Antibiotic-Specific Conformational Landscapes of a Multidrug Transporter

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Abstract

Multidrug transporters are membrane proteins that can transport an ensemble of structurally dissimilar compounds and contribute to bacterial multidrug resistance (MDR) by exporting different antibiotics from the cell. However, whether they transport different substrates through a common mechanism or via distinct substrate-dependent mechanisms remains unclear. In this work, we used single-molecule Förster resonance energy transfer (smFRET) to measure time-resolved conformational dynamics of LmrP, a multidrug transporter of the Major Facilitator Superfamily (MFS). We present high-resolution conformational landscapes of LmrP in the presence of different antibiotics. Through multi-parameter Hidden Markov Modeling (mpH^{2}MM), we uncovered transient states and quantified their sub-millisecond interconversion kinetics. We observed antibiotic-dependent heterogeneity in the conformational landscape, both in accessible states and in interconversion rates. Notably, poorly or non-transported antibiotics slow down transition kinetics, pointing to rapid state interconversion as a driver of efficient transport. This suggests that MFS MDR transporters bind and export structurally dissimilar antibiotics by relying on an array of underlying conformational states with ligand-dictated interconversion rates. This work provides novel insights into the mechanism of MDR transporters and advocates for combined structure/dynamics-based drug design when targeting their function.

Introduction

Active extrusion of drugs across the cell membrane via transport proteins is a key driver of multidrug resistance in bacterial pathogens[1–3]. By decreasing the intracellular drug concentration, multidrug transporters enable bacteria to survive upon antibiotics treatment and eventually acquire genetically encoded resistance mechanisms, such as target mutation or enzymatic degradation. A molecular-level understanding of the transport mechanism of efflux pumps is a prerequisite for the development of targeted inhibition strategies to re-sensitize resistant strains [4,5].

How a single transport protein can recognize and translocate substrates with widely diverse chemical properties such as charge, size, and shape remains a fascinating enigma and, at the same time, complicates the rational design of efflux pump inhibitors. Structural adaptation to chemically diverse substrates is observed for the prototypical efflux pump AcrB, which features distinct binding pockets and multiple substrate pathways[6–8]. AcrB and its homologs, which belong to the same structural family of Resistance-Nodulation-Division (RND) transporters, play a prominent role in the emergence of multidrug-resistant phenotypes in Gram-negative bacteria [9,10]. These tripartite pumps are absent in Gram-positive bacteria, where most drug efflux systems belong to the Major Facilitator Superfamily (MFS)[1–3,11,12].

The molecular architecture of MFS efflux pumps differs markedly from that of RND pumps. MFS transporters are composed of 12 to 14 transmembrane helical segments arranged in two bundles of six helices that surround a central binding pocket [4,13,14]. MFS proteins mediate transport via the alternating access model, where the transporter alternates between inward- and outward-facing states to sequentially expose the binding site to each side of the membrane [15]. How these multidrug efflux pumps reconcile broad ligand promiscuity with a conserved MFS fold is poorly understood: do different substrates follow distinct pathways through conformational space, or do they converge onto a common sequence of structural rearrangements leading to transport? As a corollary, how multidrug transporters distinguish between transported and non-transported binders remains unclear.

Here, we address these questions using the multidrug transporter LmrP from *Lactococcus*
lactis[16]. LmrP is a substrate/proton antiporter from the Major Facilitator Superfamily that
can export several molecules including antibiotics[17,18]. We have previously investigated
the coupling of LmrP conformational changes to protons and ligand binding using Double
Electron-Electron Resonance (DEER) distance measurements on paramagnetically
labelled protein mutants [19–21] . These studies have shown that LmrP alternates between
at least two conformational states, an inward-open (IO) and an outward-open (OO) state,
where the protonation states of key acidic residues orchestrate the transition[19] such as
Asp68, a highly conserved residue in the MFS family[22,23] .In parallel, X-ray crystallography
characterization and DEER studies suggest that LmrP binds different ligands in an OO
conformation, consistent with a "one-size-fits-all" high affinity state[24]. These data were
obtained from solid (frozen) samples and thus conformationally fixed protein samples,
thereby excluding the range of dynamically interconverting structural states, including
short-lived ones, that compose the conformational landscape of the transporter. Recent
studies on transporters have revealed that heterogeneous conformational landscapes
composed of multiple subpopulations define transport function[25–28] . Whether this
underlying complexity can be leveraged to accommodate multidrug binding and,
subsequently, multidrug transport remains an open question.

Practically, an accurate depiction of a conformational landscape requires identifying
pathways between different conformations occupied for different time periods .To identify
the different states, the pathways that connect them and the underlying kinetics of the
transitions, we turned to single-molecule Förster resonance energy transfer (smFRET)
which is uniquely positioned in that regard[29–31]. In FRET, a non-radiative energy transfer
occurs from a donor fluorophore (D) to an acceptor fluorophore (A), where both are
attached to specific sites on a protein molecule [28,32]. The efficiency of the energy transfer,
E, sensitively reports on the distance between the donor and acceptor on the nanometer
length scale[33]. Real-time tracking of E for single molecules in turn allows monitoring
protein conformational changes and dynamics at nanometric spatial resolution combined
with a wide range of accessible timescales (ns-minutes).[34] Specifically, we employed
multi-parameter fluorescence detection with Pulsed-Interleaved Excitation
characterization (PIE-smFRET)[35](**Fig.1A**) to study the conformational dynamics of LmrP

and characterize its ligand-dependent conformational landscape. Our work reveals how

distinct conformational states and their interconversion kinetics together form a fine-

grained conformational landscape that different ligands navigate in distinct ways.

93 Results

94 Charting the conformational landscape of LmrP

Based on the crystal structure of LmrP in the OO conformation and the AlphaFold2 model

in the IO conformation[36], we selected pairs of residues with a spatial separation allowing

to distinguish the two states by smFRET when using ATTO 488 (donor) and Alexa Fluor

647 (acceptor) dyes. Specifically, one pair was selected on the extracellular side (H98

and M374 in TM3 and TM11 respectively) and one at the intracellular side (K9 and K404

in TM1 and TM12) (**Fig.1B,C**). The selected residues were replaced with cysteines into a

cysteine-less LmrP to enable covalent attachment of the fluorescent dyes through their

maleimide groups. The predicted FRET efficiency values E, together with the

corresponding expected distances between the probes in both conformations, are given

in (**Table S.1**). All double cysteine mutants were functionally tested by following LmrP-

mediated transport of Hoechst33342 in inverted membrane vesicles (**Fig. S1**). Single-

molecule FRET measurements were performed on the labeled LmrP molecules in

detergent micelles, diluted to ~50-pM concentration, and freely diffusing through the

femtolitre-size probe volume of the confocal microscope (**Fig.1A**). The distribution of

FRET efficiencies for all measured molecules was plotted as burst-wise E histograms.

At pH 7, the conformational landscape of apo-LmrP is characterized by a broad

distribution of E on the extracellular side, with a maximum around $E \approx 0.45$ (**Fig. 1D, left,**

blue histogram). On the intracellular side, the histogram displays a main central state

with a maximum around $E \approx 0.55$ (**Fig.1E**, **right, blue histogram**). By comparing the

observed to expected E values (**Table S.1**), we can conclude that the apo protein at pH

7 adopts a flexible conformational state that is predominantly closed on the intracellular

side, while sampling different opening magnitudes on the extracellular side.

As changes in pH modulates the conformational equilibrium of LmrP[19], we then performed

smFRET measurements at pH 5, where we observe a shift in the distance distributions

compared to pH 7. On the extracellular side the population centers around $E \approx 0.8$,

indicating closure upon lowering the pH (**Fig. 1D, left, orange histogram**) while, on the

intracellular side, we observe the main population shifting to $E \approx 0.3$, indicating opening

(**Fig. 1E, right, orange histogram).** We confirmed this observation by coupling the

extracellular reporter with the D68N mutation, which is known to stabilize LmrP in the IO

state [19] showing an increase of the high-FRET population at both pH 7 and 5, compared

to wild-type (**Fig. S2**). These observations are in line with our previous DEER studies[19,20]

and validate the ability of smFRET to identify and distinguish different conformational

states of LmrP. Importantly, smFRET analyses up to this point have reported burst-

averaged values of E. However, if molecules interconvert rapidly between two (or more)

FRET states while diffusing through the probe volume, these states appear as averaged

histograms rather than distinct peaks, leaving conformational intermediates with potential

functional significance unresolved[37,38].

Figure 1. **smFRET charts the conformational landscape of LmrP**. **A.** Schematic illustration of the PIE

smFRET experiment setup, showing the free diffusion of labelled LmrP molecules across the focal spot of

an inverted fluorescence microscope. "D" and "A" correspond to donor and acceptor dyes, respectively. In

the PIE setup, two spatially overlapped and alternatingly pulsing lasers are shooting at the objective to

excite donor and acceptor fluorescence consecutively. Fluorescence signals of the donor and acceptor are

recorded separately, and the fluorescence lifetime and anisotropy of each dye are determined.**B and C.**

Cartoon representation of LmrP helices with 98C (TM3)-374C (TM11) in outward-open **(OO)** or outward-closed **(OC)** conformations, and LmrP 9C (TM1)-404C(TM12) in inward-open **(IO)** or inward-closed **(IC)** conformations. The accessible volumes of the fluorophores ATTO488 (donor, red) and Alexa647 (acceptor, blue) are depicted as spheres. The N- and C-terminal halves of LmrP comprise TM1–6 (light orange) and TM7–12 (wheat beige), respectively. **D and E.** Burst-wise FRET efficiency histograms showing the smFRET signatures of intracellular and extracellular distance reporter mutants. The data reveals pH-dependent conformational changes of apo LmrP, with distributions recorded at **pH 7 (blue)** and **pH 5 (orange)**.

Kinetic analysis of apo LmrP reveals transient hidden states

To characterize the conformational states underlying the burst-averaged FRET histograms and the time scales at which these states interconvert, we took advantage of the advanced capabilities of the optical setup used in this work (*see Methods*). The setup records individual photons emitted by the donor and acceptor fluorophores, after their respective pulsed excitation, on the level of individual single-molecule fluorescence bursts. This enables the use of multiparameter photon-by-photon hidden Markov modeling (mpH^{2}MM) [35,39,40] to monitor FRET changes in these bursts with sub-millisecond precision [39] (**Fig. S3**). Specifically, mpH^{2}MM infers the number of conformational states a single molecule visits within a single burst, their mean E values, the stoichiometry S, the kinetic rate constants that report on how often molecules transition between given states and an estimated mean dwell time that corresponds to the average time that a molecule stays in a particular state before transitioning to another state. By integrating state identity and dwell times, we obtain a high-resolution conformational landscape for both the intracellular and extracellular reporters.

At pH 7, a three-state model was the best fit to describe the data for the distance reporters on either side of the membrane (**Fig. 2A**). When comparing the observed mean E values for each of the three states to those predicted from structural models (**Supplementary Table 1),** we observe only partial overlap. This discrepancy indicates that the protein in solution samples other states than the OO observed by crystallography or the IO predicted by Alphafold 2 modelling. We here annotated the three FRET states according to their mean E value: a low-FRET state **L** (E = 0.1–0.4), a medium-FRET state **M** (E = 0.4–0.7), and a high-FRET state **H** (E = 0.7–1.0). A subscript (E or I) is used to specify whether the measurement is on the extracellular or intracellular reporter.

We observe that the interconversion kinetics between the three states favor the
intermediate states M_E and M_I, where the protein dwells for longer (**Fig. 2A**). There is,
however, a noticeable difference between both sides of the protein, with a dwell time of
4.7 ms for the M_E and 11 ms for M_I. The dwell times for the other visited states (L_E, L_I, H_E,
H_I) are about equivalent (~3 ms). The mismatch in dwell times observed at the
extracellular versus intracellular reporters points to intrinsic flexibility of individual
transmembrane helices, enabling faster conformational transitions at one helix end than
the other.

This is further supported by the conformational landscape observed at pH 5. A two-state
model on both sides of the protein now best describes the data (**Fig.2B**), On the
extracellular side, it displays an interconversion between state L_E and state H_E, with mean
dwell times of ~3 ms and ~5 ms, respectively. On the intracellular side, the protein mainly
dwells for about ~15 ms in FRET state L_I and shortly transitions to the FRET state H_I for
~4 ms. This differential occupancy of FRET states on each side of the transporter is
indicative of a protein that explores a broad structural space, extending beyond simple
inward–to–outward conformational transitions through rigid-body motions. Given this
complex and asymmetric conformational landscape, we sought a global descriptor of the
protein's dynamics and therefore calculated the average conformational lifetime (ACL).

The average conformational lifetime (ACL) is defined as the sum of dwell times divided
by the number of states. It provides a global measure of protein dynamics, reflecting the
mean time required to transition between states. For the apo protein at pH 7, the average
lifetime is 4.5ms, increasing to 6.9ms at pH 5. Thus, protonation of specific residues
simplifies the conformational landscape (from three to two states) leading to more stable,
longer-lived structural states. Altogether, our findings show that apo LmrP samples a
complex conformational landscape that is asymmetric in both state populations and
transition kinetics, and that this landscape is modulated by protonation.

Figure 2. Kinetic analysis of apo LmrP reveals transient hidden states. A. mpH²MM analysis of LmrP in the apo state at pH 7. *Upper panel:* E–S scatter plot of individual dwells, colored according to the state assignment of the selected mpH²MM model. Red circles indicate the mean FRET efficiency (E) of each state, and the numbers above the arrows denote the transition rate constants (s⁻¹) between FRET states. *Lower panel:* Burst-wise FRET histogram showing corrected, exclusively FRET-active bursts (see Methods). The mean E value and mean dwell time (ms) for each interconverting FRET state are indicated below. Data from extracellular distance reporters are shown on the left, and intracellular distance reporter data on the right. **B**. mpH²MM analysis of LmrP at pH 5. Data are presented using the same layout and analysis as in panel **A**.

LmrP displays ligand-dependent conformational dynamics

Next, we investigated how the conformational landscape of LmrP reorganizes in response to antibiotics binding. We performed smFRET measurements in the presence of 2mM of antibiotics, at pH 7, followed by mpH^{2}MM analyses. Four classes of antibiotics were tested: lincosamides (clindamycin), macrolides (roxithromycin), aminoglycosides (kanamycin), and β-lactams (ampicillin).

Quantitative kinetic state analysis using mpH^{2}MM shows that each antibiotic presents E-S plots for both reporters distinct from the others, indicating ligand-dependent modulation of the conformational landscape. To verify that this was not an artifact due to the presence of 2 mM solute, we performed a control experiment with 2 mM glucose, which is not recognized by LmrP due to its high polarity and hydrophilicity. A three-state model was identified as the best fit on both sides of the membrane, with a conformational dynamics and kinetic profile resembling that of the apo-state (**Fig.S4**), Thus, the changes observed relative to apo are attributable to ligand binding.

Two aspects were considered for interpreting the impact of ligand binding: (i) how the conformational landscape differs from the apo condition in terms of visited states, and (ii) whether transitions between states are faster or slower on average (i.e., changes in dwell times/rate constants). First, by examining changes in the conformational landscapes, we observed that all ligands tested lead to a simplification of the conformational landscape on one side of the transporter. Indeed, while three states were sampled on each side under apo pH 7 conditions, only two states are sampled on one side and three on the other for each ligand, suggesting that ligand binding reduces the number of accessible states and the overall conformational heterogeneity.

Interestingly, the ensemble of accessible states differs as a function of the ligand. Kanamycin (**Fig. 3A**), roxithromycin (**Fig. 3C**) and ampicillin (**Fig. 3D**) data fit a two-state model on the extracellular side and a three-state model on the intracellular side, while clindamycin binding leads to the opposite profile. Notably, these differences between ligands are not reflected in their burst-averaged histograms, supporting the importance of a proper kinetic analysis. For example, the conformational landscape of clindamycin as

revealed by the E-S scatter plot (**Fig. 3B**) differs markedly from that of the other ligands
and apo in terms of accessible structural states. However, its burst-averaged histogram
is almost identical to that of the apo, kanamycin-, and ampicillin-bound states (**Fig. 3A,**
D).

Analysis of kinetics of the transition between states also reveals ligand-dependent
modulations, with shorter dwell times for kanamycin (ACL of 3.1 ms compared to 4.6 for
apo), and longer dwell times (ACL of 5.8 ms) for roxithromycin. Clindamycin and ampicillin
show ACLs (4.0 ms and 4.6 ms, respectively) close to the apo condition. Together, these
data provide evidence that the conformational adaptation of this prototypical multidrug
efflux pump is highly diverse and ligand dependent.

Figure 3. LmrP displays distinct ligand-dependent conformational dynamics. A-D mpH²MM analysis of LmrP in the presence of a 2 mM saturating concentration of different antibiotics. *Upper panel:* E–S scatter plots of corrected dwells, colored according to the state assignment of the selected mpH²MM model. Red circles indicate the mean FRET efficiency (E) of each state, and the numbers above the arrows denote the transition rate constants (s⁻¹) between FRET states. *Lower panel:* Burst-wise FRET histograms showing post-purified, corrected bursts. The mean E value and mean dwell time (ms) for each interconverting FRET state are indicated below. Data from the extracellular distance reporter is shown on the left, and intracellular distance reporter data on the right. The chemical structure of each antibiotic is shown adjacent to the corresponding panel (obtained from PubChem).

Different kinetic profiles have different resistance profiles

We speculated that the differences between ligand-induced conformational dynamics could translate into a difference in transport efficiency, that would impact resistance. To test this, we monitored the cell growth of *L. lactis NZ9000* strain transformed with *lmrp*-containing plasmid under the control of a nisin-inducible promoter, in which the multidrug transporters LmrA and LmrCD are deleted[41,42]. The assays were done for LmrP wild-type (WT) and the transport-deficient mutant D68N, to compare the relative efficiency of antibiotic efflux as a function of cell survival at different drug concentrations[43] [44].The transport-deficient D68N mutant is expressed at levels comparable to the wild type, indicating that the observed differences in relative growth arise from differences in the transport activity of LmrP[24] (**Fig. S5**). In the presence of kanamycin (**Fig. 4A,I**), cells expressing LmrP WT required a 21-fold higher IC_{50} to inhibit growth compared to cells expressing the D68N mutant. For clindamycin, we observed 12-fold increase in IC50 for the WT, indicating a clear contribution of LmrP-mediated transport (**Fig. 4B,I**). In contrast, The IC_{50} difference for roxithromycin was modest and did not exceed 2-folds (**Fig.4C,I**) suggesting poor transport. For ampicillin, the IC_{50} ratio between WT and mutant strains was close to unity, indicating that ampicillin is not transported by LmrP under the tested conditions (**Fig. 4D,I**). Together, these results indicate that LmrP transports kanamycin and clindamycin, while roxithromycin or ampicillin are poorly or not transported (**Fig. 4I**).

We next compared antibiotic-dependent cell survival with the corresponding kinetic profiles of LmrP. Although the conformational landscape sampled by the isolated transporter in solution is unlikely to fully mirror that in cells, this comparison allows us to assess whether differences in conformational behavior are associated with functional output. Therefore, we asked two main questions: (1) Does efficient transport correlate with a specific structural sub-state(s)/ conformations? (2) Or is it rather the rate(s) of transition between states that can be correlated with transport efficiency? To facilitate this comparison, dwell times of the conformational states sampled by the extracellular and intracellular reporters were plotted alongside the cell survival curves. This representation allows visualization of which states are populated by the protein, and whether these states are long-lived, or only transiently visited. (**Fig.4E,F,G,H**).

Well-transported substrates, clindamycin and kanamycin, show different conformational
landscapes both on the intracellular and extracellular reporters, with the state M_E visited
by clindamycin-bound (**Fig.4F**) but not kanamycin-bound protein (**Fig. 4E**), while the state
L_I is visited by kanamycin-bound (**Fig. 4E**) but not clindamycin-bound LmrP (**Fig.4F**). In
contrast, kanamycin-bound protein visits the same conformational landscape (**Fig. 4E**) as
the ampicillin-bound (**Fig. 4H**). This indicates that the conformations adopted or
accessible upon binding do not correlate with efficient transport. In contrast, analysis of
the transition kinetics between states reveals an interesting feature: the only clear
difference between the conformational landscapes of kanamycin (transported) and
ampicillin (not transported) lies in the faster transitions/ shorter dwell times (< 10 ms)
observed on the intracellular side for kanamycin (**Fig 4E**) compared to ampicillin (**Fig.
4H**). Transition kinetics/dwell times of clindamycin-bound LmrP are also much faster on
the intracellular side (**Fig. 4F**), whereas roxithromycin-bound LmrP displays long dwell
times (>10ms) and slow transition kinetics (**Fig.4G**). Together, these data suggest that
fast sampling of the conformational landscape on the intracellular side upon binding might
be a prerequisite for efficient transport.

Figure 4. LmrP-mediated antibiotic resistance. (A–D) Growth inhibition assays of *L. lactis* expressing LmrP wild-type (WT, black) or the transport-deficient mutant D68N (red) in the presence of kanamycin, clindamycin, roxithromycin, and ampicillin. Non-linear regression (dose–response inhibition) was used to estimate IC_{50} values. A representative assay is shown for each antibiotic; each data point represents the mean ± SEM from n = 4–8 technical replicates. **(E-H)**: Bar charts showing the mean dwell times in ms of different conformational states obtained from mpH²MM analysis of smFRET data in the presence of each

antibiotic, for distance reporters located on the extracellular and intracellular sides of LmrP.**(I)** Bar chart summarizing the WT/Mutant fold change in IC50 values of the tested antibiotics, calculated from at least three independent biological experiments. The IC50 values were calculated using non-linear regression fit; log[inhibitor] vs response. Statistical significance was assessed using one sample t-test comparing fold changes in IC50 values of different biological replicas for WT and D68N mutant. P values are indicated as follows:$p \leq 0.02$(**), $p \leq 0.05$ (*), and $p > 0.05$ (ns).

Discussion

Our single-molecule FRET (smFRET) analysis shows that different antibiotics differentially reshape the conformational dynamics of the MFS multidrug transporter LmrP, giving rise to distinct ligand-dependent conformational landscapes.

LmrP and homologous multidrug transporters (e.g., MdfA, NorA, QacA) function as antiporters that exchange protons for substrates with protons entering from the extracellular medium and being released to the cytosol[45–47], while substrates are recruited either from the inner leaflet of the membrane[48] or directly from the cytosol[17]. Hence, the initial contact between the ligand and the transporter occurs on the intracellular side and involves different conformational adaptation for binding from different locations (inner leaflet or cytosol). Interestingly, clindamycin and roxithromycin are lipophilic, with logP values of 1.04 and 3, respectively (ChEMBL), while kanamycin and ampicillin are hydrophilic, with logP values of −7.6 and −2.0, respectively, suggesting different recruitment sites.

Remarkably, the conformational landscapes of LmrP in presence of kanamycin or ampicillin on the intracellular side are qualitatively similar to each other and to the apo condition (**Fig.2 and Fig.3**), transitioning between three states (L_I, M_I and H_I) with the M_I state more populated. In contrast, the conformational landscapes for the two lipophilic ligands depart markedly from the apo state; in the case of roxithromycin, the three states are present, but the L_I state is the most favored. For clindamycin, the L_i state is absent. This observation indicates that, on the intracellular side, binding of hydrophilic ligands results in minimal structural rearrangement, in contrast to lipophilic ligands, which induce significant conformational adaptation. Our work, together with that of others, points to a deviation from the canonical alternating access model of transport proposed for MFS that surmises that one conformational state is primed for binding and the other for release

(see Introduction). We observe that a diversity of conformations appears compatible with
substrate binding. Previous work on the multidrug transporter MdfA confirms this
observation[49]. Substrate binding of the lipophilic compound TPP^+ occurs in an occluded
conformation, as shown by DEER spectroscopy while substrate binding of hydrophilic
chloramphenicol was observed by X-ray crystallography in the inward-open
conformation[50].

However, binding does not necessarily result in transport. This work offers insight into the
distinction between transported and non-transported ligands in a promiscuous transporter
such as the MFS MDR transporter LmrP. Although the LmrP scaffold can accommodate
structurally diverse substrates, efficient transport appears to require the preservation of
structural flexibility on the intracellular side after binding. We correlated the kinetic profiles
obtained for the isolated protein with resistance assays in bacteria and observed that
higher transport efficiency was associated with a flattening of the conformational
landscape charted by the intracellular reporter. Specifically, clindamycin and kanamycin,
are both efficiently transported and induce accelerated transitions between states relative
to the apo-state at pH 7 (**Fig. 5A, B**). This is indicated by the short mean dwell times
below 6 ms for clindamycin and kanamycin (**Fig. 3A, B**), respectively, compared to up to
11 ms for the apo-state (**Fig. 2A**). On the other hand, ampicillin traps LmrP in the M_I state
for 13.2 ms (**Fig. 3D**), while roxithromycin traps it in state L_I state for 12.6ms **(Fig. 3C).**
Within the framework of this hypothesis, it is not the specific state that determines efficient
transport, but rather the height of the kinetic barrier between states. (**Fig 5**)**.** Further
studies with additional ligands, whether transported or not, are required to confirm this
hypothesis.

This study opens new avenues for developing strategies for functional control of multidrug
resistance pumps. Our results reinforce the notion that designing ligands to target a
specific conformational state (e.g., inward- or outward-facing) is unlikely to be a viable
approach, as multidrug transporters can operate effectively across a broad
conformational landscape. Instead, interfering with the dynamics of state interconversion
i.e., kinetically trapping the transporter in whichever conformation it happens to encounter
the ligand appears more promising, as stopping conformational cycling is sufficient to

block transport activity. In practical terms, such kinetic trapping could be achieved by
designing binders that primarily increase the activation barriers between conformational
states locking moving domains, stabilizing inter-domain interfaces, thereby slowing or
arresting the conformational cycle irrespective of the exact structural state that is
populated. In this context, a promising proof-of-principle approach was recently
demonstrated for the multidrug transporter NorA, where a Fab locked the transporter in a
single conformation[43], here the outward-open. Peptide derivatives designed from the
CDR3 recognition region of this Fab efficiently inhibited NorA-mediated efflux of various
drugs, including ciprofloxacin. Together, these findings, consistent with the current
understanding of the remarkable plasticity of such transporters, pave the way for novel
drug design strategies that move beyond a static structural view and incorporate the role
of dynamic structural rearrangements.

Figure 5. The conformational model of LmrP. **A-D** Schematic free-energy landscapes corresponding to the conformational cycle of LmrP with the antibiotics: kanamycin, clindamycin, roxithromycin, and ampicillin. The drug-bound profiles highlight distinct energetic preferences for conformational states which either lead to transport (low kinetic barrier indicated by a green arrow) or to inhibition (red kinetic barrier indicated by a red arrow). Horizontal dashed lines indicate the energy level of the most stable state in each condition. In-set is the cartoon illustration of the conformational landscapes of LmrP with each antibiotic. Cartoon illustration of LmrP conformations are based on outward-open LmrP structure (PDB:6T1Z), inward-open (published AlphaFold2 model), outward-occluded and extra-outward-open (Chai discovery model followed by manual fine-tuning, see *Methods*).

Materials and Methods

Bacterial strains, plasmids and growth conditions

LmrP was expressed in the *L. lactis* NZ9000 strain that is deleted of the known ABC

multidrug transporters LmrA and LmrCD[41,42]. We used the in-home pHLP5-3C plasmid, a

derivative of the *L. lactis* expression vector pNZ8048 carrying the lmrP gene coding for

the C-terminally 6 Histidine-tagged LmrP[51]. Briefly, cells were grown at 30 °C in M17 broth

supplemented with 0.5% (w/v) glucose and 5 µg/mL chloramphenicol until the OD660

reaches 0.7. The overexpression of LmrP was then induced by the addition of 1:1000

dilution of the supernatant of the nisin-producing *L. lactis* strain NZ9700[52,53]. After 2h of

induction at 30 °C, cells were harvested by centrifugation at 7000 ×g.

Design and construction of the mutants

For each distance reporter, two residues were selected to be mutated into cysteines

based on the structure of LmrP obtained by X-ray crystallography in the outward-open

conformation (PDB:6T1Z) as well as on the AlphaFold predicted model of the inward-

open conformation[36]. Cysteines were placed at the extracellular and intracellular

extremities of chosen TMs while avoiding mutating conserved residues. The expected

FRET efficiencies were estimated by simulating the fluorescent probes' accessible

volume[54] so that they are separated by a distance within the Förster radius, 56.8 Å in this

case [55]. The accessible volume (AV) clouds were simulated using the parameters given

for ATTO488 and Alexa647 in [55].

The plasmid template was first methylated using the dam methyltransferase (NEB),

following the manufacturer's recommendations and as described in[56] Mutations were

introduced by site-directed mutagenesis using the Pfu DNA polymerase (Promega) in the

in-home pHLP5 plasmid, in which an alanine had previously replaced the only

endogenous Cys270[20]. The primers were designed as described in[57]. After

transformation, plasmid DNA was extracted and verified by sequencing.

Preparation of inside-out membrane vesicles

423　As previously described[51] ,cells were washed in 50 mM HEPES pH 7. They were

424　resuspended (10 mL for each L of culture) in the same buffer supplemented with 7 mg/mL

of lysozyme from chicken egg whites (BioChemica), 10µg/mL Deoxyribonuclease I from bovine pancreas DNAse I (VWR), 10 mM MgSO4, and 50mM Dithiothreitol (DTT). After 90min incubation at 30 °C, cells were homogenized with a Potter-Elvehjem-type tissue grinder and lysed by four passes at 1000–1500 bars using a high-pressure homogenizer. Cell debris and undisrupted cells were subsequently eliminated by centrifugation at 17,000 × g. Inside-out membrane vesicles were then isolated by ultracentrifugation of the supernatant at 100,000 ×g for 2h at 4°C. Membranes were resuspended in a pH7 buffer of 100 mM HEPES, 300 mM NaCl, 20% (w/v) glycerol (5 mL buffer/L culture) and 1mM DTT.

Transport assays

The transport activity of LmrP mutants was assessed by measuring Hoechst fluorescence (EX 355 nm, EM 457 nm) decay over time at 30°C, based on previously published protocols[41,51] .Briefly, inside-out membrane vesicles of LmrP-expressing cells (~1mg proteins) were diluted in transport buffer (50 mM HEPES, 2mM MgCl2, 300 mM KCl, pH 7.4) with 0.3µM of Hoechst (Invitrogen). The addition of 3 mM of ATP-Na2 in 50 mM HEPES pH 6.8 buffer supplemented with 250 mM MgSO4 allows the generation of a proton gradient by activating the endogenous F0F1-ATPase. Active extrusion of Hoechst by LmrP is detected by a decrease of fluorescence. Addition of 1µM of the ionophore nigericin dismisses the proton gradient and Hoechst is no longer actively extruded by LmrP, causing the fluorescence to increase. Raw data were normalized for fluorescence intensities relative to the initial fluorescence prior to ATP addition.

LmrP purification and labelling for smFRET

Inside-out membrane vesicles were solubilized in a solution of 2.5% (w/V) n-dodecyl- β – D-maltoside (β-DDM) in water with 1 mM DTT. After 1.5 h incubation on a rotating wheel at 4 °C, the insoluble part was removed by ultracentrifugation at 100 000 × g for 1 h. The supernatant was batch-incubated with Ni^{2+}-nitrilotriacetate affinity resin (Ni-NTA; Qiagen; 500 µL resin/L of culture) with 10mM imidazole at 4°C for 2h. The resin was pre-equilibrated with buffer A (50mM HEPES, 150mM NaCl, 10% (w/V) glycerol, 20mM imidazole, and 0.05% (w/V) β-DDM, pH 7). After the incubation, the slurry was transferred

into a polypropylene column, the flow-through discarded, and the resin was washed with
8 CV of buffer A, supplemented with 0,5mM DTT. LmrP was then eluted by stepwise
additions of buffer B (buffer A with 250mM imidazole and 0,5mM DTT). The more
concentrated fractions were pooled together. Imidazole and DTT were removed just prior
to labelling using a desalting column (Disposable PD10 desalting columns, GE
Healthcare) previously equilibrated with labelling buffer (50mM HEPES, 150mM NaCl and
0.02% (w/V) β-DDM, pH 7.5) that was degassed in vacuum/N2 under continuous stirring.
LmrP was concentrated to approximately 100µM using a 50kDa MWCO concentrator
(Amicon, GE Healthcare) and then labelled with the donor ATTO488 (ATTO 488
maleimide, ATTO-TEC GmbH) and the acceptor Alexa647 (Alexa Fluor 647 maleimide,
Life Technologies Europe BV). 250µM Alexa647 and 100µM ATTO488 were mixed in the
degassed Labelling buffer and 50µM of LmrP was added for 2h at RT. Unreacted dye
was removed on a desalting column, and the labelled protein was run on an SDX-200
10/300 GL (GE Healthcare) size exclusion chromatography column in SEC buffer (50 mM
HEPES, pH 7, 150 mM NaCl, 10% (w/v) glycerol, and 0.02% (w/v) β-DDM). The efficiency
of the labelling was checked via UV-visible absorbance at 280nm, 501nm, and 650nm to
estimate the amount of LmrP, ATTO488, and Alexa647, respectively, after correcting the
280nm peak for the dyes' absorbance.

Confocal Multiparameter-PIE Microscope setup

The experimental measurements were recorded using a home-built multiparameter
fluorescence confocal microscope operating with pulsed-interleaved excitation [34,35,58].

Two pulsed lasers were used for excitation with excitation wavelengths of 483nm (LDH-
P-C-470, Picoquant, Berlin, Germany) and 635nm (LDH-P-C-635B, Picoquant, Berlin,
Germany) with clean-up filters for both excitation sources (Chroma ET485/20x, F49-
482,AHF Analysentechnik, Tübingen, Germany and Chroma z635/10x, Picoquant, Berlin,
Germany). Both lasers were operated with a pulse frequency of 26.67 MHz (PDL 828
"Sepia 2", Picoquant, Berlin, Germany) (and delayed by 18ns relative to each other. The
laser beams were reflected using mirrors and dichroic mirrors into an optical fiber (60FC-
4-RGBV11-47 and PMC-400Si-2.6-NA012-3-APC-150-P, Schäfter und Kirchhof,
Hamburg, Germany). After the fiber, the beams were collimated (60FC-L-4-RGBV11-4 7,

Schäfter und Kirchhoff, Hamburg, Germany), passed through a linear polarizer (Codixx

VIS-600-BC-W01, F22-601, AHF, Tübingen, Germany) and reflected into the backport of

a microscope body (X70, Olympus Belgium NV, Berchem, Belgium) using a 3mm thick

excitation polychoric (zt 405/488/561/640tpc, AHF, Tübingen, Germany) and two mirrors.

The power of the excitation sources was measured and adjusted to the desired power

before the beam entered the microscopy body using a laser power meter. Excitation

powers of 100µW and 50µW were set for the 483nm and 635nm excitation sources,

respectively. The excitation lasers were focused into the sample volume using a water-

immersion objective lens (UPLSAPO-60XW, Olympus), and the confocal spot was

focused about 10µm into the measured solution. The emission was collected through the

objective lens, transmitted through the polychroic mirror, focused through (AC254-200-A-

ML, Thorlabs, Bergkirchen, Germany) a 75 µm-pinhole (P75S, Thorlabs), and collimated

again (AC254-50-A-ML, Thorlabs). Subsequently, the emission was spectrally separated

using dichroic mirrors. The 483-nm emission (referred to as B) is reflected (Chroma

T560lpxr, F48-559, AHF), filtered (Chroma ET525/50m, F47-525, AHF). The 635-nm

emission (R) was passed through and filtered (Chroma ET705/100m, AHF). Both

emission beams are furthermore separated according to their polarization (BS252,

Thorlabs) and then detected using four avalanche photodiodes, two for the parallel ($\parallel$)

and perpendicular ($\perp$) polarization components of each spectral range (PD-100-CTE,

MPD for B$\parallel$ and B$\perp$; EG&G SPCM-AQR12/14 for R$\parallel$ and R$\perp$). The detector outputs were

routed (HRT-82, Becker & Hickl, Berlin, Germany) to and recorded with a TCSPC card

(SPC-630, Becker & Hickl). The experimental TCSPC data were recorded in a 12-bit

format encoding for the total experiment time ("macrotime"), time relative to the laser

excitation pulse ("nanotime"), and a detector identification signal. Data processing of the

recorded TCSPC data is discussed in the section Burstwise Data Processing([35,59]).

Single-Molecule FRET Data Recording

Before performing sample measurements, the alignment of the smFRET microscope was

confirmed by measuring a mixture of 5 nM each of ATTO488-COOH and ATTO655-

COOH in water. The setup was aligned to achieve brightnesses of > 100kHz for the donor

and >70kHz for the acceptor detectors with laser excitation powers of 100 and 50 µW for

514 the donor and acceptor excitation laser, respectively. The purified and double-labeled

515 LmrP protein was diluted in measurement buffer: 50 mM HEPES pH 7 (adjusted for 20°C,

516 Sigma-Aldrich), 150 mM NaCl (VWR Normapur), 0.02 % β-DDM (w/V, Carl Roth) to a

517 concentration of approximately 50 pM and to achieve a burst event frequency of 10 to 20

518 / s. For measurements in the presence of a substrate, the burst concentration-diluted

519 solution of protein was allowed to incubate with the indicated substrate concentration on

520 ice for 30 minutes. The measurement buffer was recorded without the protein to confirm

521 the absence of an excessive and potentially interfering signal contribution. This was

522 repeated for each substrate with the same concentration as in measurements with labeled

523 protein. The sample solution was recorded on an 8-well Nunc Lab-Tek chambered 1.0

524 cover glass (Thermo Fisher Scientific). The confocal observation volume was focused

525 about 10 μm above the surface of the cover glass into the sample solution. Measurements

526 were recorded at room temperature (approximately 22 °C).

527 **Burst-wise Data Processing**

528 The experimentally recorded TCSPC data were processed by converting the recordings

529 to the HDF5 format using the phconvert Python module (phconvert 2024). The data were

530 further processed using the FRETbursts module[60]. The detector identification signals and

531 detector timing ranges, which indicate the spectral range and polarization (the photon

532 records of the two polarization channels per spectral channels were summed) of the

533 detected photon, were assigned to either the donor excitation/donor emission ($D_{ex}D_{em}$),

534 donor excitation/acceptor emission ($D_{ex}A_{em}$), or acceptor excitation/acceptor emission

535 ($A_{ex}A_{em}$) to disentangle the recorded signal according to the pulsed-interleaved excitation

536 scheme. The background contribution was estimated using a single-exponential fit with a

537 30-second time window and a background threshold of less than 1.7. Individual burst

538 events were identified using an all-photon burst search with a 20-photon sliding window

539 and a minimum burst event acceptance threshold of 7-times the background count rate.

540 Burst-wise parameters, such as the burst-wise FRET efficiency *(E)* or stoichiometry *(S),*

541 were automatically calculated by the FRETbursts module (see section Calculation of

542 Burstwise Parameters and Parameter Correction)[60]. To remove bursts that only contained

543 signal from either the donor or acceptor dye, a minimum of 60 $D_{ex}D_{em}$ or $D_{ex}A_{em}$ photons

544 and at least 40 $A_{ex}A_{em}$ photons were required. The distribution of bursts in E and S was

545 assessed for the presence of a FRET-active population with S of around 0.5 which

546 indicates emissions from both the donor and acceptor dyes. This removes bursts with

547 predominantly non-emissive donor or acceptor dyes (with S of 1 and 0, respectively). The

548 photon-count filtered set of bursts was loaded into the burst mpH^2MM module for removal

549 of short-lives donor or acceptor blinking states using a four-state mpH^2MM fit [61]. All photon

550 streams ($D_{ex}D_{em}$, $D_{ex}A_{em}$, and $A_{ex}A_{em}$) were used for mpH^2MM fitting. The fit results were

551 used to filter out individual burst events in which a molecule ever visited a non-FRET-

552 active state during the transition time through the observation volume. This was achieved

553 by identifying the states with near-1 and near-0 S and removing such burst events from

554 the list of bursts. This procedure removed about half of the burst events from the

555 presented data. A second round of mpH^2MM analysis was performed with the cleaned

556 dataset to describe the dynamics of the data (see supplementary data for a step-by step

557 overview of the data processing and Hidden-Markov Model Fitting)[39,60].

558 **Calculation of Burst-wise Parameters and Parameter Correction**

559 Using the pulsed interleaved excitation (PIE) scheme for smFRET, the FRET efficiency

560 is defined as

561
$$E = \frac{I_{DA} - \alpha\, I_{AA} - \delta\, I_{DD}}{I_{DA} - \alpha\, I_{AA} - \delta\, I_{DD} + \gamma I_{DD}}$$

562 and the stoichiometry as

564
$$S = \frac{I_{DA} - \alpha\, I_{AA} - \delta\, I_{DD} + \gamma I_{DD}}{I_{DA} - \alpha\, I_{AA} - \delta\, I_{DD} + \gamma I_{DD} + I_{AA}}$$

565 Where I_{DA}, I_{AA} and I_{DD} are the background corrected number of photons detected in the

566 acceptor detection channels after donor excitation, in the acceptor detection channels

567 after acceptor excitation and in the donor detection channels after donor excitation,

568 respectively; α is a correction factor for the direct excitation of the acceptor with the 483

nm laser; δ is a correction factor for the emission crosstalk of the donor in the acceptor

channel and γ is a correction factor for the relative detection efficiency of the donor and

acceptor [34,35].To account for instrument-specific difference of donor and acceptor signal

detection efficiencies, the γ correction factor was determined by measuring a mixture of

FRET-labeled DNA standards with two different FRET efficiencies and labeled with the

same dyes as the LmrP protein (Alexa Fluor 488 and ATTO 647N) . The α and β

correction values were determined from the protein measurements itself by fitting . The

obtained E-S histogram was used to determine the corrections. The reader is referred to

Maslov et al. for details on smFRET correction factors[34,59]. The correction values were

found to be similar between different measurement repeats. The following correction

values were used for the processing of all presented datasets: α = 0.03, δ = 0.03, and γ

= 1.2 .

Analysis of Intra-Bursts Dynamics with Hidden-Markov Model Fitting

The processed and blinking-removed set of bursts was used to assess each dataset for

the presence of intra-burst dynamics. The processed and cleaned-up list of burst events

(see section Burst-wise Data Processing) were loaded into the burstH2MM module

(Harris et al., 2022) to quantify and characterize states visited by the FRET-labeled

samples. A multiparameter H^{2}MM fit was carried out (using the $D_{ex}D_{em}$, $D_{ex}A_{em}$, and

$A_{ex}A_{em}$ photon streams) with a maximum of 3200 fit iterations to a maximum of five states.

The integrated complete likelihood (ICL) criterion was assessed to predict the most likely

number of states in the model as described by Harris et al. (2022). The model with the

lowest ICL value, or where the states had a mean stoichiometry of S≈0.5, corresponding

to FRET active molecules exclusively, was selected for each condition. A model was

rejected once the returned mean values for S were markedly different from what can be

considered a FRET-active state. This is the case, for example, if an mpH^{2}MM model has

split a state into two states with different S compared to the previous model with one state

less. We rejected such models with a difference in S since no meaningful conformational

state can be inferred from such a model. We estimated the mean dwell time for each state

from the extracted transition rate constants between states. The mean state dwell time

was calculated as tau_i = 1 / ((∑k_i,j) / N_states) as the inverse sum of all exchange rates

that depopulate state i divided by the number of depopulation paths. The burst

classifications were used to visualize the distribution based on the total burst duration

(burst-wise histogram) or per-dwell basis (dwell-based scatter plot). The state-average

FRET efficiency was used to compare different conditions. The uncertainty of the

transition rates obtained by the best-fitting mpH^{2}MM model was tested with the log-

likelihood ratio test which is implemented in the burstH^{2}MM module. The 95%-confidence

interval was calculated using the likelihood ratio test, where the log-likelihood was

reduced by 1.92 from its optimal value to determine the bounds of the interval.

We accounted for the possibility that mpH^{2}MM underfitted the experimental data.

However, with a simulated molecule visiting three states, underfitting with a two-state

model cannot result in shorter state dwells (see Suppl. Notes). Thus, the observed

increase in speed of the conformational change cannot explained by methodological

artifacts but rather by an actual acceleration of the conformational landscape of the

transporter.

Cell survival assay

Mutant and WT LmrP genes were transformed into *L. lactis* strain NZ9000 ΔLmrA

ΔLmrCD[42] through electroporation. Individual colonies were grown overnight and used to

inoculate 10ml cultures of M17 (supplemented with 0.5% glucose, 5 µg/ml of

chloramphenicol), which were then incubated statically at 30 °C until reaching an OD660

of ~0.7, when expression was induced through the addition of a 1:1,000 dilution of the

supernatant of the nisin-producing *L. lactis* strain NZ900034[52]. After 2 h of expression,

cells were diluted with M17 (supplemented with 0.5% glucose, 5 µg/ml of chloramphenicol

and 1:1,000 dilution of the supernatant of the nisin-producing L. lactis strain NZ9000) to

an OD660 of 0.05. These dilute solutions were then aliquoted into 96-well plates

containing a dilution series of antibiotic within the M17 (supplemented with 0.5% glucose,

5 µg/ml chloramphenicol and a 1:1,000 dilution of the supernatant of the nisin-producing

L. lactis strain NZ9000. The plates incubated to at 30 °C, without shaking, for 16 h before

measuring the OD660 using Spark Multimode Microplate Reader(Tecan). The assays

were conducted for at least three independent times, with six replica for each antibiotic

dilution per assay. After OD normalization, the IC50 values and corresponding standard

error of the mean data were calculated from a non-linear regression fit (dose–response

inhibition model)[62,63] using GraphPad Prism 10.3.

Data availability

633 The code used to perform mpH2MM-based analysis of the presented experimental

634 smFRET data is provided on Zenodo :

635 URL: https://zenodo.org/records/19449660

636 DOI: 10.5281/zenodo.19449660

637 (https://doi.org/10.5281/zenodo.19449660)

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Author Contributions

C.G, C.M and J.H conceived the study. H.M and T.K designed the experiments. A.R performed the mutagenesis, H.M, A.R and T.K carried out the smFRET experiments and analyzed the data. C.M and H.M wrote the paper with input from all authors.

Competing Financial Interests Statement

The authors declare no competing financial interests.

B **Extracellular**
LmrP H98C-M374C
TM 3-11

Outward-open   Outward-closed

C **Intracellular**
LmrP K9C-K404C
TM 1-12

Inward-open   Inward-closed

A

τ_D = 1-10 ms

High FRET

Low FRET

Confocal spot

Quartz slide

Objective

Donor

Acceptor

Pulsed Interleaved excitation
(PIE)

D

pH7 Apo
pH5 Apo

Norm. Frequency

E

E

pH7 Apo
pH5 Apo

Norm. Frequency

E

A

pH 7

Extracellular

Intracellular

$L_E \rightleftarrows M_E \rightleftarrows H_E$

$L_I \rightleftarrows M_I \rightleftarrows H_I$

E	0.22	0.54	0.86
Mean dwell time	3.2ms	4.7ms	2.9ms

E	0.24	0.54	0.86
Mean dwell time	2.9ms	11ms	2.9ms

B

pH 5

Extracellular

Intracellular

$L_E \rightleftarrows H_E$

$L_I \rightleftarrows H_I$

E	0.31	0.78
Mean dwell time	3.2ms	4.7ms

E	0.31	0.82
Mean dwell time	14.7ms	4.1ms