Efflux pumps in *Mycobacterium tuberculosis*: What is their role in multi-drug resistance?

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INTRODUCTION

***Mycobacterium tuberculosis* infection remains a major cause of morbidity and mortality in large parts of the world and is considered to be one of the most important global health problems. Despite the use of vaccine and effective antibiotics, in 2012, an estimated 8.6 million people developed TB and 1.3 million died from the disease (including 320 000 deaths among HIV-positive people). (WHO,2013)**

Rifampicin and isoniazid are the two main drugs used in current first-line anti-TB chemotherapy. Multi-drug resistant tuberculosis (MDR-TB) is caused when *M.tuberculosis* is resistant, at least to these both drugs.
Antibiotic tolerance can be defined as the ability of bacteria to stop growing in the presence of an antibiotic, while still surviving to resume growth once the antibiotic has been removed. Even though antibiotic resistance is attributed to gene mutations in genes involved in cell wall biosynthesis, some strains have appeared with no mutations but the presence of efflux pumps, considered to be the mechanism to extrude the drugs. [1]

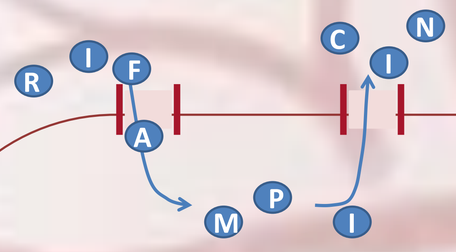
The objective of this study is to have an idea if the efflux pumps have an important role in MDR-TB and to see their inhibitors as a new possible therapies.

EFFLUX PUMPS

In the pathogen *Mycobacterium tuberculosis*, drug resistance is mainly due to chromosomal mutations in genes encoding the drug target or prodrug activating enzymes. However, drug efflux pumps could contribute to the acquisition of such mutations and explains why mutations in the target genes were not found in many low level resistant strains. Some efflux pumps have been characterized to know whether they are involved on MDR-TB and the role they play. These pumps are Tap and MmpL7 for extrusion of rifampicin and a isoniazid, respectively. [2]

Figure 1: Characteristics of Mmpl7 and Tap efflux pumps.

Pump	Gene	Transporter Family	Known Substrates	Known inhibitors	Energy source
Mmpl7	Mmpl7	RND	Isoniazid	Reserpine CCP	PMF
Tap	Rv1258c	MFS	Tetracycline Rifampycin	Piperine	PMF



Tap

In a strain of *M.bovis BCG,* used as a model for the analysis, when exposed to antibiotics, disruption of the Tap transporter (KOTap cells) or its overexpression, altered the strain susceptibility. Inactivation of Tap originated a progressive growth defect and altered morphology in *M.bovis* strains, as well as an alteration in gene expression in stationary growth phase, but not during exponential growth.

Gene regulation in KOTap cells during stationary growth phase:

- **Upregulated genes:** stress response genes and genes encoding for peptidglican components.
- **Downregulated genes**: Mainly genes of cell wall processing.

Results show that Tap is needed to maintain balanced physiological functions during late stationary phase and in latency. As bacterial growth is arrested during the stationary phase, toxic waste products accumulate inside the cell and must be cleared from the cytoplasm; so, toxics are substrates of Tap when nutrients are limited. This explains the upregulation of genes involved in general stress responses, acting as a detoxifying system. [3]

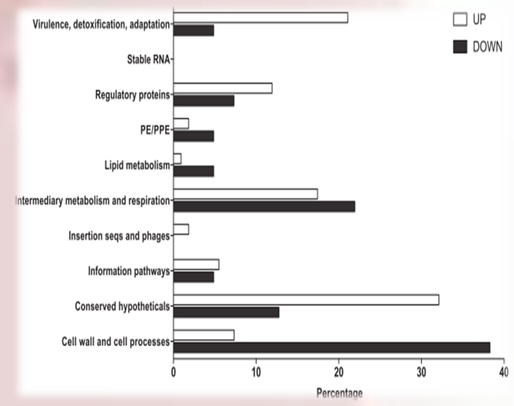


Figure 2: M.Bovis BCG gene regulation during stationary growth phase when exposed to INH.

Efflux pump inhibitors: CCP, verapamil and piperine

A Tap inhibitor could provide new strategies for TB therapy, allowing shortened treatment, but their use for TB therapy generates concerns about the pharmacological effects on cardiac conduction and disorders associated with Ca^{2+} channel blockers.[4]

Organism	MIC of piperine (mg/L)	MIC of rifampicin (mg/L) without piperine	MIC of rifampicin (mg/L) with 25 mg/L piperine	Fold reduction
M. tuberculosis H37Rv	>100	0.25	0.06	4
M. tuberculosis rif'	>100	128	16	8
M. tuberculosis clinical isolate	>100	64	16	4

Figure 3: Effects of piperine to different strains of M.tuberculosis

During TB treatment periods, exposure of a rifampicin-resistant strain to rifampicin stimulates cross-resistance, inducing resistance to ofloxacin, one of the most effective second-line anti-TB drugs, as they both are recognized by the same pump. [5]

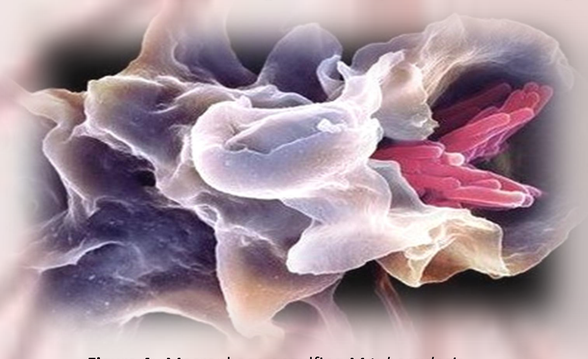
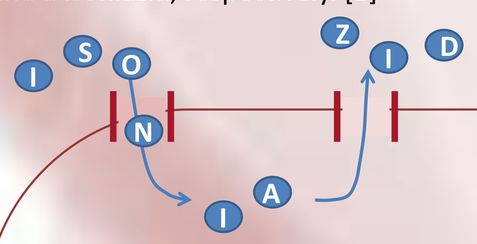


Figure 4: Macrophage engulfing *M.tuberculosis.*

Drug-tolerant bacteria are originated in macrophages dependent on the activity of bacterial efflux pumps [6].

The association between drug tolerance and intra-macrophage growth suggests that a common mechanism promotes both.



MmpL7

To determine if the resistance to INH was due to MmpL7 as active drug efflux mechanism, the accumulation of this drug was monitored in *M.smegmatis* cells carrying either pSODIT/mmpL7 and pSODIT-2 shuttle vectors from *M.tuberculosis*.

Strains were incubated with INH, and its intracellular concentration was measured at various time points. Cells harboring pSODIT-2 had a low level drug accumulation.

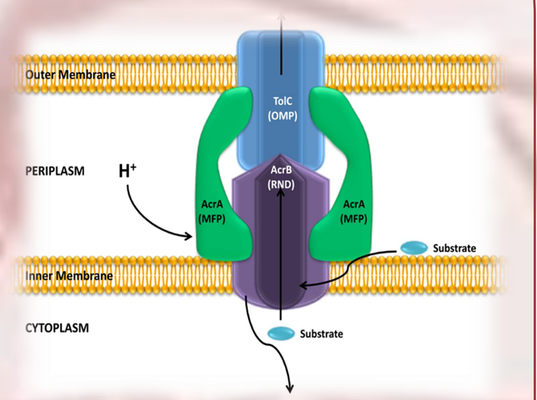
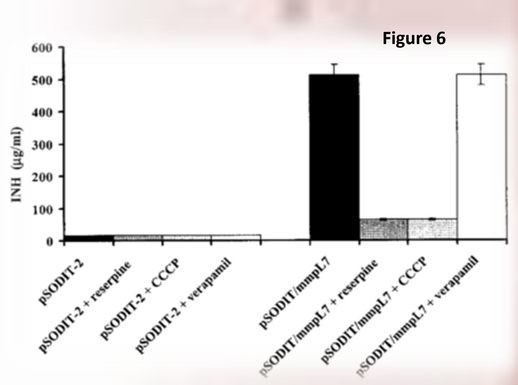
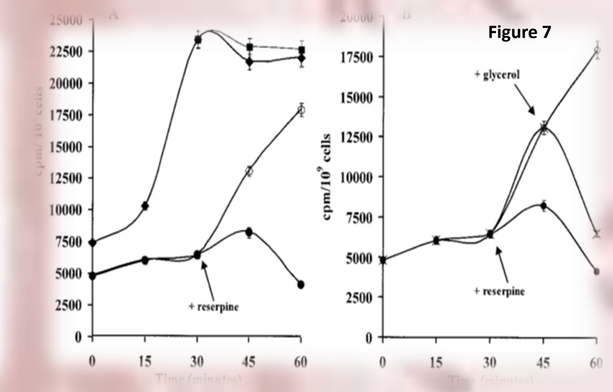


Figure 5: RND Efflux pump

Efflux pump inhibitors: reserpine, CCCP and verapamil

To determine the effect of membrane deenergization on the uptake of INH, reserpine was added to cells containing INH. Then, to test if the available source of energy could lead to drug efflux, glycerol was added 15 min after the addition of reserpine.





. **Figures 6 and 7** show the effects of efflux pump inhibitos to different strains. In Figure 7 is also represented the addition of glycerol 15 min after the addition of reserpine. *M.segmantis* expressing MmpL7, rapidly eliminated INH

Results suggest that MmpL7 is an efflux pump responsible for the INH resistance in an energy dependent process. [7]

In general, increased activity of efflux systems is responsible for conferring low-level resistance to antibiotics, contrasting with the high-level resistance caused by mutations in gene encoding for the primary targets of these antibiotics.

Consequently, the *M. tuberculosis* MmpL7 protein can utilize INH as a substrate when it is expressed in *M. segmantis*, while *M. tuberculosis* INH could compete with the natural substrate (PDIM) of MmpL7 since its principal physiological role appears to be the export of complex lipids to the cell exterior. Its overexpression also confers low-level INH resistance.

CONCLUSIONS

- The analysis of genome sequences have shown that *M. tuberculosis* has many open reading frames of putative efflux pumps. However, the role of these pumps in intrinsic and acquired resistance has not been determined as a major cause of the antibiotic resistance of mycobacteria. They are responsible for low-level resistance.
- One efflux pump is able to confer resistance to two antibiotics for its ability to recognize both as substrates. Further, it presents cross-resistance developing MDR, which alters not only first-line drugs, but also second-line anti-TB drugs. This makes the treatment period longer and more difficult.
- More research is needed, either for efflux pumps, because not all of them have been characterized, or for their inhibitors, because the known ones lead to pharmacological disorders.

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