# THE DISCOVERY OF A REVERSED ANTIBIOTIC PUMP MECHANISM IN BACTERIA AND ITS POTENTIAL FOR A TWIST IN THE GLOBAL FIGHT AGAINST MULTIDRUG-RESISTANT BACTERIAL KILLER STRAINS


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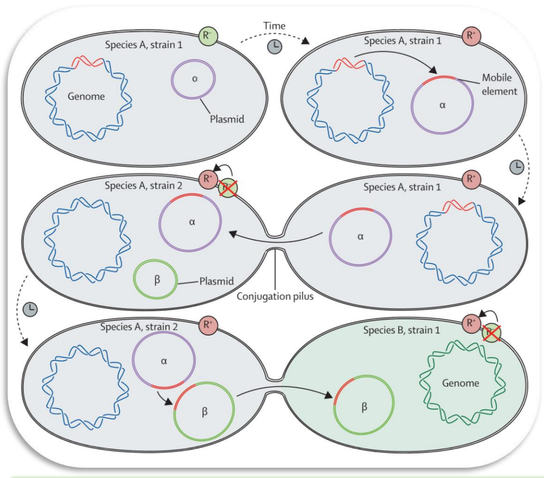
## 1. On the importance of antibiotic resistance-research

**The WHO ranks multidrug resistance as one of the three greatest risks to global human health, the others being climate change and malnutrition.**

- Failure of drug-based treatment of patients with cancer or infections by pathogenic bacteria
- Threat to global health and poses economic burden on health-care system
- Dry antibiotic pipelines of pharmaceutical companies

## 2. Spread, mechanisms, and effect of antibiotic resistance



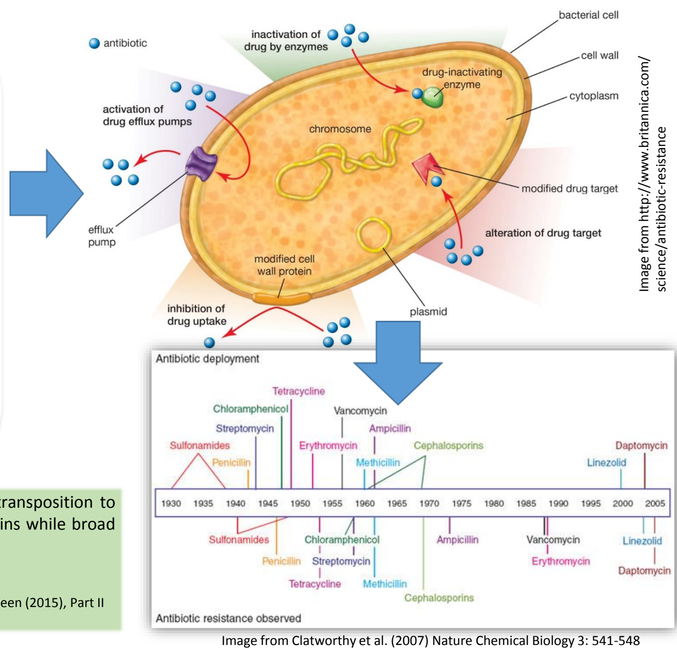




Image from http://www.britannica.com/science/antibiotic-resistance

Resistance genes (red) develop on the genome and are moved by transposition to plasmids. Narrow host-range plasmids (α) allow spread between strains while broad host-range plasmids (β) allow transfer to distantly related bacteria.
R = drug sensitive; R⁺ = drug resistant

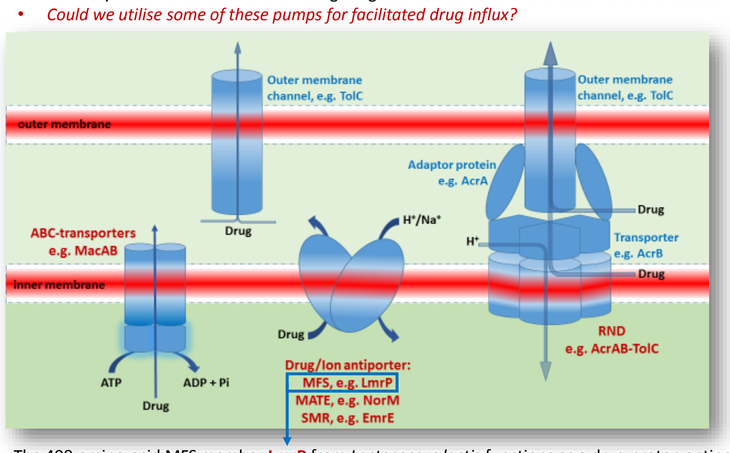
Image from Wellington et al. (2013) Lancet Infect Dis. 13:155-65; text adapted after Van Veen (2015), Part II Lecture Series.

Image from Clatworthy et al. (2007) Nature Chemical Biology 3: 541-548

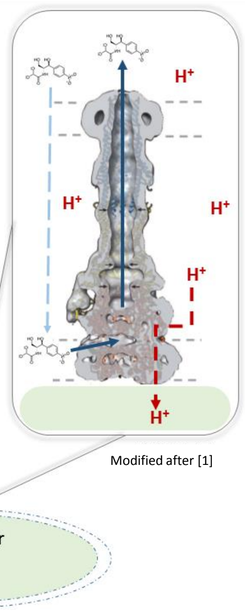
## 3. Multidrug resistance-causing efflux pumps at a glance

MDR-associated transport systems: **ATP-binding cassette (ABC)** and **ion-coupled** exporters (**4 families: SMR, MATE, MFS, RND**)

- How do multidrug transporters recognise so many different drugs?
- How do they discriminate antibiotics from vital intracellular metabolites that should not be expelled?
- Can new drugs be developed that bypass the action of multidrug transporters?
- Can they be inhibited such that existing drugs can still be used in the treatment of diseases?
- *Could we utilise some of these pumps for facilitated drug influx?*




**Substrates of AcrB:**
Chloramphenicol
Tetracycline
Minocycline
Erythromycin
Nalidixic acid
Enoxacin
Doxorubicin
Novobiocin
...



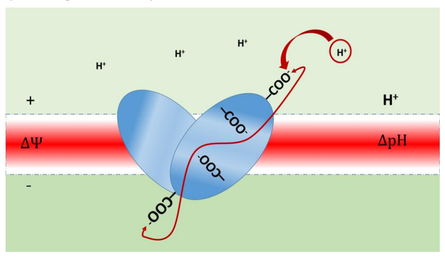

Modified after [1]

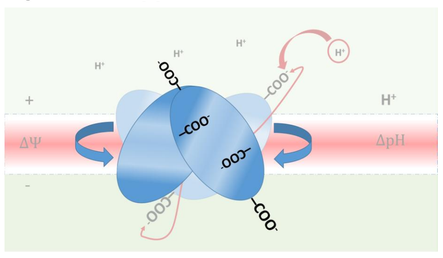
The 408-amino-acid MFS member **LmrP** from *Lactococcus lactis* functions as a drug-proton antiporter that utilises both the membrane potential (interior negative) and the chemical proton gradient (interior alkaline) of the proton-motive force to mediate the efflux of amphiphilic substrates from the cell [2-5].

## 4. For LmrP, two different models have been proposed to explain drug efflux.

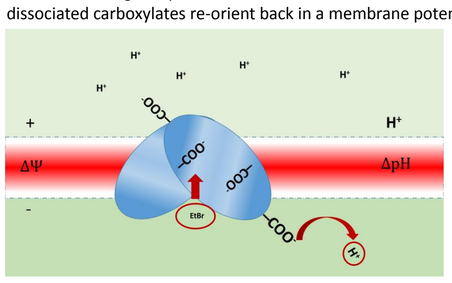
1. Cysteine cross-linking studies with LmrP C270S I34C V240C suggest that substrate binding stabilises a conformational state that is inward-facing (Model 1):

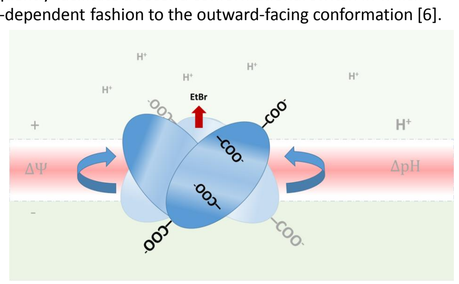
During proton-drug antiport, carboxylates in LmrP are protonated in the outward-facing conformation. This triggers a chemical proton gradient-dependent conformational switch to the inward-facing conformation [6].



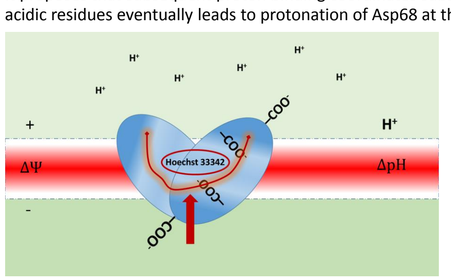

Ethidium binding and proton release at the inside surface subsequently facilitate a second conformational switch in which dissociated carboxylates re-orient back in a membrane potential-dependent fashion to the outward-facing conformation [6].

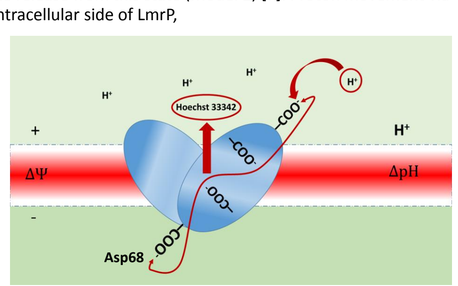


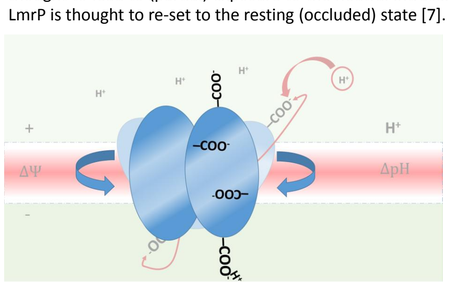

2. A study on LmrP based on distance measurements by EPR suggests that binding of Hoechst 33342 stabilises the outward-facing conformation:

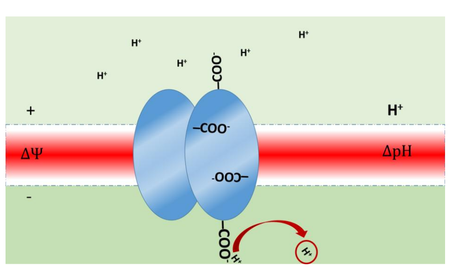
It proposes that subsequent proton binding coordinates substrate release from this state (Model 2) [7]. Proton movement via acidic residues eventually leads to protonation of Asp68 at the intracellular side of LmrP,




This induces closure of LmrP on the extracellular side with concomitant opening on the intracellular side. This opening is thought to lead to (partial) exposure to the neutral intracellular milieu, allowing for deprotonation of Asp68, at which point LmrP is thought to re-set to the resting (occluded) state [7].

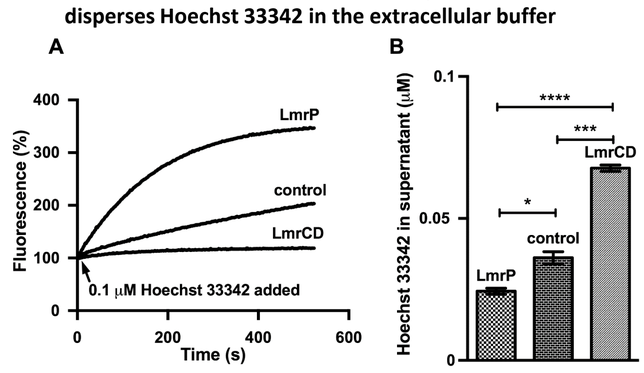



## 5. What is the gap between these two models?

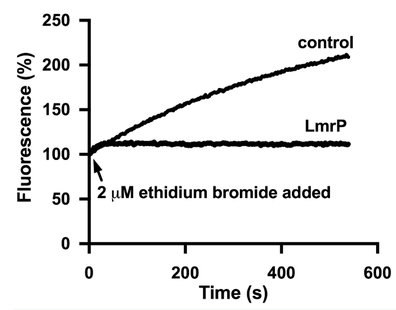
➢ **Hoechst 33342 is a hidden "Janus" amongst substrates for LmrP!**

**LmrP accumulates Hoechst 33342 in cells, whereas LmrCD disperses Hoechst 33342 in the extracellular buffer**




(A) Lactococcal cells expressing LmrP, LmrCD, or neither of these proteins (control) were first allowed to generate metabolic energy for 3 min through the addition of 25 mM glucose before Hoechst 33342 was added at a final concentration of 0.1 μM. Hoechst 33342 transport over time was followed by fluorimetry. Traces are typical for data obtained in three independent experiments using different batches of cells. (B) The concentration of Hoechst 33342 in the supernatant of the cells suspensions in (A) at 600 s was measured in triplicate after addition of 1 mg/mL calf thymus DNA (*, $p < 0.05$; ***, $p < 0.001$; ****, $p < 0.0001$) [8].

**LmrP expressing cells show reduced accumulation of ethidium compared to control cells**

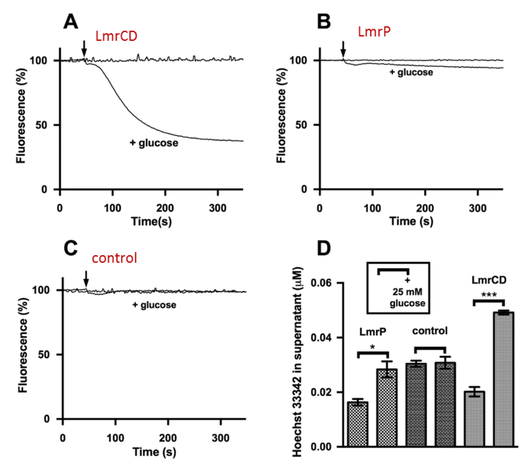



The substrate accumulation experiments described in A of left figure were performed with ethidium bromide at a final concentration of 2 μM. Traces are typical for data obtained in three independent experiments using different batches of cells [8].

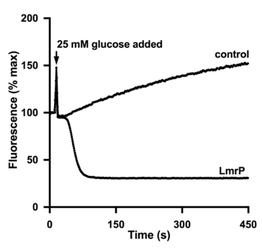
## 6. Is LmrP capable of Hoechst 33342 export at all?

**Idea: What if we pre-load de-energised (DNP-treated) cells with Hoechst 33342 before re-energising?**

**Active Hoechst 33342 efflux in cells is significant for LmrCD but weak for LmrP**



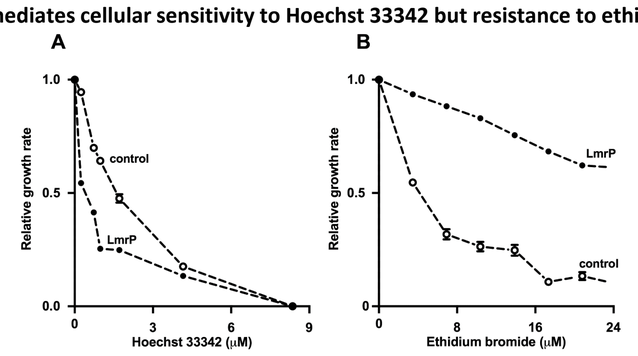

**LmrP mediates active efflux of ethidium**




The same efflux experiments (left figure) were performed with ethidium bromide at a final concentration of 2 μM. Traces are typical for data obtained in three independent experiments using different batches of cells [8].

(A-C) ATP-depleted cells with LmrCD (A), LmrP (B) or without LmrCD or LmrP proteins (control) (C) were preloaded with Hoechst 33342 at a final concentration of 0.1 μM. At the arrow, 25 mM glucose was added to one aliquot of the cells whereas in the other aliquot the cells remained de-energised. Traces are typical for data obtained in three independent experiments using different batches of cells. (D) The concentration of Hoechst 33342 in the supernatant of the cells suspensions in (A-C) at 900 s was measured in triplicate after addition of 1 mg/mL calf thymus DNA. (*, $p < 0.05$; ***, $p < 0.001$) [8].

## 7. LmrP mediates cellular sensitivity to Hoechst 33342 but resistance to ethidium




Relative growth rate of lactococcal cells containing LmrP or without LmrP expression (control) in the presence of (A) 0 to 8.4 μM Hoechst 33342, or (B) 0 to 24 μM ethidium [8].

## Conclusions

**I.**

- Multidrug transporters play a major role in the protection of (bacterial) cells against toxic compounds (antibiotics, organic acids and solvents).
- Knowledge about the architecture of
  - substrate- and modulator-binding sites and
  - energy-generating and translocating functions

  may allow to design compounds that can poison, circumvent, or even foster the activity of these transport proteins.

**II.**

- A stabilisation of an outward-facing state by Hoechst 33342 but inward-facing state by ethidium can now be explained in the light of LmrP's opposite transport directions for these substrates.
- Both, Hoechst 33342 and ethidium, bind to the transporter at the side of the membrane from where transport is initiated.
- The uptake of Hoechst 33342 by LmrP is a novel feature amongst multidrug transporters.
- Substrate uptake by multidrug transporters might be exploited therapeutically to selectively target cells and tissues.

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