ATP-dependent substrate transport by the ABC transporter MsbA is proton-coupled

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ATP-binding cassette transporters mediate the transbilayer movement of a vast number of substrates in or out of cells in organisms ranging from bacteria to humans. Current alternating access models for ABC exporters including the multidrug and Lipid A transporter MsbA from Escherichia coli suggest a role for nucleotide as the fundamental source of free energy. These models involve cycling between conformations with inward- and outward-facing substrate-binding sites in response to engagement and hydrolysis of ATP at the nucleotide-binding domains. Here we report that MsbA also utilizes another major energy currency in the cell by coupling substrate transport to a transmembrane electrochemical proton gradient. The dependence of ATP-dependent transport on proton coupling, and the stimulation of MsbA-ATPase by the chemical proton gradient highlight the functional integration of both forms of metabolic energy. These findings introduce ion coupling as a new parameter in the mechanism of this homodimeric ABC transporter.
TP-binding cassette (ABC) multidrug exporters are embedded in the plasma membrane and actively extrude cytotoxic drugs from the cell. They play a critical role in the failure of pharmacological treatment of microbial diseases and cancers, affect drug pharmacokinetics in mammals and are a prime target for clinical research. Some of these transporters, including the mammalian multidrug resistance P-glycoprotein ABC1 and its bacterial homologues MsbA and LmrA, transport lipids and chemotherapeutic drugs from the inner leaflet of the plasma membrane to the outer leaflet and extracellular environment.

ABC exporters are thought to utilize the free energy from ATP-binding and hydrolysis at two nucleotide-binding domains (NBDs) to transport substrates via a translocation pathway that is formed by two membrane domains (MDs). In ABC1, these four domains are fused on a single polypeptide, whereas in bacterial MsbA and LmrA, an MD is fused to an NBD in a half-transporter that homodimerizes to form the full transporter. Current structural and biochemical data support an ‘alternating access’ model in which the substrate-binding sites in the MDs are exposed to either side of the membrane, as the transporter alternates between inward-facing and outward-facing conformational states. The transition from the inward-facing conformation to the outward-facing conformation is governed by ATP-binding-associated NBD dimerization, often referred to as ‘the power stroke’, after which ATP hydrolysis and ADP-and-Pi-release-dependent NBD dissociation reinserts the transporter to the inward-facing conformation. However, many important details of this mechanism remain to be elucidated. MsbA transports cytotoxic agents and the Lipid A anchor of lipopolysaccharides, and is an essential transporter in many Gram-negative bacteria. Here we show for Escherichia coli MsbA that ATP binding and hydrolysis are insufficient to drive drug transport in the absence of an electrochemical proton gradient. We conclude that proton coupling is essential in the nucleotide-dependent power stroke in MsbA.

**Results**

**Studies in intact cells.** Energy coupling by MsbA was first studied in ATP-depleted Lactococcus lactis cells with a very low internal ATP concentration of ~7 µM (ref. 21) that were preloaded with 2 µM ethidium by reversed transport by MsbA. A steady state was reached, the addition of glucose raised the intracellular ATP concentration to ~9 mM (ref. 21), and initiated a significant ethidium efflux activity by wild-type MsbA (MsbA-WT) compared with the non-expressing control (Fig. 1c,d). Surprisingly, ethidium efflux was also observed for cells containing MsbA-MD (Fig. 1c,d), a truncated form of MsbA-WT that lacks the NBD and that is expressed in a similar orientation and at a moderately elevated level (117%) in the plasma membrane compared with MsbA-WT (Fig. 1a,b). To investigate the possibility that transport by MsbA-MD in these cells is dependent on an electrochemical proton gradient, also referred to as the proton motive force (Δp, interior positive and acidic), or one of its components, the transmembrane pH gradient (ΔpH) and electrical membrane potential difference (Δψ), measurements of ethidium efflux by MsbA-MD were repeated in cells in which the magnitude and composition of the Δp (= Δψ – ZΔpH in which Z is approximately equal to 58 mV at 20°C) was manipulated with the ionophores nigericin and valinomycin. The results show that ethidium efflux by MsbA-MD was completely inhibited in the presence of the Δψ only. In contrast, significant efflux was observed in the presence of the ΔpH only (Fig. 1e). The results for MsbA-WT (Fig. 1f) showed similarities with those for MsbA-MD, and both were clearly different from non-expressing control cells for which no ethidium efflux was observed (Fig. 1g). Previous studies in cells highlighted the dependency of ethidium efflux by MsbA-WT on ATP binding and hydrolysis; the efflux activity is strongly inhibited by impairment of the MsbA-ATPase activity down to 4–6% of WT activity through the deletion of the Walker A lysine residue at position 382 (ΔK382 mutation). Indeed, although the expression level of MsbA-ΔK382 was only slightly below that of MsbA-WT (77%; Fig. 1a), the ethidium transport activity of the mutant was strongly inhibited (Fig. 1c,d). Taken together, these findings suggest that MsbA-mediated ethidium efflux is dependent on both the electrochemical proton gradient and ATP hydrolysis.

Proton-coupled substrate transport in proteoliposomes. To investigate the dependence of transport activity of MsbA on the electrochemical proton gradient in the absence of nucleotides and other components, MsbA-WT, MsbA-MD, MsbA-ΔK382 and the transport-inactive triple mutant MsbA-DED (D41N in transmembrane helix (TMH) 1, E149Q in TMH 3 and D252N in TMH 5) were affinity-purified and reconstituted in proteoliposomes prepared from E. coli phospholipids. Unlike whole cells, spheroplasts and plasma membrane vesicles, these proteoliposomes are devoid of cytoplasmic constituents and alternative primary-active and secondary-active transporters, allowing studies on the transport and energetics of purified MsbA proteins in the absence of energy-transducing transport processes. The MsbA proteins incorporated equally well in proteoliposomes and were present in an inside-out orientation (Fig. 2a,b). Purified MsbA-WT and MsbA-MD samples used for the reconstitution experiments were examined by LC-MS/MS mass spectrometry. This analysis confirmed the lack of the native NBD in the MsbA-MD protein (Fig. 2c). The Mascot database was also searched against the UniProt L. lactis subsp. lactis database, which demonstrated insignificant levels of contaminating membrane transporters and ABC NBDs (Supplementary Data 1), below 0.01% for MsbA-WT and 0.7% for MsbA-MD when the exponentially modified protein abundance index was used as a measure for the protein abundance.

To study the functionality of the MsbA proteins in the proteoliposomes, a ΔpH (interior acidic) was generated by pH jump (Fig. 3). In this method, 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 pH jump (pHin 6.8/pHout 8.0). This pH difference was sustained by dissociation of NH4+ in the lumen of the proteoliposomes and the outward diffusion of NH3. The Δψ (interior positive) was imposed by diffusion of SCN– from the lumen down an outwardly directed chemical gradient ([SCN–]in/[SCN–]out = 100 mM versus 1 mM). No changes in ethidium fluorescence were observed upon imposition of ΔpH and/or ΔpH in liposomes lacking MsbA proteins (Fig. 4a) or containing inactive MsbA-DED (Fig. 4b). These results are consistent with the mass spectrometry data showing the absence of contaminating membrane transporters in our protein preparations (Fig. 2c and Supplementary Data 1). However, for both MsbA-WT (Fig. 4c) and MsbA-MD (Fig. 4d), ethidium transport in the proteoliposomes with the imposed ΔpH (interior acidic) was significantly higher, more than fivefold for MsbA-WT compared with the equilibration level in the no-gradient controls (pHin 6.8/pHout 6.8 and pHin 8.0/pHout 8.0). These results point to concentrative ΔpH-dependent accumulation of ethidium. In contrast, uptake of ethidium by MsbA-WT and MsbA-MD was not stimulated in the presence of a reversed ΔpH (ΔpHREV, interior alkaline), which was imposed by the passive diffusion of acetic acid from the lumen of the proteoliposomes.
Upon the imposition of the $\Delta c$ plus $\Delta pH$ ($\Delta p$, interior positive and acidic), ethidium transport was above control but was reduced compared with the activity obtained in the presence of the $\Delta pH$ only (Fig. 4c,d). As these results suggested that the imposed $\Delta c$ (interior positive) was inhibitory for ethidium transport in proteoliposomes, the effect of reversed $\Delta c$ ($\Delta c_{REV}$, inside negative) was tested. The $\Delta c_{REV}$ was imposed in the proteoliposomes by the electrogenic downhill diffusion of $K^+$ from the lumen to the external buffer by valinomycin (added at 10 nmol (mg of protein)$^{-1}$; Fig. 3), and was found

Figure 1 | Ethidium efflux in intact cells. (a) Immunoblot probed with anti-polyhistidine tag antibody (left) shows that MsbA-MD and MsbA-$\Delta K382$ are expressed in the plasma membrane of L. lactis (5 $\mu$g total membrane protein per lane) at 117% and 77% of MsbA-WT, respectively, and that these proteins are absent in control cells (Ctrl). The migration of molecular mass markers is indicated. Histogram (right) shows MsbA signal intensities. (b) Availability of the cytosolic NH$_2$-terminal His-tag in MsbA-WT and MsbA-MD to cleavage by proteinase K (+PK) at the external side of right-side-out (RSO) or inside-out (ISO) membrane vesicles (3 $\mu$g protein per lane). Incubation without the protease (-PK) served as control. Uncleaved His-tag was detected on immunoblot (left). Signal intensities are shown in the histogram (right). (c) Efflux of monovalent cationic ethidium was initiated by the addition of 20 mM glucose (Glc) as a source of metabolic energy to ATP-depleted cells that were preloaded with 2 $\mu$M of the dye. Efflux was observed for MsbA-WT but not for non-expressing control or MsbA-$\Delta K382$, which exhibits a strongly reduced ATPase activity due to the absence of the catalytic Walker A lysine residue. Remarkably, ethidium efflux was also observed for a truncated form of MsbA-WT that lacks the NBD (MsbA-MD). (d) Histogram shows significance of fluorescence levels in (c) at $t = 400$ s. (e–g) Ethidium efflux from cells containing MsbA-MD (e), MsbA-WT (f) or no MsbA proteins (g) to which ionophores nigericin ($\Delta c$ only, interior negative), valinomycin ($\Delta pH$ only, interior alkaline) or both (no $\Delta p$) were added at concentrations of 1.0 and 0.1 $\mu$M, respectively, 3 min prior to the addition of the glucose. Data represent observations in 3 or more independent experiments with independently prepared batches of cells. Values in histograms are expressed as mean $\pm$ s.e.m. (one-way analysis of variance; $^* P < 0.05$; $^{**}* P < 0.01$; $^{***}* P < 0.001$; $^{****}* P < 0.0001$).
to stimulate ethidium transport in the proteoliposomes, also when combined with the ΔpH (interior acidic), yielding ΔpH_{rev} = Δψ_{rev} - ΔpH (Fig. 4e). No increase in ethidium fluorescence was observed under these conditions in liposomes lacking MsbA proteins (Fig. 4f). When taken together in the physiological context of the cell (ΔpH, interior negative and alkaline), these findings indicate that the ΔpH (interior alkaline) supports ethidium efflux by MsbA-WT and MsbA-MD, whereas the Δψ (interior negative) inhibits this activity.

Proton-coupled substrate transport by MsbA proteins was also observed for the neutral antibiotic chloramphenicol. 20. The 100-fold dilution of (proteo)liposomes in dilution buffer...
Proton coupling is functionally linked to ATP hydrolysis. In view of the finding that ethidium transport by MsbA is dependent on ATP hydrolysis (Fig. 1c) and components of the Δp (Figs 1f and 4c,e), the relationship between these two forms of metabolic energy was further studied in proteoliposomes. For this purpose, ethidium uptake in MsbA-WT-containing proteoliposomes was measured in the absence or presence of the imposed Δp (inside acidic; pH_{in} 6.8/pH_{out} 8.0) in buffer containing 2.5 mM Mg-ATP or non-hydrolysable nucleotide analogue AMP-PNP. Remarkably, the ATP did not initiate ethidium accumulation in the absence of the Δp, nor did the nucleotide enhance accumulation against the inwardly directed drug concentration gradient that is driven by a ΔpH across the membrane. These data suggest that the conformational changes in MsbA-WT associated with ethidium transport can occur in the absence of ATP in a reaction driven by a ΔpH and ΔpH_{rev} (interior negative, ΔpH or in empty liposomes without MsbA proteins (Fig. 5a,b). Thus, the proton dependence of MsbA-mediated transport is observed for two different substrates, chloramphenicol and ethidium, with different charge profiles containing inside-out oriented MsbA-WT or MsbA-MD as described in the main text. Inclusion of DNA in the lumen allows the recording of the fluorescence emission of accumulated ethidium.

Proton-coupled ethidium efflux by MsbA-WT is inhibited by the 8.0; Fig. 6b,c) or in which the ΔpH was not imposed (pH_{in}/pH_{out} set at 8.0/8.0; Fig. 6d). In these experiments, the local pH near the MsbA-NBD at the external side of the membrane remained constant. These data suggest that the conformational changes in MsbA-WT associated with ethidium transport can occur in the absence of ATP in a reaction driven by a ΔpH and ΔpH_{rev}. However, when ion gradients are imposed in the presence of ATP, proton coupling becomes functionally linked to ATP binding and hydrolysis, which are required to drive the dimerization and dissociation of the NBDs during the propagation of the transport cycle. Although the MsbA-ΔK382 mutant can operate in a ΔpH-dependent manner in the absence of ATP, the addition of ATP traps this mutant in an ATP-bound state and renders it transport-inactive (Fig. 6e) in an analogous manner as observed in ATP-containing cells (Fig. 1c,d). This inhibitory trapping was mimicked by the addition of the non-hydrolysable AMP-PNP to MsbA-WT (Fig. 6a). The inhibitory effect of AMP-PNP on ΔpH-dependent ethidium accumulation in the proteoliposomes was not observed for MsbA-MD lacking the NBD (Fig. 6f).

MsbA-WT is more efficient than MsbA-MD. The observations on active drug transport by MsbA-MD raise questions about the functional importance of ATP binding and hydrolysis in full-length MsbA. The direct comparison of the transport activities of MsbA-WT and MsbA-MD in cells show that MsbA-WT catalyses the ATP-coupled transport of chloramphenicol to lower intracellular steady-state levels than MsbA-MD (Fig. 1c,d). When cell growth was measured in the presence of the MsbA substrate erythromycin, a protein expression caused significant shifts in the erythromycin concentration at which the growth rate is half-maximal (IC_{50}), from 0.004 μM or the non-expressing control to 0.393 μM (P = 0.008) for MsbA-WT and 0.094 μM (P = 0.003) for MsbA-MD; the IC_{50} for MsbA-ΔK382 (0.051 μM) was close to control (Fig. 6g). Hence, the enhanced efficiency of efflux by full-length MsbA compared with the NBD-less protein was also found in the ability of the MsbA proteins to confer cellular resistance to the antibiotic erythromycin. The ATP-dependent dimerization of the NBDs with closure of the substrate-binding cavity towards the inside surface of the membrane facilitates capture of substrate from the cellular interior and/or inner membrane leaflet, and enables efflux against a larger drug concentration gradient and/or lipid–water partition coefficient. The ATP dependence therefore enhances the directionality of the transport reaction.

**Discussion** Although MsbA is an ABC transporter that mediates substrate transport in an ATP-dependent manner, the experiments in intact cells and proteoliposomes prepared from _E. coli_ phospholipids demonstrate for the first time that the ATP-binding-associated power stroke during drug transport is assisted by proton coupling via apparent drug–proton antiport. The dissipation of the ΔpH (interior alkaline) in cells by the addition of nigericin blocks MsbA-WT-mediated ethidium efflux (Fig. 1f). Conversely, the artificial imposition of the ΔpH (inside acidic) in proteoliposomes initiates (i) the accumulation of ethidium and chloramphenicol by purified, inside-out oriented MsbA-WT above the equilibration level (Figs 4c and 5a,b) and (ii) proton efflux in a chloramphenicol-dependent manner (Fig. 5c). The role of the ΔpH in MsbA-mediated transport in cells is also supported by the observations on the erythromycin efflux by MsbA-MD against the inwardly directed drug concentration gradient that impairs growth of the non-expressing control cells (Fig. 6g). Proton-coupled ethidium efflux by MsbA-WT is inhibited by the

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**Figure 3 | Schematic showing methods for artificial imposition of electrochemical ion gradients in proteoliposomes.** The ΔpH (interior positive), Δp (interior acidic), ΔpH (interior positive and acidic), ΔpH_{rev} (interior negative), ΔpH_{rev} (interior negative and acidic), or ΔpH_{rev} (interior alkaline) were imposed by 100-fold dilution of proteoliposomes containing inside-out oriented MsbA-WT or MsbA-MD as described in the main text. Inclusion of DNA in the lumen allows the recording of the fluorescence emission of accumulated ethidium.
**Figure 4 | Ethidium transport in proteoliposomes.** (a–d) Ethidium transport in DNA-loaded empty liposomes (a) or proteoliposomes containing the MsbA-DED triple mutant (b), MsbA-WT (c) or MsbA-MD (d) with imposed ΔpH (pH<sub>in</sub> 6.8/pH<sub>out</sub> 8.0), Δψ (interior positive), proton-motive force (Δp = Δψ − ΔpH in which Z equals −58 mV at 20 °C), Δψ<sub>REV</sub> (pH<sub>in</sub> 8.0/pH<sub>out</sub> 6.8), or in the absence of ion gradients (pH<sub>in</sub> 6.8/pH<sub>out</sub> 6.8, termed No gradient and pH<sub>in</sub> 8.0/pH<sub>out</sub> 8.0, No gradient B/8). No gradient B/8 for (a,b,d) was very close to the No gradient control, and is not shown for clarity of presentation. The 5-fold accumulation of ethidium by MsbA-WT is indicated in the fluorescence versus time graph in (c). (e,f) Effect of the imposition of a reversed Δψ<sub>REV</sub> (interior negative) without or with the ΔpH (interior acidic) (Δψ<sub>REV</sub> = Δψ<sub>REV</sub> − ZΔpH) on ethidium transport in proteoliposomes containing MsbA-WT (e) or empty liposomes (f). Data represent observations in three or more independent experiments with independently prepared batches of proteoliposomes. Values in histograms show significance of fluorescence levels at steady-state, and are expressed as mean ± s.e.m. (one-way analysis of variance; *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001).

Δψ (interior negative) in cells (Fig. 1f) and equivalent Δψ (interior positive) in the proteoliposomes (Fig. 4c). Together with the observed stimulation of transport in proteoliposomes by the Δψ<sub>REV</sub> (interior negative; Fig. 4e), the data point to apparent electrogenic antiport of ethidium <sup>+</sup> and nH<sup>+</sup> with n<1. Thus, two or more ethidium <sup>+</sup> molecules are exchanged per H<sup>+</sup> which is consistent with the presence in this type of ABC transporter of two cavities at the MD–MD interface that are related by twofold pseudosymmetry and that can be separated by mutation<sup>[10,24,26,27]</sup>. Proton-coupled transport is associated with the MD of MsbA; the observations on ΔpH dependence for MsbA-WT in intact cells and proteoliposomes could all be reproduced using MsbA-MD that lacks the NBD (Figs 1e and 4d).

Evidence was obtained that proton coupling operates in conjunction with a functional catalytic cycle at the NBDs when nucleotide is present. First, ethidium efflux in metabolically active cells containing mM concentrations of ATP<sup>[22]</sup> was inhibited by the MsbA-ΔK382 mutation (Fig. 1c). As the NBDs are conformationally coupled to the MDs, the reduced rate of ATP hydrolysis in the mutant will cause more persistent binding of the nucleotide, which in turn will block the propagation of the catalytic cycle, and, hence, inhibit transport. Second, for MsbA-WT this transport reaction in proteoliposomes was significantly inhibited by the inclusion of the non-hydrolysable ATP analogue AMP-PNP in the external buffer (Fig. 6a). Third, ΔpH (interior acidic)-dependent ethidium accumulation in proteoliposomes by MsbA-ΔK382 was inhibited by the addition of Mg-ATP to the external buffer where the NBDs reside (Fig. 6e). Fourth, the addition of ATP or AMP-PNP had no effect on ΔpH (interior acidic)-dependent ethidium accumulation in proteoliposomes containing MsbA-MD without the NBD (Fig. 6f). Finally, the imposition of a ΔpH stimulated the MsbA-WT ATPase activity in proteoliposomes (Fig. 6b–d). The dependence of drug transport on the genotype of the expressed or reconstituted MsbA proteins demonstrates that the drug transport activity is not dependent on auxiliary proteins but on MsbA itself. This conclusion is consistent with the mass spectrometry analysis demonstrating insignificant levels of contaminating membrane transporters or NBDs in our protein preparations (Fig. 2c).
Protons can have different roles in the mechanisms of membrane transporters. A role of $H^+$ in primary-active transport was previously described for the P-type $Ca^{2+}$-ATPase (SERCA), in which protons neutralize $Ca^{2+}$-coordinating carboxylates following substrate dissociation, essentially giving primary-active transmembrane proton-$Ca^{2+}$ antiport. $H^+$ binding and movement in proton-coupled secondary-active transporters are also known to induce changes in electrostatic and hydrogen-bonding interactions between interhelix side chains that underlie the conformational transitions associated with proton–substrate symport and antiport. The finding of proton-coupled transport by MsbA suggests that similar mechanistic principles are relevant for ABC exporters. Indeed, recent structural studies on the antibacterial peptide ABC exporter McjD from L. lactis conclude that the conformational transitions required for substrate transport might not all be dependent on ATP binding and hydrolysis. The MsbA data share similarities with observations on the dual mode of energy coupling by the arsenite and antimonite-translocating ArsB protein from E. coli, which acts as a secondary-active metallloid–proton antiporter, but when associated with the ArsA ATPase subunit can utilize ATP for improved extrusion efficiency. The findings for MsbA-MD are reminiscent to those described for the MD of the ABC exporter LmrA from L. lactis, which catalyses apparent ethidium–proton symport, illustrating that the coupled transport of substrate and protons is more widespread among ABC exporters. Our conclusions introduce proton coupling as a new parameter in the mechanism of MsbA, and point to the existence of proton-coupled conformational transitions in its transport cycle. This work is of fundamental importance for our understanding of how ABC exporters operate.

Methods

Bacterial strains and plasmids. The drug-hypersensitive L. lactis strain NZ9000 $\Delta$lnrA $\Delta$lnrCD strain devoid of the endogenous ABC multidrug transporters LmrA and LmrCD was used as a host for expression vector pNZ8048-derived plasmids containing a chloramphenicol resistance marker gene, nisin-inducible nisA promoter, and His-tagged wild-type (WT) or mutant MsbA gene, or truncated MsbA gene encoding the MD only.

Construction of MsbA mutants. To express N terminally His-tagged MsbA-MD, the corresponding region of the msbA gene from E. coli was PCR-amplified from pNZMsbA15 with the forward primer 5′-GGAGGCACTCACCATGGGC-3′ and the reverse primer 5′-GGAGGCACTCACCATGGGC-3′ and the reverse primer 5′-GGAGGCACTCACCATGGGC-3′ to insert a TAA stop codon after the codon for N346, equivalent to H353. The MsbA-WT gene encoding the MD only was followed by ligation of the DNA fragment into the linearized vector pNZ8048 downstream of the nisA promoter, yielding pNZMsbA-MD. For the generation of...
Figure 6 | Relationship between ATP dependence and proton coupling by MsbA proteins. (a) Effect of the presence of 2.5 mM Mg-ATP or non-hydrolyzable nucleotide analogue AMP-PNP on imposed ΔpH (pH_{in} 6.8/pH_{out} 8.0)-dependent ethidium transport by MsbA-WT in DNA-loaded proteoliposomes. Histogram shows significance of fluorescence levels at steady-state. (b,c) MsbA-WT ATPase activity in proteoliposomes in which the ΔpH (pH_{in} 6.8/pH_{out} 8.0) was dissipated in the presence of nigericin (leading to pH_{in} 8.0/pH_{out} 8.0) (b). This action of nigericin was confirmed using proteoliposomes (pH_{in} 6.8/pH_{out} 8.0) loaded with the pH probe BCECF (brown trace), the fluorescence emission of which was enhanced by the increase in the lumen pH from 6.8 to 8.0 by the addition of the ionophore at t = 0 s (orange trace) (c). (d) MsbA-WT ATPase activity in proteoliposomes in the presence of an imposed ΔpH (pH_{in} 6.8/pH_{out} 8.0) or its absence (pH_{in} 8.0/pH_{out} 8.0). Note that the pH near the NBD of MsbA (at the external side of the proteoliposomes) remains constant in the experiments displayed in (b) and (d). (e,f) Experiments as described in (a) in proteoliposomes containing MsbA-ΔK382 (e) or MsbA-MD (f). (g) Erythromycin resistance in cells expressing MsbA-WT (blue squares), MsbA-MD (red circles), MsbA-ΔK382 (green squares) compared to non-expressing control cells (black triangles). Maximum specific growth rate (μ_{max}) was determined at each erythromycin concentration and is presented as a percentage of μ_{max} in the absence of erythromycin. The error bars for some of the data points in (g) were too small to be displayed, and are hidden behind the data point symbols. Data represent observations in 3 or more independent experiments with independently prepared batches of proteoliposomes or cells. Values in histograms are expressed as mean ± s.e.m. (one-way analysis of variance except for (b,d) unpaired student-t test; *P<0.05; **P<0.01; ****P<0.0001).
pNZMsbA-D (D41N E149Q D252N) the following primers were used: D41N (forward) 5'-GCCAGCACTCCTGATTGCTGATC-3', (reversed) 5'-AGGCGCCTGCTGCTGCTGAGGATGTTA-3'; E149Q (forward) 5'-CTGGCTGGACTGCGG-3', (reversed) 5'-AGGACCATGTGACACAGTAGATC-3'; D252N (forward) 5'-CAGCTTCAATGCGCTATGCGCT-3', (reversed) 5'-GTAGCGTGGAGGAGCTGCTG3'. The DNA was sequenced to ensure that only the intended changes were introduced.

**Growth conditions and protein expression.** *L. lactis NZ9000* ΔmraA ΔmrcC was grown overnight in M17 medium (Difco) supplemented with 0.5% glucose and 5 mmol l⁻¹ chloramphenicol at 30 °C to an OD₆₀₀ of 0.5–0.6. For protein expression, cells harbouring pNZMsbA, pNZMsbA-MD, pNZMsbA-AK382 (ref. 16), pNZMsbA-D or pNZ8048 (empty vector) were incubated for 1 h at 30 °C in the presence of a 1:1,000 dilution of the culture supernatant of nisin-A-producing *L. lactis* NZ9007 corresponding to a nisin A concentration of ~10⁻⁹ mg ml⁻¹ (ref. 38), unless stated otherwise.

**Ethidium transport in de-energized cells.** *L. lactis NZ9000* ΔmraA ΔmrcC cells expressing MbaA, MbaA-AK382 or MbaA-MD and non-expressing control cells were grown to an OD₆₀₀ of 0.6, and protein expression was induced for 1 h at 30 °C by 10 µg ml⁻¹ nisin A. Cell pellets from 50 ml culture were harvested by centrifugation (6,500 g for 10 min at 4 °C) and washed with ice-cold washing buffer (50 mM KPi, pH 7.0, containing 5 mM MgSO₄). To deplete intracellular ATP levels, cells were incubated with 0.5 µM of the protonophore 2,4-dinitrophenol for 30 min at 30 °C. The protonophore was removed by centrifugation, followed by washing of cells with the washing buffer. Finally, the cells were resuspended in washing buffer to an OD₆₀₀ of 5.10⁻⁹. Membrane vesicles were isolated by ultracentrifugation. To remove any DNA contamination from the lipid bilayer. Finally, liposomes were extruded 11 times through a 400-nm polycarbonate filter to form unilamellar vesicles of homogenous size and destabilized by the step-wise addition of Triton X-100 which was followed at OD₅₄₀ (ref. 38). For reconstitution, purified protein was reduced with the detergent-destabilized liposomes in a 1/50 ratio (w/w) and incubated at room temperature (RT) for 30 min. Detergent was then removed using polysorbene bio-beads (Bio-Bead SM-2, Bio-Rad). For this purpose, Bio-Beads were pre-washed three times with methanol, then with chloroform, and finally with 8 mg ml⁻¹ Bio-Beads for 2 h at 37 °C and then 8 mg ml⁻¹ Bio-Beads for 2 h at 4 °C. For reconstitution, purified protein was reduced with the detergent-destabilized liposomes in a 1/50 ratio (w/w) and incubated at room temperature (RT) for 30 min. Detergent was then removed using polysorbene bio-beads (Bio-Bead SM-2, Bio-Rad). For this purpose, Bio-Beads were pre-washed three times with methanol, then with chloroform, and finally with 8 mg ml⁻¹ Bio-Beads for 2 h at 37 °C and then 8 mg ml⁻¹ Bio-Beads for 2 h at 4 °C. For reconstitution, purified protein was reduced with the detergent-destabilized liposomes in a 1/50 ratio (w/w) and incubated at room temperature (RT) for 30 min. Detergent was then removed using polysorbene bio-beads (Bio-Bead SM-2, Bio-Rad). For this purpose, Bio-Beads were pre-washed three times with methanol, then with chloroform, and finally with 8 mg ml⁻¹ Bio-Beads for 2 h at 37 °C and then 8 mg ml⁻¹ Bio-Beads for 2 h at 4 °C. For reconstitution, purified protein was reduced with the detergent-destabilized liposomes in a 1/50 ratio (w/w) and incubated at room temperature (RT) for 30 min. Detergent was then removed using polysorbene bio-beads (Bio-Bead SM-2, Bio-Rad). For this purpose, Bio-Beads were pre-washed three times with methanol, then with chloroform, and finally with 8 mg ml⁻¹ Bio-Beads for 2 h at 37 °C and then 8 mg ml⁻¹ Bio-Beads for 2 h at 4 °C. For reconstitution, purified protein was reduced with the detergent-destabilized liposomes in a 1/50 ratio (w/w) and incubated at room temperature (RT) for 30 min. Detergent was then removed using polysorbene bio-beads (Bio-Bead SM-2, Bio-Rad). For this purpose, Bio-Beads were pre-washed three times with methanol, then with chloroform, and finally with 8 mg ml⁻¹ Bio-Beads for 2 h at 37 °C and then 8 mg ml⁻¹ Bio-Beads for 2 h at 4 °C.
we were diluted 100-fold in Buffer i (10 mM K-HEPES (pH 8.0), 10 mM Tris-Cl and 100 mM K2SO4) to impose the ΔpH (interior positive and acidic), Buffer ii (10 mM Tris-Cl (pH 8.0), 10 mM Tris-Cl and 100 mM K2SO4) to impose the ΔpH (interior positive), Buffer iii (10 mM K-HEPES (pH 6.8), 10 mM Tris-Cl, 50 mM (NH4)2SO4 and 100 mM K2SO4) to impose the ΔpH (interior positive). In experiments with the ΔpH(Rev) proteoliposomes in Buffer 2 (see under ‘Reconstitution of purified MsbA proteins’) were diluted 100-fold into Buffer iv (10 mM NMG-HEPES (pH 6.8), 10 mM Tris-Cl and 50 mM (NH4)2SO4) in the presence of 10 mM per mg protein of valinomycin to impose the ΔpH(Rev) (interior negative). Buffer v (10 mM K-HEPES (pH 8.0), 10 mM Tris-Cl and 50 mM K2SO4) to impose the ΔpH (interior acidic), or Buffer vi (10 mM NMG-HEPES (pH 8.0) and 10 mM Tris-Cl) in the presence 10 mM per mg protein valinomycin to impose the ΔpH(Rev) (interior negative and acidic). After 30 s of recording, ethidium bromide (2 μM) was added and fluorescence was measured as a function of time in an LS 55B luminescence spectrometer (Perkin-Elmer Life Sciences) with excitation and emission wavelengths of 500 and 580 nm with slit widths of 10 and 5 nm, respectively.

In control experiments, proteoliposomes were diluted 100-fold in the buffer in which they were prepared (pH6.8/10 mM Tris-Cl and 100 mM K2SO4) to impose the ΔpH. Proteoliposomes prepared in Buffer 1 were diluted 20-fold in Buffer ii as described under ‘Substrate transport in proteoliposomes’ (Fig. 6b). To dissipate the ΔpH, the ATPase reaction was started by the addition of 2.5 mM Mg-ATP (high grade Sigma) and the mixture was kept on ice for 5 min before the measurements of ATPase activity. The OD600 was determined. Pi release between t and t+1 min was measured with wavelengths for excitation at 502 nm and emission at 525 nm, and the samples were subsequently incubated at 0 °C for 10 min. The reaction was terminated by the addition of 10 mM phenylmethylsulfonyl fluoride (from stock in ethanol), after which 3 x SDS-PAGE sample-loading buffer and 1 mM DTT were added. The samples were incubated at RT for 10 min and analysed on immunoblot as described under ‘Preparation of inside-out membrane vesicles’.

**Cytotoxicity assays.** L. lactis expressing MsbA-WT, MsbA-MD or MsbA-AK382, and non-expressing control cells were grown as described under ‘Growth conditions and protein expression’ at 30 °C in 96-well plates in the presence of a range of erythromycin concentrations. Nisin A was added at a concentration of 5 pg/ml to induce protein expression, and growth was monitored by measuring OD600 in a Versamax plate reader (Molecular Devices Wokingham, UK) at 30 °C. The maximum specific growth rate (μmax) was determined from the change in OD600 over time, by fitting the data to Nt = N0eμmaxt. In which Nt and N0 are the cell densities at times t and 0, respectively. The μmax of the cells grown in the absence of drug was set at 100% to calculate relative growth rates (Fig. 6g).

**Statistical analyses.** Significance of data obtained with whole cells and proteoliposomes was tested by one-way analysis of variance. Differences in proteinase K and ATPase results were assessed using the unpaired student’s t-test. Asterisks directly above bars in the histograms refer to comparisons with control; asterisks above lines refer to specific comparisons: *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.

**Data availability.** Data that support the findings of this study have been deposited in the University of Cambridge data repository with the accession code 1810/255838 (https://www.repository.cam.ac.uk/handle/1810/255838) or available from the corresponding author upon reasonable request.

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