growth of the knockout, allowing structure-function studies. All tested variants are fully functional in LB medium in the absence of antibiotics (Supplementary Fig. 7), and variants are present in the OM as probed by western blots (Fig. 3 and Supplementary Fig. 7). A double mutant with changes in residues interacting with the phospholipid head group in MD simulations (D149A, D152A) has no growth defect. Strikingly, mutation of a third phospholipid-interacting residue (E146) results in a strong phenotype for the triple mutant (E146A/D149A/D152A) (Supplementary Fig. 7a), suggesting that Glu146 is particularly important or alternatively, that all head group-interacting residues need to be removed to compromise function. Single-substitution variants designed to make the aqueous channel more hydrophobic (E64L, R117L) are indistinguishable from wild-type MlaA, consistent with the fact that these residues are not conserved and suggesting that the overall properties of the channel are important rather than individual residues. With respect to invariant residues, most single substitutions have no (P49A, Y195A, R199E) or modest (N21A, N28A) effects on MlaA function (Fig. 3b), demonstrating that the MlaA fold is robust. Interesting exceptions are changes in Asp33 in H1 and Arg178 at the bottom of H6, both of which eliminate MlaA activity and suggest that the salt bridge formed by these two residues is crucial for function. The most illuminating loss-of-function phenotype is caused by the double mutant Y138C/W170C. The double cysteine "lock" variant was made to prevent pore enlargement via disulphide bond formation. The functionality of the double mutant is recovered in the presence of the reducing agent β -mercaptoethanol (Fig. 3c), providing strong support for the requirement of conformational shifts of the pore loop and/or H6 for pore enlargement. The Y138C/W170C mutant is not extracted using Sodium N-lauroyl sarcosine (sarkosyl) (Supplementary Fig. 7e), indicating it is located in the OM²⁵. Moreover, the single Cys mutants Y138C and W170C have no phenotype (Supplementary Fig. 7a), excluding the presence of mixed disulphides with *e.g.* the N-terminal cysteine residue as a cause for the growth defect of the Y138C/W170C mutant. Finally, changes in residues exposed to the periplasmic space (Q204A, R205L) have moderate phenotypes, suggesting the interaction with MlaC may be partially disrupted. Our results also confirm the fundamentally different character

of the KpMlaA* variant²⁴, since its phenotype is clearly more severe than that of the MlaA knockout (Fig. 3b and Supplementary Fig. 7). To supplement the *in vitro* data, we also tested the competitive fitness of wild type KpMlaA and the knockout *in vivo*, using a *Salmonella* typhoid fever mouse model. As shown in Fig. 3d, the knockout has a significant competitive disadvantage relative to wild type, with a competitive index (CI)²⁶ of 0.21, confirming that MlaA is important *in vivo*¹²⁻¹⁶.

Our structural and computational data identify MlaA as a monomeric α -helical OM protein, contrasting with both Wza²⁷ and VirB10²⁸ that form oligomeric water-filled pores in the OM. The interaction with OmpF reveals that MlaA is embedded in the inner leaflet of the OM which, together with the donut architecture, is crucial for function. The thickness of the MlaA donut is ~ 20 Å, *i.e.* one leaflet of the OM, and this ensures that inner leaflet phospholipids cannot gain access to the central channel (Fig. 4a). By contrast, the walls of MlaA do not cover the OM outer leaflet, allowing outer leaflet phospholipids to diffuse to the center of MlaA to bind at the ridge (Fig. 2 and 4). Due to their very large size, LPS molecules are not substrates for MlaA. Phospholipid binding is followed by diffusion of the substrate towards the periplasmic space, following enlargement of the pore that result from rearrangements of the pore loop, helix H6, or both (Fig. 4b; transition from state 1 to 2). The postulated conformational change could be spontaneous or a result of the binding of MlaC to the periplasmic face of MlaA. It is likely that the phospholipid moves from MlaA into the MlaC binding site⁵ without being exposed to the periplasmic space (Fig. 4a). The architecture of MlaA, specifically the physical separation of the central channel and the inner leaflet, also provides a strong argument against involvement of the Mla system in anterograde phospholipid transport to the OM⁵. The striking phenotype of the Asn26-Phe27 Δ MlaA* variant (Fig. 3b) is most likely due to a disruption in helix H1 and/or its interaction with the pore loop, resulting in a breach in the MlaA wall (Fig. 4b; state 3). This would "short-circuit" the system and now allow lateral access of inner leaflet phospholipids to the central channel (Fig. 4b; state 3 arrow), followed by a rapid, reversed flow to the outer leaflet driven by the steep concentration gradient of phospholipids between both leaflets.

The intriguing question why Enterobacterial MlaA proteins are present in stable complexes with OmpF and OmpC remains. Our crystal structures suggest that all functionality required for removal of OM phospholipids is contained within MlaA. When expressed without a signal

sequence and lipid anchor, KpMlaA_{cyto} (Methods) is still membrane-associated but does not co-purify with OmpF (Supplementary Fig. 2a), consistent with MlaA now being localised in the IM. Interestingly, this MlaA variant severely aggregates in mild detergents in which MlaA-porin complexes are stable (Methods), suggesting MlaA might not be stable on its own in the lipid bilayer. Alternatively, the porins might function as a scaffold for correct positioning or to otherwise enable MlaA to carry out its function (Supplementary Fig. 4c).

Unlike lipid flippases or floppases²⁹⁻³⁰, MlaA functions in an energy-independent fashion. While we argue that the MlaA* variant would function as a scramblase²⁹⁻³⁰ in a regular phospholipid bilayer, wild type MlaA represents a different class of lipid transport protein because it removes phospholipids from the OM via bypassing the inner leaflet, *i.e.* MlaA is a phospholipid translocation channel. Once returned to the IM via MlaC and MlaBDEF, the phospholipids likely join the general pool and may be transported back to the OM. The way in which this occurs is the biggest mystery in bacterial lipid homeostasis^{1,2}, but could perhaps mechanistically resemble steps in the Mla pathway.

Methods

Cloning, expression and purification of MlaA proteins. Genomic DNAs from *Klebsiella pneumoniae*, *Serratia marcescens*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Acinetobacter baumannii* and *Neisseria meningitidis* were used to amplify *KpmlaA*, *SmmlaA*, *PamlA*, *EcmlaA*, *AbmlaA* and *NmmlaA* genes (UniProt: A0A0W8AQT6, A0A0U8E5M0, Q9I043, A0A1J0HDI0, V5VGD0 and A0A0Y6GKR8 respectively). Amplification products were cloned under the control of an arabinose inducible promoter via NcoI-XbaI (except for *AbmlaA* that was via *EcoRI-XbaI*) into pBAD24³¹. Primers were designed to add a C-terminal hexahistidine tag during PCR amplification. Expression plasmids were transformed into an *E. coli* C43 (DE3) strain³² lacking cytochrome bo₃ ubiquinol oxidase (encoded by the *cyoABCD* operon) to facilitate OM protein purification due to the intrinsic affinity of the Cyo complex for metal affinity columns. Cells were grown in low salt LB (LB Broth low salt granulated (Melford Biolaboratories Ltd), consisting of Tryptone 10 g/l, NaCl 5 g/l and yeast extract 5 g/l. Protein expression was induced with 0.1 % of arabinose added at an OD₆₀₀ of 0.6, followed by further incubation of the cultures at 37°C whilst shaking at 150 rpm during 3-4 hours. After cell disruption (Constant Systems 0.75 kW operated at 20-23,000 psi), the membrane fraction was harvested by centrifugation (45 min at 42,000 rpm using a 45 Ti Beckman rotor). The

processing of small-scale, 1 litre cultures demonstrated that of the tested orthologs, only KpMlaA and SmMlaA were stable and produced in yields high enough to warrant scale-up to 6-8 liters of cells. Typically, membranes from eight litres of cells producing KpMlaA or SmMlaA were solubilized in 100 ml Tris-buffered saline (TBS) with 0.5% DM (n-Decyl- β -D-Maltopyranoside; Anatrace) and 0.5% DDM (n-Dodecyl- β -D-Maltopyranoside; Anatrace) during 1 hour at 4°C with stirring. Insoluble particles were removed by centrifugation (30 min 42,000) and the supernatant loaded onto an 8-10 ml nickel column previously equilibrated with 5 column volumes (CV) of wash buffer (TBS containing 10% glycerol, 25mM imidazole and 0.15% DDM) and then washed with 15-20 CV of the same buffer. Finally, proteins were eluted with 3 CV of wash buffer supplemented with 250 mM imidazole. KpMlaA was buffer exchanged into 10 mM HEPES pH7.5, 150 mM NaCl, 5% glycerol and 0.4% C8E4 (Tetraethylene Glycol Monoethyl Ether; Anatrace) using an Amicon Ultra-15 Centrifugal Filter Unit (100,000 kD cut-off). SmMlaA was further purified by size-exclusion chromatography run (HiLoad 26/600 Superdex 200, GE healthcare) using 10 mM Tris-HCl pH 8, 150 mM NaCl, 10% glycerol and 0.4% C₈E₄. To assess the purity of the proteins, samples were run on 4-12% NuPAGE SDS gels (Life Technologies). Both proteins co-purified in stable complexes with the *E. coli* trimeric general diffusion porin OmpF (Supplementary Fig. 2a), confirmed via analysis of gel bands by mass spectrometry (data not shown). KpMlaA-OmpF and SmMlaA-OmpF complexes were concentrated to at least 10 mg/ml and flash-frozen in liquid nitrogen.

Purification of KpMlaA_{cyto}. *KpmlaA*-pBAD24 plasmid was used as a PCR template, and primers were designed to remove the signal sequence and the anchor cysteine and to introduce the NcoI and XhoI restriction sites. The PCR product was cloned in pET28b. The expression plasmid was transformed into an *E. coli* BL21 (DE3) strain. Cells were grown in low salt LB (LB Broth low salt granulated (Melford Biolaboratories Ltd), consisting of Tryptone 10 g/l, NaCl 5 g/l and yeast extract 5 g/l. Protein expression was induced with 1mM of IPTG added at an OD₆₀₀ of 0.6, followed by further incubation of the cultures at 37°C whilst shaking at 150 rpm during 3-4 hours. After cell disruption (Constant Systems 0.75 kW operated at 20-23,000 psi), the membrane fraction was harvested by centrifugation (45 min at 42,000 rpm using a 45 Ti Beckman rotor). Membranes from one litre of cells producing KpMlaA_{cyto} were solubilized in 100 ml Tris-buffered saline (TBS) with 0.5% DM and 0.5% DDM during 1 hour at 4°C with stirring. Insoluble particles were removed by centrifugation (30 min 42,000) and the supernatant loaded onto an 8-10 ml nickel column previously equilibrated with 5 column

volumes (CV) of wash buffer (TBS containing 10% glycerol, 25mM imidazole and 0.15% DDM) and then washed with 15-20 CV of the same buffer containing 30 mM imidazole. Finally, the protein was eluted with 3 CV of wash buffer supplemented with 250 mM imidazole. KpMlaA_{cyto} was further purified by size-exclusion chromatography run (HiLoad 26/600 Superdex 200, GE healthcare) using 10 mM Tris-HCl pH 8, 150 mM NaCl, 10% glycerol and 0.05% DDM. Under these conditions KpMlaA_{cyto} severely aggregated and eluted in the void volume. Replacing the mild detergent DDM with the harsher detergent n-Dodecyl-N,N-Dimethylamine-N-Oxide (LDAO Anatrace, 1.5% for extraction, 0.2% for the Nickel column and 0.05% for the size-exclusion) allowed us to purify stable KpMlaA_{cyto}. Under these conditions, this shorter MlaA version does not form complexes with OmpF (Supplementary Fig. 2a), probably because it is located in the IM. As a control, during the LDAO purification the supernatant after the first spin (corresponding to the soluble protein fraction) was loaded onto a Nickel column but no KpMlaA_{cyto} was present in the elution.

Purification of KpMlaA-Y138C/W170C

The purification was performed as described above for KpMlaA with one modification; prior to the protein extraction in 0.5% DM and 0.5% DDM the IM was solubilized twice in 20 mM HEPES pH 7.5 plus 0.5% sodium N-lauroyl sarcosine²⁵ and the sample was centrifuged each time for 30 min at 42000 rpm. The pellet was collected and processed as described above via extraction with DM and DDM.

Cloning, co-expression and purification of KpMlaA-OmpK36 complex. *KpmlaA* was subcloned from the above mentioned pBAD24-derivative into pBAD322c³³ via NcoI-XbaI sites. The gene for the *K. pneumoniae ompC* ortholog, (coding for OmpK36; UniProt F2VN85), was cloned into a pBAD24 vector. The *E. coli* Omp8 strain, an expression host lacking the major outer membrane proteins including OmpF and OmpC³⁴, was electroporated with the expression vectors OmpK36 (Chl^R) and KpMlaA (pBAD24; amp^R), plates and cultures were supplemented with chloramphenicol (34 µg/ml) and ampicillin (100 µg/ml) to maintain both plasmids. Expression and purification were carried out as described above for KpMlaA with one modification; prior to the protein extraction in 0.5% DM and 0.5% DDM the IM was solubilized once in TBS plus 0.5% sodium N-lauroyl sarcosine²⁵ and the sample was centrifuged for 30 min at 42000 rpm. The pellet was collected and processed as described above.

Crystallisation and structure determination of KpMlaA-OmpF, SmMlaA-OmpF and KpMlaA-OmpK36 complexes. Sitting drop vapour diffusion crystallization trials were set up with a Mosquito Crystallization robot (TTP Labtech) using the commercial screens MemGold1 and MemGold2 (Molecular Dimensions). For the SmMlaA-OmpF samples only, reservoirs were diluted with 50% glycerol to a final concentration of 10%. Screens were performed at 4°C for KpMlaA-OmpF and 22°C for SmMlaA-OmpF and KpMlaA-OmpK36. Initial hits (KpMlaA-OmpF: 0.05 M sodium sulfate, 0.05 M lithium sulfate, 0.05 M Tris pH 8.5 and 35 % PEG 400; SmMlaA-OmpF: 0.32 M lithium chloride 0.1M Sodium citrate pH 5.5 and 14% PEG 400; KpMlaA-OmpK36: 0.04 M magnesium chloride hexahydrate, 0.05 M sodium chloride, 0.1 M HEPES pH7.5 and 32 % PEG400) were optimized by hanging-drop vapor diffusion. Crystals were cryoprotected, when needed, by increasing the PEG 400 concentration to at least 20% and flash-frozen in liquid nitrogen. Diffraction data were collected at 100 K at the Diamond Light Source (DLS; ED Table 1). Datasets were integrated with XDS³⁵ or DIALS (for the KpMlaA-OmpK36 dataset)³⁶ and the space groups were determined with Pointless. Datasets were scaled using Aimless³⁷⁻³⁸. The KpMlaA-OmpF phase problem was solved by molecular replacement using Phaser³⁹ with OmpF PDB (2ZFG) as a search model, showing clear electron density for KpMlaA. Phenix AutoBuild^{40,41} was run to partially build KpMlaA, followed by rounds of manual building using Coot⁴² and refinement with Phenix⁴³. Subsequently, the SmMlaA-OmpF phase problem was solved via molecular replacement using KpMlaA-OmpF as a search model. In this case, refinement was carried out with non-crystallographic symmetry (NCS) restraints. OmpK36 (1OSM) and KpMlaA-OmpF coordinates (manually modified to contain just one KpMlaA monomer) were used for the KpMlaA-OmpK36 molecular replacement and refinement was carried out in Phenix as described above. For all models, TLS refinement was included with each protein chain taken as a separate group. MolProbity⁴⁴ was used to validate protein geometry and PyMOL⁴⁵ for the visualization of the protein structures. The Alignment sequences were made with Aline⁴⁶ and the conservation residues were analysed with ConSurf²². The void volumes were calculated with Hollow⁴⁷.

KpMlaA-OmpF cocrystallisation with DMPG. To gain insight into how phospholipids interact with MlaA we cocrystallised KpMlaA-OmpF with 1,2-Dimyristoyl-sn-glycero-3-phosphoryl-rac-glycerol (DMPG). Crystallization trials were set up as described for KpMlaA-

OmpF by adding DMPG (1,2-Dimyristoyl-sn-glycero-3-phosphoryl-rac-glycerol; Anatrace) from a 12.5 mM stock solution in 10% C8E4 to a final concentration of 2.5 mM. Initial hits (0.05 M sodium sulfate, 0.05 M lithium sulfate, 0.05 M Tris pH 8.5 and 35 % PEG 400) were optimized by hanging-drop vapor diffusion. Diffraction data were collected at Diamond Light Source (ED Table 1) and processed as described above. KpMlaA-OmpF was used as a search model during molecular replacement. No clear electron density was observed that could correspond to a DMPG molecule, however density resembling partially ordered lipopolysaccharide molecules (LPS) was present close to OmpF. These LPS molecules were built in coot⁴² adding, sequentially, the individual monomers of the LPS structure followed by linking the molecules. eLBOW⁴⁸ was used to calculate the restraints of the different units, and the model was refined in Phenix⁴³. As before, the model was validated for protein geometry using MolProbity⁴⁴.

Functional assays. Single and double mutations were generated using the Q5® Site-Directed Mutagenesis (New England BioLabs) following the manufacturer's directions. Primers were designed using the NEBaseChanger tool (<http://nebasechanger.neb.com>). Mutations were introduced into the *KpmlaA*-pBAD24 vector, and the *KpmlaA* mutant alleles were PCR amplified and cloned, under the control of an IPTG inducible promoter, into the low copy vector pNDM220⁴⁹ via EcoRI-BamHI. All the mutants were sanger sequenced (Eurofins Genomics) to confirm that the desired DNA sequence was correctly introduced. A pNDM220 vector carrying the WT *kpmLaA* gene was also constructed. For the functional assay strains BW25113 (WT) and JW2343-1 (*ΔmLaA754::kan*) from the Keio collection⁵⁰ were used. First, JW2343-1 was transformed with pCP20, which provides FLP recombinase to remove KanR and afterwards the *ΔmLaA* strain was cured of pCP20 at 42 °C⁵¹. *EcmlaA* flanking primers were designed to check that the deletion of *EcmlaA* was successful. BW25113 and the cured JW2343-1 strains were transformed with the desired pNDM220 plasmids and plated onto LB plus 50 µg/ml of ampicillin. 10-fold serial dilutions of the transformed strains were prepared (starting culture at OD₆₀₀ of 0.5) and spotted with a free-floating pin tool (2 mm diameter pins, V&P scientific) on solid agar plates with 0.5 mM IPTG and 50 µg/ml ampicillin (supplemented with 120 µg/ml chlorpromazine or 1.2 µg/ml doxycycline as required) in 96-well format using a Biomek FX robot (Beckman Coulter UK Limited, High Wycombe, UK) incubated at 37°C and photographed after 18 hours. The results from these *in vitro* experiments, shown in Figure 3 and Supplementary Figure 7, suggest that drugs targeting the Mla pathway would potentiate various antibiotics via increasing the permeability of the OM.

397

398 **Western blot analysis.** 5ml of LB plus ampicillin (50 µg/ml) and IPTG (0.5 mM) were
399 inoculated with the desired strain and grown to an OD₆₀₀ of 1, then centrifuged and solubilised
400 in 250µl of BugBuster protein extraction reagent (Novagen), following incubation for 25
401 minutes at room temperature with vigorous shaking and finally spun down for 10 minutes at
402 14000 rpm in a microcentrifuge. The solubilised material was analysed by western blot using
403 Roche Anti-His6-peroxidase mouse monoclonal antibody (per manufacturer's specifications)
404 and developed with SuperSignal® West Pico Chemiluminescent Substrate (Thermo
405 Scientific). Chemiluminescent activity was recorded with ChemiDoc Imaging System (Bio
406 Rad). All the assays have been performed at least in duplicate.

407

408 **Competitive fitness experiments in vivo.** All animal experiments were approved (license
409 2239, Kantonales Veterinäramt Basel) and performed per local guidelines (Tierschutz-
410 Verordnung, Basel) and the Swiss animal protection law (Tierschutz-Gesetz). We used the
411 virulent strain *Salmonella enterica* serovar Typhimurium SL1344⁵² as parental strain for
412 constructing a *Salmonella* $\Delta mlaA$ mutant. We tested its in vivo fitness in the mouse typhoid
413 fever model using competitive infections. Female 8-12 weeks old BALB/c mice were
414 intravenously infected with a mixture of some 1000 colony-forming units each of SL1344 and
415 SL1344 $\Delta mlaA$. The exact inoculum dose for each strain was determined by plating. Four days
416 post infection, mice were sacrificed and spleen homogenates were prepared. Bacterial loads of
417 SL1344 and SL1344 $\Delta mlaA$ were determined and the competitive index was calculated as
418 $(\text{output}\Delta mlaA / \text{outputSL1344}) / (\text{input}\Delta mlaA / \text{inputSL1344})^{26}$. Wild-type fitness would result
419 in a competitive index of 1, values below 1 indicate compromised *in vivo* fitness of the $\Delta mlaA$
420 mutant. Statistical significance was tested using two-tailed t-test on log-transformed
421 competitive index data (which are normally distributed)²⁶. We estimated sample size by a
422 sequential statistical design. We first infected 2 mice based on effect sizes and variation
423 observed in our previous study²⁶. Data analysis suggested that 2 additional mice would be
424 sufficient to determine statistical significance with sufficient power. To ensure this was the
425 case, we tested an additional 3 mice (see text). We did neither randomize nor blind the
426 experiments, as this is irrelevant for competitive infections where a wild-type *Salmonella*
427 reference is included in each infected mouse.

428

429

Molecular dynamics simulations. The OmpF-MlaA assembly was embedded in a lipid bilayer and solvated with ~48000 water molecules and neutralized with a 150mM concentration of NaCl salt, bringing the all-atom system size to ~350,000 atoms. Moreover, the MlaA protein was simulated in absence of a OmpF protein as well, inserted in a 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE) bilayer. Both the coarse grained (CG) and the all-atom simulations were carried out using the GROMACS 5.1.2 package⁵³. The CHARMM 36 force field was employed to obtain the parameters for the lipids, waters, ions and the protein in the atomistic simulations⁵⁴. For the CG simulations, the MARTINI 2.0 force field was used for lipids, while the elastic network model with standard MARTINI constraints was used for the protein and the polarizable water model was used for the solvent⁵⁵⁻⁵⁷. Concerning the LPS, a Re-LPS (KDO₂-lipid A) lipid model, i.e., a minimal biologically active endotoxin molecule, was used in the CG simulations⁵⁸, with KDO being 3-deoxy-D-manno-2-octulosonic acid. The appropriateness of a CG representation for systems forming water channels in membranes was shown previously⁵⁹. Although additional lipids such as Cardiolipin, Phosphatidylglycerol (PG) and Phosphatidylserine (PS) are present in a small quantity in the inner leaflet of Gram-negative bacterial outer membranes, they were neglected in the present work for the sake of simplicity. All simulations were carried out at 303 K temperature to prevent gel phase formation of the lipid bilayer.

For the atomistic simulations, we used a 2 fs timestep with a Verlet integrator. A Nose-Hoover thermostat^{60,61} and Parrinello-Rahman barostat⁶² was employed for maintaining a canonical ensemble. Standard CHARMM36 settings were used to calculate the forces. Furthermore, electrostatic forces were calculated with a Particle Mesh Ewald (PME) method using a cutoff of 1.2 nm⁶³. The London dispersion effects were treated with a switch function with switch at 1 nm and a gradual cutoff at 1.2 nm. For the equilibration, the structure was first minimized and then short (1 ns) simulations were carried out in which the protein backbone and the lipids were successively relaxed. A final 100ns of simulation with all restraints removed was used as the last step of the equilibration process. The atomistic simulations were used to calculate various parameters for the lipid bilayer (Supplementary Fig. 8), including order parameters for the lipid tails, the Radial Distribution Function (RDF) for ions with the lipid heads, bilayer thickness across the box and Area per Lipid (APL) for each leaflet. The Radial Distribution Function ($g(r)$) is the distance distribution of the ions from the phosphate head group to the sodium and the chloride ions. The deuterium (D) order parameters (S_{CD}) calculate the anisotropy of the C-D bonds in the acyl chains of the lipid. Deuteriums are introduced into the lipid by substituting the hydrogens bound to the acyl carbons. S_{CD} is a measure of the

simulation average of the angle the C-D vector makes to the lipid bilayer normal and provides an insight into the flexibility of the lipid tail⁶⁴. Finally, the stability of the MlaA monomer in the simulation was tested by calculating its RMSF over the equilibration period, demonstrating that the structure of MlaA is very stable during the simulation (Supplementary Fig. 8f).

In the CG simulations, a time step of 20 fs was used for generating the trajectories. A v-rescale thermostat⁶⁵ and the Berendsen barostat⁶⁶ were made use of for maintaining a canonical ensemble. In addition, a switch function was employed to model dispersion terms. For the electrostatic terms, a cutoff of 1.2 nm was used and for the dispersion terms the switch occurred at 0.9 nm to end in a smooth cutoff at 1.2 nm. Similar to atomistic simulations, the CG simulations were also subjected to first a minimization step and then successively relaxed simulations (10 ns) in which the constraints on the proteins and the lipids were lowered to a final step of equilibration simulations for 1 μ s without any restraints.

An orientation map was prepared for the lipids of the bilayer from the simulated trajectories (Figs. 2c and d and Supplementary Figs. 5a and b). Each disk in this map represents the average lipid property in each grid space around the protein. The vector drawn at the centre of each disk shows the average tilt of the lipids with respect to the outer leaflet plane normal. Thus, an angle of 90 degrees represents a completely horizontal orientation for the lipid and a longer vector, while an angle of zero degrees implies a vertical orientation and a vector of length zero. The size of the disk corresponds to the relative density of the lipid in each grid space, *i.e.* it is a measure of occupancy of that site. Importantly, the colour of the disk represents the average depth of the lipid head from the centre of the membrane.

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Data availability

Coordinates and structure factors have been deposited in the Protein Data Bank with accession codes 5NUO for KpMlaA-OmpF, 5NUP for KpMlaA-OmpK36, 5NUQ for SmMlaA-OmpF and 5NUR for KpMlaA-OmpF-LPS.

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Author contributions

B.v.d.B and J.A-R. purified MlaA proteins, crystallised MlaA-OmpF complexes, and determined crystal structures. J.A-R. constructed MlaA variant proteins and conducted *in vitro* functional assays. S.S.K carried out and analysed MD simulations. U.K. supervised the computational studies. A.B. collected X-ray diffraction data and maintained the Newcastle Structural Biology Laboratory. B.C. carried out competitive fitness experiments, supervised by D.B. B.v.d.B and J.A-R designed experiments and B.v.d.B wrote the paper, with input from all co-authors.

Competing interests

The authors declare no competing financial interests.

Figures

Figure 1 | MlaA is a monomeric α -helical OM protein with a central channel. **a, b,** Cartoon representations from the OM plane (**a**) and from the outside of the cell (**b**) for KpMlaA-OmpF (left panel) and SmMlaA-OmpF (right panel). One monomer each for MlaA and OmpF is shown in rainbow colouring (N-terminus; blue). The hydrophobic core of the OM is shown in grey. **c,** Cartoon superposition of KpMlaA (rainbow) and SmMlaA (light green) with structural elements labelled and the channel location indicated with an asterisk. The right panel shows a side view of KpMlaA as in (**a**) with transparent surface. **d,** Surface views in rainbow colouring from the outside of the cell (left panel) and from the periplasmic side (right panel). **e-g,** Electrostatic surfaces (-15 kT/e to 15 kT/e) of the KpMlaA-OmpF dimer generated via PBEQ solver⁶⁷ viewed from the outside of the cell (**e**), the plane of the OM (**f**) and the periplasmic space (**g**). All crystal structure figures were generated with Pymol⁴⁵.

Figure 2 | MlaA binds OM phospholipids. **a**, Profile of the central MlaA channel generated with HOLE⁶⁸, including variations along the atomistic MD trajectories. Representative positions and orientations of the phospholipid at the start (grey) and end (yellow) of the simulations are indicated. **b**, MD snapshot from the OM plane after 200 ns atomistic simulation, showing the POPE molecule at the start and end of the simulation (oxygen, red; nitrogens, blue). MlaA is shown as a cartoon and coloured light blue. Phosphate head groups are shown as olive and green balls for the outer and inner leaflets respectively, water molecules as small blue spheres. The centre of the channel is indicated by the orange line. Residue Asp152 interacting with the POPE head group is labelled. **c**, **d** Interaction diagrams obtained from the second half of 2 μ s coarse-grained MD simulations for Re LPS (**c**) and POPE (**d**). The Z-coordinate of the phospholipid is represented by a coloured sphere, with the sphere diameter

proportional to the occupancy at that site. The OmpF trimer is indicated by the central green circle and the individual MlaA monomers by black circles. The length and direction of the arrows represent the tilt angles of the phospholipid acyl chains (see Methods). All figures and movies from simulations were generated with VMD⁶⁹.

Figure 3 | Functional analyses of MlaA. **a**, cartoon views from the extracellular side (left panel) and from the OM plane showing the locations of mutated residues, coloured by ConSurf conservation score. **b**, *In vitro* complementation of growth defects on LB agar plates in the presence of doxycycline. Cells were spotted in serial dilutions. Growth of the WT strain is compared to strains with a deletion of *EcmlaA* and complemented with an "empty" low copy number vector (*EcmlaAΔ*), or with the same vector harbouring wild type His-tagged *KpmlaA* (+) or different *KpmlaA* mutant alleles. The bottom panels show His-tag western blots of total membranes, indicating all variant proteins are expressed in the OM. Panels are representative of 4 experiments for the growth assays and 2 experiments for the western blots. **c**, *In vitro*

complementation of the Y138C/W170C mutant in the absence and presence of reducing agent. Panel is representative of 3 experiments. **d**, Competitive Index (CI) of the $\Delta mlaA$ strain in a Salmonella typhoid fever mouse model. Each circle represents data from one mouse from a total of two independent experiments ($n = 5$; **, $P < 0.01$). The dashed line corresponds to wild-type fitness. Statistical significance was tested using two-tailed t-test on log-transformed competitive index data.

Figure 4 | MlaA is an outer leaflet phospholipid translocation channel. **a**, Schematic overview of the MlaA transport mechanism, viewed from the OM plane. Access of inner leaflet phospholipids (PL) to the channel is prevented by the MlaA walls surrounding the central channel. By contrast, outer leaflet PL can access the channel and are removed from the OM via transfer to MlaC in the periplasmic space. **b**, Schematic views from the outside of the cell showing the relative locations of H1 (blue), H6 (orange) and the pore loop (yellow) using the same colour code as in **a**. State 1 represents the crystal structures, in which MlaA has a small,

754 hydrophilic pore. After rearrangements of the pore loop (arrow), helix H6 or both, MlaA has
755 an enlarged pore that is amphipathic (state 2). State 3 shows the putative disruption of H1 in
756 the N26-F27 Δ MlaA* variant, allowing lateral access of inner leaflet phospholipids to the
757 central channel (arrow). The polar part of the amphipathic pore is coloured red, the
758 hydrophobic part grey. For clarity, the translocating phospholipid molecule is not shown.