

Role of mycobacterial efflux transporters in drug resistance: an unresolved question

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Abstract

Two mechanisms are thought to be involved in the natural drug resistance of mycobacteria: the mycobacterial cell wall permeability barrier and active multi-drug efflux pumps. Genes encoding drug efflux transporters have been isolated from several mycobacterial species. These proteins transport tetracycline, fluoroquinolones, aminoglycosides and other compounds. Recent reports have suggested that efflux pumps may also be involved in transporting isoniazid, one of the main drugs used to treat tuberculosis. This review highlights recent advances in our understanding of efflux-mediated drug resistance in mycobacteria, including the distribution of efflux systems in these organisms, their substrate profiles and their contribution to drug resistance. The balance between the drug transport into the cell and drug efflux is not yet clearly understood, and further studies are required in mycobacteria.

Introduction

Intrinsic drug resistance and efflux pumps

Bacterial resistance to antibiotics typically involves drug inactivation or modification, target alteration, or decrease in drug accumulation associated with decrease in permeability and/or increase in efflux (Li & Nikaido, 2004). Other resistance mechanisms include inhibition of the activation of pro-drugs, such as the unique antitubercular compounds isoniazid (INH) and pyrazinamide (PZA), into active drugs (Raynaud *et al.*, 1999; Scior *et al.*, 2002). Bacterial resistance may be an intrinsic (natural) feature of an organism, or may result from spontaneous mutations or the acquisition of exogenous resistance genes (Hogan & Kolter, 2002; Normark & Normark, 2002). If a bacterium displays intrinsic resistance, then every member of the corresponding bacterial species is resistant in the absence of any additional genetic alteration. For example, all mycoplasma are resistant to β -lactam antibiotics because their cell wall does not contain peptidoglycan. Similarly, many enteric bacterial species are not very susceptible to hydrophobic antibiotics such as macrolides, because such antibiotics do not penetrate the outer membrane of these organisms efficiently (Nikaido,

2000). Intrinsic drug resistance was thought to be a passive mechanism, based on the absence of the drug target or on the lack of permeability of the bacteria to a given drug. However, it is becoming increasingly evident that the intrinsic resistance of many bacteria to antibiotics depends on the constitutive or inducible expression of active efflux systems (Li *et al.*, 1994; Nikaido, 2001; Ryan *et al.*, 2001). A typical example is *Pseudomonas aeruginosa*, which was long thought to be poorly susceptible to a large range of antibiotics of different classes because of the low level of permeability of its outer membrane to drugs. However, disruption of the gene encoding the MexB pump dramatically increases the susceptibility of *P. aeruginosa* to β -lactams, tetracyclines, fluoroquinolones (FQs) and chloramphenicol (Li *et al.*, 1995). Similarly, disruption of the gene encoding the MdrL transporter in *Listeria monocytogenes* decreases the MIC of cefotaxime by a factor of 10, suggesting that the intrinsic resistance of *L. monocytogenes* to cephalosporins may be because of mechanisms other than the production of penicillin-binding proteins with a low affinity for this subclass of β -lactams (Mata *et al.*, 2000).

Antibiotics can serve as inducers, regulating the expression of efflux pumps at the level of gene transcription by interacting with regulatory systems (Roberts, 1996).

Transporters may also be overexpressed as a result of mutations in these regulators (Zgurskaya & Nikaido, 2000), and may therefore play an important role in both intrinsic and acquired drug resistance.

The *mtr* operon of *Neisseria gonorrhoeae* encodes an energy-dependent efflux pump system composed of the MtrCMtrDMtrE cell envelope proteins (Maness & Sparling, 1973; Hagman *et al.*, 1995), which transport antimicrobial agents. Mutations in the divergently transcribed *mtrR* gene (encoding a transcriptional repressor of *mtrCDE*) lead to overexpression of this efflux pump, conferring resistance to several antimicrobial and hydrophobic agents, including penicillins, macrolides and rifamycins (Hagman & Shafer, 1995; Lucas *et al.*, 1997).

Exposure to FQs typically leads to the generation of resistant *P. aeruginosa* strains with mutations in genes encoding topoisomerases and efflux proteins (Oh *et al.*, 2003). Target site mutations in tandem with mutations in the efflux regulatory genes tend to increase the levels of FQ resistance in *P. aeruginosa* (Nakajima *et al.*, 2002).

Efflux pumps also contribute to FQ resistance in *Streptococcus pneumoniae* (Broskey *et al.*, 2000), where 45% of ciprofloxacin-resistant clinical isolates are because of an efflux mechanism (Brenwald *et al.*, 1998). In this species, resistance to FQs can be reached in a stepwise manner (Janoir *et al.*, 1996; Tankovic *et al.*, 1996). First, mutations in *parC* confer low-level resistance and additional mutations in *gyrA* lead to higher levels of resistance (Pan *et al.*, 1996). At the moment, PmrA is the only known quinolone efflux pump in *S. pneumoniae*, but its role in high-level resistance remains unclear (Gill *et al.*, 1999; Zeller *et al.*, 1997). Efflux proteins other than PmrA may contribute to efflux-mediated FQ resistance in pneumococci (Brenwald *et al.*, 2003). In clinical isolates, efflux seems to contribute only to low-level resistance in the presence or absence of *parC* mutations and such efflux rarely occurs in the presence of *gyrA* mutations (Pestova *et al.*, 2002). Efflux-derived high-level resistance, in the absence of topoisomerase mutations, is extremely rare in both clinical isolates and in isolates selected *in vitro* (Webber & Piddock, 2003). However, a recent study reported that the incubation of *S. pneumoniae* with subinhibitory concentrations of norfloxacin for long periods of time results in the acquisition of high-level resistance to quinolones in the absence of topoisomerase mutations and that this resistance is based entirely on efflux (Daporta *et al.*, 2004).

McMurry *et al.* (1980) provided the first evidence that resistance to antibiotics involves active efflux. Since then, efflux mechanisms have been recognized to be major players in bacterial drug resistance (Nikaido, 1994; Levy, 2002), which is a great cause of concern in the numerous pathogenic strains that have developed multidrug resistance (MDR) phenotypes (Levy, 2002). Some efflux pumps dis-

play marked substrate specificity, but many transporters are polyspecific and extrude a plethora of structurally unrelated drugs (Neyfakh, 2002). These MDR transporters can pump a wide variety of noxious compounds out of the cell, providing the bacteria with an ideal means of escaping conventional antibiotic therapies. They therefore constitute potentially valuable targets in the search for new inhibitors restoring the efficacy of conventional treatments (Lomovskaya & Watkins, 2001a).

Bacterial drug efflux pumps have been classified into five families (Li & Nikaido, 2004). Two of these families are large superfamilies of ancient origin: the ATP-binding cassette (ABC) superfamily and the major facilitator superfamily (MFS). The other three families are smaller and developed more recently: the small multidrug resistance (SMR) family, the resistance-nodulation-cell division (RND) family, and the multidrug and toxic compounds extrusion (MATE) family. The proteins of the MFS, SMR, RND and MATE families are secondary transporters in which drug efflux is coupled with proton (H^+) influx. These pumps are often referred to as H^+ -drug antiporters. In contrast, members of the ABC family of multidrug efflux pumps, which are often considered primary transporters, make use of ATP as an energy source. The genome of *Mycobacterium tuberculosis* contains genes encoding drug efflux transporters from all these families (<http://www.membranetransport.org>).

The burden of mycobacterial drug resistance

Tuberculosis (TB), caused by *Mycobacterium tuberculosis*, remains a leading cause of mortality worldwide (Neralla & Glassroth, 2003). *Mycobacterium tuberculosis* displays the unusual property of being able to survive in a latent form in the granuloma of an asymptomatic host, later becoming reactivated and causing active disease (Stewart *et al.*, 2003; Zhang, 2004). Control of this disease has therefore become a major challenge, despite the availability of effective drugs and of the Bacillus Calmette-Guerin (BCG) vaccine. Resistance has been found against all first-line anti-TB agents and many second-line agents (i.e. FQs). Moreover, *M. tuberculosis* strains resistant to multiple anti-TB drugs are becoming increasingly common (Espinal, 2003). MDR *M. tuberculosis* strains seem to have accumulated independent mutations in genes encoding either the drug target or the enzymes involved in drug activation (Zhang & Telenti, 2000; Wade & Zhang, 2004). However, the genetic basis of resistance to some anti-TB agents is not fully known (Rattan *et al.*, 1998). For example, streptomycin resistance emerges through mutations in *rrs* and *rpsL*, leading to a change in the binding site for streptomycin in the ribosome. However, such changes have been identified in just over half the strains studied to date (Honore & Cole, 1994; Sreevatsan *et al.*, 1996). Mutations in the *pncA* gene, encoding pyrazinamidase, are

Table 1. Putative mycobacterial drug efflux pumps associated with reduced susceptibility to antibacterial agents

	Microorganisms	Resistance	Resistance because of	References
Pump family				
MFS				
LfrA	<i>M. smegmatis</i>	FQs, ethidium bromide, acriflavine and cetyltrimethylammonium bromide	Gene amplification (multicopy plasmid)	Takiff <i>et al.</i> (1996)
Tet(V)	<i>M. smegmatis</i>	Tetracycline	Multicopy plasmid	De Rossi <i>et al.</i> (1998b)
Tap	<i>M. tuberculosis</i> , <i>M. fortuitum</i>	Aminoglycosides and tetracycline	Multicopy plasmid	Ainsa <i>et al.</i> (1998)
P55	<i>M. bovis</i>	Aminoglycosides and tetracycline	Multicopy plasmid	Silva <i>et al.</i> (2001)
Rv1634	<i>M. tuberculosis</i>	FQs	Multicopy plasmid	De Rossi <i>et al.</i> (2002)
Rv1258c	<i>M. tuberculosis</i> clinical strain resistant to RIF, ofloxacin INH, minomycin	RIF, ofloxacin	Overexpression after drug induction	Siddiqi <i>et al.</i> (2004)
SMR				
Mmr	<i>M. tuberculosis</i>	Tetraphenyl phosphonium, ethidium bromide, erythromycin, acriflavine, safranin O, and pyronin Y	Multicopy plasmid	De Rossi <i>et al.</i> (1998a)
RND				
mmpL7	<i>M. tuberculosis</i>	INH	Multicopy plasmid	G. Riccardi (unpublished data)
ABC superfamily				
PstB	<i>M. smegmatis</i>	FQs	Overexpression in a ciprofloxacin-resistant mutant	Bhatt <i>et al.</i> (2000)
DrrAB	<i>M. tuberculosis</i>	Tetracycline, erythromycin, ethambutol, norfloxacin, streptomycin, chloramphenicol and anthracyclines	Multicopy plasmid	Choudhuri <i>et al.</i> (2002)
Rv2686c-2687c-2688c	<i>M. tuberculosis</i>	FQs	Multicopy plasmid	Pasca <i>et al.</i> (2004)

MFS, major facilitator superfamily; SMR, small multidrug resistance; RND, resistance-nodulation-cell division; INH, isoniazid; RIF, rifampicin; FQ, fluoroquinolone; *M. smegmatis*, *Mycobacterium smegmatis*; *M. tuberculosis*, *Mycobacterium tuberculosis*; *M. bovis*, *Mycobacterium bovis*.

thought to be the major mechanism of PZA resistance in *M. tuberculosis*, but resistant strains containing the wild-type gene have been described (Mestdagh *et al.*, 2000). Moreover, although the primary targets of activated INH are enzymes involved in the biosynthesis of cell wall mycolic acids, mutations associated with changes in these targets have been shown to contribute to INH resistance, but cannot entirely account for this resistance (Slayden & Barry, 2000).

The intrinsic resistance of *M. tuberculosis* to most antibiotics is generally attributed to the low permeability of the mycobacterial cell wall, because of its specific lipid-rich composition and structure. This low permeability, which limits drug uptake, seems to be one of the main factors involved in resistance (Brennan, 2003; Niederweis, 2003). Along with cell wall permeability, active efflux systems also provide resistance by extruding the drug molecules that enter the cell. The intracellular concentration of a given drug depends on the balance between its influx and efflux. It is therefore of prime importance to increase our understand-

ing of the processes of drug influx through porins and drug efflux via drug transporters, if we are to combat drug resistance in mycobacteria (Niederweis, 2003).

Several mycobacterial drug efflux pumps have been identified and characterized experimentally (Table 1, Viveiros *et al.*, 2003). This review focuses on the current state of research in the field of multidrug efflux mechanisms in mycobacteria.

MFS drug transporters

The MFS comprises one of the largest families of membrane transporters. MFS permeases possess 12 or 14 putative or established transmembrane segments and transport many different compounds, including simple sugars, oligosaccharides, inositols, drugs, amino acids, nucleosides, organophosphate esters, Krebs cycle metabolites, and a large variety of inorganic anions and cations (Saier *et al.*, 1999). Some MFS transporters may confer drug resistance on bacteria

(Poelarends *et al.*, 2002; Wigfield *et al.*, 2002; Godreuil *et al.*, 2003; Bannam *et al.*, 2004). A number of plasmid-encoded MDR transporters have been described for the major human pathogen *Staphylococcus aureus*, including the closely related MFS members QacA and QacB (Littlejohn *et al.*, 1992). QacA provides high-level resistance to various toxic organic cations, including intercalating dyes (e.g. ethidium bromide) and a number of commonly used antiseptics and disinfectants such as cetrimide, benzalkonium chloride and chlorhexidine. Thus, *S. aureus* seems to have found a way of circumventing the antiseptics and disinfectants prevalent in the hospital environment by recruiting a system already well adapted for the export of such compounds (Mitchell *et al.*, 1999; Brown & Skurray, 2001). The expression of *qacA* is regulated by the transacting repressor protein QacR. The binding of QacR to chemically unrelated cations leads to expression of the QacA pump in *S. aureus*. QacR belongs to the TetR family of transcriptional repressor proteins, all of which possess a helix-turn-helix DNA-binding domain at their N-terminal end and have highly divergent C-termini thought to be involved in the binding of inducers (Rouch *et al.*, 1990). Studies of QacR, which has been crystallized with six different drugs, have increased our understanding of the binding of structurally diverse drugs to the binding site of this molecule. These studies have shown that there are separate but linked drug-binding sites within the protein (Schumacher *et al.*, 2001). The *qacA-qacR* locus commonly occurs on the multiresistance plasmids that are frequently isolated from clinical strains of *S. aureus* (Paulsen *et al.*, 1998). A survey of 98 clinical isolates of methicillin-resistant *S. aureus* revealed that 70% of the strains were antiseptic-resistant and one-third carried a transmissible plasmid containing the *qacA* gene (Noguchi *et al.*, 1999), which could be transferred into plasmid-free *S. aureus* strains, thereby also playing an important role in acquired drug resistance.

Bioinformatic analysis of the *M. tuberculosis* genome has identified 16 open reading frames encoding putative drug efflux pumps belonging to the MFS (De Rossi *et al.*, 2002). Most of the functional drug efflux pumps characterized to date in the mycobacteria also belong to this superfamily.

Mycobacterial MFS efflux pumps transporting FQs

Bacterial type II DNA topoisomerase, DNA gyrase and topoisomerase IV are the targets of FQs (Chen & Lo, 2003). Unlike most other bacterial species, *M. tuberculosis* lacks topoisomerase IV and produces only DNA gyrase: a tetramer consisting of two A and two B subunits, encoded by the *gyrA* and the *gyrB* genes, respectively (Madhusudan *et al.*, 1994). The most important mechanism of resistance to FQs in clinical isolates of *M. tuberculosis* involves mutations in the

quinolone resistance-determining region of *gyrA* (Ginsburg *et al.*, 2003). These mutations are found in 42–85% of FQ-resistant clinical isolates (Sullivan *et al.*, 1995). No clinical isolates of *M. tuberculosis* have yet been shown to have mutations in *gyrB* (Ginsburg *et al.*, 2003; Cheng *et al.*, 2004). However, *gyrA* mutations are not found in a significant proportion of FQ-resistant *M. tuberculosis* strains, suggesting that the resistance of these strains may be because of an active drug efflux pump mechanism. However, FQ efflux has not been demonstrated *in vitro* or in clinical isolates of *M. tuberculosis*.

In mycobacteria, two MFS efflux pumps have been associated with FQ transport: the LfrA efflux pump of *Mycobacterium smegmatis* and the Rv1634 efflux pump of *M. tuberculosis*.

The LfrA protein was the first functional efflux pump to be described in the genus *Mycobacterium*. Overproduction of the protein leads to low-level resistance to FQ, acridine and some quaternary ammonium compounds in *M. smegmatis* (Liu *et al.*, 1996; Takiff *et al.*, 1996). The LfrA efflux pump is similar to QacA in *S. aureus* (Paulsen *et al.*, 1996), but not to the NorA of this bacterium (Neyfakh *et al.*, 1993), even though both proteins preferentially pump out hydrophilic quinolones.

LfrA seems to play an important role in resistance to ciprofloxacin in *M. smegmatis* only if its expression is increased. Two possible mechanisms may lead to higher levels of LfrA: regulatory mutations leading to constitutive expression, or transitory induction by the substrates of this pump. The contribution of the LfrA protein to ciprofloxacin resistance in *M. smegmatis* was studied in our laboratory by constructing strains bearing an *lfrA* gene inactivated by an insertion (Sander *et al.*, 2000). Disruption of the *lfrA* gene decreased the MIC of ethidium bromide and acriflavine by a factor of eight and increased ethidium bromide accumulation. Also, it produced a twofold decrease in the MICs of ciprofloxacin, doxorubicin and rhodamine. These results were recently confirmed by Li *et al.* (2004). Thus, LfrA appears to play an important role in the intrinsic resistance of *M. smegmatis* to ethidium bromide and acriflavine, but not in intrinsic resistance to ciprofloxacin. This disparity in the effects of disrupting *lfrA*, with larger changes in MIC for ethidium bromide than for ciprofloxacin, may be because of ethidium bromide efficiently enhancing expression of the *lfrA* gene or acting as a good substrate for the LfrA efflux pump. Alternatively, unidentified efflux pumps other than LfrA may extrude ciprofloxacin in *M. smegmatis*. Consistent with this idea, Li *et al.* (2004) found that an ethidium bromide-resistant derivative of an LfrA-deficient strain was less susceptible than the wild-type strain to ethidium bromide, acriflavine, ciprofloxacin and norfloxacin. Furthermore, the resistant mutant accumulated smaller amounts of drug than its parental strain, suggesting that a

new, unidentified efflux pump is probably responsible for the phenotype of the LfrA-deficient/ethidium bromide-resistant strain (Li *et al.*, 2004). Characterization of this new *M. smegmatis* mutant might clarify the issue of LfrA and FQ resistance. The LfrA efflux pump is currently thought to have no effect on susceptibility to FQs in the absence of *lfrA* overexpression.

Fluoroquinolones are probably not the preferred or natural substrate of LfrA. There is some debate as to the natural function of bacterial FQ-MDR transporters, which may be involved in the export of naturally occurring toxic environmental agents or cell-associated metabolites (Neyfakh, 1997). Antibiotic-producing microorganisms use efflux pumps to export the antibiotics they produce rapidly, to protect the producing cell against their effects. In these organisms, efflux pumps evolved as a protective mechanism and recognize antibiotics as their natural substrates. For example, 29 different tetracycline resistance (*tet*) genes have been characterized in antibiotic-producing *Streptomyces* strains and 18 of these genes encode efflux pumps (Chopra & Roberts, 2001).

Efflux pump genes often form part of an operon, the expression of which is controlled by a regulatory gene. Excessive expression may be deleterious because of the direct, physical disruption of membrane integrity or the undesirable export of essential metabolites: a potential side effect of the broad substrate specificity of these pumps. Several local regulatory proteins, including the BmrR activator in *Bacillus subtilis*, the QacR repressor in *S. aureus*, and the TetR and EmrR repressors in *Escherichia coli*, have been shown to mediate increases in the expression of drug efflux pump genes by directly sensing the presence of the toxic substrates exported by their cognate pump (Grkovic *et al.*, 2002).

Examination of the sequence of the region upstream from the *lfrA* gene revealed the presence of an open reading frame encoding a putative polypeptide of 195 amino acids, LfrR, homologous to several transcriptional regulators of the TetR family (Li *et al.*, 2004). It is noteworthy that many known local repressors of multidrug pumps belong to the TetR family. The *lfrR* and *lfrA* genes seem to be organized into an operon potentially controlled by the transcriptional regulator LfrR. Indeed, deletion of the *lfrR* gene has been shown to increase *lfrA* expression, increasing the resistance of the strain to ciprofloxacin, norfloxacin, ethidium bromide and acriflavine (four- to 16-fold increases in MICs) (Li *et al.*, 2004).

There is no known homolog of the *lfrA* gene in the *M. tuberculosis* genome (De Rossi *et al.*, 2002). Other mechanisms or other efflux pumps may therefore be involved in resistance to FQ in *M. tuberculosis*.

In an analysis of the *M. tuberculosis* genome sequence in which we searched for MFS drug transporters, we identified

16 open reading frames encoding putative drug efflux pumps (De Rossi *et al.*, 2002). One of these putative pumps, Rv1634, decreases susceptibility to various FQs when over-expressed in *M. smegmatis*, and accumulation data suggest that this pump is involved in norfloxacin and ciprofloxacin efflux (De Rossi *et al.*, 2002). This demonstrates the presence of a novel and specific FQ efflux transporter in *M. tuberculosis*. Rv1634, cloned into different vectors, increased the MIC of *M. smegmatis* to ciprofloxacin by a factor of two to four (0.12 vs. 0.24–0.48 $\mu\text{g mL}^{-1}$). Rv1634 also decreases susceptibility to ciprofloxacin by a factor of eight in *Mycobacterium bovis* BCG (E. De Rossi, unpublished data). Gene inactivation experiments are underway in our laboratory to define the role of Rv1634 in the intrinsic susceptibility to FQ of *M. tuberculosis*.

High-level FQ resistance is generally associated with mutations in genes encoding the primary targets: topoisomerase IV and DNA gyrase. This resistance may be increased if combined with mutations increasing FQ efflux (Bush & Goldschmidt, 2000). In both cases, quinolone exposure leads to the selection of spontaneous mutants present in large bacterial populations that have chromosomal mutations altering the target protein or increasing pump expression. The newer FQs, which recognize both molecular targets, may be more potent, thereby reducing the probability of developing resistance.

Mycobacterial MFS efflux pumps transporting tetracycline

Tetracyclines display broad-spectrum activity against numerous pathogens, including Gram-positive and Gram-negative bacteria, and atypical organisms. These compounds are bacteriostatic, and act by binding to the bacterial 30S ribosomal subunit and inhibiting protein synthesis (Zhanel *et al.*, 2004). Tetracyclines are active against clinical isolates of several species of rapidly growing mycobacteria, including *Mycobacterium fortuitum*, *M. smegmatis* and *Mycobacterium mucogenicum*, for which 50% of clinical isolates are susceptible. These agents are, however, less effective in clinical conditions against other atypical mycobacteria, such as *Mycobacterium chelonae* and *Mycobacterium abscessus*. Preliminary studies have shown that these species contain a number of resistance determinants probably involved in tetracycline resistance (Pang *et al.*, 1994; Wallace *et al.*, 2002).

Tetracycline efflux pumps have been described in many bacterial species (Roberts, 1996; Chopra & Roberts, 2001). In this section, we will focus on the tetracycline efflux pump Tet(V) isolated from *M. smegmatis*, and on Tap, an efflux pump isolated from *M. fortuitum*.

Overexpression of the Tet(V) efflux pump in *M. smegmatis* increases the MIC of tetracycline by a factor of two to four

and doubles that of chlortetracycline but does not change the MIC of the tetracycline derivatives doxycycline and minocycline (De Rossi *et al.*, 1998b). The sequence of *tet(V)* displays little similarity to the sequences of other genes encoding well-known tetracycline transporters from Gram-positive microorganisms. The distribution of the *tet(V)* gene among *Mycobacterium* spp. has been investigated by PCR. *M. smegmatis* and *M. fortuitum* were the only species tested that appeared to have a *tet(V)* gene (De Rossi *et al.*, 1998b).

The *M. fortuitum* Tap efflux pump and its *M. tuberculosis* Rv1258c homologue (Ainsa *et al.*, 1998) confer resistance to tetracycline and aminoglycosides, including streptomycin, a major drug in TB treatment. The production of Rv1258c from a multicopy plasmid resulted in a fourfold increase of the MIC levels of tetracycline and an eightfold increase of the MIC levels of streptomycin. Deletion of the Rv1258c gene from the *M. bovis* BCG chromosome increased susceptibility to these two drugs, confirming the involvement of this efflux pump in the intrinsic resistance of *M. bovis* and *M. tuberculosis* to tetracycline and streptomycin (J. A. Ainsa, unpublished result). A correlation was recently identified between drug resistance and Rv1258c gene transcription levels in a clinical *M. tuberculosis* isolate resistant to rifampicin (RIF; MIC = 40 µg mL⁻¹), ofloxacin (MIC = 4 µg/mL⁻¹), INH (MIC = 2 µg mL⁻¹) and minomycin (MIC = 2 µg mL⁻¹) (Siddiqi *et al.*, 2004). RT-PCR showed that the level of Rv1258c transcript increased by a factor of 10 if this isolate was grown in the presence of RIF and by a factor of six if it was grown in the presence of ofloxacin. Neither RIF nor ofloxacin has been reported to be a substrate of the Tap efflux pump (Ainsa *et al.*, 1998). These results suggest that efflux pumps may be induced in a general manner in response to drugs in mycobacteria, consistent with the hypothesis that efflux pumps are involved in detoxification (Fernandes *et al.*, 2003). The P55 efflux pump from *M. bovis* and *M. tuberculosis* has also been associated with low-level resistance to tetracycline and aminoglycosides (Silva *et al.*, 2001).

Other mycobacterial MFS efflux pumps

The *M. tuberculosis* H37Rv *efpA* gene encodes a putative efflux protein (EfpA), 55 670 Da in size. The deduced amino acid sequence of EfpA has a predicted secondary structure similar to that of members of the QacA transporter family (QacA TF), which mediates resistance to antibiotics and chemicals in bacteria and yeast. The predicted EfpA sequence included all the transporter motifs characteristic of the QacA TF, including those associated with proton-antiporter function and those specific to drug transporters. However, it was not possible to demonstrate a link between *efpA* and drug resistance in *M. tuberculosis* (Doran *et al.*, 1997). The expression of this gene increased in response to

INH treatment (Wilson *et al.*, 1999) and thiolactomycin treatment (Betts *et al.*, 2003). The INH-mediated induction of *efpA* raises the question as to whether the protein encoded by this gene transports molecules relevant to mycolic acid production. If EfpA mediates an essential function in mycolate biosynthesis, then it may be an appropriate novel drug target. Waddell *et al.* (2004) found that the *efpA* gene was also induced in response to several other compounds with antimycobacterial activity (isoxyl, tetrahydrolipstatin and three compounds from the Southern Research Institute, Birmingham, AL). They therefore suggested that the activation of this gene might be part of a general response of efflux pumps to the accumulation of drugs or toxic compounds in the intracellular environment.

Deletion of the *efpA* homologue in *M. smegmatis* resulted in a twofold increased susceptibility to ethidium bromide, gentamicin and FQs, and an eightfold increased susceptibility to acriflavine. Unexpectedly, the mutant also showed a fourfold decreased susceptibility to rifamycins and chloramphenicol, and a twofold decreased susceptibility to INH and erythromycin in comparison with the wild type (Li *et al.*, 2004). The *efpA* mutant grew more slowly than the wild-type parental strain, so its greater susceptibility may be because of impaired growth (Li *et al.*, 2004). The role of EfpA in drug resistance and its substrate specificity remain unclear. However, as the overexpression of *efpA* confers ethidium bromide resistance on *M. smegmatis* (E. De Rossi, unpublished data) and the deletion of this gene increases susceptibility to ethidium bromide (Li *et al.*, 2004), it seems likely that ethidium bromide is a suitable substrate of the encoded pump.

SMR family drug transporters

SMR family pumps are prokaryotic homo-oligomeric or hetero-oligomeric transport systems. The subunits of these systems are 100–120 amino acids long and contain four membrane-spanning helices. The functionally characterized members of the SMR family catalyse efflux by means of a drug; the H⁺ antiporter mechanism, in which proton motive force (PMF) is used to induce the expulsion of various drugs, generally cationic in nature (Jack *et al.*, 2001). One of these pumps, EmrE, has been cloned from *E. coli* on the basis of its ability to confer resistance to ethidium bromide and methyl viologen, and has been extensively characterized (Yerushalmi *et al.*, 1995). The three-dimensional structure of the EmrE multidrug transporter was recently determined. The most remarkable feature of this structure is that it is an asymmetric homodimer (Ubarretxena-Belandia *et al.*, 2003). Ninio *et al.* (2004) used two independent approaches to demonstrate that the first loop of EmrE, connecting TM1 and TM2, faces the periplasm of

cells, whereas the carboxy-terminus of the protein faces the cytoplasm.

Another SMR multidrug pump from *P. aeruginosa*, similar to EmrE, has been characterized and shown to play an important role in the intrinsic resistance of *P. aeruginosa* to ethidium bromide, acriflavine and aminoglycoside antibiotics (Li *et al.*, 2003).

Only one protein (Mmr, Rv3065) of the SMR family has been described in *M. tuberculosis* (De Rossi *et al.*, 1998a). The *M. tuberculosis* chromosomal gene *mmr*, inserted into a multicopy plasmid, renders *M. smegmatis* less susceptible to tetraphenyl phosphonium (TPP⁺), ethidium bromide, erythromycin, acriflavine, safranin O and pyronin Y. Experiments on TPP⁺ accumulation have shown that Mmr actively extrudes TPP⁺, in a process driven by the PMF. The presence of *mmr*-like genes in other *Mycobacterium* species (*M. simiae*, *M. goodii*, *M. marinum* and *M. bovis*) has been demonstrated by Southern hybridization (De Rossi *et al.*, 1998a). The Mmr protein from *M. tuberculosis* was recently produced in *E. coli*, in which it conferred resistance to ethidium bromide, acriflavine and methyl viologen. The purified Mmr protein has also been demonstrated to function as a proton/drug antiporter *in vitro* (Ninio *et al.*, 2001). Bioinformatic analysis of sequenced mycobacterial genomes has revealed that *mmr* homologues are also present in macrolide-susceptible strains, such as *Mycobacterium leprae*, *Mycobacterium avium* and *M. smegmatis* (Doucet-Populaire *et al.*, 2002). Thus, these genes are unlikely to play a crucial role in high-level macrolide resistance. Interestingly, the deletion of the *mmr* homologue in *M. smegmatis* increases susceptibility to cationic dyes and FQ, but not to erythromycin (Li *et al.*, 2004).

RND drug transporters

The members of the RND family are transport proteins with 12 transmembrane spans and include a number of multidrug efflux proteins of particularly broad substrate specificity. RND transporters have been found in all major kingdoms of living organisms, but seem to be involved in drug resistance only in Gram-negative bacteria. In these bacteria, some RND multidrug efflux systems require two auxiliary constituents: a membrane fusion protein and an outer membrane protein (Tseng *et al.*, 1999). These two proteins may act together, enabling the bacterium to transport drugs across both membranes of the cell envelope directly into the external medium. The AcrAB/TolC drug efflux pump of *E. coli* provides a prototype for such export systems, with AcrB constituting the membrane pump itself, AcrA the membrane fusion protein and TolC the outer membrane component (Nikaido & Zgurskaya, 2001). This structural organization allows the extrusion of substrates directly into the external medium, bypassing the periplasm.

The substrate specificity of the AcrAB system is unusually broad. It extrudes cationic, neutral and anionic substances. Examination of the crystal structure of AcrB suggested that RND transporters may also pump substrates out from the periplasm (Murakami *et al.*, 2002). These findings are consistent with the periplasmic loops of RND transporters being critical for substrate determination and suggest that these loops contain multiple binding sites for structurally unrelated compounds.

This complex construction of RND multidrug efflux pumps in Gram-negative bacteria contrasts sharply with most of the efflux pumps of Gram-positive bacteria, which are simpler in organization and have only one component located in the cytoplasmic membrane, like the pumps of the MFS. Although mycobacteria cluster phylogenically with Gram-positive bacteria, they are structurally more similar to Gram-negative bacteria, as they are protected by an outer lipid bilayer formed of mycolic acids and a cell envelope composed of noncovalently bound lipids and glycolipids. Mycobacteria have the thickest known biological envelope, which includes pore proteins that span the outer membrane (Brennan, 2003; Niederweis, 2003).

All the drug efflux pumps identified in mycobacteria to date are simple transporters located in the cytoplasmic membrane. The lack of identification in mycobacteria of multisubunit pumps similar to those in Gram-negative bacteria is surprising, although something new is emerging.

The genome sequence of *M. tuberculosis* contains 15 genes encoding putative transmembrane proteins predicted to be transport proteins of the RND superfamily (<http://www.membranetransport.org>). These proteins appear to be confined to mycobacteria and have therefore been designated MmpL (mycobacterial membrane proteins, large). The MmpL proteins are similar to each other in both sequence and structure. They each comprise about 950 amino-acid residues and are predicted to contain 12 membrane-spanning α helices. These MmpL proteins have two large extracytoplasmic domains, which probably face the periplasmic space: the first, comprising about 140 residues, is located between transmembrane segments 1 and 2; the second, located between transmembrane segments 7 and 8, is between 300 and 400 residues long. The C-terminal residues are located in the cytoplasm. In four cases, the MmpL genes are preceded in the operon by sequences encoding MmpS proteins, which are small mycobacterial membrane proteins with a hydrophobic α helix close to the NH₂-terminus, and COOH-terminal domains of about 120 residues predicted to be exposed to the exterior. The hydrophobic nature of the Mmp proteins and the close association of their genes with genes encoding proteins involved in lipid metabolism suggest that these proteins may be involved in the transport of fatty acids (Tekaiia *et al.*, 1999). We found sequence similarities between the

MmpL7	-----MPSPAGRLHRIYIRLKKSSPDCRATITSGSADGQRRSPRLTN	43
AcrB	MPNFFIDRPIFAWVIAIIIMLAGGLAILKLPVAQYPTIAPPVITISASYPG-ADAKTVQD	59
	: ** * : : : : : : : * . * . * : : :	
MmpL7	LLVVAAWVAAVIANLLLTFTQAEPHDTSPALLPQDAKTAAATSRIAQAFFGTGSNAIAY	103
AcrB	TVTQVIEQNMGIDNLMYSSNSDSTGTQITLTFESGTDADIAQVQVQNKQLQAMP LLP	119
	: . . * ** : : : : . * . : : * * : : : : : :	
MmpL7	LVVEGGSTLEPQDPYDAAVGALRAD-TRHVGSVLDWWSDPVTAPLGTSP-----DG	155
AcrB	QEVQQQGVSVSEKSSSSFLMVGVINTDGTMTQEDISDYVAANMKDAISRTSGVGDVQLFG	179
	* : . . : . . : . * . : : * * : : * : : . . . : *	
MmpL7	RSATAMVWLGEAGTTQAESLDAVRSLRQLPPSEGLRASIVPAITNDMPMQITAWQS	215
AcrB	SQYAMRIWMNPENLKFQLTPVDVITAKAQNQAAGQLGGTTPPVKGQQLNASIIAQTR	239
	. : : * : . : . . : * : : : : * . : . . . * . : : : . * *	
MmpL7	ATIVTVAAVIAVLLLLR-ARLSVRAAAIVLLTAD-----LSLAVA	254
AcrB	LTSTEEFGKILLKVNQDGSRLVRDVAKIELGGENYDIIAEFNGQPASGLGIKLATGANA	299
	* . . * : : : : * : * . * : * . : : : : : * *	
MmpL7	WPLAAVVRGHDWGTDSVFSWTLAAVLTTIGTITAATMLAARLGS DAGHSAAPTYRDSLPAF	314
AcrB	LDTAAAIRAELAKMEPFPSGLKIVPYDTPFVKISIEHVVKTLVEAILVFLVMYFL	359
	** : * . . : . * . * : . . : . : :	
MmpL7	A-LPGACVAIFTGPLLLAR---TPALHGVGTAGLGVF-VALAASLTVPALIALAGASRQ	369
AcrB	QNFRATLIPTIAVPVLLGTFAVLAAGFSGINTLTMFGMVLAIGLLVDDAIVVVENVERV	419
	: . : . : : * : * . * . * . * : * : * . * * * : : : . . *	
MmpL7	LPAPTTGAGWTGRSLPVSSASALGTAAVLAICMLPIIGMRWGAENPTRQGGQAVLPGN	429
AcrB	MAEEGLPPKEATRKSQMGQIQGALVGIAMVLSAVFVPMMA-FFGGSTGAIYRQFSITIVSAM	478
	: . . : * * : . . : * * * : : : : : * : * * . : . .	
MmpL7	ALPDVVVKSARDLRDPAALIAIN-----QVSHRLVEVP	463
AcrB	ALSVLVALILTPALCATMLKPIAKGDHGEKKGFFGWFNRMFEKSTHHYDTSVGILRST	538
	** . : * : : * . : : : : : : : : : : : : : : :	
MmpL7	GVRKVESAAWPAGVPWTDASLSSAAGRLADQLGQQAGSFVPAVTAIKSMKSIIEQMSG--	521
AcrB	GRYLVLVLIIVVGMAVLFVRLPSSFLPDEDQGVFMTMVQLPAGATQERTQKVLNEVTHYY	598
	* * . : : . * * : ** : : * : : : : : : : :	
MmpL7	---AVDQLDSTVNVTLAGARQAQQYLDPLAAARNLKNKTTEELSEYLETIHTWIVGFTNC	578
AcrB	LTKEKNVSEVFAVNGFGFAGRGQNTGIAFVSLKDWADRPGEENKVEAITMRATRAFSQI	658
	: : : * . . * . * : : : : : : : : : : : * :	
MmpL7	PDDVLCMTAMRKVIEPYDIVVTG---MNELSTGADRISAISTQTMASALSAPRMVAQMR-	633
AcrB	KDAMVFAFNLPALVELGTATGDFDFELIDQAGLGHEKLTQARNQLLAEAAKHPDMLTSVRP	718
	* : : : . * . . : : : . * : : : . * : : : . * * : : *	
MmpL7	SALAQVRSFVPKLETTIQDAM-----PQIAQASAMLKNLSADFADTG-----EGG	678
AcrB	NGLEDTPQFKIDIDQEKALQALGSINDINTTLGAAWGGSYVNDFIDRGRVKKVYVMSEAK	778
	. * : . * : : : : : : : : : : : * * * * *	
MmpL7	FHLRDKDLADPSYRHVRESMFSSDGTATRLFLYSDGQLDLAAAARAQQLAAG-----	732
AcrB	YRMLPDDIGDWYVRAADGQMPVPSAFSSSRWEYGSPLERYNGLPSMEILGQAAPGKSTG	838
	: : : * : * * . . . * . . . : : : * . : : : : *	
MmpL7	KAMKYGSLVDSQVTGG-----AAQIAAAVRDALIHDAVLLAVILLTVVALASMWRGA	785
AcrB	EAMELMEQLASKLPTGVGYDWTGMSYQERLSGNQAPSLYAISLIVVFLCLAALYESWSIP	898
	: * : . : * : : * : : * : : * : : * : : * : : *	
MmpL7	VHGAAVG-VGVLASYLALGVSIALWQHLLDRELNALVPLVSFAVLASCGVPYLVAGIKA	844
AcrB	FSVMLVVP LGVIGALLAATFRGLTNDVYFQVGLLTTIGLSAKNAILIVEFAKDLMDKEGK	958
	. * : * : : * * : : : : : * : : . . * * . * :	
MmpL7	GRIADEATGARSKG-----AVSGRGAVAPLALGGVFGAGLVLVSGGSFVSLSQIGTVV	898
AcrB	GLIEATLDAVRMLRPLIMTSLAFILGVMLPVISTGAGSGAQNAGVTGVMGGMVTATVLA1018	
	* * . * : : : . * * . * . . . * . * : : . .	
MmpL7	VLGLGLVITVQRAWLPTTPGRR-----	920
AcrB	IFFVPVFFVVRRRFRSKNEDIEHSHTVDHH	1049
	: : : * : * * . . .	

Fig. 1. Alignment of MmpL7 from *Mycobacterium tuberculosis* and AcrB from *Escherichia coli*. Identical residues are indicated by asterisks, while conservative amino acid substitutions are indicated by dots. Dashes represent gaps inserted to optimize the protein alignment. The alignment was performed by CLUSTALW at <http://www.expasy.org>

mycobacterial MmpL proteins and the *E. coli* AcrB transporter protein, and, to a lesser extent, between the MmpS proteins and the membrane fusion protein AcrA. Pairwise alignment of amino acid sequences showed that MmpL7 is 14% identical and 46% similar to AcrB (Fig. 1). Furthermore, the hydrophobic profile of the MmpL7 protein, described above, is very similar to that observed in proteins belonging to RND family.

We therefore suggest that the MmpS proteins of *M. tuberculosis* may be analogous to AcrA (the membrane fusion protein) and the MmpL proteins may be analogous to the AcrB efflux pump. As for *E. coli* TolC, the outer membrane component may be encoded elsewhere on the chromosome (Poole, 2004).

In *M. tuberculosis*, MmpL7 catalyses the export of phthiocerol dimycocerosate (PDIM), a lipid constituent of the outer membrane (Camacho *et al.*, 2001). Inactivation of the *mmpL7* gene attenuates *M. tuberculosis* virulence in the mouse model (Camacho *et al.*, 1999). Upstream from the *mmpL7* gene is the *fadD28* gene, which encodes an acyl-CoA synthase probably involved in the release and transfer of mycocerosic acid from mycocerosic acid synthase to diols. The *fadD28* and *mmpL7* genes display coupled transcription. A strain with an insertion in *mmpL7* produces a dimycocerosate (DIM) molecule that is primarily retained in the cytosol or the cytoplasmic membrane. In contrast, a strain with an insertion in the *fadD28* gene produces no detectable DIM. We can therefore conclude that the FadD28 protein is directly involved in the biosynthesis of DIM (Camacho *et al.*, 2001).

The heterologous production of MmpL7 in *M. smegmatis* confers a high level of resistance to INH (MIC 32 times higher than wild type). This phenotype is completely reversed if *fadD28* and *mmpL7* are expressed simultaneously, suggesting that DIM and INH compete for the same MmpL7 transporter (G. Riccardi, unpublished data).

In addition to MmpL7, signature-tagged transposon mutagenesis experiments have shown that MmpL2 and MmpL4 are also required for the growth of *M. tuberculosis* in mouse lungs (Camacho *et al.*, 1999).

Converse *et al.* (2003) and Domenech *et al.* (2004) recently demonstrated that MmpL8 is required for the transport of a precursor of the sulfated glycolipid SL-1 and for sustained bacterial growth and persistence in mice, as an MmpL8 mutant was only weakly pathogenic in mice. It has also been reported that the gene encoding MmpL8 is induced as part of a general response to toxic compounds (Waddell *et al.*, 2004). Betts *et al.* (2003) used DNA microarrays to investigate the response of *M. tuberculosis* to treatment with three mycolic acid biosynthesis inhibitors: INH, thiolactomycin and triclosan. Eight genes were induced in response to all triclosan treatments. Only one of these eight genes (*mmpL6*) was also upregulated by the two other inhibitors tested, displaying induction by treatment

with the MIC of thiolactomycin for 2 h (Betts *et al.*, 2003). As MmpL6 is a putative transporter of the RND superfamily, these authors suggested that it might take part in an efflux or detoxification system by which *M. tuberculosis* limits the effects of INH, thiolactomycin and triclosan.

Complete analysis of the remaining MmpL proteins will reveal whether the *M. tuberculosis* MmpL proteins are involved in the efflux of anti-TB drugs and/or play important roles in pathogenesis.

ABC drug transporters

The ABC transporters constitute a large superfamily of multisubunit permeases that transport various molecules (ions, amino acids, peptides, drugs, antibiotics, lipids, polysaccharides, proteins, etc.), using ATP as energy source (Schmitt & Tamp, 2002). These transporters are important virulence factors in bacteria because they are involved in nutrient uptake and the secretion of toxins and antimicrobial agents (Davidson & Chen, 2004).

ABC transporters appear to consist of at least four domains: two membrane-spanning domains (MSDs) and two nucleotide-binding domains (NBDs). Each of the highly hydrophobic MSDs consists of six putative transmembrane segments that form the pathway through which the substrate crosses the membrane. The two NBDs lie at the periphery of the cytoplasmic face of the membrane. They bind ATP and couple ATP hydrolysis to substrate translocation (Kerr, 2002).

The human P-glycoprotein, encoded by the *MDR1* gene at the 7q21.1 locus, is the ABC transporter conferring resistance to the widest variety of anticancer agents. P-glycoprotein is therefore a highly attractive molecular target in attempts to limit drug resistance in human malignancies (Kuwano *et al.*, 2002).

Remarkably, few prokaryotic ABC-type efflux proteins are involved in the transport of multiple drugs. Our understanding of the structure and function of ABC transporters has been considerably improved by crystallization and determination of the structure of EC-MsbA, a lipid *E. coli* flippase that transports lipid A, which is itself a major component of the bacterial outer membrane (Chang & Roth, 2001). In EC-MsbA, the MSDs are composed of six α helices, which form the translocation pathway. The NBDs are located at the cytoplasmic face of the membrane, where they are unlikely to contribute to the translocation pathway, but may be involved in the changes in the conformation of the MSD during ATP hydrolysis. Comparison of the structures of VC-MsbA, the *Vibrio cholerae* homologue (Chang, 2003), and EC-MsbA from *E. coli* has revealed that these transporters share a conserved core of transmembrane α helices, but differ in the relative orientations of their NBDs (Schmitt & Tamp, 2002). Upon

substrate recognition, MsbA transporters are thought to undergo a conformational change that increases the capacity of the NBDs to bind ATP.

Genes encoding ABC transporters occupy about 2.5% of the *M. tuberculosis* genome (Braibant *et al.*, 2000). Based on structural similarities to the typical subunits of ABC transporters present in all living organisms, at least 37 complete and incomplete ABC transporters have been identified in *M. tuberculosis* (Braibant *et al.*, 2000). Only a few of these transporters have been characterized and shown to be involved in drug resistance in *M. tuberculosis*.

M. tuberculosis contains a putative doxorubicin-resistance operon, *drxAB*, similar to that in *Streptomyces peucetius* (Guilfoile & Hutchinson, 1991). The *DrrAB* genes expressed in *M. smegmatis* confer resistance to a broad range of clinically relevant antibiotics, including tetracycline, erythromycin, ethambutol, norfloxacin, streptomycin and chloramphenicol. The resistant phenotype is reversed by treatment with reserpine or verapamil, both of which are known to inhibit pumps (Choudhuri *et al.*, 2002). Studies have suggested that the principal physiological role of the *Drr* proteins of *M. tuberculosis* may be the export of complex lipids to the exterior of the cell. This hypothesis is supported by the finding that an insertion in the *drxC* gene results in the production of small amounts of DIM, present principally in the bacterial cytosol or plasma membrane. The *DrrC* protein therefore seems to be involved in the transport of phthiocerol dimycocerosates (Camacho *et al.*, 2001). A library of signature-tagged transposon mutants of *M. tuberculosis* was constructed and screened for low levels of multiplication in mouse lungs. One of the 16 mutants with attenuated virulence had an insertion in the *drxC* gene, indicating that the *DrrC* protein is involved in virulence (Camacho *et al.*, 1999).

Bhatt *et al.* (2000) have shown that the ciprofloxacin resistance of a laboratory-generated *M. smegmatis* mutant with high levels of transcription and chromosomal amplification of a gene encoding a putative nucleotide-binding subunit of an ABC family transporter (*pstB*) results primarily from active efflux. The gene concerned is similar to the *phoT* gene (Rv0820) of *M. tuberculosis*, suggesting that this protein may also be involved in phosphate import in prokaryotes in conditions of phosphate starvation. The authors demonstrated that this mutation increases phosphate uptake and that inactivation of this gene in *M. smegmatis* results not only in the loss of high-affinity phosphate uptake, but also in hypersensitivity to fluoroquinolones and to structurally unrelated compounds (Banerjee *et al.*, 2000). These findings suggest that a single ABC transporter may be involved in both the active efflux of FQ and phosphate transport in *M. smegmatis*.

The role of ABC transporters in intrinsic resistance to FQ in *M. tuberculosis* is beginning to attract the attention of the

research community. As in *M. smegmatis*, the *phoT* gene of *M. tuberculosis* may be involved in FQ resistance, but this hypothesis remains to be tested.

Most of the prokaryotic genes encoding ABC transporters are organized into operons containing NBDs and MSDs as separate subunits requiring assembly into a biologically active transporter. We recently focused our attention on the Rv2686c–2687c–2688c operon. The 5' and 3' ends of the Rv2687c ORF overlap with the stop codon of Rv2688c and the translation start codon of Rv2686c, respectively, suggesting that these three genes may be cotranscribed. Rv2686c and Rv2687c each have six putative transmembrane segments, whereas the Rv2688c protein possesses an NBD and is probably involved in ATP hydrolysis. We have demonstrated that the *M. tuberculosis* Rv2686c–Rv2687c–Rv2688c operon encodes an ABC transporter responsible for FQ efflux when produced from a multicopy plasmid. This operon confers resistance to ciprofloxacin (8 × MIC) and, to a lesser extent, to norfloxacin (2 × MIC) when over-expressed in *M. smegmatis*. The level of resistance decreases in the presence of three efflux pump inhibitors: reserpine, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and verapamil (Pasca *et al.*, 2004) (efflux pump inhibitors are discussed in more detail in the next section). CCCP may have an indirect effect on this ATP-driven efflux pump because CCCP-induced proton gradient uncoupling also depletes the intracellular ATP pool. A similar level of resistance has been found in *M. bovis* BCG, transformed with the Rv2686c–2687c–2688c operon. This demonstrates that these genes are truly responsible for FQ efflux (G. Riccardi, unpublished data).

Indirect evidence of drug transport in mycobacteria

Several compounds deplete the energy source used to drive transport and can therefore block the activity of an efflux pump. The use of such inhibitory compounds provides evidence of drug transport and information about the energy source involved in that transport. The most widely used inhibitors are CCCP (a PMF uncoupler), *ortho*-vanadate and reserpine (two inhibitors of ATP-dependent efflux pumps), and verapamil (a well-known inhibitor of human P-glycoprotein and bacterial efflux pumps). These compounds generally abolish the increase in resistance produced by efflux pumps, as described above for the Rv2686c–2687c–2688c efflux pump.

One study has demonstrated that INH accumulation in *M. smegmatis* is increased by the addition of the PMF uncoupler, CCCP, and an inhibitor of ATP-dependent efflux pumps, *ortho*-vanadate (Choudhuri *et al.*, 1999); this provides evidence that both PMF- and ATP-dependent

Table 2. Evidence for efflux or drug accumulation of antituberculosis drugs in mycobacteria

Drug	Species	Evidence	Hypothesis	References
INH	<i>Mycobacterium smegmatis</i>	Increase of INH accumulation in presence of CCCP or <i>ortho</i> -vanadate	PMF and ATP-driven efflux pumps may be involved in INH efflux	Choudhuri <i>et al.</i> (1999)
	<i>Mycobacterium tuberculosis</i>	INH resistance can be induced gradually; reserpine decrease induced INH resistance	INH-inducible efflux pumps may be involved in INH efflux	Viveiros <i>et al.</i> (2002)
PZA	<i>Mycobacterium smegmatis</i>	Able to convert PZA into the active compound POA; intrinsically PZA resistant	There must be a very active POA efflux mechanism	Zhang <i>et al.</i> (1999)
RIF	<i>Mycobacterium tuberculosis</i> , <i>Mycobacterium smegmatis</i> , <i>Mycobacterium aurum</i>	Increase of RIF accumulation in the presence of reserpine, reversed by glucose	Suggestive of energy-dependent active efflux of RIF	Piddock <i>et al.</i> (2000)
	<i>Mycobacterium tuberculosis</i>	RIF induces Rv1258c expression in a <i>Mycobacterium tuberculosis</i> clinical strain resistant to RIF, ofloxacin, INH and minomycin	Contribution of efflux pumps to RIF resistance	Siddiqi <i>et al.</i> (2004)
	<i>Mycobacterium tuberculosis</i>	CCCP and reserpine did not affected FQ resistance levels	Efflux pumps unlikely to be involved in baseline FQ resistance	Piddock & Ricci (2001)
FQ	<i>Mycobacterium tuberculosis</i>	Ofloxacin induces Rv1258c expression in a <i>Mycobacterium tuberculosis</i> clinical strain resistant to RIF, ofloxacin, isoniazid and minomycin	Contribution of efflux pumps to ofloxacin resistance	Siddiqi <i>et al.</i> (2004)

INH, isoniazid; PZA, pyrazinamide; RIF, rifampicin; FQ, fluoroquinolone; ATP, adenosine triphosphate; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; POA, pyrazinoic acid.

extrusion systems are involved in INH efflux in this micro-organism (Table 2). As only 50%–60% of INH-resistant *M. tuberculosis* strains have mutations in genes encoding a catalase-peroxidase (*katG*), an enzyme of the mycolic acid pathway (*inhA*) and a β -ketoacyl-acyl carrier protein synthetase (*kasA*), conventional wisdom dictates that other mechanisms contribute to INH resistance (Slayden & Barry, 2000). Bardou *et al.* (1998) demonstrated that INH transport in *M. tuberculosis* is mediated by a passive mechanism, but the role of drug accumulation in intrinsic and acquired INH resistance has not been evaluated. Viveiros *et al.* (2002) reported that reserpine, a plant alkaloid that inhibits ATP-dependent efflux pumps, decreases the level of INH resistance in INH-induced resistant *M. tuberculosis* strains. The demonstration that high-level resistance to INH can be induced gradually in INH-susceptible strains of *M. tuberculosis* via a reserpine-sensitive mechanism that does not involve a mutation (Viveiros *et al.*, 2002) is consistent with the hypothesis that induced resistance might involve an efflux pump mechanism (Table 2). It was not determined whether the resistance was induced by an increase in expression of the pump gene, resulting in the presence of more pump units in the membrane or by another mechanism. These results suggest that efflux pumps are almost certainly involved in INH resistance in *M. tuberculosis*, but further experimental work is required to determine the proteins involved.

PZA is one of the most important drugs used to treat TB. PZA is a pro-drug that enters *M. tuberculosis* by passive diffusion and is converted to the active compound pyrazi-

noic acid (POA) by a nicotinamidase/pyrazinamidase enzyme encoded by the *pncA* gene (Zhang *et al.*, 1999). Hirano *et al.* (1997) reported that 72%–97% of PZA-resistant *M. tuberculosis* clinical isolates carry a mutation in the coding region of the *pncA* gene or in its putative promoter region. In contrast, Raynaud *et al.* (1999) reported that isolates with a high level of PZA resistance lacked mutations in the *pncA* gene. Other mechanisms may therefore be involved (PZA uptake, *pncA* regulation, POA efflux or mutations in the yet unidentified targets for POA) in PZA-resistant strains with no mutations in the *pncA* gene. The intrinsically PZA-resistant *M. smegmatis* converts the pro-drug into POA, but does not accumulate POA, because of a very active POA efflux mechanism (Table 2) (Zhang *et al.*, 1999). Furthermore, the EmrAB-TolC efflux pump has been shown to contribute to POA resistance in *E. coli* (Schaller *et al.*, 2002).

RIF is another potent antituberculous drug. Most RIF-resistant isolates ($\approx 95\%$) carry mutations in the *rpoB* gene, which encodes the β subunit of RNA polymerase. Piddock *et al.* (2000) analysed the levels of RIF accumulation in *M. tuberculosis*, *Mycobacterium aurum* and *M. smegmatis*, and reported that accumulation increased in the presence of the efflux pump inhibitor reserpine, suggesting that efflux pumps are also involved in the transport of this drug. If active efflux occurs, then de-energized cells accumulate more of the substance concerned than do energized cells. The small effect of the efflux inhibitor was consistent with an efflux pump present at low levels in wild-type bacteria. In the case of *M. aurum* and *M. smegmatis*, the addition of glucose (which can be metabolized to provide

energy) antagonized the effect of reserpine, indicating that RIF is pumped out of the cell in an energy-dependent process (Table 2).

Finally, Piddock & Ricci (2001) showed that CCCP and reserpine do not change the MICs of FQs in *M. tuberculosis*, suggesting that efflux pumps are not involved in determining baseline FQ-MICs (Table 2).

Concluding remarks

The use of efflux systems by bacteria as a means of eliminating antibacterial compounds was first suggested in 1978 (Levy & McMurry, 1978), with the description of membrane-bound antibiotic transport proteins in Gram-negative bacteria that expelled tetracyclines from *E. coli* (Ball *et al.*, 1980; McMurry *et al.*, 1980). The discussion of the relative importance of decreases in permeability and increases in efflux in the development of resistance is gradually changing. Burns *et al.* (1985) suggested that decreases in sensitivity to chloramphenicol, in the absence of chloramphenicol acetyltransferase, might be because of a decrease in influx. Nikaido & Nakae (1979) had previously suggested that Gram-negative bacteria can develop a high level of drug resistance as a result of mutations causing alterations in porin proteins, decreasing passive drug diffusion. However, Nikaido (1994) later observed that permeation barriers alone rarely produce significant resistance. It is now generally accepted that efflux pumps are becoming increasingly important as a mechanism of resistance, both alone and in synergy with changes in outer membrane permeability (Nikaido & Nakae, 1979; Levy, 1992; Nikaido, 1994, 2000, 2001; Zgurskaya & Nikaido, 2000; Ryan *et al.*, 2001; Li & Nikaido, 2004; Poole, 2004). One natural role of efflux pumps in prokaryotic and eukaryotic cells is the elimination of toxins from the cell. This protective function enables bacterial cells to survive in hostile environments, including those containing antibiotics, during the treatment of infections. The upregulation of efflux systems by physiological induction and spontaneous mutation can significantly decrease the intracellular concentration of many antibiotics, reducing their clinical efficacy. For this reason, many academic and pharmaceutical programmes have focused on identifying inhibitors of the efflux systems of Gram-negative and Gram-positive bacteria that could potentially be used in combination with antibiotics, to improve efficacy and abolish resistance (Aeschlimann *et al.*, 1999; Lomovskaya & Watkins, 2001a, b; Gibbons *et al.*, 2003; Renau *et al.*, 2003). Recent studies have demonstrated that the overproduction of efflux pumps is responsible for drug resistance in 14%–75% of clinical isolates of *P. aeruginosa* (Aeschlimann, 2003). One study has shown that *P. aeruginosa* mutants lacking efflux pumps are susceptible to most clinically useful antibiotics, including FQs, β -lactams and aminoglycosides

(Morita *et al.*, 2001). These findings have stimulated research on efflux pump inhibitors (Coban *et al.*, 2004). The inhibition of efflux pumps significantly decreases the level of intrinsic resistance to a similar extent to gene inactivation. In comparison, deletion of the *mexAB-oprM* genes (encoding an RND-type efflux pump) from the *P. aeruginosa* chromosome decreases resistance to levofloxacin by a factor of eight; resistance can also be decreased to this extent by treatment with the inhibitor MC-207 110 (Coban *et al.*, 2004). These results suggest that combining pump-specific inhibitors with efflux-sensitive antibiotics may be of value for treatment. However, no inhibitors that are not toxic to eukaryotic cells have yet been identified.

It has been shown that reserpine, a plant alkaloid that inhibits mammalian multidrug efflux systems, can also inhibit some bacterial efflux systems, thereby rendering bacteria more susceptible to antibiotics (Mullin *et al.*, 2004). For example, reserpine inhibits the NorA efflux pump in *S. aureus*, so any reserpine-mediated increase in either FQ or multidrug susceptibility is considered to indicate the involvement of NorA-type efflux pumps in Gram-positive bacteria (Brenwald *et al.*, 1997).

Recent research into efflux mechanisms in mycobacteria, using laboratory strains, has provided promising insights, but the relevance of the efflux mechanism to the resistance of clinical strains is only just beginning to become clear. Indeed, most research in this area has involved transferring hypothetical efflux genes into a heterologous host (*M. smegmatis*) and demonstrating that the overexpression of these genes increases resistance (see Table 1). New experimental approaches are needed to assess the involvement of efflux pumps in intrinsic/acquired drug resistance in mycobacteria. Recent studies, such as that by Siddiqi *et al.* (2004), are therefore marking a change in studies of efflux pumps in clinical isolates of *M. tuberculosis*. Siddiqi showed that the transcription of the Rv1258c gene increases upon induction by RIF and ofloxacin in a clinical multidrug-resistant *M. tuberculosis* isolate, suggesting that efflux pumps are involved in MDR in *M. tuberculosis* (Siddiqi *et al.*, 2004). However, although several mycobacterial pumps have been characterized, the clinical consequences of efflux-mediated resistance are mostly unknown because of variable levels of expression and the lack of specific markers for use in laboratory practice.

Early studies of drug accumulation in intact cells suggested that drug-resistant mycobacteria accumulated fewer drugs than the susceptible parental strains and these results were often attributed to the lower cell envelope permeability in resistant cells (Hui *et al.*, 1977). However, some of these drug accumulation data probably require reinterpretation because of the higher levels of drug efflux in drug-resistant strains, according to current understanding of drug influx and efflux processes across the mycobacterial cell envelope.

Our comprehension of mycobacterial efflux pumps has progressed considerably in the last few years. Studies of the genome sequence of *M. tuberculosis* have suggested that this species contains several genes encoding putative drug efflux transporters (<http://www.membranetransport.org>). Extensive clinical and research work around the world has resulted in the characterization of many of these efflux pumps. We now know that efflux pumps are involved in intrinsic mycobacterial drug resistance, as shown by Siddiqi *et al.* (2004). The question of the importance of the role played by these transporters in intrinsic and acquired antibiotic resistance remains unresolved, but will no doubt be addressed by future studies.

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