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A Review

Transport mediated antibiotic resistance in *Mycobacterium tuberculosis*

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The incredible complexity of the mycomembrane represents a challenge for antibiotics to penetrate and effectively kill *Mycobacterium tuberculosis* (*M. tb*), the causative organism of human tuberculosis (TB).Additionally, intrinsic resistance mechanisms that either degrade or modify antibiotics further reduce the effectiveness of anti-TB drugs. To overcome the problem of permeation, it is important that transport mechanisms across the mycobacterial cell envelope are well understood. Porins required for nutrient uptake are sparsely represented in *M. tb*, and mutations in porin encoding genes are known to modulate antibiotic susceptibilities in *M. tb.* Along with the reduction in influx, upregulation of efflux pumps, and mutations leading to their overexpression are frequent in clinical isolates of drug-resistant TB. Since the present treatment strategy involves a lengthy regimen with high dose drug combinations, the study of transport proteins, becomes crucial to developing innovative treatment regimens to significantly improve patient outcomes. This review presents the state-of-art knowledge about transporters that help *M. tb* maintain nutrient uptake from the host and extrude toxic compounds, including antibiotics. These transporters could not only serve as potential targets to shorten the treatment regimen for drug susceptible TB, but could also be of utility in developing treatments for drug resistant infections.

Keywords: Drug resistance, Efflux Pumps, *Mycobacterium tuberculosis*, PE_PPE family, Transporters

Tuberculosis - Current status

Tuberculosis (TB) is an ancient disease, persisting invincibly in the face of modern interventions in medical technology, and an army of antibiotics. Despite advances in our understanding of its biology, how *M. tb* can flourish in a host with complex immunocompetence still largely remains a conundrum. In 2022, about 10.6 million people fell sick with TB, with an estimated 1.3 million deaths. India alone accounts for a quarter of new cases of TB (Global Tuberculosis Report - 2023, WHO). To treat drug-susceptible TB, a combination of 4 drugs, Isoniazid, Rifampicin, Ethambutol, and Pyrazinamide, is prescribed. When the infection cannot be treated with isoniazid and rifampicin, the infecting strain is categorized as multi-drug resistant (MDR), which is a significant public health issue that has a detrimental impact on both individuals and medical systems. The updated Bacterial Priority Pathogen list featuring antibiotic-resistant groups has categorized Rifampicin-resistant *M. tb* as a critical priority pathogen. MDR-TB is treated with a novel regimen containing Bedaquiline, Pretomanid, Linezolid, and Moxifloxacin (BePaLM). Further resistance to fluoroquinolones, along with any of the BePaL drugs, is referred to as extremely drug-resistant (XDR) TB, and a more extended regimen of remaining effective drugs is designed (WHO consolidated guidelines on tuberculosis, 2022, WHO). These regimens need a longer course of therapy spanning 6-12 months. Treatment of MDR- and XDR-TB involves the use of medications with a high toxicity profile. Patients may also have major side effects and have worse treatment outcomes.

M. tb - The armament

The secret of the enigma that is *M. tb*, lies in its unique physiological features - the cell envelope being a formidable barrier, and an unmatched potential to develop resistance *via* multiple mechanisms to drugs designed to kill it. Antibiotics that pass through the first layer of defence and enter the bacterium can be inactivated by enzymatic cleavage. The *M. tb* β-lactamase, BlaC, is regarded as an extended-spectrum β-lactamase because of its broad substrate specificity and variable affinities to β-lactam antibiotics. In addition to cleavage, antibiotics can be rendered inactive through modification, such as

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acetylation and methylation. Apart from modifying the drug or the drug target, efflux pumps offer an alternative resistance mechanism by expelling drugs with a wide range of specificity, leading to full-blown resistance, summarised in (Fig. 1). The innate resistance of *M. tb* to numerous classes of antibiotics and the ever-increasing number of drug-resistant strains threaten progress in limiting the spread of the disease. Mutations leading to upregulation or overexpression of efflux pumps have been observed in clinical isolates of MDR-TB and XDR-TB. It appears that canonical mutations, along with modified permeability through altered influx and efflux, play out to amplify the resistance phenotype. Taking into consideration the significance of intrinsic as well as the acquired capability to control transport across the cell envelope, we focus on transporters as an adjunct target to the available repertoire of drugs, increasing their efficacy and shortening the complicated regimen of treatment.

Transport potential of *M. tb*

The mycobacterial cell envelope facilitates cell division, influx of nutrients, efflux of metabolic by-products and toxic compounds, and response to host immunologic assaults. Maintaining homeostasis in the dynamic milieu of immune cells requires intricate regulation of the cell envelope composition. The multi-layered structure comprises the capsule, the cell wall core, and the cell membrane. The capsular coat of pathogenic *Mycobacterium* is populated by polysaccharides, mainly glucan, decorated with proteins and the surface lipid phthiocerol dimycocerosate (PDIM). These proteins and PDIMs modulate host responses by either manipulating immuno-signaling or masking the pathogen-specific signatures present in the cell wall. The outer membrane, called the mycomembrane, the arabinogalactan, and the peptidoglycan (PG) constitute the cell wall core. Then, there is the cell membrane made of glycerophospholipids

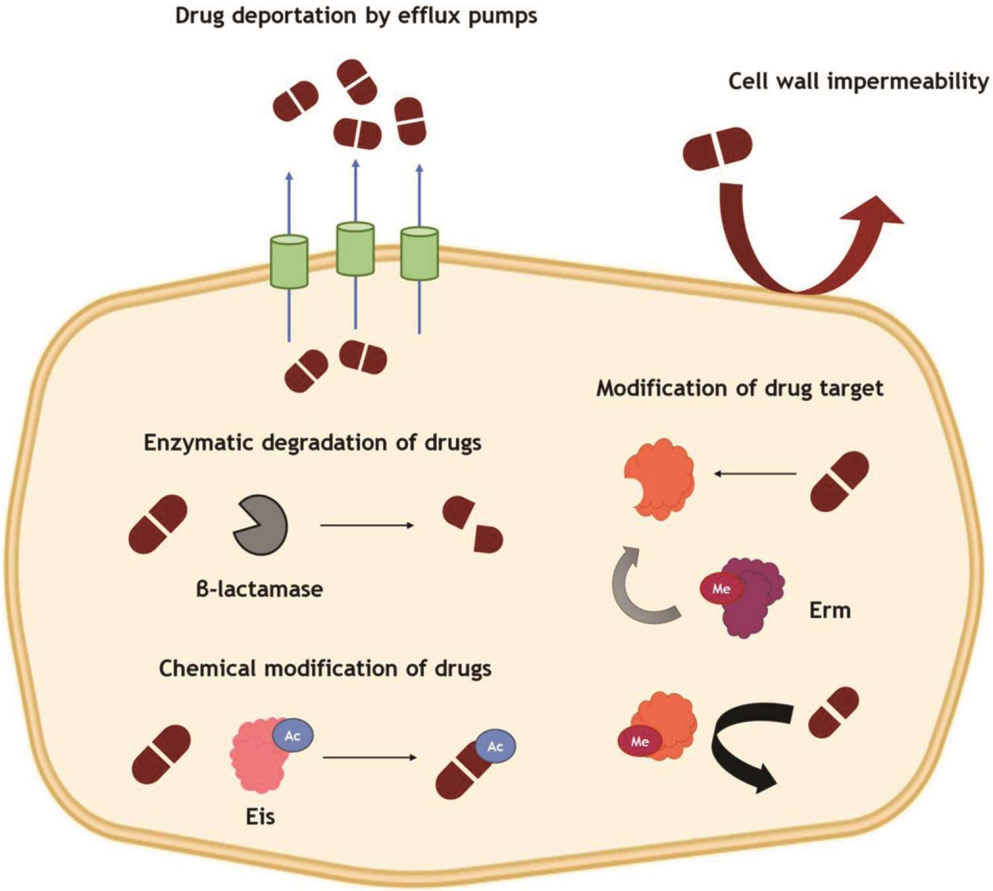

Fig. 1 — Intrinsic mechanisms of resistance in *M. tb. (*A) Drug efflux *via* transporters; (B) Cell wall impermeability conferred by mycomembrane; (C) Enzymatic degradation of β-lactam antibiotics by β-lactamase, BlaC; (D) Inactivation of aminoglycosides by Enhanced Intracellular Survival (Eis) protein-mediated acetylation; and (E) Erythromycin Resistance Methylase (Erm37) encodes an enzyme that methylates a specific site in 23S rRNA, prevents antibiotic binding

and actinomycetes-exclusive phosphatidylinositol mannosides (PIMs) and their derivatives. The architecture of the cell envelope allows the least permeability among other related groups of bacteria. A lipid-rich envelope should enable passive diffusion of the hydrophobic substrate. However, passive diffusion is directly associated with membrane fluidity, which, in the case of *Mycobacteria*, is restricted due to the presence of lengthy saturated hydrocarbon chains. In contrast, hydrophilic compounds need to be assisted by porins, energy-independent water-filled channels. Trias *et al.* provided the first evidence for the presence of porins in *Mycobacteria* in 1992. They reconstituted liposomes with *M. Chelonei* cell wall extract and identified a 59 kDa protein to cause significant swelling of liposomes and confirmed to function as a porin by a single-channel experiment. Subsequently, they reported the presence of porin in the cell wall of *M. smegmatis*. To this date, porin function has been attributed to only a few candidate genes in the *Mycobacterium* genus. *ompATb* is restricted to pathogenic species and was identified due to its homology to the OmpA family of outer membrane proteins in Gram-negative bacteria. The *ompATb* deletion mutant had reduced permeability to hydrophilic small molecules and virulence in macrophages and mice. Its expression was found to be upregulated in low pH, conferring adaptation to survive in phagosomes[1]. The structure of OmpATb was elucidated by NMR spectroscopy, and it was found that the protein does not form transmembrane β-barrel as typically expected for its porin function. Instead, it is a mixed α/β globular structure of two independently folded domains with the possibility of forming an oligomer[2]. However, Song *et al.* could not validate the porin function of OmpATb and reported its involvement in mediating ammonia secretion to counter the acidic environment[3]. Stahl *et al.* identified MspA as a porin and noted three other nearly identical members: MspB, MspC, and MspD. Deletion of *mspA* led to reduced permeability to Cephalosporin, a β-lactam, by a factor of nine and to glucose by a factor of four[4]. Expression of MspA in *M. bovis* and *M. bb* accelerated glucose uptake by two-fold and increased susceptibility to β-lactam antibiotics, resulting in a 16-fold reduction in minimum inhibitory concentration (MIC)[5]. The structure of MspA, the first for a mycobacterial outer protein, was established by Faller *et al.* two decades ago and constitutes two consecutive β-barrels forming a homooctameric conformation with a central channel[6].

Rv1698, exclusive to mycolic-acid-containing bacteria, was identified to form a water-filled channel through a lipid bilayer and restored antibiotic susceptibility towards Ampicillin and Chloramphenicol in the *mspA* mutant[7]. CpnT is another *M. tb* outer membrane protein constituted by an N-terminal channel forming domain for nutrient uptake and a C-terminal necrosis-inducing secreted toxin domain[8]. Clinical drug-resistant strains of *M. tb* in China have been found to carry nonsynonymous single nucleotide polymorphisms in *cpnT*[9]. A transposon mutant of *M. bovis* BCG disrupted with an insertion in *cpnT* was found resistant to multiple antimycobacterial drugs, including Isoniazid, Ethambutol, Streptomycin, Rifampicin, and Fluoroquinolones. Interestingly, the expression of MspA was sufficient to restore the drug susceptibility of the mutant[10]. Hence, channel proteins of the outer membrane regulate antibiotic susceptibility in *Mycobacterium sp.* None of the proteins that were identified exhibit any homology, and *mspA* does not have any homolog in *M. tb.* It is possible that *Mycobacterium* channel proteins can multitask to regulate nutrient uptake and eliminate toxic molecules. Low porin density on the envelope and mutations leading to loss of such proteins confer resistance to toxic compounds. The channel proteins identified are not essential for survival, indicating that multiple channel proteins could be involved in the uptake of particular nutrients, or the major ones are yet to be discovered. An extensive hydrophobic barrier with restricted mobility across the cell envelope reduces the availability of antibiotics below their effective concentrations. Consequently, the medication prescribed involves doses that are toxic and have significant side effects.

The presence of protein machinery capable of efflux further contributes to resisting antibiotic action. In silico analysis indicates that the *M. tb* H37Rv genome encodes 266 putative transporters (TransportDB2.0 http://www.membranetransport.org/transportDB2/index.html). Transport systems exist to ensure the uptake of nutrient substrates and detoxification of metabolic by-products or environmentally acquired toxins. Channels, primary or active transporters, secondary transporters, and group translocators are the major classes of transporters. Channels are energy-independent and transport down a chemical or electrical gradient of

molecules such as water, small hydrophilic molecules, and specific ions. Transport through primary transporters is coupled to ATP hydrolysis, while secondary transporters utilize proton/sodium motive force to drive transport along an electrochemical gradient. Further, each class is comprised of families and sub-families classified on the basis of phylogeny, function, and substrate specificity. Of concern to us are the families associated with antibiotic/ drug tolerance or resistance – commonly known as drug efflux pumps. In the *Mycobacterium* sp., these families are a) ATP-binding cassette (ABC) superfamily comprising two transmembrane domains, each made up of a bundle of transmembrane-helical segments linked to two cytoplasmic nucleotide-binding domains, making up the simplest functional unit of an ABC transporter, b) Major facilitator superfamily (MFS) having two symmetrical transmembrane domains each of six transmembrane helices connected by a flexible loop, c) Resistance-nodulation-cell division (RND) family, which also has a 2-fold symmetry with a periplasmic domain and a transmembrane domain of 11-12 helices in each half, d) Small multidrug resistance (SMR) family which forms 4 transmembrane helices, and e) multidrug and toxic compound extrusion (MATE) family, which contain two bundles of transmembrane helices. The skeletal structure of each family of transporters is conserved, while each member varies in amino acid sequence within the family. Homologs of members of these families of transporters across bacterial genera share sequence identity relative to their phylogenetic distance from each other. The AlphaFold predicted structure of Rv1217c, an ABC transporter implicated in antimycobacterial export, shows a complete structural overlap with its putative homologs in *Streptomyces coelicolor* (Amino acid identity 40.7%), *Bacillus subtilis* (identity 26.8%) and *Streptococcus pneumonia* (identity 24.9%) (Fig. 2), indicative of a conserved 3D fold despite low sequence identity. Members involved in clinical resistance are known to efflux multiple classes of antibiotics. While promiscuity exists among efflux pumps, the ability to discriminate between nutrients and toxins and to be discrete enables survival. The structure of only a few transporters is known for *M. tb,* which limits our understanding of mechanisms of substrate recognition, an important aspect of design-specific efflux pump inhibitors. Transporters discussed in the review, along with their location within the cell envelope, have been depicted in (Fig. 3). To this date, no such inhibitors are in clinical use, although Verapamil (a calcium channel blocker) and Reserpine, have shown promising outcomes *in vitro*. The knowledge of structure would not only help in the design of small molecules that inhibit antibiotic binding but also target energy mechanisms or dismantle the functional assembly of the transporter.

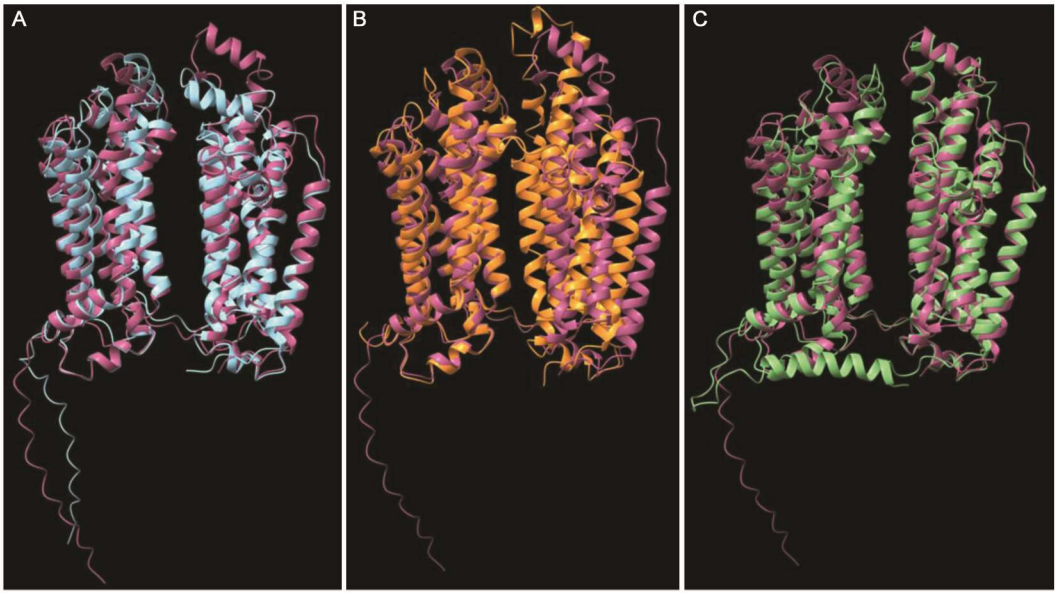

Fig. 2 — Structural overlap of Rv1217c (pink) over homologs in (A) *Streptomyces coelicolor* (blue): RMSD between 354 pruned atom pairs is 1.157 Å; (B) *Bacilli subtilis* (orange)*:* RMSD between 235 pruned atom pairs is 1.134 Å; and (C) *Streptococcus pneumoniae* (green)*:* RMSD between 293 pruned atom pairs is 1.329 Å across 535 atom pairs. Models generated by AlphaFold, representation by Chimera (https://www.cgl.ucsf.edu/chimera/)

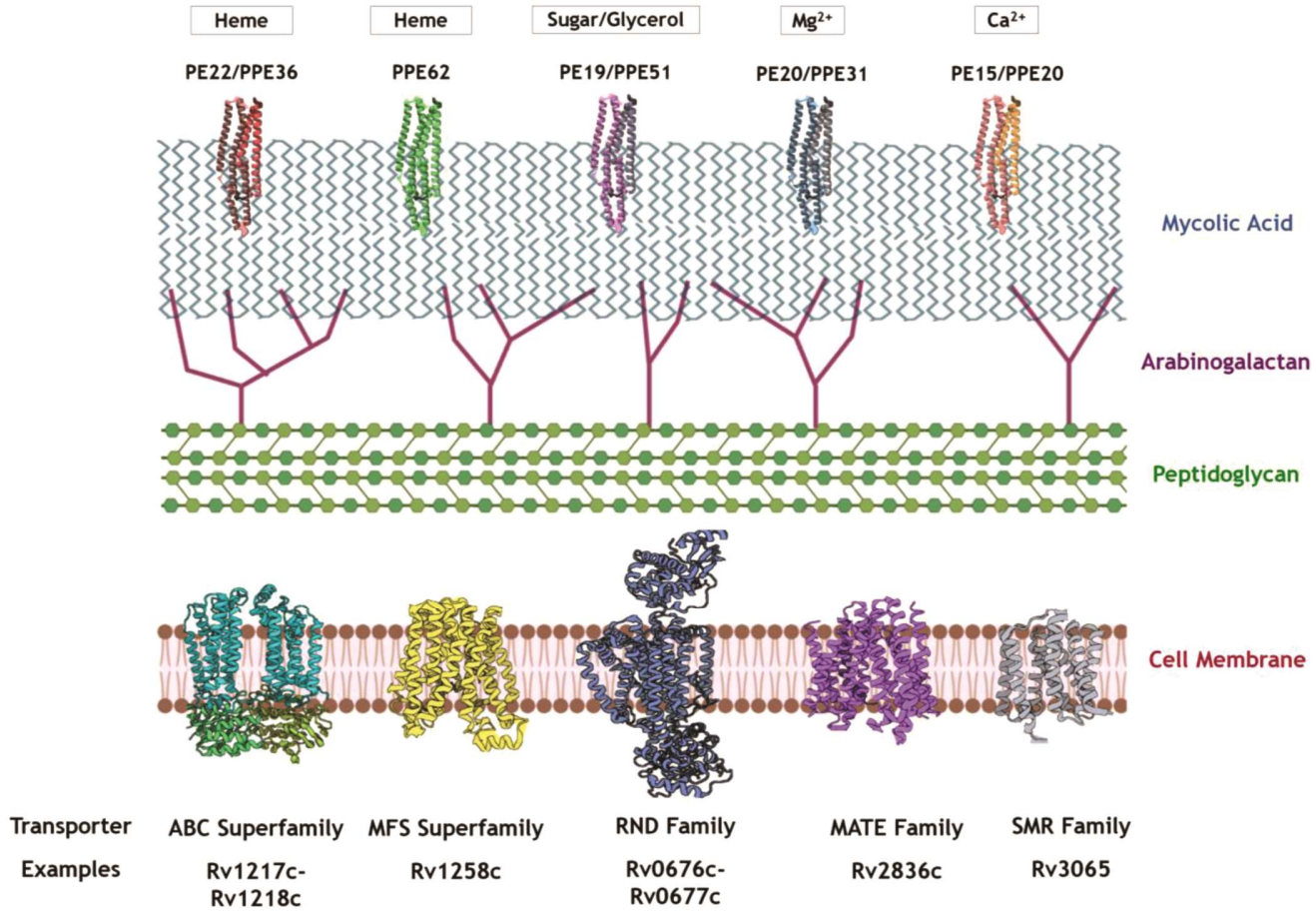

Fig. 3 — Schematic representation of outer membrane proteins of the PE_PPE and cell membrane transporter families

Efflux pumps implicated in drug-resistant *M. tb* clinical isolates

Members of the ABC and MFS families of transporters have been observed to be involved in the efflux of multiple antimycobacterial drugs. DrrAB (*Rv2936-Rv2937)*, a member of the ABC transporter family of *M. tb,* which consists of DrrA (*Rv2936*), an ATP-binding protein, and DrrB (*Rv2937*), a membrane integral protein, contributes to rifampicin-monoresistance in clinical isolates in addition to classical mutations in the *rpoB* gene[11]. Overexpression of *drrA* and *drrB* has been frequently observed in MDR-TB and XDR-TB clinical isolates[12–14].

Rv1217c-Rv1218c, another member of the ABC transporter superfamily, has been observed to be overexpressed at the transcriptional level in the MDR-TB clinical isolates. An increase in MIC of Rifampicin was observed on the overexpression of *Rv1217c* and *Rv1218c*, while an increase in MIC of Isoniazid was observed on the overexpression of only *Rv1218c* in MDR-TB clinical isolates[15].

An ABC efflux pump consisting of *Rv1456c-Rv1457c-Rv1458c* showed overexpression at the transcriptional level in MDR- and XDR-TB clinical isolates[16]. A greater than four-fold increase in the expression of *Rv0842*, *bacA*, *Rv2333, efpA, drrB*, and *drrC* genes was observed in Rifampicin monoresistant isolates, implying a role for these pumps in Rifampicin resistance[11]. RNA expression analysis of MDR- and XDR-TB clinical isolates showed increased levels of *Rv0933* (*pstB*) and decreased expression levels of *Rv2687c* and *Rv2688c*, suggesting a putative role of Rv2687c and Rv2688c in the influx of antibiotics[17,18].

Rv1258c, a tap-like pump and member of the MFS superfamily, was observed to be overexpressed in the presence of Bedaquiline (BDQ) in BDQ-resistant *M. tb* clinical isolates[19]. RNA sequencing and Whole Genome Sequencing of Ofloxacin-resistant isolates showed significant overexpression and a role for *Rv0191*, another member of the MFS superfamily, in Fluoroquinolone resistance[20].

Studies on MDR- and XDR-TB clinical isolates manifest that a single efflux pump can extrude various unrelated antibiotics, as demonstrated by the overexpression of genes encoding efflux pumps in the presence of different antimicrobials, listed in (Table 1). Studying the differences in virulence and antibiotic resistance mechanisms among clinical isolates can help identify novel targets and alternative mechanisms of resistance, necessitating the urgent validation of the role of efflux pumps in drug-resistant clinical *M. tb* isolates.

Role of efflux pumps in antimycobacterial resistance

Despite our knowledge of their presence in the *M. tb* genome since 1998, mechanistic details about the functioning of mycobacterial efflux pumps are in their nascent phase. Rv1877, a putative MFS efflux pump, has been implicated in antimycobacterial resistance[21]. Ectopic expression of *Rv1877* in *E. coli* increased the MICs for Sparfloxacin, Nalidixic acid, Levofloxacin (2x), Ofloxacin, and Apramycin as compared to controls. In addition, the accumulation of Ofloxacin was significantly lower in cells expressing

Antimycobacterial Drugs	Efflux pump genes overexpressed in clinical drug-resistant *M. tb* isolates	Transporter Family	References
Isoniazid (INH)	*Rv1218c* *Rv0933* *Rv2936/37* *Rv1819c* *Rv1456c/57c/58c* *Rv0194* *Rv1634* *Rv1410c* *Rv1250* *Rv1258c* *Rv2846c* *Rv2459* *Rv2333c* *Rv0849* *Rv0507* *Rv0676c/77c* *Rv3065* *Rv2836c* *Rv2936/37/38* *Rv1217c/18c* *Rv0194* *Rv1819c* *Rv1456c/57c/58c*	ABC Family MFS Family RND Family SMR Family MATE Family ABC Family	[14], [15], [16], [18], [19]
Rifampicin (RIF)	*Rv1250* *Rv2333c* *Rv2846c* *Rv1258c* *Rv1410c* *Rv1634* *Rv2459* *Rv0849* *Rv0676c* *Rv0507* *Rv3065* *Rv2836c* *Rv0842* *Rv1819c* *Rv1410c* *Rv1258c*	MFS Family RND Family SMR Family MATE Family Undetermined ABC Family	[11], [13], [15], [16], [18], [19], [21]
Ethambutol (EMB)	*Rv1634* *Rv2459* *Rv2846c* *Rv0676c* *Rv3065*	MFS Family RND Family SMR Family	[19]

(*Contd*.)

Table 1 — Efflux pumps overexpressed in drug-resistant clinical isolatesof *M. tb* (*Contd*.)

Anti-TB Drugs	Efflux pump genes overexpressed in clinical drug-resistant *M. tb* isolates	Transporter Family	References
Kanamycin (KAN)	*Rv1258c*	MFS Family	[17]
	Rv1456c/57c/58c	ABC Family	
	Rv1819c		
Streptomycin (STR)	*Rv1410c*	MFS Family	[16], [19]
	Rv0676c	RND Family	
	Rv3065	SMR Family	
	Rv0655		
Fluoroquinolones (FQ)	*Rv3102c*	ABC Family	[20]
	Rv1463		
	Rv0191	MFS Family	
	Rv1819c	ABC Family	
	Rv1273c		
	Rv1258c		
Bedaquiline (BDQ)	*Rv1410c*		
	Rv1634	MFS Family	
	Rv2459		
	Rv2846c		
	Rv0676c	RND Family	[19], [32], [33]
	Rv0507		
	Rv3065	SMR Family	

Rv1877, signifying a role for this gene in resistance against fluoroquinolone. *Rv1877* expression in *trans* increased the biofilm formation in *E. coli*, indicating its putative function in biofilm formation[22]. Similarly, the expression of *Rv1273c*, a putative ABC transporter, increased tolerance to fluoroquinolones and β-lactams in *E. coli* and *M. smegmatis*. *M. smegmatis* expressing *Rv1273c* also increased the MICs for Isoniazid and Rifampicin. *E. coli* and *M. smegmatis* expressing *Rv1273c* showed a 30% and 94% increase in biofilm formation, respectively, pointing to the role of the gene in biofilm formation[23]. Efflux systems contribute to biofilm formation *via* the transport of extracellular polymeric substances and/or quorum sensing/quorum quenching molecules, extrusion of toxins and harmful metabolites, aggregating adhesion to surfaces or other cells, and indirectly regulating genes involved in biofilm formation. The studies on *Rv1877* and *Rv1273c* implicate their role in forming biofilms, which collectively efflux diverse classes of chemical compounds and show increased resistance against antibiotics.

EfpA (*Rv2846c*), a highly conserved efflux pump in slow-growing (*M. tb*, *M. leprae*, and *M. avium*) and fast-growing mycobacteria (*M. smegmatis* and *M. fortuitum*) belongs to the MFS family of transporters and shows high relatedness with the members of the QacA transporter family. Overexpression of *efpA* in *M. smegmatis* showed an appreciable increase in the MIC of Ciprofloxacin and Moxifloxacin; similar results were observed upon tunable expression of *efpA*, revealing its essential role in resistance against fluoroquinolones[24]. EfpA is an essential pump in *M. tb*, as demonstrated by the knock-down of *efpA* using CRISPR interference. This knock-down reduced the viability of *M. tb* and changed the morphology of cells into an elongated phenotype with extension in length up to 4-7 μM with branched and blebbing morphology, suggesting irregularities in the cell division when *efpA* expression is diminished[25]. A recent study by Wang *et al*. on its cryo-EM structure showed three lipid binding sites in EfpA and its resemblance to the lysophospholipid transporter MFSD2A, suggesting its role as a lipid flippase[26]. Together, all these studies predict that EfpA should be pursued as a lipid transporter and must be validated further. Perturbation of *efpA* might cause irregularities in the lipid content of the mycobacterial cell envelope with concomitant reduction in the uptake of antibiotics, which may be one of the mechanisms of EfpA in antimycobacterial resistance.

Apart from the efflux of antibiotics, toxins, adhesins and quorum sensing molecules, the P-type ATPases, regulate the intracellular concentration of metal ions essential in various fundamental processes and are involved in heavy-metal resistance. These are a class of membrane proteins that consist of multiple domains such as an actuator, nucleotide binding, and phosphorylation domain, which are cytoplasmic domains, and a transport domain embedded in the membrane. They get activated by autophosphorylation

at a conserved aspartate residue and regulate the concentration of various metal ions against their electrochemical gradient *via* hydrolysing ATP. P-type ATPases are conserved and abundant in *M. tb*, with genome analysis identifying 12 such proteins thus far[27]. Rv2856 (NicT), a putative P-type ATPase nickel/cobalt transporter (NiCoT family) from *M. tb,* is involved in the resistance against Isoniazid, Ofloxacin, Norfloxacin, and Nalidixic acid, as observed upon the overexpression of *Rv2856* in *M. smegmatis*, which lacks an *Rv2856* homologue. *Rv2856* expression did not alter the sensitivity towards Kanamycin, Ethambutol, Tetracycline, Doxycycline, Neomycin and Apramycin, which is reduced in the presence of a sub-inhibitory concentration of Ni^{2+}, surmising role of Rv2856 in cross-resistance to antibiotics[28]. Similarly, Rv3270, a P-type ATPase known for its role in combating Zn^{2+} and Mn^{2+} metal ion toxicity in *M. tb,* has been shown to influence the expulsion of structurally unrelated drugs and enhance biofilm formation in *E. coli* and *M. smegmatis* upon its overexpression. The MICs for Levofloxacin, Apramycin, and Rifampicin are increased in the presence of a sub-inhibitory concentration of Zinc ($ZnSO_4$), postulating the role of Zn^{2+} in enhancing the efflux activity, strengthening its role in antimycobacterial resistance[29]. Taken together, all these studies imply the role of drug-metal cross-resistance in regulating the exclusion activity of efflux pumps *via* electrochemical gradient modulation.

P55 (Rv1410c), an MFS efflux pump, has been shown to be associated with various instances of resistance against antimycobacterials such as fluoroquinolones, Isoniazid, Rifampicin, and Bedaquiline[11,12,14,19,20,30]. *Rv1410c* is present in an operon with *Rv1411c* encoding the lipoprotein LprG, and may participate in the transport of lipids across the cell membrane. Deletion of the P55 homolog in *M. bovis* BCG rendered the strain susceptible to different toxic compounds, including Rifampicin and Clofazimine, and to some cell wall-targeting compounds such as Ethambutol, Vancomycin, and Bacitracin, denoting its role in lipid transport or cell wall formation as observed in other studies[31].

The mycobacterial membrane protein Large (MmpL) and mycobacterial membrane protein-small (MmpS) proteins form an efflux pump belonging to the RND superfamily of transporters and are involved in various physiological processes such as lipid transport, siderophore transport, and iron acquisition. The MmpL5 (Rv0676c)/MmpS5 (Rv0677c) efflux pump has been associated with resistance against Bedaquiline and Clofazimine in various *M. tb* clinical isolates[19,32,33].

Regulation of efflux pump expression

Uncontrolled expression of efflux systems can be harmful to bacteria as they are involved in various critical physiological processes such as lipid transport, the exclusion of toxic by-products, and other harmful compounds. Hence, the regulation of their expression is crucial for bacterial survival. Understanding the mechanism of regulation of efflux pumps involved in antibiotic resistance in *M. tb* is a subject of active investigation, as described below.

The TetR (tetracycline repressor) family of transcriptional regulators (TFTRs) are extensively associated with instances of antibiotic resistance and consist of an N-terminal helix-turn-helix (HTH) DNA binding motif and a C-terminal ligand-binding pocket. They are the best-studied regulators of efflux pumps in *M. tb*, and genome analysis shows the presence of 52 TetR regulators[34]. *Rv3066*, a gene located immediately downstream of *Rv3065* (*mmr*), encodes an efflux pump of the small multidrug resistance (SMR) protein family. This protein has been shown to negatively regulate the expression of the Rv3065 efflux pump *via* binding to a 14-bp palindromic inverted repeat upstream of the *Rv3066* gene. Structural analysis of this protein revealed a structure consistent with the architecture of TFTRs, with a multidrug-binding pocket containing five aromatic residues, indicating the binding of ligands through aromatic stacking interactions[35]. Similarly, the TetR regulators Rv3249c and Rv1816 have been demonstrated to recognise the promoter and intragenic regions of *mmpL* genes, thereby regulating their expression[36].

The efflux pump encoded by *Rv1217c-Rv1218c* is also regulated by a TetR-like regulator Rv1219c, whose open reading frame is located immediately upstream to *Rv1218c*, as demonstrated by the binding of Rv1219c to the intergenic and intragenic regions of the *Rv1217c/18c/19c* operon. Structural analysis of Rv1219c revealed an entire helical structure with two domains and a large cavity surrounded by seven aromatic residues, indicating aromatic stacking interactions in the C-terminal of Rv1219c, suggesting a putative multi-drug binding pocket[37]. Another TetR regulator, Rv1255c, has been implicated in adaptation

to hypoxia-induced dormancy, increased Isoniazid tolerance and efflux *via* positive regulation of genes encoding efflux pumps[38].

MSMEG_3765, the *M. smegmatis* orthologue of *M. tb Rv1685c,* has been observed to be induced on exposure to acid-nitrosative stress and regulate the transcription of *MSMEG_3762/63/65* operon, an orthologue of *M. tb Rv1687c/86c/85c*, suggesting the role of Rv1685c as a regulator of the Rv1687c/Rv1686c efflux pump[39]. In docking studies, Rifampicin and Ciprofloxacin have been observed to prevent the binding of *MSMEG_3765* to its operator site, thereby inhibiting the induction of efflux pump expression[40]. This observation, however, needs experimental validation.

Mutations in the *Rv0678* encoding a regulator, have been associated with resistance against Bedaquiline and Clofazimine[32,33]. Rv0678 is a MarR-like family transcriptional regulator that forms a dimeric two-domain molecule that binds to the promoters of and regulates *MmpL5-MmpS5*, *MmpL4-MmpS4*, and *MmpL2-MmpS2*[41].

WhiB7, a member of the *M. tb* WhiB family of transcriptional regulators, contains a redox-sensitive [2Fe-2S] cluster and has been shown to be involved in upregulating the *Rv1473c* encoded efflux pump which is involved in resistance against macrolides[42].

The involvement of Lsr2 (Rv3597c, leprosy serum reactive clone 2), a small (~12 kDa), basic, histone-like, nucleoid-associated transcriptional regulator, has been demonstrated in the regulation of the IniBAC efflux pump[43], which confers resistance against Isoniazid and Ethambutol. Lsr2 shows an unusual mechanism of regulation as it binds non-specifically to the AT-rich 5′-untranslated region with a preference for circular DNA and forms large oligomers. Lsr2 inhibits DNase I activity, inhibits transcription*in vitro*, and introduces a modest degree of supercoiling into a relaxed plasmid, suggesting its role in DNA compacting and restricting DNA accessibility[43].

Since they are involved in various physiological processes such as virulence, lipid transport, and antibiotic efflux and are critical to bacterial survival, it is essential to study the mechanisms by which the expression of efflux pumps is regulated. Compounds that induce high levels of expression, silence the expression of, or block the function of the regulator protein could be lethal for *M. tb*, and are candidates for anti-TB drug development.

Identification of novel targets - The GWAS approach

The utilisation of whole genome sequencing approaches has significantly advanced our understanding of the possible drug resistance mechanisms of the disease by offering meaningful information on the evolution and dissemination of resistance mutations. Genome-wide association studies (GWAS) seek to uncover the relationship between genotypes and phenotypes through testing for variations in the frequency of genetic variants between individuals who are ancestrally similar but phenotypically distinct. Determining the underlying source of heritable phenotypes and using the associations identified to serve as an impetus for research into potential therapeutic interventions are the primary motives that drive GWAS. Considering how well human GWAS has performed in determining possible therapeutic targets and comprehending the aetiology of complex diseases such as type 2 diabetes, microbiological genome analysis has increasingly drawn the attention of infectious disease experts since it can discover variants in the genomes of microorganisms and attribute the variants to clinically important traits such as drug resistance.

Resistance in *M. tb* arises from changes in the genome, such as single nucleotide polymorphisms (SNPs), insertions and deletions in genes coding for drug targets or involved in drug metabolic pathways, or from upregulation of efflux pumps. A breakthrough in the quick, easy, and standardised treatment of drug-resistant tuberculosis infections is the use of GWAS to identify genomic changes between sensitive and resistant strains of the disease. Farhat *et al*. sequenced the genomes of 116 *M. tb* clinical isolates and discovered a mutation in *ponA1* that imparted a twofold greater MIC to rifampicin than wild-type bacteria[44]. Zhang *et al*. analysed 161 genomes and identified 72 new genes and 28 intergenic regions associated with resistance. They demonstrated that SNPs within two intergenic regions upstream of the known resistance genes *thyA-Rv2765* and *thyX-hsdS.1* contributed to the upregulation of a *lacZ* construct gene expression in *M. smegmatis*, indicating that these mutations might underlie p-aminosalicylic acid resistance by overexpressing downstream genes[9]. Desjardins *et al*., with the help of whole genome sequencing data published by Zhang *et al.* and Cohen *et al.*, identified a novel drug resistance mechanism to D-cycloserine, a second-line TB

drug[45,46]. They discovered that loss-of-function mutations in the *ald* (*Rv2780*) gene, which encodes L-alanine dehydrogenase, were linked to unexplained drug resistance. It featured a remarkably high number of distinct loss-of-function mutations that seemed to follow a pattern that is consistent with convergent evolution. GWAS, with a large data set of 10,288 clinical isolates along with resistance quantification *via* MIC estimation of 13 antimicrobials, enabled the CRyPTIC Consortium to uncover resistance-conferring cofactors that are not currently documented[47]. Thus, the increased availability of whole-genome sequences from phenotypically different strains of *M. tb*, along with improved GWAS methods, is enabling the identification of novel drivers of unexplained resistance. For these newly discovered determinants to be useful in combating clinical cases of drug resistance, experimental validation and investigation of their role in conferring resistance is needed. Interestingly, from the GWAS, it is evident that members of the PE-PPE family contribute to resistance to a range of antimicrobial drugs. Previous studies focused on assigning genotypic characters to drug-resistant clinical strains have also uncovered the contribution of the PE_PPE family (described below) to resistance. Tantivitayakul *et al.* observed that the *PE/PPE* genes had significantly higher homoplastic SNPs among the 8 different functional groups studied that included genes involved in lipid metabolism, virulence, and cell wall metabolism[48]. Genomic sequence analysis to identify gene interactions associated with drug resistance found that, in most cases, a member of this family is associated with a drug target. *katG, rpoB*, and *embB,* conferring Isoniazid, Rifampicin, and Ethambutol resistance, respectively, are all associated with *PPE54*, while *embA* is associated with *PPE68*[49].

The PE_PPE family

The *PE_PPE* family constitutes a unique set of 168 genes found among the estimated 4173 genes in the *M. tb* genome, and approximately 7–10% of the genome's coding capacity is contributed by these genes. Members of this family were identified based on the presence of either a conserved proline-glutamic acid (PE) or proline-proline-glutamic acid (PPE) motif within the highly conserved N-terminal domain of the protein, which is around 100 or 180 residues long, respectively[50,51]. Although they are conserved in each family, their N-terminal domains are not identical. On the other hand, the C-terminal domains exhibit a great deal of variation in both length and sequence. *PE_PPE* genes are unique to the *Mycobacterium* genus. Although they are present in both saprophytic and pathogenic strains of *Mycobacteria*, comparative genomic studies have revealed that these genes are expanded in the slow-growing and pathogenic strains of the bacterium. The *M. tb* complex and other *Mycobacteria*, including *M. leprae*, *M. marinum*, and *M. avium*, contain the greatest number of *PE_PPE* genes. This suggests that these genes have undergone beneficial evolutionary selection, particularly in pathogenic *Mycobacteri*a, and that pathogenic species of *Mycobacteria* adapted to an intracellular and pathogenic lifestyle facilitated by the expansion of the *PE_PPE* family. The ESAT-6 cluster regions (*ESX*) that encode the mycobacterial type VII secretion system, have been tightly linked to the *PE_PPE* gene families. Some protein products of these genes are surface-bound, while others are secreted. Irrespective of their localisation, PE_PPE proteins must first pass through the inner membrane of mycobacteria using their cognate ESX type VII secretion system. PE_PPE proteins have also been known to modulate the host immune responses and benefit the intracellular survival of *M. tb*[52–55]. It is evident that *M. tb* employs proteins of the PE_PPE family to develop resistance to a range of stressors that are present in the intracellular environment of the host cell. Here, we focus on the transport capability of this unique protein family.

PE_PPE - Non-canonical *M. tb* transporters

Recent reports demonstrate that due to their structural ability, the PE/PPE proteins, have a potential role in importing small molecules into the cell's interior. The cell surface localized PPE36 and PPE62 have been shown to specifically bind heme, and PPE36 is essential for growth in a medium with heme as the sole iron source. This study provided the first direct evidence that members of this protein family bind to small molecules and are involved in nutrient acquisition, although their precise role is unclear[56]. PPE64, the protein product of one of the *M. tb* genes significantly upregulated in the presence of heme, has been shown to form a channel in a lipid bilayer[57]. PE20 and PPE31 have been observed to interact with each other and are required for growth in magnesium-limiting conditions[58]. The channel-like activity specific to Ca^{2+} uptake has been demonstrated

for PE15-PPE20 as well[59]. Two elegant studies deciphered the role of these proteins in the uptake of sugar molecules in *M. tb*, specifically, disaccharides, glycerol, and glucose. They determined the function of PPE51 and PE19 in nutrient uptake and demonstrated that PPE51 and PE19 physically interact with one another, revealing that these proteins are secreted together, are localised to the outer membrane, and create a porin-like channel to transfer nutrients[58,60] (Fig. 3). Deletion of the operonic *PPE50-PPE51* from *M. tb* $mc^2$6230 confers tolerance to both Isoniazid and Rifampicin[61]. Therefore, it seems possible that PE-PPE family members can combine in multiple permutations that power *M. tb* to survive hypoxia, low pH, and host immune responses, along with antibiotics, all at once. Considering that the *M. tb* cell wall lacks traditional porins for transport, the PE_PPE family proteins seem to offer a useful alternative for these restrictions. Although the available data points to the PE_PPE family members as being able to facilitate nutrient import, it is also possible that other molecules, such as anti-TB drugs, may be transported through the pore-like channels these proteins might generate. This offers the tantalising possibility of these proteins playing a prominent role in tolerance/resistance to antibiotics, a premise that requires experimental validation.

Conclusion

Transport across the cell envelope is an important function for the survival of any organism and, hence, represents a major drug target. A number of efflux pumps are known to be associated with antimycobacterial tolerance and resistance in *M. tb*. However, the mechanism of efflux of anti-TB drugs is not understood in its molecular detail, which restricts our ability to design specific efflux pump inhibitors. Moreover, our knowledge of the mechanisms of entry of drugs through the mycomembrane remains limited. Innovations such as toxic substrate-analogue or substrate-antibiotic conjugates would open a new avenue of antimycobacterials and could be explored only if the nitty-gritty of import proteins is known. This review sheds light on the major findings regarding the transport potential and its regulation in *M. tb*. Questions such as the following are prerequisites to designing effective antimycobacterial drugs and shortening the treatment regimen: a) How does *Mycobacterium* maintain a near-impermeable envelope and still ensure a selective supply of nutrients within the hostile host environment? b) How is the expression of transport proteins regulated? c) What is the binding mechanism of antibiotics to efflux pumps and their regulators? d) Are efflux pumps as non-specific as they seem in pumping out antibiotics of unrelated groups, or is there conserved specificity in the binding sites of antibiotics among different efflux proteins? An important piece of information still missing from the field is the structure of drug-bound transporters. The structural biology of transporters in *M. tb* is still in its emerging phase and requires immediate attention as antibiotic tolerance has become a limiting factor in combating Tuberculosis.

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Conflict of interest

All authors declare no conflicts of interest.

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