Protonation drives the conformational switch in the multidrug transporter LmrP

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Multidrug antiporters of the major facilitator superfamily couple proton translocation to the extrusion of cytotoxic molecules. The conformational changes that underlie the transport cycle and the structural basis of coupling of these transporters have not been elucidated. Here we used extensive double electron-electron resonance measurements to uncover the conformational equilibrium of LmrP, a multidrug transporter from Lactococcus lactis, and to investigate how protons and ligands shift this equilibrium to enable transport. We find that the transporter switches between outward-open and outward-closed conformations, depending on the protonation states of specific acidic residues forming a transmembrane protonation relay. Our data can be framed in a model of transport wherein substrate binding initiates the transport cycle by opening the extracellular side. Subsequent protonation of membrane-embedded acidic residues induces substrate release to the extracellular side and triggers a cascade of conformational changes that concludes in proton release to the intracellular side.

In an increasing number of cases, antibiotic resistance of bacterial strains relies on the expression of specialized molecular pumps called multidrug transporters1. In addition to their association with a pressing clinical issue, these polyspecific exporters of cytotoxic molecules present a fascinating variation on the classic membrane-transport theme. Although most membrane transporters are specific to a single substrate, multidrug transporters can recognize a vast range of structurally diverse molecules and efficiently extrude them2. Furthermore, multidrug exporters share a common evolutionary origin with substrate-specific importers, indicating that a similar scaffold has evolved to either export or import substrates.

A general mechanism for membrane transport was first proposed in 1966 (ref. 3) and later coined the term ‘alternating access model’4. In this model, the transporter alternates between two different structural states that enable binding of the substrate on one side of the membrane and subsequent release on the other. Vectorial translocation across the bilayer entails conformational changes to switch accessibility of the binding site. Although a wealth of biochemical and structural data lends support to the general concept of alternating access, the underlying structural mechanics have yet to be defined for each transporter class (reviewed in ref. 5).

The major facilitator superfamily (MFS) of transporters groups together ion-coupled secondary transporters of sugars, amino acids, drugs, nucleosides and a variety of organic and inorganic anions and cations5. They share a conserved common architecture consisting of two symmetry-related bundles of six-transmembrane (TM) helices encoded by a single polypeptide. The two bundles cradle the substrate in a binding site at their interface. Crystal structures of MFS members capture conformations described as ‘inward-open’2,8, i.e., open to the intracellular medium; ‘outward-open’, where the substrate can access the protein from the extracellular medium only; or ‘occluded’10–12, shielding the substrate-binding pocket from both membrane sides. Together, these structures stimulated the rocker-switch model for alternating access, wherein the N- and C-terminal halves of the protein rock back and forth against each other through rigid-body motions811. However, molecular dynamics simulations14,15 and biophysical studies16,17 indicate that the structural changes most likely involve important local motions as well. Pioneering work on lactose permease (LacY) culminated in a detailed mechanistic model developed in the framework of a high-resolution structure and complemented by analysis of conformational dynamics18. In this model, LacY sequentially binds proton and substrate in an outward-facing conformation19, transitions into an occluded intermediate and then adopts an inward-facing conformation that enables substrate and proton release. Sugar binding induces proton transfer between essential glutamate residues, triggering the opening to the intracellular side and subsequent proton and sugar release20. Deprotonation, which is driven by the transmembrane proton gradient, triggers the return to the outward-open conformation21,22.

In contrast, the conformational cycle of MFS multidrug resistance (MDR) exporters has not been elucidated. The only available crystal structure of an MFS-MDR exporter, that of the Escherichia coli EmrD, profiles a transporter in a doubly occluded conformation with a hydrophobic cavity located in the bilayer11. However, hardly any functional studies exist for this transporter to enable mechanistic interpretation of the structure. Building on the LacY model, the E. coli MdfA and L. lactis LmrP multidrug antiporters have been proposed to operate in a reciprocal fashion, binding their substrates from a high-affinity, inward-open conformation and switching to a low-affinity, outward-open conformation to allow substrate release23,24. Critical to proton and substrate transport is a group of acidic residues located mainly in TM segments. Several studies demonstrated that the accessibility of these residues can be modulated by substrate binding and identified the stoichiometries of substrate–to–proton transport19,25,26. However, a detailed understanding of how protein conformational motion couples ion gradients to substrate translocation and the sequence of events that defines the transport mechanism are missing.

Here we address these central elements of multidrug transport through extensive double electron-electron resonance (DEER; also known as pulsed electron-electron double resonance (PELDOR))
Figure 1 | The extracellular side of LmrP closes at low pH. DEER distance distributions between labeled cysteine pairs located on the extracellular ends of TM helices. (a–l) At pH 8 (blue curves), a single population (i.e., >70%) is observed for all distances. At pH 5 (red curves), a substantial decrease in the distances is observed for pairs between the N-terminal (TM1–6) and C-terminal (TM7–12) bundles: TM1–TM10 (a), TM1–TM11 (b), TM4–TM7 (c), TM4–TM10 (d), TM4–TM11 (e), TM5–TM7 (f), TM5–TM10 (g) and TM5–TM11 (h). (i–l) In contrast, no effect is observed for distances within the same bundle: TM1–TM5 (i), TM7–TM10 (j), TM7–TM11 (k), TM10–TM11 (l). Distributions were normalized. r indicates the interspin distance, and P(r) indicates the distance probability. Residue numbers and positions of cysteine pairs are depicted on an LmrP model based on the crystal structure of the E. coli homolog EmrD (Online Methods) viewed from the extracellular side. Targeted helices are highlighted in orange, with TM numbers indicated atop. Asterisks denote peaks resulting from partial aggregation observed in some samples upon concentration.

RESULTS

Mapping LmrP conformations with EPR probes

To directly follow the conformational dynamics of LmrP, we carried out DEER measurements on spin-labeled cysteine pairs placed on the extra- and intracellular sides of LmrP revealed a highly dynamic transporter that shifts between multiple conformational states in equilibrium. This conformational equilibrium is regulated by protonation of key transmembrane residues, defining a structural switch. Together the data support a mechanistic model of drug extrusion that sheds new light on the alternating access mechanism for MFS multidrug transports.

LmrP conformational equilibrium is modulated by pH

LmrP shows major structural differences on its extra- and intracellular sides at the two pH values. Distance distributions for 12 spin label pairs on the extracellular side of the transporter reveal large amplitude distance changes that are collectively consistent with a closing of this side of the transporter at lower pH. Distance distributions obtained at pH 8 (Fig. 1) typically show one major population, with the main peak accounting for 70–90% of the total area under the curve. At pH 5, all of the distances measured between the N-terminal (TM1–6) and C-terminal (TM7–12) halves of the transporter display a second population, with distances typically 10 Å shorter (Fig. 1a–h). Distances measured within the same bundle show little to no pH dependence (Fig. 1i–l), except for TM1–5 (Fig. 1i). Notably, the conformation observed at pH 8 is also partly populated at pH 5, indicating the coexistence of two LmrP conformers under acidic conditions. Thus, although LmrP adopts an outward-open conformation at pH 8, we observe a coordinated closing of the transporter at pH 5, producing an outward-closed conformation.

Conformational differences are concomitantly observed at the intracellular side (Fig. 2). For most distances measured between the two halves of LmrP, the observed changes indicate an overall opening on the intracellular side at pH 5 (Fig. 2a–f) compared to pH 8 (Fig. 2a–f). Distances measured within the same bundle remain largely unaffected (Fig. 2g,h), and a concerted shortening of the intra- and extracellular distances between TM5 and TM11 is observed at pH 5 (Figs. 1h and 2f). For distances spanning both halves of LmrP, four out of six report a single conformation on the intracellular side at low pH (Fig. 2a–f), in contrast to the bimodal distributions observed for all of the reporters on the extracellular side (Fig. 1a–h).

We interpret this data as revealing that LmrP is in equilibrium between at least two conformational states that alternately open and close each side of the transporter. The fact that two or more conformations are observed on one side (for example, extracellular distances at pH 5) and one conformation is detected on the other (for example, intracellular distances at pH 5) indicates limited...
conformational coupling between the helical movements on both ends of the protein and thus suggests that the structural changes do not strictly follow rigid-body motions. Shifting the pH does not alter spin label mobility, as deduced from continuous-wave electron paramagnetic resonance line shapes (Supplementary Figs. 4 and 5), indicating that the distance changes cannot merely be attributed to spin-label rotamer rearrangements but rather reflect TM domain movements. In addition, the position and amplitude of the distance distributions were not markedly altered by variations of the background slope that change the r.m.s. deviation between data and fits by up to 10% (Online Methods and Supplementary Fig. 6).

Protonation of acidic residues stabilizes distinct states

We then investigated the molecular basis for pH sensing in LmrP. Previous mutagenic analysis has identified a number of acidic residues that may be implicated in substrate binding, proton coupling or both. To establish that the conformational transitions deduced from DEER distance distributions correlate with protonation of acidic side chains, we carried out a titration experiment where the extracellular TM5–TM10 distance (mutant LmrP L160C T310C) was monitored at eight different pH values ranging from 4.5 to 8 (Supplementary Fig. 7). A two-population fit of the distance distributions clearly shows a gradual shift in the equilibrium from an outward-open to outward-closed conformation as the pH is reduced, with the population ratio inverting at pH 4.8 (Supplementary Fig. 7).

To identify the transmembrane acidic residues whose protonation induces conformational changes, we mutated Asp68, Asp128, Asp142, Asp235 and Glu327 (Fig. 3b) to asparagine or glutamine, thereby mimicking permanent protonation. These point mutants were then introduced into the LmrP^{L160C T310C} background to monitor the extracellular and intracellular conformations of LmrP, respectively (Supplementary Figs. 8 and 9). Our measurements indicate that distance distributions can be altered by the D68N or E327Q substitutions (Fig. 3c) and, to a lesser extent, the D128N substitution (Supplementary Fig. 8). We observe that the D68N mutation induces the outward-closed conformation at pH 8, a state only seen at pH 5 in the wild-type (WT) background. At pH 5, the D68N mutant shows almost a single outward-closed population, with the outward-open conformation being nearly absent. On the intracellular side, this mutation also locks the transporter in a pH 5–like state, with the TM5–TM11 distance showing a single distribution at pH 8 identical to that observed for WT LmrP at pH 5. We conclude that protonation of Asp68, located at the interface with the intracellular medium, stabilizes the outward-closed–inward-open conformation following a global conformational change. Thus, the reported functional importance of this aspartate31–36 reflects a critical role in driving conformational changes.

In contrast, the E327Q mutation restricts LmrP to the outward-open conformation. The short distance component, which reflects the outward-closed conformation, is almost absent in the L160C–T310C distance distribution, even at pH 5. The mutation does not alter the pH-induced shift in equilibrium at the pair monitoring the intracellular side. This indicates that the conformational equilibrium can be partly uncoupled between the extracellular and intracellular ends.

Figure 3 | The conformational equilibrium of LmrP is coupled to the protonation of acidic residues. (a) Distance distributions of the extracellular reporter pair L160C–I310C (TM5–TM10) were determined at eight different pH values ranging from 4.5 to 8 (color gradient from red to blue). To quantify the variation in population ratios as a function of pH, fits were carried out assuming a two-component Gaussian distance distribution (Supplementary Fig. 7 and Online Methods). At low pH, the short component (centered at 36 Å) dominates the bimodal distance distributions; at high pH, the long component (centered at 47 Å) is dominant. (b) Key acidic residues depicted on an EmrD-based model of LmrP: Asp68 (bottom of TM2), Asp128 (bottom of TM4), Asp142 (center of TM5), Asp235 (top of TM7) and Glu327 (center of TM10). (c) A protonation mimetic of key acidic residues can block the conformational switch. The single mutations D68N and E327Q were combined with the double cysteine mutations L160C–I310C and V137C–S349C, which served as extracellular and intracellular reporters, respectively. Distance measurements at pH 5 (red curves) and pH 8 (blue curves) in the absence (dashed line) and presence (solid line) of each mutation reveal the structural consequence of permanent protonation of these essential acidic residues.

Figure 2 | Acidic pH opens the intracellular side of LmrP. DEER distance measurements on the intracellular side of LmrP, performed at pH 8 (blue curves) and pH 5 (red curves). (a–h) Distances were measured either between the N-terminal and C-terminal bundles, as shown in TM1–TM7 (a), TM2–TM7 (b), TM3–TM10 (c), TM4–TM7 (d), TM4–TM8 (e) and TM5–TM11 (f), or within the same bundle, as shown in TM1–TM2 (g) and TM8–TM11 (h). Residue numbers and positions are depicted on an LmrP model based on the crystal structure of the E. coli homolog EmrD viewed from the intracellular side. Asterisks denote aggregation peaks.
Figure 4 | Hoechst 33342 binding restricts TM8 conformational flexibility and stabilizes the outward-open conformation. (a,b) Distance distributions measured from the extracellular end (I256C) (a) and the center (Cys270) (b) of TM8 to the extracellular end of TM10 (I310C) at pH 8 (blue curves), pH 8 in the presence of 1 mM Hoechst 33342 (green curves) and pH 5 (red curves). The presence of substrate restricts the distributions to a single population, whereas TM8 adopts multiple conformations in apo-LmrP, both at pH 5 and pH 8. (c) Effect of substrate binding (1 mM Hoechst 33342) on the extracellular conformation at pH 8 in the absence of a functional mutation; in combination with D68N; in combination with E327Q. The effect of the D68N mutation is partly reversed by the presence of the substrate.

Notably, protonation of Asp128 (D128N) substantially increases the population of outward-closed state at pH 5, whereas the intracellular distance reporter is less affected (Supplementary Fig. 8). We interpret this result as suggesting that Asp128 acts cooperatively with other protonation-dependent events as the effects of D128N are only observed at low pH. Mutation D142N has a moderate effect on the intracellular reporter at pH 8, favoring the long distance. For D235N, no marked change is observed relative to the WT background.

Together, our DEER data show that the conformational equilibrium between two discrete, pH-dependent structural states can be altered by protonation of single acidic residues: protonation of Glu327 stabilizes the outward-open state, whereas protonation of Asp68 shifts the equilibrium toward the outward-closed state.

Substrate binding stabilizes the outward-open conformation
Protonation of acidic side chains must be triggered from the extracellular side and must be coupled to binding and translocation of substrate. To assess the effect of substrate binding on LmrP conformational equilibrium, we measured all of the interhelical distances in the presence of the well-characterized LmrP substrate Hoechst 33342 at pH 8. We observed no major changes in most distance distributions (Supplementary Figs. 10 and 11), with a notable exception for spin label pairs involving TM8 (Fig. 4a,b). For these spin label pairs, substrate addition leads to narrowing of the distance distributions to a unique population, with more than 90% of the proteins in a ligand-bound conformation. Addition of 1 mM of kanamycin, a molecule not transported by LmrP, does not affect the distance distributions (Supplementary Fig. 12), demonstrating that these conformational changes are substrate specific.

Distance distributions involving labels on TM8 (i.e., I256C or C270) show multiple conformations at both pH 8 and pH 5 (Fig. 4a,b), suggesting a flexible helix. Consistent with this interpretation, Hoechst 33342 binding decreases spin label mobility on TM8, with the strongest effect in close proximity with the presumed ligand cavity, as proposed in the crystal structure of EmrD (Supplementary Fig. 13). Taken together, these results show that, under basic conditions, TM8 becomes constrained in the presence of ligand.

To identify the conformational state stabilized by substrate binding, we monitored the L160C–T310C distance (TM5–TM10) as a reporter (outward-open versus outward-closed) in either a WT, D68N or E327Q background (Fig. 4c). At pH 8 in the presence of 1 mM Hoechst 33342, both WT LmrP and LmrPE327Q are in the outward-open conformation. Remarkably, the presence of the ligand tends to partly reverse the conformational (closing) effect of the D68N mutation, increasing the population of the outward-open conformation, consistent with the conclusion that substrate binding stabilizes this state.

DISCUSSION
The main finding of this study is that the multidrug MFS transporter LmrP alternates between at least two conformations, outward-open and outward-closed, in a transition driven by the change in the protonation state of one or more transmembrane acidic residues. Therefore, this finding suggests that a proton gradient would power the structural transitions required for transport by driving proton transfer between key acidic residues. The side chain of Asp68, which is the most conserved residue in the MFS family and has been proposed to be involved in proton transfer and lipid-protein interaction in LmrP, would act as a master conformational switch. Although this residue is located far from the putative substrate-binding site, its mutation leads to transport inhibition and decreased substrate binding in a number of MFS transporters. Protonation of Asp68, as the culmination of proton translocation from the extracellular side, induces an outward-closed conformation (Fig. 3). The network of acidic residues important for propagation of conformational changes includes membrane-embedded Glu327, which is required for ligand binding and transport and is believed to interact directly with the substrate. Protonation of this residue stabilizes an outward-open conformation but has little or no influence on the intracellular side. On the basis of these data we propose that, under gradient conditions, a proton (or protons) will travel down from Glu327 to Asp68, leading to a transition from the outward-open to outward-closed state and thus creating an efficient conformational switch powered by the proton motive force. Proton passage is likely to involve other transmembrane residues of functional importance, such as Asp235, Asp142 and Asp128, as suggested by previous mutagenic studies and the moderate but notable effects observed by mimicking protonation of these residues.

Although the DEER data was obtained under constant pH conditions, it can be extended to a model of drug export by LmrP in the presence of a pH gradient (Fig. 5). In this model, proton translocation along the transmembrane charge network can only occur subsequent to substrate binding to prevent uncoupled proton transport, which would short the transmembrane ion gradient. Therefore, the resting state (i.e., in the absence of substrate but in the presence of a proton gradient) cannot be the outward-open conformation as this conformation is likely to allow proton entrance and protonation of the binding site. We propose that the resting state is a proton-occluded state that nevertheless is open to substrate binding from the inner leaflet of the bilayer (Fig. 5). Indeed, DEER distance distributions demonstrate the closing of the extracellular end of the TMs (Fig. 1) at acidic pH, which is typically encountered on the extracellular side under transport conditions. The intracellular pH is neutral to slightly alkaline, which, according to our data, should lead to a closing of the intracellular side (Fig. 2). Because the conformational changes we identify here do not involve rigid body motion of TMs, the two sides of the transporter have structural
Therefore, opening of the extracellular end could be due, in the presence of the gradient, to the cooperative effect of substrate binding and Glu327 protonation. Protonation of the binding site will in turn reduce substrate binding affinity, promoting substrate release into the extracellular medium (state IV). Coupling of protons and substrate could be achieved by competition for the same binding residues, as proposed for EmrE<sup>39</sup>, although a more indirect structural coupling is also feasible, as proposed recently for MdfA<sup>30</sup> and the multidrug transporter NorM<sup>30</sup> of the multidrug and toxic compound extrusion (MATE) family.

In our model, substrate-induced opening of the extracellular side initiates the conformational changes by enabling protons to access the network of acidic residues (Asp235-Glu327-Asp142-Asp128) across the membrane all the way to the intracellular side, where protonation of Asp68 leads to closing of the extracellular side of the TM bundle (Fig. 5). At this stage of the transport cycle, both the deprotonation of Glu327 (Fig. 3) and protonation of the key residues on the intracellular end (Asp128 and Asp68; Fig. 3 and Supplementary Fig. 8) favor the outward-closed–inward-open state, which is stabilized under acidic conditions (Figs. 1 and 2). In a cellular context, this state would expose Asp68 to the neutral pH of the cytoplasm, leading to its deprotonation and thus intracellular proton release. Subsequently, the transporter would switch back to the resting state; overall one substrate has been extruded, and one or several protons have been imported, in agreement with our current knowledge of extrusion stoichiometry in LmrP.

Our model differs fundamentally from existing models for MDR MFS transporters. In the current alternating access model, the substrate-bound, high-affinity conformation corresponds to the outward-open state for an importer and to the inward-open conformation for an exporter, as proposed for the <i>E. coli</i> MFS multidrug transporter MdfA<sup>30</sup>. In the latter model, we expect the substrate to stabilize an inward-open conformation and not an outward-open conformation, which contradicts our findings. Notably, crystal structures of the NorM transporter bound to three different substrates all captured an outward-open state<sup>39</sup>, suggesting that such conformation is compatible with high-affinity binding, at least in the case of MATE multidrug transporters.

Although the extra and intracellular parts form distinct structural domains, appropriate coupling between these domains must be realized to achieve protein function, i.e., a conformational change triggered on one side must be transmitted to the other during the transport cycle. Disruption of such coupling (as in the E327Q mutant) leads to transport impairment. As already proposed in ref. 18, reciprocity between opening on the extracellular side and the intracellular side may thus not be obligatory.

High-resolution crystal structures will be needed to adequately describe the local interactions that stabilize different LmrP conformations and in particular to elucidate how protonation of Glu327 and Asp68 trigger conformational changes. The role of Asp68 in driving conformational transitions may be divergent between MFS transporters. Although our results reveal a direct role of Asp68 protonation in stabilizing the inward-facing conformation, it should be noted that for LacY, Asp68 is not described as a proton binding site<sup>40</sup>. Identifying the specific polar networks involved in the interactions with Asp68 may highlight conserved and divergent conformational switching mechanisms between MFS transporters. We have previously proposed that interactions between Asp68 and the lipid environment could be important in regulating substrate transport<sup>30</sup>, suggesting that the bilayer could have an important effect on LmrP conformational dynamics. Therefore, it will be critical to test the model proposed here in membrane-like systems, such as proteoliposomes or nanodiscs, to elucidate the effect of lipids on the proton-dependent conformational changes of the transporter.

A simple multidrug transport mechanism emerges from our studies. To be transported by LmrP, substrates must merely (i) bind...
in the ligand pocket and (ii) subsequently catalyze proton entrance (i.e., by opening of the extracellular side). The simplicity of this mechanism thus provides the basis for how structurally diverse molecules can be extruded by a single transporter. It will be particularly interesting to investigate whether this model can be generalized to other pH gradient–driven members of the MFS family.

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METHODS

Methods and any associated references are available in the online version of the paper.

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Author contributions
M.M., C.M., H.S.M. and C.G. were involved in experimental design. M.M. and C.M. performed mutagenesis, expression, activity, purification and labeling experiments. M.M., R.A.S., S.M. and H.S.M. performed EPR measurements. R.A.S. and H.M. performed DEER and continuous-wave data analysis. C.G. and C.M. performed molecular modeling. C.G. and H.M. oversaw all aspects of the experiments and manuscript preparation. All authors participated in interpreting the data and writing the paper.

Competing financial interests
The authors declare no competing financial interests.

Additional information
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**ONLINE METHODS**

**LmrP homology models.** For each of the three structural templates (EmrD, Protein Data Bank (PDB) code 2GPP; LacY, PDB code 2V8N; FusC, PDB code 3O7Q), the sequence of LmrP was aligned using multiple alignments of LmrP and orthologs together with the sequences of the template and its orthologs. Initial alignments were generated using ClustalW and then manually adjusted to (i) prevent insertion and deletion in the TM helices and (ii) avoid introduction of charged residues facing the lipid tails. Subsequent LmrP-template sequence alignments were then used to generate molecular models with MODELLER (Supplementary Figs. 1 and 2). Figures were prepared with Chimera.

**Design and construction of the mutants.** Cysteine-replacement residues were selected to be located at the extracellular or intracellular end of a chosen TM region by using the homology model while avoiding mutation of conserved residues. The mutations were introduced in C-terminally His-tagged LmrP in a derivative of the E. coli PCR4 Blunt-TOPO vector (Invitrogen) by site-directed mutagenesis using the QuickChange Lightning kit (Stratagene). Unless otherwise stated, the endogenous Cys270 was previously replaced by an alanine using the same method. After transformation, plasmid DNA was extracted and verified by sequencing. The lmrP gene fragment containing the desired mutation was then introduced into the pHLP5-3C vector, a derivative of the L. lactis expression vector pHLP5 containing C-terminally His-tagged LmrP.

**Bacterial strains, plasmids and growth conditions.** The L. lactis NZ9000 strain was used as a host for pHLP5-3C-based plasmid expression, as described previously. Briefly, cells were grown at 30 °C in M17 medium supplemented with 0.5% glucose and 5 mg mL⁻¹ chloramphenicol until the OD₆₀₀nm reached 0.8. Overexpression of LmrP mutants was then induced by addition of 1:1,000 dilution of the supernatant of the nisin-producing L. lactis strain NZ9700. After 2 h of induction, cells were harvested by centrifugation at 5,000g.

**Preparation of inside-out membrane vesicles.** Cells were washed in 50 mM HEPES, pH 7, and resuspended (10 mL for each L. casei in the same buffer containing 5 mg mL⁻¹ of lysozyme and 10 μg mL⁻¹ of DNase I. After 1-h incubation at 30 °C, cells were broken by four passes at ~15,000 psi using a high-pressure homogenizer. Cell debris and undisrupted cells were subsequently removed by a 20-min centrifugation at 17,000g. Inside-out membrane vesicles were then isolated by ultracentrifugation at 125,000g for 1 h at 4 °C and resuspended in 50 mM HEPES, pH 7, 100 mM NaCl and 10% (v/v) glycerol (10 mL per L of cells). Vesicles were then frozen in liquid nitrogen and stored at −80 °C for further use.

**Transport assay.** The transport activity of the LmrP mutants was assayed as described previously. Briefly, inside-out membrane vesicles of LmrP-expressing cells (~2 mg of LmrP) were incubated 5 min in transport buffer (50 mM HEPES, 2 mM MgCl₂, 300 mM KCl, pH 7.4) at 30 °C in the presence of 0.1 μM Hoechst 33342 (Invitrogen). Addition of 2 mM Mg²⁺-ATP allowed generation of a proton-motive force by activating the endogenous F₀/F₁ ATPase, thereby initiating LmrP transport activity. Fluorescence generation of a proton-motive force by activating the endogenous F₀/F₁ ATPase, thereby initiating LmrP transport activity. Fluorescence spectros...