Energetics of lipid transport by the ABC transporter MsbA is lipid dependent

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The ABC multidrug exporter MsbA mediates the translocation of lipopolysaccharides and phospholipids across the plasma membrane in Gram-negative bacteria. Although MsbA is structurally well characterised, the energetic requirements of lipid transport remain unknown. Here, we report that, similar to the transport of small-molecule antibiotics and cytotoxic agents, the flopping of physiologically relevant long-acyl-chain 1,2-dioleoyl (C18)-phosphatidylethanolamine in proteoliposomes requires the simultaneous input of ATP binding and hydrolysis and the chemical proton gradient as sources of metabolic energy. In contrast, the flopping of the large hexa-acylated (C12-C14) Lipid-A anchor of lipopolysaccharides is only ATP dependent. This study demonstrates that the energetics of lipid transport by MsbA is lipid dependent. As our mutational analyses indicate lipid and drug transport via the central binding chamber in MsbA, the lipid availability in the membrane can affect the drug transport activity and vice versa.
The passive movement of phospholipids between the leaflets in a membrane bilayer is extremely slow, in the order of hours to days, as shown in model membranes. This is, at least in part, due to the energetically unfavourable movement of the hydrophilic headgroup across the hydrophobic core of the bilayer. For this reason, the cell requires membrane-embedded enzymes that facilitate the transbilayer movement of lipids within seconds. Examples of mammalian phospholipid transporters include (i) the calcium-activated nTMEM16 scramblase that catalyses the shuffling of phospholipids between the inner and outer leaflets of the plasma membrane independent of metabolic energy, (ii) the type IV P-type ATPase flippases that catalyse the inward translocation of phospholipids from the outer leaflet to the inner leaflet of the plasma membrane, and (iii) the lipid ATP-binding cassette (ABC) flippase ABCB4 for which in vivo data indicate a role in the outward translocation of phosphatidylcholine (PC) and its biliary secretion from hepatocytes into the canaliculi.

Phospholipid transport is equally important in microorganisms. The dimeric ABC exporter MsbA is an essential lipid transporter in Gram-negative bacteria. In vivo observations point to a role of MsbA in the translocation of phospholipids and core Lipid-A from their point of synthesis at the inner leaflet of the plasma membrane to the outer leaflet. In further steps at the outer leaflet of the plasma membrane, a polysaccharide moiety (O-antigen) is ligated to core Lipid-A to form full-length lipopolysaccharides (LPS). The Lipid-A (endotoxin) domain of LPS is a unique, glucosamine-based phospholipid that serves as the hydrophobic anchor of LPS and is the bioactive component of the molecule that is associated with Gram-negative septic shock. After it is formed, LPS is transported across the periplasm into the outer leaflet of the outer membrane by the LPS translocase LptB2FGCADE. But exactly how MsbA mediates the transport of naturally abundant lipids and how this reaction is related to MsbA’s ability to transport amphiphilic drugs has not been investigated in detail. Here we study the energetics of lipid transport by purified MsbA in proteoliposomes and compare the lipid and drug transport activities in a mutagenesis approach.

**Results**

**Energetics of lipid flippase activity.** We conveniently express *Escherichia coli* MsbA in *Lactococcus lactis*, a Gram-positive bacterium that lacks Lipid-A and LPS as well as an *E. coli*-like periplasm and outer membrane. We use these cells as a source of MsbA for lipid transport studies in proteoliposomes (Fig. 1). As (i) a previous study detected the transport of phospholipids and Lipid-A by MsbA in a 32P and 14C-acetate labeling approach in *E. coli* cells, (ii) PE is the predominant phospholipid in *E. coli* making up 70–80% of the total phospholipid pool, and (iii) advanced studies by native mass spectrometry detected the binding of PE to MsbA, we established a transport assay for physiologically relevant long-acyl-chain (2 × C18) PE in proteoliposomes in which MsbA was incorporated in an inside-out fashion (Fig. 1a). Lipids labelled with fluorescent nitrobenzoxadizole (NBD) have frequently been used in transport studies with ABC transporters. However, as MsbA mediates the efflux of fluorescent dyes and dithionite, the intrinsic membrane permeability of which reduces the ability to discriminate between labelled lipids in the outer and inner leaflet of the membrane. In our assay, 1 in 17 PE molecules in the proteoliposomes contained a non-invasive biotin moiety that was covalently linked to the ethanamine head group of the lipid (Fig. 1b). We detected PE flopping by inside-out oriented MsbA as a relocation of the lipid from the outer to the inner leaflet of the membrane. The reduction in the amount of external biotin-PE over time was quantified from the emission of membrane-impermeable fluorescence-tagged avidin when a quencher in complex with the avidin is displaced by the biotin moiety of the PE (Fig. 1a). The potential sources of metabolic energy that can drive substrate transport by MsbA, ATP and the transmembrane chemical H+ gradient (ΔpH), were applied using well-established methods. Briefly, proteoliposomes prepared in buffer pH 6.8 were diluted in buffer pH 8.0, imposing a difference between the interior pH and external pH by a pH jump (pHint 6.8/pHout 8.0) that was stabilised by the dissociation of NH4+ in the lumen of proteoliposomes and the outward diffusion of NH3. Using the fluorescent pH indicator BCECF trapped in the proteoliposomal lumen, measurements of the interior pH over time demonstrate that this pH difference was sustained for the 20 min duration of the biotin-lipid floppase assays (Supplementary Fig. 1). Where required, ATP was included in the external buffer. Remarkably, a significant PE floppase activity for MsbA-WT was observed only in the simultaneous presence of 5 mM Mg-ATP and the ΔpH (pHint 6.8/pHout 8.0) (Fig. 1c). The increased biotin-PE signal obtained with the addition of Triton-X100 to disrupt these proteoliposomes at the end of the floppase assay (Fig. 1d), demonstrates that PE flopping is based on the translocation of lipid to the inner leaflet of the membrane. This conclusion is strengthened by the finding that free biotin is neither transported by MsbA, nor liberated from biotin-PE by spontaneous hydrolysis during the floppase assay (Supplementary Fig. 2). No significant PE floppase activity was observed in MsbA-ΔLipid-A containing proteoliposomes in the presence of ATP without the ΔpH (pHint 6.8/pHout 6.8 and pHint 8.0/pHout 8.0) or the ΔpH (pHint 6.8/pHout 8.0) only (Fig. 1c), indicating the importance of the simultaneous presence of both forms of metabolic energy. Furthermore, PE floppase activity was not detected in empty liposomes (Fig. 1e) or proteoliposomes containing MsbA-ΔK382 that lacks the catalytic Walker A lysine residue and has strongly reduced ATPase activity (Fig. 1f). A previous cryo-electron microscopic (cryo-EM) study identified the quinoline compound G907 as a specific MsbA inhibitor that binds to and stabilises the inward-facing MsbA dimer, thereby preventing the transition to the outward-facing conformation. We synthesised G907 (Supplementary Methods) and tested its efficacy in our biotin-PE transport assay. At a 100 nM concentration (5x the reported in-vitro IC50), G907 completely inhibited active biotin-PE transport in MsbA-WT-containing proteoliposomes (Fig. 1c), thus confirming that this activity is mediated by MsbA. These findings indicate that inside-out oriented MsbA transports biotin-PE from the outer leaflet to the inner leaflet of the proteoliposomal membrane. This floppase activity is based on apparent H+/biotin-PE antiport and is facilitated by ATP binding and hydrolysis in the presence of a ΔpH (interior acidic in the proteoliposomes). The lipid transport assays were repeated using the relatively large *E. coli* hexa-acetylated (C12-C14) Lipid-A that is biotin-labelled on the disaccharide core (Fig. 2a). Different from biotin-PE, significant biotin-Lipid-A transport was observed in the proteoliposomes with ATP only; this activity was not affected by the imposed ΔpH (interior acidic) (Fig. 2b and Supplementary Fig. 1). Therefore, the transport of Lipid-A and PE has different energetic requirements.

**Similar pathways for lipids and drugs.** Recent cryo-EM and crystal structures of the MsbA-Lipid-A complex suggest that R78 (transmembrane helix (TMH) 2), R148 (TMH3), Q256 (TMH5), R296, K299 (TMH6) and interspersed carboxyl residues D41

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*References are omitted for brevity.
(TMH1), E149 (TMH3) and D252 (TMH5) form a ring of polar interactions with the 1 and 4′ phosphate group and the glucosamine, ester and amide groups connecting the acyl chains in Lipid-A8,23,25 (Fig. 3). Similar acidic and basic residues coordinate Lipid-A binding in the cryo-EM structure of the LPS translocase LptB2FGC26. We tested the effect of mutations of the ‘ring’ residues in MsbA on lipid and drug transport. First, we focussed on the arginine residues in the ring and found a deficiency in active Lipid-A and PE transport in proteoliposomes for a triple mutant MsbA containing R78A R148A R296A substitutions (referred to as MsbA-TripRA) (Figs. 1g and 2b); the K299A mutation did not inhibit PE transport (Fig. 1h). Moreover, we tested the impact of the arginine substitutions on MsbA-mediated lipid transport in E. coli WD2 cells. These cells contain a genome-
The binding domains in lipid transport by MsbA.

In vivo experiments point to a requirement for two out of the three arginine side chains and functional nucleotide-binding domain dimer (in orange and slate blue) and simultaneous proton conduction (blue arrow) by the membrane domains (MD) (in light orange and light blue) provide metabolic energy for the transport of PE from the outer leaflet of the membrane to the inner leaflet.

Second, we focussed on carboxyl residues D41, E149 and D252 that, in inward-facing MsbA, are interspersed between R78, R148 and R296 and located in the vicinity of the polar headgroup of bound Lipid-A (Fig. 3). In our lipid transport assays, we observed that the D41N, E149Q and D252N triple mutation (referred to as MsbA-DED) is tolerated in biotin-PE transport in proteoliposomes (Fig. 1i). However, MsbA-DED showed considerably reduced lipid transport in E. coli WD2 cells (Supplementary Figs. 3 and 4). These in vivo experiments point to a requirement for two out of the three arginine side chains and functional nucleotide-binding domains in lipid transport by MsbA.

In addition to lipids, MsbA transports small molecules like ethidium, erythromycin and chloramphenicol\(^{10,11,15}\). The efflux of ethidium can conveniently be monitored in \(L.\) \textit{lactis} cells from the decrease in fluorescence emission when the dye dissociates from intracellular nucleic acids\(^{27}\). In contrast to the observations for Lipid-A and PE, the R78A R148A double mutant and TripRA mutant showed a significantly enhanced efflux of ethidium compared to MsbA-WT (Fig. 5a).

Analysis of protein expression levels and the rate of ethidium transport as a function of the dye concentration identified an increased ethidium binding affinity of MsbA as the underlying mechanism for the enhanced efflux activity (Fig. 5b and Supplementary Figs. 5 and 6). Hence, the arginine side chains facilitate the binding of Lipid-A and PE in the chamber but inhibit the binding of ethidium.
Disaccharide core of our biotin-Lipid-A (Fig. 2a) does not interfere with the transporters in which the residues are located. The biotin tag in the ester and amide groups connecting the acyl chains in Lipid-A (in green stick with the same arginine and carboxyl residues as in form salt-bridges within each half-transporter (indicated by circles with interactions of Lipid-A with the ring residues.

Discussion

During cell growth and division of Gram-negative bacteria, phospholipids and core Lipid-A are synthesised in the inner membrane with the two half-transporters in light blue and light orange. The basic residues are important for the interactions with the two anionic phospho-glucosamine moieties and 1 and 4 phosphate group (in orange spheres) and the glucosamine, ester and amide groups connecting the acyl chains in Lipid-A (in green stick representation). The colours of the residue numbers refer to the half-transporters in which the residues are located. The biotin tag in the disaccharide core of our biotin-Lipid-A (Fig. 2a) does not interfere with the interactions of Lipid-A with the ring residues.

Lipid-A. The basic residues are important for the interactions with PE but inhibit the interactions with cationic ethidium. Instead, ethidium transport is facilitated by the presence of the acidic residues.

In our Lipid-A and PE transport assays, we measured the metabolic energy-dependent floppase activity of purified MsbA in proteoliposomes, prepared with E. coli lipids and egg yolk PC, as a relocation of biotinylated lipid from the external membrane surface of proteoliposomes to the internal membrane surface. The biotin-lipid remaining in the outer leaflet of the membrane is detected by the binding of membrane-impermeable avidin (Fig. 1a). Control experiments show that free biotin is neither transported by MsbA, nor liberated from the biotinylated lipids in the time course of the assays (Supplementary Fig. 2). In further control experiments, the lipid floppase activity (i) does not occur in empty liposomes but requires functional MsbA protein, (ii) is inhibited by the MsbA inhibitor G907, and (iii) is affected by the ring residues in the substrate binding chamber that are known to coordinate Lipid-A binding (Figs. 1–5). The location of the biotin tag in the disaccharide core of our biotin-Lipid-A (Fig. 2a) ensures that the biotin does not interfere with the interactions of Lipid-A with the ring residues (Fig. 3a, b). Given the biotin-labelling of 1 in 17 PE molecules in our proteoliposomal membranes, the excess of unlabelled PE over biotin-Lipid–A, and the chemical differences between the biotinylated lipids and the native lipids they mimic, the measured PE and Lipid-A transport activities most likely underestimate the in-situ activity of MsbA as a lipid floppase.

The findings in this study represent important advancements in our understanding of lipid transport by MsbA. First, the process of Lipid-A transport from the plasma membrane to the outer membrane in E. coli requires two major transport systems, MsbA and LptB2FGCADE, the activity of which could be intrinsically connected. As summarised in the schematic in Fig. 6a–c, our results demonstrate that MsbA can mediate the transport of Lipid-A and PE in the absence of auxiliary proteins. Therefore, MsbA functions as an independent lipid floppase in the plasma membrane that supports LptB2FGCADE, and possibly also the MlaFEDB phospholipid translocases, in antegrade lipid trafficking from the outer leaflet of the plasma membrane to the outer membrane. The amount of LPS, and its underacetylated forms, versus phospholipid that is ultimately transported to the outer membrane might, therefore, be regulated at the level of MsbA. Second, we show that the activity of ATP-dependent biotin-PE transport in proteoliposomes is strongly stimulated by the imposition of a ΔpH (interior acidic) (Figs. 1 and 6c). The repetition of these measurements with long-acyl-chain (2 x C18) NBD-PE also demonstrates the importance of the ΔpH + ATP in the MsbA-mediated transport of this lipid (Supplementary Fig. 7), and extends previous observations on the low ATP-dependent transport activity of MsbA for long-acyl-chain NBD-PE in proteoliposomes. As the efficient transport of ethidium, chloramphenicol and erythromycin by MsbA requires the input of ATP and ΔpH (Fig. 6b), in which the ATPase reaction is stimulated by the ΔpH, the energetics of PE transport is most similar to that of small-molecule transport. On the other hand, the Lipid-A transport reaction is ATP-dependent without a noticeable contribution of the ΔpH (Figs. 2 and 6a). The requirement for ATP binding and hydrolysis in Lipid-A and PE transport is in agreement with the phospholipid and Lipid-A-stimulated ATPase activity of purified MsbA and the dimerisation of the nucleotide-binding domains in the MsbA dimer upon Lipid-A binding. We conclude that the role of the ΔpH in ATP-dependent lipid transport by MsbA is independent of the biotin modification but is linked to the structural properties of the transported lipid. Third, our data suggest that lipids and drugs share similar pathways in MsbA. Both types of substrates are transported via the central substrate binding chamber of MsbA where they interact with the same ring of side chains in the substrate binding chamber. R78, R148, R296, K299, and interspersed D41, E149 and D252 are known to form hydrophilic interactions with the two anionic phospho-glucosamine moieties in Lipid-A in inward-facing MsbA and to rotate away in the...
outward-facing state, thus enabling electrostatic interactions between R78-D41 and R296-E149 and facilitating lipid release (Fig. 3). These basic and acidic residues are important for Lipid-A transport in our experiments (Figs. 2, 4 and 6a). The arginine residues in the ring also support the transport of the smaller PE ligand that contains a single phosphodiester moiety but no glucosamine groups (Figs. 1 and 6c). In agreement with the PE data, basic residues contribute to the coordination of the phosphodiester moiety of phospholipid substrates in the crystalised lipoxygenase enzyme LOX34 and periplasmic phospholipid transport protein Ttg2D35 from Pseudomonas aeruginosa. Reported molecular dynamics simulations for the phospholipid scramblase nhTMEM16 from Nectria haematococca also point to a role of basic residues in the coordination of phospholipid binding36. Although the R78, R148, R296 residues in the ring are essential for PE transport, they are inhibitory for ethidium
transport. In particular, the ethidium efflux rate and apparent binding affinity increased with the removal of basic residues in the MsbA-R78A R148A and MsbA-TripRA mutants (Fig. 5). The replacement of K299 in the ring by A (MsbA-K299A) showed a similar enhancement of ethidium transport (Fig. 5a) but did not affect the transport of PE (Fig. 1h). The binding sites for PE and ethidium are, therefore, partially overlapping. As our biotin-PE contains C18 acyl chains, whereas structural evidence suggests that the interior binding chamber can accommodate Lipid-A with C12 and C14 acyl chains,23, the possibility exists that, similar to small molecules, only the headgroup of biotin-PE is accommodated in the central binding chamber. The acyl chains would remain in the phospholipid bilayer. Related mechanisms were proposed for phospholipid flipping by eukaryotic type IV P-type ATPases,4,37 and the transport of lipid-linked oligosaccharide by the ABC floppase PglK from Campylobacter jejuni.28 However, with two acyl chains in PE versus 6 acyl chains in Lipid-A, the acyl chain region in PE is much less densely packed and, due to the rotational freedom around the sp3 hybridised carbon atoms, it might have sufficient conformational flexibility to be accommodated in the chamber.

In the transport of ethidium by MsbA, the carboxylates in the inward-facing state (Fig. 5). We suggest that, following the binding of cationic ethidium near the ring carboxylates in the inward-facing state, proton binding to these transport. In particular, the ethidium efflux rate and apparent binding affinity increased with the removal of basic residues in the MsbA-R78A R148A and MsbA-TripRA mutants (Fig. 5). The replacement of K299 in the ring by A (MsbA-K299A) showed a similar enhancement of ethidium transport (Fig. 5a) but did not affect the transport of PE (Fig. 1h). The binding sites for PE and ethidium are, therefore, partially overlapping. As our biotin-PE contains C18 acyl chains, whereas structural evidence suggests that the interior binding chamber can accommodate Lipid-A with C12 and C14 acyl chains,23, the possibility exists that, similar to small molecules, only the headgroup of biotin-PE is accommodated in the central binding chamber. The acyl chains would remain in the phospholipid bilayer. Related mechanisms were proposed for phospholipid flipping by eukaryotic type IV P-type ATPases,4,37 and the transport of lipid-linked oligosaccharide by the ABC floppase PglK from Campylobacter jejuni.28 However, with two acyl chains in PE versus 6 acyl chains in Lipid-A, the acyl chain region in PE is much less densely packed and, due to the rotational freedom around the sp3 hybridised carbon atoms, it might have sufficient conformational flexibility to be accommodated in the chamber.

In the transport of ethidium by MsbA, the carboxylates in the ring are essential for transport activity (Fig. 5). We suggest that, following the binding of cationic ethidium near the ring carboxylates in the inward-facing state, proton binding to these carboxylates in the outward-facing conformation aids in the dissociation of the bound ethidium towards the extracellular environment and the reorientation of MsbA to the inward-facing state (Fig. 6b). The transport cycle is completed by the deprotonation of the carboxyl residues in the cellular interior and binding of ethidium. In this way, the ATP-dependent ethidium transport reaction in cells is facilitated by the imposition of the ΔpH (interior alkaline) across the plasma membrane. Proton coupling in ATP-dependent biotin-PE transport will involve protonatable groups other than D41, E149 and D252.

In conclusion, our findings demonstrate MsbA-mediated Lipid-A and PE transport with different energy requirements and provide important details in the structural mechanisms proposed for MsbA based on X-ray crystallography and cryo-EM techniques. MsbA transports lipids and amphiphilic drugs out of cells by shared translocation pathways. The lipid availability in the membrane can, therefore, affect the drug transport activity and vice versa. This conclusion will also be relevant for other multidrug transporters that can transport drugs and lipids as alternative substrates.

Materials and methods

Compound synthesis. The synthesis of biotin-labelled E. coli Lipid-A from the glutaryl glucose linker-containing Lipid-A derivative and biotin-dPEG4™ hydrazide (Invitrogen) is described by Fujimoto et al.24. The selective MsbA

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**Fig. 5 Ethidium transport by MsbA in L. lactis. a** ATP-depleted cells were preloaded with 2 μM ethidium after which ethidium efflux was initiated by the addition of glucose (+Glc) as a source of metabolic energy.11,15 Active efflux by MsbA-WT was abolished in the presence of 2.5 μM of the MsbA inhibitor G907 (0.7x the reported in-vivo IC50 in cells).23 MsbA-mediated ethidium efflux was inhibited by the triple DED mutations in the substrate binding chamber and was enhanced by K299A and sequential R-to-A replacements in the chamber. Fluorescence traces are the mean of three independent experiments. Values in histograms show significance of fluorescence levels near 600 s. All mutants were equally well expressed as MsbA-WT in the plasma membrane (Supplementary Figs. 5 and 6). b Effect of R-to-A mutations on the apparent affinity (Km) of MsbA for ethidium in the transport reaction. Histogram data represent observations in three experiments (n = 3) with independently prepared batches of cells and are expressed as mean ± s.e.m. (one-way analysis of variance; **P < 0.01; ***P < 0.001; ****P < 0.0001). Asterisks above the square brackets refer to comparisons with MsbA-WT, whereas asterisks above the horizontal line refer to comparisons with the non-expressing control.
inhibitor G907 was synthesised by the procedure reported by Ho et al. and is described in full in the Supplementary Methods.

Bacterial strains, mutagenesis and protein expression. The drug-hypersensitive L. lactis NZ9000 ΔlmtA ΔlmtCD\(^\text{\textregistered}\) lacking expression of the endogenous multidrug transporters LmrA and LmrCD, was used for the expression of His\(_6\)–tagged MsbA proteins from nisin-inducible pNZ0048 plasmids in the nisin controlled expression (NICE) system (NIZO Food Research, The Netherlands). The expression of MsbA proteins from arabinose-inducible pBAD24 plasmid (ATCC product 87399) in E. coli W2D2 cells is described below under Cell growth experiments.

For site-directed mutagenesis of MsbA, the pGEM-SEI(+) (Promega)-derived cloning vector pGEM-MsbA containing coding regions for an amino-terminal His\(_6\)-tag and thrombin cleavage site followed by the wild-type MsbA-TripRA or D41N E149Q D252N (referred to as MsbA-DED) were diluted into bacteria. The mutant genes were sequenced and previously described mutants were sequenced in full in the Supplementary Methods.

Preparation of inside-out membrane vesicles. Lactococcal cells from 2 L culture were harvested at 4 °C by centrifugation (13,000 × g, 10 min). The cell pellet was washed once (100 mM K\(_2\)PO\(_4, \text{pH} 7.0\)) and resuspended in 25 mL of the same buffer. Chicken egg white lysozyme (3 mg mL\(^{-1}\), Sigma-Aldrich) was added together with half a tablet of Complete Protease Inhibitor Cocktail (Sigma-Aldrich) and the mixture was incubated for 30 min at 30 °C. To lyse the cells, the mixture was passed twice through a cell disrupter (Basic Z 0.75-kW Benchtop Cell Disrupter, Constant Systems) at 20,000 p.s.i. Subsequently, 10 µM D\(_{\text{18}}\) and 10 mM Mg\(_2\)O were added, and the resultant mixture was incubated for 30 min at 30 °C to digest DNA and RNA. In all, 15 mL K\(_2\)EDTA (pH 7.0) was then added, and the mixture was centrifuged for 40 min at 13,000 × g and 4 °C. The supernatant containing the membrane vesicles was transferred to a clean tube and centrifuged for 1 h at 125,000 × g and 4 °C. The membrane vesicle pellet was resuspended in 50 mM K\(_2\)Pi buffer (pH 7.8) containing 10% (v/v) glycerol to a total membrane protein concentration of 60 mg mL\(^{-1}\). The membrane vesicle suspension was stored in aliquots in liquid nitrogen. The presence of MsbA proteins in membrane vesicles was assessed on western blot probed with a 1:1000 (v/v) dilution of primary mouse antibody (Sigma-Aldrich, cat. no.: A4416). The histidine-tagged MsbA signals were detected by chemiluminescence using SuperSignal™ West Pico PLUS Chemiluminescent Substrate (ThermoFisher Scientific) and Odyssey Fc Imaging System (Li-Cor Biosciences).

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7.0, 0.1 M NaCl, 5% (v/v) glycerol, 0.05% (w/v) DDM and 150 mM imidazole (pH 8.0). The purified protein was kept on ice and used for further experiments immediately.

Preparation of proteoliposomes containing biotinylated lipids. Purified MsbA proteins were reconstituted in proteoliposomes in an inside-out fashion. For this purpose, 8 mg of liposomes were prepared from (i) acetone-ether-washed total lipid extract from E. coli, (ii) egg yolk phosphatidylcholine, and (iii) 18:1 Biotinyl Cap PE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-cap biotinyl), referred to as biotin-PE (all purchased from Avanti Polar Lipids Inc.) mixed at a ratio of 3:1:0.2 (w/w/w) in chloroform. For the preparation of biotin-Lipid-A containing proteoliposomes, the biotin-PE was replaced by 0.5% (w/v) biotin-Lipid-A24 (stored at 0.5 mg mL⁻¹ in chloroform:methanol 9:1). The solvent was evaporated from the lipid mixture as described above and a lipid monolayer was deposited on an aqueous subphase (N₂ gas) and the lipid monolayer was hydrated in Buffer 1 (20 mM KPi, 100 mM NH₄SCN, 50 mM K₂SO₄, pH 6.8) at a concentration of 4 mg mL⁻¹. Using a Liposofast Basic extruder (Avestin), lipids were extruded 11 times through a 400 nm polycarbonate filter to form unilamellar liposomes of homogenous size. The extruded liposomes were destabilised by adding Triton X-100 until the maximum OD₅₄₀ was just passed. For reconstitution, 160 μg of purified protein was mixed with destabilised liposomes in a ratio of 1:50 (w/w) to a final concentration of 80 μg mL⁻¹. The mixture was left shaking gently at RT for 30 min. To remove detergent, the proteoliposomes were successively incubated with polystyrene bio-beads (Bio-Bead SM-2, Bio-Rad): 80 mg mL⁻¹ at room temperature for 2 h, 8 mg mL⁻¹ at 4 °C for 2 h, and finally 0.5 mg mL⁻¹ at 4 °C for 18 h. Before use, the bio-beads were pre-washed 3 times with methanol, once with ethanol, four times with ultrapure water and once with Buffer 1. Ready-made proteoliposomes were harvested by centrifugation at 165,000 × g and 4 °C for 20 min and resuspended in 200–300 μL Buffer 1. Samples were kept on ice and immediately used in further experiments.

Biotin-lipid floppase assays. MsbA-containing proteoliposomes and empty liposomes containing 3% (w/w) biotin-PE or 0.5% (w/v) biotin-Lipid-A were diluted 50-60-fold in Buffer 1 in which they were prepared (no gradient control) or Buffer 2 (20 mM KPi, 100 mM KSCN, pH 8.0) to impose a ΔpH (interior acidic). All buffers were pre-warmed at 30 °C and supplemented with 5 mM MgS₂O₄ and, where indicated, 5 mM Mg·ATP. Following dilution, the proteoliposomes were incubated for 20 min at 30 °C. Subsequently, 9 to 15 samples of 10 μL of each liposome population were mixed with 90 μL working solution of Pierce™ Fluorescence Biotin Quantiitation Kit. The mixture was incubated at RT for 10 min, and the fluorescence intensity was measured in a CLARIOstar plate reader (BMG Labtech) with excitation and emission wavelengths of 494 nm and 520 nm, respectively. For biotin-Lipid-A analyses, the F₃₅₅₆/F₃₈₇₅₆ ratios were divided by the F₃₅₅₆/F₉₅₀ for each data point × 100%, where p corresponds to MsbA-containing proteoliposomes, 1 corresponds to empty liposomes (without MsbA protein), e corresponds to the treatment with ATP or ΔpH+ATP and c corresponds to the control treatment without metabolic energy. For biotin-PE analyses, F₉₅₀/F₃₅₅₆ and F₉₅₀/F₃₈₇₅₆ ratios for the different treatments (ATP, ΔpH+ and ΔpH−ATP) were compared with the control treatment (without metabolic energy set at 100%), where p corresponds to MsbA-containing proteoliposomes, 1 corresponds to liposomes without MsbA protein, and F₉₅₀ and F₃₅₅₆ correspond to the observed fluorescence plateau before and after dithioflavin addition.

Statistics and reproducibility. All experiments were performed at least three times using three independent batches of cells and proteoliposomes. Significance of data was tested by one-way analysis of variance and Tukey’s multiple group comparisons. Asterisks shown in histograms and scatter dot plots either refer to comparisons with control (above the bar) or comparisons of specific pairs (horizontal brackets): ***p < 0.005; **p < 0.01; *p < 0.05; **p < 0.001; ****p < 0.0001.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

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Data availability
Data that support the findings of this study have been deposited in the University of Cambridge research repository Apollo with link https://doi.org/10.17863/CAM.75215 (ref. 4) or are available from the corresponding author upon reasonable request. The source data for the figures and supplementary figures are included in Supplementary Data 1. The DNA sequences of pNZ-MsbA, pGEM-MsbA and pBAD-MsbA plasmid and the wild-type and mutant mbtA genes, as well as the nucleotide sequences of the MsbA proteins, are included in Supplementary Data 1. Requests for unique materials should be addressed to the corresponding author. The plNZ2 plasmids utilise the nisin controlled expression (NICE) system, which was obtained from NIZO Food Research, The Netherlands.
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Author contributions

D.G., H.S., C.G., Y.T., T.N. and H.W.v.V. planned and performed cloning, protein expression and reconstitution, lipid flippase and drug resistance and drug transport experiments. A.S. and K.F. planned and performed the synthesis of bioin-Lipid-A. S.M.R. and D.R.S. planned and performed the synthesis of MsbA inhibitor G907. H.W.v.V. and D.G. wrote the manuscript with input from the other authors. Supplementary Information on G907 synthesis was written by S.M.R. and D.R.S.

Competing interests

The authors declare no competing interests.

Additional information

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