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MINIREVIEW

Energy Metabolism and Drug Efflux in *Mycobacterium tuberculosis*

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The inherent drug susceptibility of microorganisms is determined by multiple factors, including growth state, the rate of drug diffusion into and out of the cell, and the intrinsic vulnerability of drug targets with regard to the corresponding antimicrobial agent. *Mycobacterium tuberculosis*, the causative agent of tuberculosis (TB), remains a significant source of global morbidity and mortality, further exacerbated by its ability to readily evolve drug resistance. It is well accepted that drug resistance in *M. tuberculosis* is driven by the acquisition of chromosomal mutations in genes encoding drug targets/promoter regions; however, a comprehensive description of the molecular mechanisms that fuel drug resistance in the clinical setting is currently lacking. In this context, there is a growing body of evidence suggesting that active extrusion of drugs from the cell is critical for drug tolerance. *M. tuberculosis* encodes representatives of a diverse range of multidrug transporters, many of which are dependent on the proton motive force (PMF) or the availability of ATP. This suggests that energy metabolism and ATP production through the PMF, which is established by the electron transport chain (ETC), are critical in determining the drug susceptibility of *M. tuberculosis*. In this review, we detail advances in the study of the mycobacterial ETC and highlight drugs that target various components of the ETC. We provide an overview of some of the efflux pumps present in *M. tuberculosis* and their association, if any, with drug transport and concomitant effects on drug resistance. The implications of inhibiting drug extrusion, through the use of efflux pump inhibitors, are also discussed.

Tuberculosis (TB), caused by *Mycobacterium tuberculosis*, remains a global health problem, causing 8.8 million incident cases and 1.1 million deaths in 2012 (1, 2). In many countries, the TB epidemic continues unabated in the face of combination chemotherapy which involves the administration of at least four drugs. The most significant barrier to the eradication of TB is the rapid emergence of multidrug-resistant (MDR) and extensively drug-resistant (XDR) TB, which has rendered current treatments ineffective and placed an enormous patient management burden on TB control programs. Treatment of drug-resistant TB is costly and requires the use of highly toxic drugs, leading to numerous side effects. Moreover, the spread of MDR strains makes for an alarming situation which provides an ideal breeding ground for further, more advanced forms of drug resistance (3). Given the limited number of anti-TB drugs currently available and the duration of treatment required to achieve cure, there is a global need for the discovery of novel drugs with rapid sterilizing activity against active and dormant bacteria. These drugs should ideally shorten treatment duration and reduce the pill burden (2, 4). Moreover, maintaining the fidelity of the current antibiotics and further understanding the mechanism of emergence of drug resistance require immediate attention to address this growing problem.

Suboptimal intracellular concentrations of drugs often lead to transient drug tolerance, which may be a precursor to chromosomally encoded, stable drug resistance. There is a growing body of evidence that suggests that *M. tuberculosis* retains the capacity to extrude drugs from the cell, resulting in drug tolerance effects (reviewed in reference 5). In many cases, these processes are postulated to be dependent on the proton motive force (PMF) and the presence of sufficient ATP concentrations within the cell. This suggests that the activity of the mycobacterial electron transport chain (ETC) under different conditions plays a key role in determining the inherent susceptibility of *M. tuberculosis* to various drugs. In this review, we provide an overview of the mycobacterial ETC and discuss this component of *M. tuberculosis* metabolism as a target for novel antitubercular agents. We also highlight the importance of energy metabolism in mediating drug tolerance through efflux.

THE MYCOBACTERIAL ETC

In bacteria, the ETC is integrally involved in the generation of energy via oxidative phosphorylation. The mycobacterial components involved in oxidative phosphorylation and energy production have been extensively reviewed, and the reader is referred to these for more-detailed information (6–12). Electrons enter and are shunted through the ETC in a variety of ways, depending on the source of growth substrates and the availability of terminal electron acceptors. Under aerobic conditions, oxygen is used in the final electron transfer steps, and under anaerobic conditions, nitrate or fumarate can be used (Fig. 1). Electron transport in mycobacteria is initiated through the activity of various NADH dehydrogenases (NDH) and succinate dehydrogenases (SDH), which transfer electrons to menaquinone, a lipophilic redox carrier (7, 13, 14). Electrons are then passed to various cytochrome oxidases, which are dependent on oxygen availability (Fig. 1) (7, 15, 16). Of particular note is the fact that the mycobacterial ETC, like many bacterial ETCs, is branched and displays an extensive capacity to utilize numerous electron donors and acceptors for adapting to decreasing levels of oxygen tension and the availability

Published ahead of print 10 March 2014

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doi:10.1128/AAC.02293-13

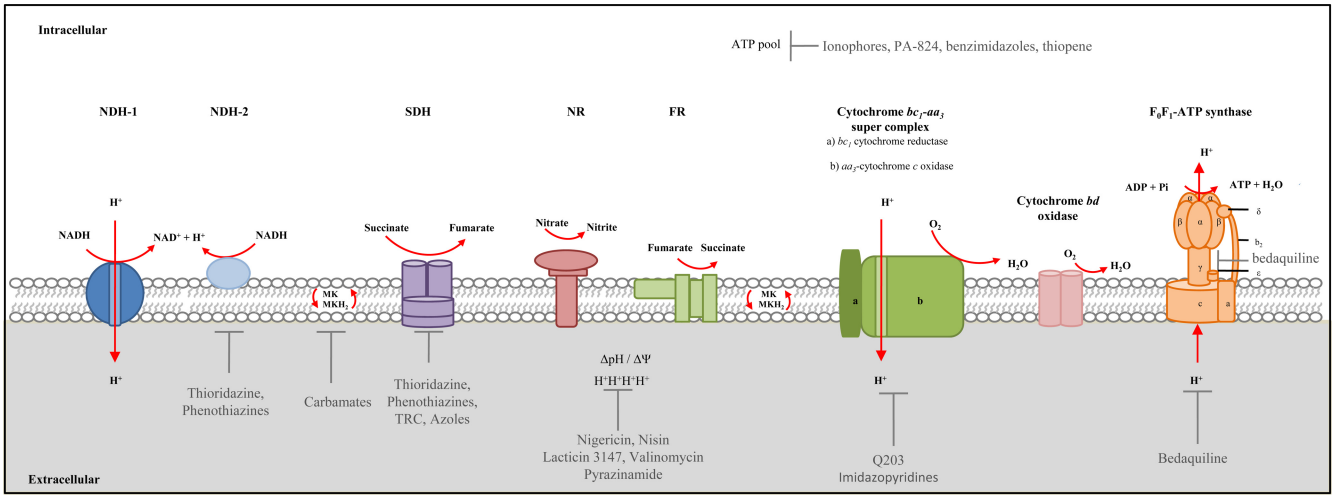

FIG 1 The mycobacterial electron transport chain. Proposed aerobic and hypoxic/anaerobic pathways are shown (7, 9). NDH-1, NDH-2, and succinate dehydrogenase (SDH) are electron donors which reduce menaquinone (MK) to menaquinol (MKH_2). Subsequently, MKH_2 becomes oxidized, transferring electrons to the terminal electron acceptors through the activity of cytochrome oxidases, nitrate reductase (NR), and fumarate reductase (FR). ATP production via the F_1F_0-ATP synthase is fuelled by translocation of protons (H^+). The F_1F_0-ATP synthase consists of two regions, namely, the hydrophobic integral membrane region (F_0) composed of 3 subunits designated a, b, and c and the hydrophilic region (F_1), which extends into the cytoplasm and is composed of 5 subunits designated α, β, δ, γ, and ε (7, 45–47). Energy production may be targeted by anti-TB compounds at multiple points involved in ATP synthesis as shown. These include the transmembrane proton gradient (ΔpH)—targeted by compounds such as nigericin, nisin, lacticin 3147, valinomycin, and pyrazinamide (PZA), resulting in disruption of ATP homeostasis. Compounds resulting in a depletion of the ATP pool include phenothiazines, ionophores (shown in the diagram), PA-824, benzimidazoles, thiophene, and imidazopyridines (Q203)/bedaquiline—through direct/indirect effects on the ETC. Other components of energy metabolism targeted by compounds include NDH-2 (thioridazine and phenothiazine), SDH (thioridazine, phenothiazine, triclosan, and azoles), MK biosynthesis (carbamates), Q203, and the F_1F_0-ATP synthase (bedaquiline). Vertical red arrows indicate proton pumping components of the ETC that directly translocate protons into the extracellular matrix.

of different reducing equivalents. The roles of various components of the ETC are detailed below.

ELECTRON DONORS

Analysis of the genome sequence of *M. tuberculosis* H37Rv reveals two types of NDHs in mycobacteria, namely, type I NDH (NDH-1), encoded by the *nuoABCDEFGHIJKLMN* operon, and type II NDH (NDH-2), encoded by either the *ndh* gene or the *ndhA* gene (17, 18). In mycobacteria, the reduction in expression of *nuoB*, encoding a subunit of NDH-1, during oxygen deprivation suggests that NDH-1 is required preferentially under aerobic conditions (19), while use of NDH-2 is favored under anaerobic/nonreplicating conditions (20). Treatment of mycobacteria with NDH-2-specific inhibitors, such as trifluoperazine, results in blockage of initiation of the ETC under anaerobic conditions, implying that NDH-2 is the dominant NDH involved in anaerobic respiration under the conditions tested (20). However, the dispensability of the entire *nuoABCDEFGHIJKLMN* operon under aerobic conditions (20) suggests that NDH-2 can operate as an electron donor irrespective of oxygen availability. The NuoG subunit of NDH-1 has been identified in a forward genetic screen to be essential for inhibiting macrophage apoptosis and in virulence in the murine model of TB infection (21). The precise mechanism of apoptosis inhibition is not yet understood, but more-recent work suggests that it involves the neutralization of NOX2-derived reactive oxygen species (22). In this regard, the suppression of apoptosis leads to reduced distribution of tubercle bacilli between cells found within the lung and this results in decreased proliferation of *M. tuberculosis*-specific naive T cells (23). Transposon mutagenesis has identified NDH-1 to be dispensable for growth *in vitro*, and in this context, the apparent essentiality of NuoG for macrophage apoptosis does not seem to be directly related to its role in energy metabolism (24). Saturating transposon mutagenesis predicts that NDH-1 and NDH-2 are dispensable for growth of *M. tuberculosis in vitro* (24). Furthermore, an *ndhA* mutant is able to colonize mouse lungs, confirming that this enzyme is not essential for pathogenesis (25).

SDH, or succinate:menaquinone oxidoreductase, is another enzyme responsible for the donation of electrons to the quinone pool (13). Transposon mutagenesis confirmed that SDH is essential for survival of *M. tuberculosis in vitro* (24). This enzyme predominantly acts in the citric acid cycle, where it oxidizes succinate to fumarate, thereby donating electrons to the quinone pool (26, 27). It has been recently demonstrated that, through remodelling of the tricarboxylic acid cycle, the activity of SDH is essential for the metabolic adaptation to hypoxia, maintenance of membrane potential, and ATP synthesis, indicating that this is a key enzyme for persistence and therefore represents a potential new drug target (27).

MENAQUINONE

Quinones are lipid soluble electron carriers that are responsible for the transfer of electrons between the components of the ETC (7, 28). In mycobacteria, the predominant quinones are menaquinones (MK), in contrast to the case in *Escherichia coli*, which utilizes both menaquinone and ubiquinone (14, 29–31). In *M. tuberculosis*, MK is synthesized from the precursor chorismate through a series of reactions catalyzed by enzymes encoded by the *menABCDEFG* operon. Recently, an array of MenA inhibitors which display bactericidal activity against nonreplicating *M. tuberculosis* was developed (32). These observations suggest that MK needs to be continuously resynthesized to maintain membrane potential.

Of the compounds developed, carbamates have shown significant bacteriostatic activity against nonreplicating *M. tuberculosis* (Fig. 1) (32). Due to the importance of the MK pool in maintaining the ETC and subsequent ATP synthesis, this biosynthetic pathway represents an important target for anti-TB drug design.

ELECTRON ACCEPTORS

Electrons from reduced menaquinol can be transferred to either of two terminal oxidases: (i) the cytochrome *bd*-type menaquinol oxidase or (ii) the aa_3-type cytochrome *c* oxidase (via the cytochrome bc_1 reductase complex). It has been suggested that the mycobacterial aa_3-type cytochrome *c* oxidase and cytochrome bc_1 reductase form a supercomplex (7, 16). The *M. tuberculosis* genome encodes a putative cytochrome *bd* oxidase which, in *Mycobacterium smegmatis*, has been shown to play an important role in the adaption to a reduced oxygen environment (15, 17). The *M. tuberculosis* genome also encodes a membrane-bound respiratory/assimilatory *narGHI*-encoded nitrate reductase (NR) which is responsible for nitrate-associated respiration and assimilation (17, 33–36). Analysis of clinical *M. tuberculosis* isolates revealed that NarG plays an important role in fitness in macrophages (37), consistent with the detection of transcripts from this operon within granulomas from lungs of TB patients (38) and in the guinea pig model of TB infection (39). The NR is also required for survival of *M. tuberculosis in vitro* under anaerobic conditions of nonreplicating persistence (40) and for the protection of *M. tuberculosis* against acidic conditions during hypoxia (41). These data suggest that respiration using nitrate may be an important mechanism for adaption under various conditions of stress. In support of this hypothesis, the *nirBD*-encoded nitrite reductase has recently been shown to play an important role in nonreplicating persistence of *M. tuberculosis* (34, 42). Considering other alternate electron acceptors, transcriptional analysis revealed that *frdA*, a gene encoding the flavoprotein subunit of the fumarate reductase, is upregulated under hypoxic conditions in *M. tuberculosis* and plays a vital role in maintenance of an energized membrane (43).

ATP SYNTHESIS AND THE F_1F_0-ATP SYNTHASE

Substrate-level phosphorylation involves the production of ATP by the utilization of free energy produced during various steps in metabolic pathways and provides a fast source of ATP through a process that is not dependent on external electron acceptors (44). In contrast, during oxidative phosphorylation, ATP is produced through the activity of the F_1F_0-ATP synthase enzyme, which is coupled to the PMF. *M. tuberculosis* has been classified as an obligate aerobe; as such, it would be dependent on oxidative phosphorylation for growth and survival during pathogenesis. The structure of F_1F_0-ATP synthase in various prokaryotes is conserved and consists of two regions, namely, the hydrophobic integral membrane region (F_0) and the hydrophilic region (F_1), extending into the cytoplasm (Fig. 1) (45–47). The F_0 region is composed of 3 subunits designated a, b, and c, whereas the F_1 region is composed of 5 subunits designated α, β, δ, γ, and ε (45, 46). ATP is synthesized by the $\alpha_3\beta_3$-hexamer through rotation of the γ-ε pair (48). Rotation of subunits ε and γ is coupled to rotation of the c-ring upon proton translocation (Fig. 1) (48, 49). The mycobacterial F_1F_0-ATP synthase is encoded by a single operon, *Rv1303-atpBEFHAGDC-Rv1312* (17). Recently, BlaI (Rv1846c) was identified as a transcriptional regulator of this operon (50). Treatment with ATP synthase inhibitors results in increased expression of *blaI*, suggesting a role for this regulator in response to stress (51). The mode of regulation of *blaI* in *Staphylococcus aureus* has been elucidated. BlaI has a structure that is similar to that of penicillin binding proteins and is able to act as a transcriptional repressor in response to antibiotic treatment (52). In this regard, it has been shown that BlaI (Rv1846c) in *M. tuberculosis* responds to antibiotic treatment and is released from its cognate operator sequences to allow gene expression (50). BlaR (Rv1845c), a zinc-dependent metalloprotease, has been hypothesized to play a role in cleaving itself and BlaI during derepression of the operon (50, 52). More recently, transcriptional regulators *Rv1773c* and *Rv3405c* have been identified as regulators for *Rv1303*, the first gene of the *atpBEFHAGDC* operon (53).

DRUGS THAT TARGET THE ETC

Recent drug discovery efforts have led to numerous compounds which have shown great promise in the treatment of TB due to their ability to eliminate *M. tuberculosis* in various preclinical models and early clinical trials. Among these is the discovery of TMC207 (bedaquiline), which kills *M. tuberculosis* by inhibition of the membrane-bound F_1F_0-ATP synthase complex, resulting in depletion of cellular ATP levels and eventual death of the organism (49, 54–56). Use of this drug, commonly known as bedaquiline, resulted in decreased time to smear conversion during a phase IIb randomized trial (54, 57). Bedaquiline kills *M. tuberculosis* by interacting with the hydrophobic region of subunit c, as well as with subunit ε (48), of the F_1F_0-ATP synthase and does not cross-react with the human ATP synthase complex (56, 58). Inhibition of c-ring rotation due to disruption in the c-ring:ε subunit interaction results in inhibition of ATP production and subsequent cell death (48). The efficacy of bedaquiline in clinical trials confirms that targeting energy metabolism during TB infection may be promising, particularly for nonreplicating organisms, as there is documented evidence that ATP is essential for the viability of nonreplicating persistent mycobacteria (20, 55, 59). Moreover, the ability to eliminate subpopulations of persisting organisms provides an opportunity for tissue sterilization, thereby minimizing the risk for recrudescent disease through reactivation of persisting bacteria. The ability of these organisms to maintain an energized membrane potential in the face of prolonged quiescence is critical to their survival and highlights the importance of understanding the physiology of *M. tuberculosis* with regard to energy metabolism (9). In this context, ATP synthesis has become a focus area for the identification of new drug targets in mycobacteria (60).

The PMF is an important aspect in the final production of ATP via the F_1F_0-ATP synthase (20). The PMF is established through the development of the transmembrane proton gradient (ΔpH) which occurs when electrons move through the ETC and results in the establishment of membrane potential (Δψ) (reviewed in references 6 and 7). The proton gradient generated through oxidative phosphorylation drives ATP synthesis via the F_1F_0-ATP synthase which is responsible for the conversion of the electrochemical potential energy generated by the PMF into chemical energy in the form of ATP (45). Since oxidative phosphorylation is the main source of energy production in mycobacteria, ATP synthase represents the key enzyme involved in ATP production in mycobacteria.

Studies of valinomycin and nigericin treatment (inhibitors of Δψ and ΔpH, respectively) revealed that in both actively replicat-

ing and hypoxic nonreplicating bacilli, death occurs via decreased ATP levels in a dose-dependent manner (20). Nisin, a lantibiotic produced by *Lactococcus lactis*, has been shown to dissipate $\Delta\psi$ and ΔpH, thereby disrupting energy metabolism (20, 61). Lantibiotics such as nisin and lacticin 3147 form pores in the mycobacterial cell membrane which result in dissipation of $\Delta\psi$ (Fig. 1) (several studies have investigated the effect of these compounds in mycobacteria [61–65]). Although nisin is able to dissipate the membrane potential and decrease ATP levels in mycobacteria, the MIC values for various mycobacterial strains are very high and not comparable to those of current anti-TB drugs (62). This poor inhibitory activity of nisin has been attributed to its low solubility at pH 7, in contrast to lacticin 3147, which is soluble under such conditions and demonstrates greater activity against mycobacteria, thus warranting further investigation as a potential anti-TB drug (62). Another compound shown to target $\Delta\psi$ is pyrazinamide (PZA), where treatment results in a decrease in ATP levels, most likely the resulting effect of diminished membrane potential (66). Unlike current first- and second-line anti-TB drugs, PZA has been shown to be active against both replicating and nonreplicating mycobacteria (67). This was highlighted in a recent study, where nutrient-starved *M. tuberculosis* displayed increased susceptibility to PZA due to the decreased membrane potential (68). Furthermore, PZA treatment of mice infected with *M. tuberculosis* significantly reduces the release of proinflammatory cytokines and chemokines, suggesting that PZA has important host-directed effects (69).

Due to the importance of ATP for cellular viability, the components involved in the process of ATP production represent viable drug targets which, in combination with current anti-TB drugs, could be used for effective treatment. A number of existing compounds deplete cellular ATP levels and have subsequent bactericidal effects on replicating and nonreplicating mycobacteria (Fig. 1). These compounds include *n*-decanesulfonylacetamide (DSA) and nisin. DSA is the lead compound of the β-sulfonylacetamide class of antimicrobials and has been shown to be active *in vitro* against replicating *M. tuberculosis* as well as against anaerobic *M. bovis* BCG (70–73). It has been proposed that DSA interferes with components of the respiratory chain, thereby disrupting energy metabolism (73).

Another compound targeting energy metabolism is PA-824, which is currently in human clinical trials as an anti-TB drug (57, 74–76). PA-824, a bicyclic nitroimidazole, has the ability to kill both replicating and hypoxic, nonreplicating *M. tuberculosis* through a multifaceted mechanism that involves inhibition of mycolic acid biosynthesis and respiratory poisoning through intracellular release of nitric oxide, which is postulated to inhibit the final stages of electron transfer in cytochrome *c* oxidase (76).

More recent efforts have yielded a novel class of imidazo[1,2-*a*]pyridine amide (IPA) compounds (77, 78) that prevent proliferation of *M. tuberculosis* by inhibition of the cytochrome bc_1 reductase complex in the mycobacterial respiratory chain (77, 79) (Fig. 1). These compounds bind the QcrB subunit and induce bacterial cell death by abrogating electron flow through the ETC, resulting in reduced ATP synthesis under aerobic and anaerobic conditions (79). Two independent studies identified QcrB as the target for IPAs through the generation of spontaneous resistant mutants carrying various substitutions at the Thr313 residue (77, 79). The lead compound from this series, Q203, displays potent killing of *M. tuberculosis* in axenic culture, in macrophages, and in the murine model of TB infection, with a spontaneous mutation rate in the order of 10^{-8} (79). Q203 is well tolerated in mice and now awaits further analysis in clinical trials.

Phenothiazines, such as chlorpromazine and thioridazine (THZ) (Fig. 1), are a group of clinically relevant compounds that are predicted to target the ETC through inhibition of NDH-2 (18). THZ is an old neuroleptic agent that has demonstrated activity in killing drug-susceptible and drug-resistant *M. tuberculosis* in various model systems *in vitro* and *ex vivo* and in the murine model of TB infection (80–84). In the promising development, THZ demonstrated therapeutic benefit in treatment of XDR-TB patients in Argentina and is currently being used in trials in India (reviewed in reference 85). In a recent study, THZ activity was shown to be independent of the bacterial growth phase; i.e., THZ is effective against actively replicating bacilli, semidormant bacilli, and nonreplicating persisters (86). That study also demonstrated a low mutation frequency in *M. tuberculosis*, suggesting a delay in the development of THZ resistance (86). While retaining the ability to inhibit the ETC in mycobacteria, THZ also has the potential to directly inhibit efflux of drugs (87). In addition to phenothiazines, which target the mycobacterial NDH-2, clofazimine (CFZ)—a rhiminophenazine—is subject to reduction by NDH-2 and, upon subsequent oxidation, leads to the formation of reactive oxygen species (88), which presumably contributes to its efficacy in mice (89).

Numerous studies have investigated the efficacy of novel combinations of drugs by incorporating new and existing compounds which have demonstrated activity against bacterial targets involved in energy metabolism. These combinations have demonstrated efficacy that is comparable or enhanced in comparison to the current anti-TB regimens, and the potential to shorten the current treatment period has been highlighted. A recent 14-day early bactericidal activity (EBA) study demonstrated the benefit of adding PA-824 to a regimen containing moxifloxacin (MXF) and PZA to shorten treatment duration (89). Bedaquiline–PZA–PA-824 and bedaquiline–PA-824–MFX combinations have demonstrated greater efficacy than rifampin (RIF)-isoniazid (INH)-PZA in reducing CFU (90). Tasneen et al. also demonstrated that drug combinations containing bedaquiline showed higher efficacy with respect to relapse prevention in a mouse model (89). In a separate study, numerous combinations of bedaquiline, PA-824, CFZ, and PNU-100480 were shown to be more effective than a RIF-INH-PZA combination (90).

ENERGY METABOLISM AND DRUG EFFLUX

Active transport of drugs and xenobiotics through the activity of efflux pumps (EPs) is well documented in bacteria and points to an important role for this process in phenotypic drug tolerance and the subsequent emergence of drug resistance due to reduced intracellular drug concentrations (5). A key feature of these systems is their dependence on the PMF or the availability of ATP, which inextricably links drug efflux to energy metabolism and the ETC (5, 91, 92). EPs adopt a diversity of structures in bacteria and can be classified into different families based on overall secondary structure and the molecules transported. These include, but are not limited to, the major facilitator superfamily (MFS), small multidrug resistance (SMR) family, resistance/nodulation/cell division (RND) family, the ATP-dependent ABC-type superfamily of transporters, and the multidrug and toxic compound extrusion (MATE) family (91, 93, 94).

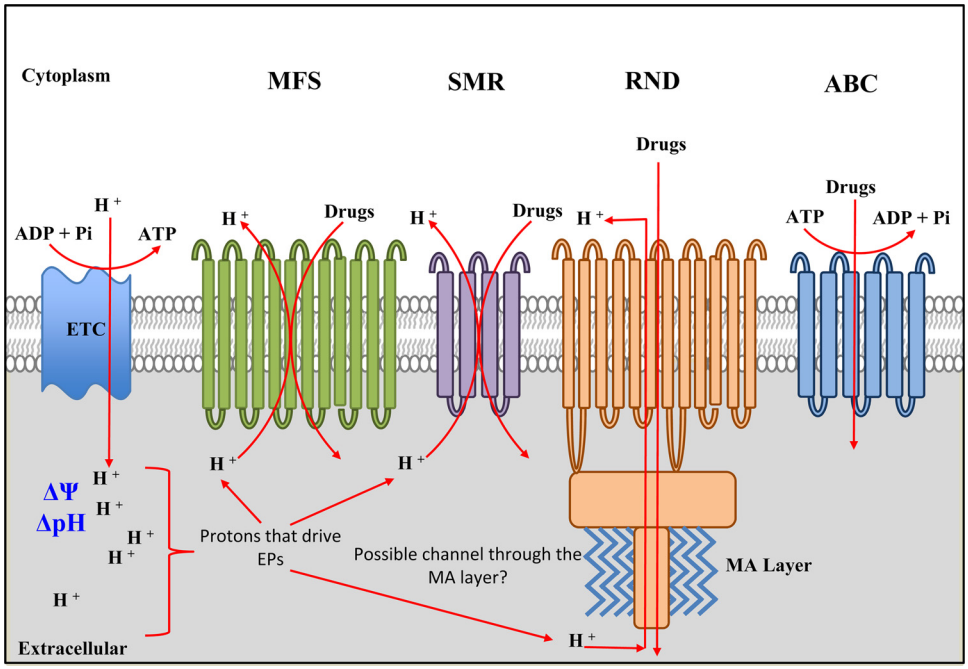

FIG 2 Efflux pumps in *M. tuberculosis*. The EPs dependent on the PMF and ATP are shown. The mycobacterial ETC generates a transmembrane proton gradient (ΔpH) resulting in membrane potential (Δψ); ΔpH and Δψ together constitute the PMF. Protons translocated into the pseudoperiplasmic space are used by EPs to extrude drugs. The major facilitator superfamily (MFS) EPs are made of integral membrane proteins with 12 to 14 transmembrane regions, while the small multidrug resistance (SMR) EPs contain 4 to 6 transmembrane domains. Both systems use protons from the periplasm, and the members differ in their requirements for ΔpH and Δψ (see the text for details). The resistance nodulation/cell division (RND) EPs are also integral membrane proteins, and members of this family from Gram-negative bacteria associate with other proteins to form a multisubunit complex that spans both the inner and outer membranes. Hence, there is a possibility that a similar structure occurs in mycobacteria, where the members of this group of EPs span the pseudoperiplasmic space and associate with the mycolic acid (MA) layer, possibly through OmpA (outer membrane protein A)-like homologues. RND proteins also require protons—although these may originate from outside the cell—and the PMF. ABC transporters require ATP for the active drug extrusion and, as such, are also dependent on energy production in the cell. Not shown in the figure are the members of the fifth class of EPS, termed the multidrug and toxic compound extrusion (MATE) proteins; while some of these are dependent on PMF and protons, the majority of characterized members operate via sodium influx (93).

The widely distributed P-glycoprotein, encoded by the *mdr* gene, is one of the first ABC-type transporters implicated in drug efflux and has been implicated in the extrusion of numerous drugs in different organisms (91). The members of this family of transporters require the energy of ATP hydrolysis for active efflux of drugs (Fig. 2) (95, 96). In contrast, the MFS, SMR, RND, and MATE-type EPs require an energized membrane and the PMF (ΔpH and ΔΨ) for activity (reviewed in reference 91). The pumps often operate as drug-metabolite/proton symporters, antiporters, or uniporters. Schematic representations of these EPs are shown in Fig. 2. The MFS-type EPs can be characterized into 6 evolutionarily related subfamilies, including those containing 14 or 12 transmembrane segments, sugar importers, phosphate ester antiporters, and other transporters (97–99). It has been proposed that these large membrane-associated proteins have evolved via intragenic tandem gene duplication to give rise to a widely distributed, structurally diverse family of proteins (97). MFS EPs require the presence of protons in the periplasm and couple efflux with proton translocation to the cytoplasm (100) (Fig. 2). However, these systems differ in their dependence on the ΔpH and ΔΨ; for example, the *bmr*-encoded multidrug EP in *Bacillus subtilis* requires a strong ΔpH to drive the extrusion of ethidium bromide through a electroneutral drug/proton antiport mechanism (91).

The Smr staphylococcal multidrug efflux protein and *E. coli* EmrR represent well-characterized EPs that belong to the SMR family, which is constituted of four transmembrane-containing transporters (91, 101). Smr from *Staphylococcus aureus* requires the ΔpH and ΔΨ for efficient transport of a wide range of drugs and other small compounds (102). Reconstitution experiments with both Smr and Emr confirm that these EPs require the PMF and operate through a drug/proton antiport mechanism (103). The RND proteins constitute the third family of broad substrate EPs that require the PMF for activity and are structurally more complex than SMR EPs, containing 12 transmembrane domains and various loop regions (104, 105). Unlike the MFS family of EPs, these proteins are phylogenetically closely related, suggesting that they evolved from a single founding member (91). In addition to being autonomous transporters, the members of the RND family of proteins interact with other transport proteins in Gram-negative bacteria such as membrane fusion proteins (MFPs) and outer membrane factors (OMFs) to facilitate the transport of a variety of substrates (Fig. 1) (105–107).

The genome of *M. tuberculosis* retains multiple homologues of the major families of PMF-dependent EPs described above and, in addition, encodes numerous ABC-type transporters or hypothetical proteins with homology to transporters (17, 108, 109). This complex multiplicity of EPs, detailed in Table 1, illustrates the ability of the tubercle bacillus to transport a variety of toxic compounds or antibiotics and has important implications for drug resistance in TB infection. The key feature of note is that many of

TABLE 1 Putative efflux pumps in *M. tuberculosis*[a]

Gene(s)	Description	Transporter family[b]	Energy source	Drugs effluxed	Differentially regulated in clinical isolates	Differentially regulated by indicated drug(s)	Reference(s)
Rv0037c	Probable conserved integral membrane protein	MFS	ATP		Yes		110
Rv0194	Drug transport transmembrane ATP-binding protein	ABC	ATP				17, 111
mmpS5 (*Rv0677c*)	Membrane protein MmpS5	RND	PMF	TET			17, 112
Rv0849	Probable conserved integral membrane protein	MFS	PMF	β-Lactams			113
Rv1218c	Probable tetronasin transport ATP-binding protein	ABC	ATP	β-Lactams			113
Rv1250	Probable drug transport integral membrane protein	MFS	PMF		Yes		110
Rv1258c	Conserved membrane transport protein	MFS	PMF	INH, RIF, EMB, OFL, β-lactams	Yes	INH, RIF, OFL	17, 114, 110, 115, 116, 113-117
Rv1272c	Probable drug transport transmembrane ATP-binding protein	ABC	ATP				17, 118
Rv1273c	Probable drug transport transmembrane ATP-binding protein	ABC	ATP		Yes		17, 110, 118
itrA (*Rv1348*)	Probable drug transport transmembrane ATP-binding protein	ABC	ATP				17, 118
itrB (*Rv1349*)	Probable drug transport transmembrane ATP-binding protein	ABC	ATP				17, 118
Rv1410c	Aminoglycosides/tetracycline transport integral membrane protein	MFS	PMF		Yes	INH, RIF	110, 115
Rv1456c-Rv1458c	Integral membrane proteins	ABC	ATP		Yes (in drug-resistant isolates)		17, 119
Rv1463	Conserved transmembrane ATP-binding protein	ABC	ATP				17
Rv1634	Drug efflux membrane protein	MFS	PMF		Yes		17, 109, 110, 120, 121
Rv1686c	Probable conserved ATP-binding protein	ABC	ATP		Yes (in drug-resistant isolates)		122
Rv1687c	Probable conserved ATP-binding protein	ABC	ATP		Yes		110
Rv1747	Conserved transmembrane ATP-binding protein	ABC	ATP	INH			17, 118
Rv1877	Conserved membrane protein	MFS	PMF	TET, KAN, erythromycin			17, 120, 118, 121
bacA (*Rv1819c*)	Drug transport transmembrane ATP-binding protein, vitamin B_{12} acquisition	ABC	ATP			INH, RIF	17, 123, 124, 115
Rv2209	Probable conserved integral membrane protein					OFL	124
Rv2333c	Conserved membrane transport protein	MFS	PMF	TET, spectinomycin	Yes		17, 110, 120, 125
jefA (*Rv2459*)	Conserved integral membrane transport protein	MFS	PMF			INH, EMB	17, 126, 124, 120, 127
Rv2477c	Probable macrolide transport ATP-binding protein	ABC	ATP			OFL	124
Rv2686c	Antibiotic transport membrane leucine- and alanine- and valine-rich protein	ABC	ATP	CIP			17, 118, 128
Rv2687c	Antibiotic transport membrane leucine- and valine-rich protein	ABC	ATP	CIP			17, 118, 128
Rv2688c	Antibiotic transport ATP-binding protein	ABC	ATP	CIP		STR	17, 124, 118, 128
Rv2994	Conserved membrane protein	MFS	PMF			STR	17, 124, 120, 121
Rv3000	Possible conserved transmembrane protein	ABC	ATP		Yes		110
Rv3239c	Conserved integral membrane transport protein	MFS	PMF			RIF	17, 129, 120
Rv3728	Conserved two-domain membrane protein	MFS	PMF			INH, RIF, EMB	17, 124, 120
drrA (*Rv2936*)	Daunorubicin-dim (dimycocerosate) transport ATP-binding protein ABC transporter DrrA	ABC	ATP	TET, STR, EMB, RIF	Yes		17, 110, 130, 118, 131
drrB (*Rv2937*)	Daunorubicin-dim transport membrane protein ABC transporter DrrB	ABC	ATP	TET, STR, EMB	Yes		17, 110, 118, 131
drrC (*Rv2938*)	Daunorubicin-dim transport membrane protein ABC transporter DrrC	ABC	ATP	TET, STR, EMB		STR, EMB	17, 124, 118, 131
efpA (*Rv2846c*)	Integral membrane efflux protein	MFS	PMF	possibly INH		INH	17, 124, 121, 132-133
emrB (*Rv0783*)	Possible multidrug resistance integral membrane efflux protein EmrB	MFS	PMF	RIF	Yes		17, 110, 132, 134

iniA (*Rv0342*)	Isoniazid inducible gene protein IniA	membrane protein		INH, EMB	Yes	17, 110, 135, 136
iniB (*Rv0341*)	Isoniazid-inducible gene protein IniB	membrane protein		INH		17, 135, 136
iniC (*Rv0343*)	Isoniazid-inducible gene protein IniC	Membrane protein		INH		17, 135, 136
mmpL3 (*Rv0206c*)	Probable conserved transmembrane transport protein MmpL3	RND	PMF			17, 137
mmpL4 (*Rv0450c*)	Probable conserved transmembrane transport protein MmpL4	RND	PMF		Yes	110
mmpL5 (*Rv0676c*)	Probable conserved transmembrane transport protein MmpL5	RND	PMF	TET		17, 112
mmpL7 (*Rv2942*)	Probable conserved transmembrane transport protein MmpL7	RND	PMF	INH	Yes	17, 110, 128, 137
mmpL11 (*Rv0202c*)	Probable conserved transmembrane transport protein MmpL11	RND	PMF			17, 137
mmr (*Rv3065*)	Integral membrane efflux protein	SMR	PMF	Erythromycin, β-lactams		INH, EMB 17, 109, 124, 113, 121
pstB (*Rv0933*)	Phosphate transport ATP-binding protein	ABC	ATP	INH, RIF, EMB, CIP		17, 130, 117, 118, 138, 139

[a] CIP, ciprofloxacin; EMB, ethambutol; INH, isoniazid; KAN, kanamycin; OFL, ofloxacin; RIF, rifampin; STR, streptomycin; TET, tetracycline.
[b] Transporter families were identified by literature searches (relevant cases are cited in the text) and from the genomic annotation/comparisons (http://genolist.pasteur.fr/TuberculList/ and http://tuberculist.epfl.ch/).

these transport systems require either the PMF or ATP, thus linking their activity with metabolic flux through the ETC and the maintenance of an energized membrane.

Further associations between the PMF and drug efflux in mycobacteria have been made through various studies which demonstrated that dissipation of the PMF by treatment with efflux pump inhibitors (108, 114, 126, 140) can reverse low levels of resistance to TB drugs. Early work with ^{14}C-labeled RIF suggests that this drug may be extruded from mycobacteria by the activity of PMF-dependent efflux pumps—a process that can be marginally reversed by the addition of reserpine, an EP inhibitor (141). In drug-resistant strains, exposure to efflux pump inhibitors such as verapamil, reserpine, Phe-Arg-β-naphthylamide (PAβN), carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), and 2,4-dinitrophenol (DNP) restores susceptibility to anti-TB drugs such as RIF and ofloxacin (OFL) (129, 142, 143). Inclusion of verapamil in drug susceptibility assays results in a marked decrease in the MIC for bedaquiline and clofazimine (144). Knockout studies have further implicated efflux pumps such as Rv1218c, Rv3065, Rv0849, and Rv1258c as important mechanisms of resistance to various chemical classes of drug compounds in *M. tuberculosis* (145, 146). A recent study demonstrated increased expression of 15 EP-encoding genes, from various classes, in drug-susceptible and drug-resistant strains compared to reference, laboratory grown strains (110). These data suggest that infection in the human host presumably drives expression of these genes to transport/detoxify noxious compounds during pathogenesis, with the concomitant benefit of drug extrusion. Similarly, the expression of 10 EP-encoding genes—Rv3065 (*mmr*), Rv2938 (*drrC*), Rv1819c (*bacA*—recently implicated in vitamin B_{12} acquisition [123]), Rv2209, Rv2459, Rv2477, Rv2688, Rv2846 (*efpA*), Rv2994, and Rv3728—is differentially induced during *in vitro* drug treatment of clinical isolates with standard TB drugs (124), as detailed further in Table 1, suggesting a complex induction pattern of these genes during short-course chemotherapy. While the regulatory mechanisms governing these gene expression changes have not been completely described, there is some evidence for transcriptional regulation of EPs. In this regard, it has been shown that WhiB7, a redox-sensitive transcriptional activator, plays an important role in mediating intrinsic drug resistance in mycobacteria, in some cases through direct regulation of EPs (147–149). Similarly, resistance to INH has been associated with the IniB-IniA-IniC efflux system (Table 1), which is regulated by the MtrAB two-component system (150), and the *mmr*-encoded EP is regulated by a TetR-type repressor (151). With respect to the evolution of drug resistance, transcriptional profiling of longitudinal isolates from drug-compliant patients, with MANU1, CAS, and Beijing spoligotypes, revealed upregulation of various EPs/multidrug resistance proteins, including Rv3065 (*mmr*), Rv2936 (*drrA*), Rv2397, Rv1686c, and Rv1687c, pointing to a role for these proteins in the evolution of drug resistance during treatment (122). Increased expression of genes encoding various efflux pumps upon exposure to RIF has been observed in *M. tuberculosis* which, in some cases, has been coupled with an increase in RIF and OFL tolerance. This phenomenon could be reversed by the addition of efflux pump inhibitors, highlighting the role of efflux in drug resistance (114–116, 129, 152). A similar result was observed during macrophage infection, where efflux pump activity was coupled to RIF and INH tolerance in *M. tuberculosis* (153). In RIF-monoresistant and -susceptible *M. tuberculosis* isolates, initial

low-level resistance to INH (reversible by the addition of efflux pump inhibitors) preceded the development of drug resistance-conferring mutations (126). Recently, it has been demonstrated that two EP-encoding genes, those encoding Rv2936 and Rv0783 (Table 1), are associated with RIF resistance in RIF-monoresistant clinical isolates. Overexpression of these two genes, but not of that encoding Rv0933, resulted in increased RIF resistance in *E. coli* (130). Similarly, low-level efflux-induced azithromycin resistance in *M. avium* was followed by reversible high-level resistance, thought to be due to acquisition of mutations (140).

These data suggest that the initial efflux activity allows antibiotic tolerance in mycobacteria, enabling resistance-causing mutations to arise and/or be selected for. In this context, the production of ATP via the F_1F_0-ATP synthase would be essential for the function of these systems. Since efflux pumps seem to play an important role in contributing to drug tolerance, which may then lead to drug resistance, the driving energetic force behind these pumps represents an additional point of vulnerability with respect to targeted drug design in mycobacteria. Studies using PMF and ATP synthase inhibitors have demonstrated the role of the PMF and ATP in INH efflux in *M. smegmatis* (135). Although efflux has been studied in *M. tuberculosis*, the role of the driving force of these pumps in drug resistance remains poorly investigated. Direct coupling of PMF and ATP to drug efflux—and consequent drug resistance—has been investigated in *E. coli*, *B. subtilis*, *L. lactis*, *Streptococcus pneumoniae*, and a host of other organisms (reviewed in references 91, 97, 104, 105, 154, 155, 156, 157, and 158). Considering the success in targeting the ETC in TB drug development, together with the demonstrated importance of efflux as an active process contributing to drug resistance, the role of energy metabolism in drug resistance and inherent susceptibility in *M. tuberculosis* merits further investigation. The recent demonstration that iron-sulfur (Fe-S) cluster biogenesis is related to intrinsic susceptibility of bacteria to aminoglycosides due to the use of an alternate pathway for Fe-S biogenesis, which results in perturbations in the ETC, leading to reduced PMF and altered drug uptake (159), is consistent with this. Little is known about the energy requirements of mycobacteria during infection, although it has been demonstrated that ATP levels are important for survival in actively replicating as well as nonreplicating mycobacteria, demonstrating the importance of maintaining ATP levels under conditions of growth and survival (20, 55). These ATP levels and associated PMF are essential for *M. tuberculosis* to extrude drugs via PMF-dependent EPs.

The presence of numerous potential regulators involved in the control of cellular ATP levels suggests that there may be different mechanisms involved in the regulation of ATP synthesis. It has been demonstrated that, although ATP levels drop significantly during the shift down to a nonreplicating state, the decreased levels of cellular ATP are essential for viability since treatment with bedaquiline resulted in a loss of viability. *M. smegmatis* displays upregulation of F_1F_0-ATP synthase in response to antibiotic stress. For example, an increase in F_1F_0-ATP synthase abundance was observed upon the exposure of *M. smegmatis* to ethambutol (EMB). Furthermore, among the other proteins upregulated in response to EMB, 23% were related to energy metabolism (160). Similarly, treatment with β-lactam antibiotics resulted in increased expression of F_1F_0-ATP synthase in *M. tuberculosis* (51). These data suggest that antibiotic treatment may inflict physiological stresses on bacteria that impose a requirement for greater energy production. In the case of β-lactam antibiotics, it is unclear whether their effect on the cell wall results in a perturbation of the PMF which is compensated for by an upregulation of the genes encoding the ATP synthase. There have been no extensive studies showing the response of pathways involved in energy metabolism to anti-TB treatments such as RIF; this could provide novel insight into cellular responses or adaptations occurring within the cell leading to drug resistance or identification of targets for drug development.

CONCLUDING REMARKS

The ETC and associated PMF are essential components for energy production through the generation of ATP, which is required for metabolic processes within the cell. The ETC has gained recent prominence in TB drug development through the discovery of numerous compounds that target this pathway such as bedaquiline and Q203. However, in addition to the obvious effects of inhibiting the ETC, a secondary effect of targeting this pathway would be a reduction in the activity of the various PMF/ATP-dependent EPs present in *M. tuberculosis*. These effects may accelerate cell death through higher intracellular concentrations of drugs and reduced extrusion of toxic metabolites, with an added benefit of reducing transient drug tolerance and consequent drug resistance. Various studies now point to potentially positive therapeutic effects of using EP inhibitors such as verapamil to increase the potency of drugs and limit the acquisition of drug resistance. Energy metabolism, including the regulation thereof, represents an ideal component of metabolism to mine for new drug targets.

ACKNOWLEDGMENTS

This work was supported by grants from the Medical Research Council of South Africa, the National Research Foundation (NRF), and the University of the Witwatersrand and an International Early Career Scientist Award from Howard Hughes Medical Institute (to B.D.K.). Funding was also received from the Wellcome Trust (WT087383MA to T.C.V.) NRF (NRF81776 to T.C.V).

We thank Christopher Ealand, Bhavna Gordhan, Monique Williams, Edith Machowski, and Nicole Narrandes for helpful discussions and suggestions.

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