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MINIREVIEW

# Efflux as a mechanism for drug resistance in *Mycobacterium tuberculosis*

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## Abstract

Tuberculosis remains an important global public health problem, with an estimated prevalence of 14 million individuals with tuberculosis worldwide in 2007. Because antibiotic treatment is one of the main tools for tuberculosis control, knowledge of *Mycobacterium tuberculosis* drug resistance is an important component for the disease control strategy. Although several gene mutations in specific *loci* of the *M. tuberculosis* genome have been reported as the basis for drug resistance, additional resistance mechanisms are now believed to exist. Efflux is a ubiquitous mechanism responsible for intrinsic and acquired drug resistance in prokaryotic and eukaryotic cells. *Mycobacterium tuberculosis* presents one of the largest numbers of putative drug efflux pumps compared with its genome size. Bioinformatics as well as direct and indirect evidence have established relationships among drug efflux with intrinsic or acquired resistance in *M. tuberculosis*. This minireview describes the current knowledge on drug efflux in *M. tuberculosis*.

## Introduction

The high mortality and morbidity associated with tuberculosis (TB), especially in poor countries, is one of the features characterizing tuberculosis as a major public health concern worldwide. In the absence of a more effective vaccine, chemotherapy is one of the main tuberculosis control tools. There is an important increase in the prevalence of tuberculosis cases in several settings with multidrug- and extensively-drug resistance rates on the rise (World Health Organization, 2010b). Multidrug-resistant tuberculosis (MDR-TB) is caused by strains of *Mycobacterium tuberculosis* that are resistant to at least rifampicin and isoniazid, two key drugs in the treatment of the disease. Extensively drug resistant tuberculosis(XDR-TB), on the other hand, is caused by strains of *M. tuberculosis* that, in addition to being MDR, are also resistant to any quinolone and to one of the three injectable second-line drugs: kanamycin, capreomycin or amikacin (Migliori *et al.*, 2007). Patients with MDR-TB are treated following the recommendations of the WHO according to defined parameters (World Health Organization, 2010a). There are no official specific recommendations for the treatment of patients with XDR-TB, although positive experiences have been reported (Bonilla *et al.*, 2008). Patients with XDR-TB have fewer options for treatment and risk higher mortalities, especially in HIV-coinfected persons, as has been reported earlier (Gandhi *et al.*, 2006).

Although new candidate drugs are under development, it will probably take several years until one new anti-tuberculosis drug becomes available, stressing the need to better understand the mechanisms of resistance to the currently available drugs and to reduce the incidence of drug-resistant cases (Dye, 2009).

Intrinsic drug resistance in *M. tuberculosis* has been attributed to a combination of a highly impermeable mycolic acid-containing cell wall and an active drug efflux mechanism (Jarlier & Nikaido, 1994; De Rossi *et al.*, 2006). Acquired drug resistance, on the other hand, does not occur by horizontal gene transfer as in other microorganisms, because *M. tuberculosis* lack plasmids and genomic DNA transfer has not been described, occurring instead by spontaneous mutations in specific target genes rendering the bacteria resistant to a given drug (Ramaswamy & Musser, 1998). This has to be distinguished from the clinical definition of acquired drug resistance, defined as resistance to one or more drugs in *M. tuberculosis* strains recovered from patients who have received previous anti-tuberculosis treatment (World Health Organization, 1997).

Our knowledge of drug resistance in *M. tuberculosis* has increased considerably in the last 20 years. Gene mutations

**Table 1.** Efflux pumps for anti-tuberculosis drugs

| Drug efflux pump | Transporter family | Drug | References |
|---|---|---|---|
| Rv1258c | MFS | STR RIF, OFX, INH* | Aínsa *et al.* (1998), Jiang *et al.* (2008) |
| Rv1410c | MFS | STR,INH, RIF | Silva *et al.* (2001), Jiang *et al.* (2008) |
| Rv1634 | MFS | FQ | De Rossi *et al.* (2002) |
| Rv2459 | MFS | INH, EMB | Gupta *et* al. (2010a, b) |
| Rv2846c (EfpA) | MFS | INH, ETH | Wilson *et al.* (1999) |
| DrrABC | ABC | EMB, FQ, STR | Choudhuri *et al.* (2002) |
| Rv2686c-Rv2687c-Rv2688c | ABC | FQ | Pasca *et al.* (2004) |
| Rv0194 | ABC | STR | Danilchanka *et al.* (2008) |
| MmpL | RND | INH | Pasca *et al.* (2005) |

STR, streptomycin; RIF, rifampicin; OFX, ofloxacin; INH, isoniazid; FQ, fluoroquinolones; ETH, ethionamide; EMB, ethambutol.

in several *loci* have now been characterized as the main basis for drug resistance (Zhang & Telenti, 2000). However, in a certain proportion of clinical isolates, resistance cannot be explained by the presence of gene mutations as it occurs in up to 30% of isoniazid-resistant or in around 5% of rifampicin-resistant clinical isolates of *M. tuberculosis*, suggesting that other mechanisms of drug resistance must exist (Louw *et al.*, 2009). Among these, efflux has been proposed as the basis for drug resistance in clinical isolates lacking previously described gene mutations (Escribano *et al.*, 2007; Spies *et al.*, 2008)

Drug efflux, where a transporter is capable of extruding multiple drugs without apparent common structural similarity, was first described in eukaryotic cells. (Ambudkar *et al.*, 1992, 1999). Soon, it became apparent that multidrug efflux systems were also present in several microorganisms (McMurry *et al.*, 1980; Nikaido, 1998; Paulsen & Lewis, 2001).

More recently, drug efflux has been described as an important mechanism for intrinsic and acquired drug resistance in numerous prokaryotic and eukaryotic cells (Levy, 1992; Nikaido & Zgurskaya, 1999; Li *et al.*, 2003, 2004; Webber & Piddock, 2003; Piddock, 2006a). Drug efflux has also been associated with pathogenicity, virulence, biofilm formation and quorum sensing (Piddock, 2006b; Bina *et al.*, 2009; Chan & Chua, 2010; Høiby *et al.*, 2010). Not all the currently available antituberculosis drugs, however, are considered as substrates of efflux mechanisms (Palomino *et al.*, 2009).

Analysis of the available bacterial genomes has shown that putative drug efflux pumps (EPs) constitute 6–18% of all transporters found in any given bacterial cell. *Mycobacterium tuberculosis* presents one of the largest numbers of putative EPs compared with its genome size (Paulsen *et al.*, 2001). Consequently, active drug efflux systems have been shown to be present in mycobacteria (Sander *et al.*, 2000; Pasca *et al.*, 2005), extruding structurally and functionally unrelated compounds, and for this reason, they are also known as MDR EPs. The mechanisms for the induction and regulation of these EPs are not yet fully understood. Moreover, their implication in clinical drug resistance needs to be completely elucidated (Putman *et al.*, 2000). This minireview summarizes our current knowledge on EPs in *M. tuberculosis* taking into account bioinformatics as well as direct and indirect evidence (Table 1).

## Drug efflux in the genus *Mycobacterium*

In *Mycobacterium smegmatis* as well as in other mycobacteria, the cell wall, rich in mycolic acids, functions as an efficient barrier limiting the access of several molecules including antibiotics. However, this is not enough for explaining the intrinsic drug resistance of these microorganisms (Li *et al.*, 2004).

Although efflux mechanisms have been studied in several mycobacteria (Aínsa *et al.*, 1998; Nomura *et al.*, 2004; Ramon-Garcia *et al.*, 2006; Rodrigues *et al.*, 2008), for practical reasons, *M. smegmatis* has been used as the model system for expressing heterologous putative EP genes and to study the efflux mechanism itself (Liu *et al.*, 1996; Takiff *et al.*, 1996; De Rossi *et al.*, 1998a; Silva *et al.*, 2001; Pasca *et al.*, 2005; Kim *et al.*, 2008).

The first EP characterized in mycobacteria was the LfrA present in *M. smegmatis* that, expressed in multicopy plasmids, confers low-level resistance to fluoroquinolones, ethidium bromide, acridine and some quaternary ammonium compounds (Liu *et al.*, 1996; Takiff *et al.*, 1996).

Other EPs initially characterized in mycobacteria were TetV, conferring resistance to tetracycline (De Rossi *et al.*, 1998a), and Tap, which confers low-level resistance to aminoglycosides and tetracycline when overexpressed in *M. smegmatis* (Aínsa *et al.*, 1998). These reports can be considered as precursors of the different studies of drug efflux in *M. tuberculosis* that will be described below.

## Putative EPs in *M. tuberculosis*

EPs, also known as transporters, have been classified into five superfamilies: ATP-binding cassette (ABC), major facilitator super-family (MFS), resistance nodulation division (RND), small multidrug resistance (SMR) and multidrug

and toxic-compound extrusion (MATE). While MFS, SMR, RND and MATE members are secondary transporters, typically energized by the proton motive force ($H^+$ or $Na^+$), members of the ABC superfamily use ATP as the energy source and are considered as primary transporters (Tseng *et al.*, 1999; Putman *et al.*, 2000; Cattoir, 2004; Li & Nikaido, 2009; Saier *et al.*, 2009).

The ABC superfamily is a large and ancient family that consists of 52 subfamilies comprising uptake or efflux systems for a wide range of substrates including drugs, sugars, amino acids, carboxylates, metal ions and peptides (Paulsen *et al.*, 2001). ABC transporters have been associated with the acquisition of drug resistance in mycobacteria. The gene cluster *drrA-drrB-drrC*, with high similarity to an ABC exporter of daunorubicin in various *Streptomyces* species, determines resistance to a broad range of clinically relevant antibiotics when overexpressed in *M. smegmatis* (Guilfoile & Hutchinson, 1991; Choudhuri *et al.*, 2002). Another ABC transporter coded by the genes Rv2686c-Rv2687c-Rv2688c when overexpressed in *M. smegmatis* increased by eightfold the minimum inhibitory concentrations (MIC) of ciprofloxacin when the entire operon was overexpressed and by fourfold when only Rv2686c was overexpressed (Pasca *et al.*, 2004).

In *M. tuberculosis*, genes coding for ABC transporters represent 2.5% of its entire genome and by sequence analysis at least 12 putative EP genes have been identified: Rv0194, Rv1218c-Rv1217c, Rv1273c-Rv1272c, Rv1348-Rv1349, Rv1456c-Rv1457c-Rv1458c, Rv1473, Rv1667c-Rv1668c, Rv1686c-Rv1687c, Rv1819, Rv2477, Rv2688c-Rv2687c-Rv2686c and *drrA-drrB-drrC* (Braibant *et al.*, 2000).

A novel ABC multidrug EP was identified in *M. tuberculosis* while investigating the molecular mechanisms for resistance to β-lactam antibiotics (Danilchanka *et al.*, 2008). Rv0194 was identified not only as an EP involved in resistance to β-lactams in *M. tuberculosis*, but it was also found that low-level expression of *rv0194* increased the resistance of *Mycobacterium bovis* BCG to several antibiotics.

More recently, Balganesh *et al.* (2010) characterized another new major ABC transporter in *M. tuberculosis* found to be responsible for the efflux of a wide variety of substrates including novobiocins, pyrazolones, biarylpiperazines, bisanilinopyrimidines, pyrroles and pyridones. MICs of these compounds were decreased by four- to eightfold in mutants lacking *Rv1218c* compared with the wild-type strain.

Until now, however, the role of ABC EPs in conferring clinically relevant resistance to multiple drugs has not been described (Piddock, 2006a).

Bioinformatics tools (http://www.membranetransport.org/) have identified up to 20 potential EP genes belonging to the MFS drug transporters in the *M. tuberculosis* genome (Saier *et al.*, 2009). Furthermore, by sequence and motif similarity to EPs present in several microorganisms, at least 16 putative EP genes of this family have been identified in *M. tuberculosis:* Rv0037c, Rv0191, Rv0783c, Rv0849, Rv1250, Rv1258c, Rv1410c, Rv1634, Rv1877, Rv2333c, Rv2456c, Rv2459, Rv2846c (*efpA*), Rv28994, Rv3239c and Rv3728 (De Rossi *et al.*, 2002). The Tap protein and LfrA mentioned before are EPs belonging to the MFS family conferring resistance to tetracycline and fluoroquinolones, respectively (Takiff *et al.*, 1996; Aínsa *et al.*, 1998). Also, P55, the protein encoded by the Rv1410c gene, was characterized as a multidrug EP of the MFS family in *M. tuberculosis* and *M. bovis* conferring resistance to streptomycin and tetracycline (Silva *et al.*, 2001). P55 has been recently been shown to require the cell surface LprG lipoprotein to function properly (Farrow & Rubin, 2008), with both proteins being critical for the survival of *M. tuberculosis* during infection (Bigi *et al.*, 2004).

The Mmr protein of *M. tuberculosis* has been identified as a multidrug EP of the SMR family that confers resistance to acriflavine, ethidium bromide and erythromycin in *M. smegmatis* when expressed in a multicopy vector (De Rossi *et al.*, 1998b).

EPs of the RND family are most commonly found in Gram-negative bacteria. In *M. tuberculosis*, the *mmpL7* gene that encodes a putative RND transporter confers a high-level resistance to isoniazid when overexpressed in *M. smegmatis* (Pasca *et al.*, 2005). This resistance was reversed in the presence of EP inhibitors. More recently, on characterizing several azole-resistant spontaneous mutants of *M. tuberculosis* and *M. bovis* BCG, an increased econazole efflux and an increased transcription of *mmpS5-mmpL5* genes that encode a hypothetical EP of the RND family were also found. Furthermore, it has been demonstrated that upregulation of these genes was linked to mutations in either the Rv0678 gene, its hypothetical transcriptional regulator or in its putative promoter/operator region (Milano *et al.*, 2009). Interestingly, the *M. tuberculosis* genome revealed the presence of 15 putative transmembrane proteins, predicted to belong to the RND family (http://genolist.pasteur.fr/TuberCuList/). Because these proteins showed some characteristics restricted to mycobacteria they were designated MmpL (mycobacterial membrane proteins, large).

Transporters belonging to the MATE family have not been reported in mycobacteria, being more common in *Escherichia coli* and *Vibrio* sp. (Li & Nikaido, 2004).

## Overexpression and antimicrobial resistance profile

Using *M. smegmatis* or *M. bovis* as expression hosts and plasmids carrying genes coding for putative EPs in *M. tuberculosis*, increased resistance to several drugs has been reported. Using this approach, Rv1258c and Rv1410 (P55) encoding MFS transporters, produced resistance to tetracycline and aminoglycosides. Similarly, overexpression

of Rv1634, another MFS family member, was found to confer resistance to fluoroquinolones (Silva *et al.*, 2001; De Rossi *et al.*, 2002).

The study by Ramon-Garcia *et al.* (2007) showed that the *stp* gene (Rv2333c) from *M. tuberculosis* conferred resistance to spectinomycin and tetracycline when expressed in *M. bovis* BCG. Overexpression studies have also shown that Rv0194 that codes for an ABC-type EP conferred MDR in *M. bovis* BCG and *M. smegmatis* and also reduced the accumulation of ethidium bromide in the latter (Danilchanka *et al.*, 2008).

More recently, using recombinant strains of *M. tuberculosis* H37Ra, the overexpression of Rv2459 (*jef*A) led to an increase in the MICs of isoniazid and ethambutol. These MIC values decreased again when the bacteria were grown in the presence of the EP inhibitors carbonyl cyanide m-chlorophenyl hydrazone (CCCP) and verapamil. Bioinformatics analyses have also shown a close relation of the JefA protein with drug EPs in other clinically relevant bacteria (Gupta *et al.*, 2010b).

## Drug-induced EPs

Although the overexpression of genes coding for putative EPs has been a widely used strategy, exploring drug-induced alterations in gene expression has also been used to associate several EPs with different drugs.

Using this approach, Siddiqi *et al.* (2004) reported a MDR clinical isolate of *M. tuberculosis* that, when grown in the presence of subinhibitory concentrations of rifampicin and ofloxacin, showed increased transcription of the gene Rv1258c that encodes a tap-like EP. Although drug resistance-associated mutations were detected in *gyrA* and *rpoB*, it was hypothesized that the high level of resistance to rifampicin and ofloxacin could reflect an additional overexpression of the gene Rv1258c. Similar results were obtained in another study with a clinical MDR isolate in which the expression of Rv1258c and Rv1410c was significantly increased in the presence of rifampicin or isoniazid (Jiang *et al.*, 2008). Another study that supports the involvement of Rv1258c as a putative EP in *M. tuberculosis* has been reported by Sharma *et al.* (2010), showing that piperine, a *trans–trans* isomer of 1-piperoyl-piperidine, inhibited the clinically overexpressed Rv1258c gene.

One of the first studies to use genome-wide expression analysis in the presence of an antibiotic was reported by Wilson and colleagues. In this study, using microarray hybridization and induced gene expression, it was found that isoniazid and ethionamide increased the expression of *efpA*, a member of the MFS family of EPs (Wilson *et al.*, 1999). A more recent study by Gupta *et* al. (2010a), using a DNA microarray with 25 drug EP genes of *M. tuberculosis*, found overexpression of 10 genes after exposure to various anti-tuberculosis drugs. These included Rv3065 and Rv2938, already reported as active drug EPs in *M. tuberculosis* in previous studies, and eight other EP genes reported for the first time (Rv1819, Rv2209, Rv2459, Rv2477c, Rv2688, Rv2846, Rv2994 and Rv3728). The simultaneous overexpression of the EP genes Rv2459, Rv3728 and Rv3065 was associated with resistance to the combination of isoniazid and ethambutol that, along with streptomycin, were identified to group together, signaling their probable importance in the development of MDR in *M. tuberculosis*. An interesting study has reported the induction of high-level resistance to isoniazid (up to 20 $\mu g\,mL^{-1}$) in *M. tuberculosis*, which could be reduced 100-fold by subinhibitory concentrations of reserpine, supporting the argument that induced drug resistance might be due to an EP mechanism (Viveiros *et al.*, 2002).

Finally, expression of Rv0341, Rv0342 and Rv0343 (*iniB, iniA* and *iniC*, respectively) is induced by isoniazid or ethambutol. It has been proposed that *iniA*, albeit not a typical EP, could be functioning like an MDR-pump system (Colangeli *et al.*, 2005).

The use of EPs inhibitors as a possible alternative or adjuvant in antituberculosis therapy has been considered as an interesting option since some time ago. Shortening the duration of tuberculosis treatment and reducing the spread of drug resistance are high priorities for the control of the disease. The effects of EP inhibitors in reducing the resistance to antibiotics have been clearly shown in other bacteria (Aeschlimann *et al.*, 1999). Considering tuberculosis, Amaral *et al.* (2008) have recently argued on the possibility of using thioridazine, an inhibitor of EPs, in the treatment of MDR/XDR-TB. Thioridazine has been found previously to be active in killing intracellular MDR *M. tuberculosis* at concentrations below those normally present in the plasma of patients (Ordway *et al.*, 2003). Similarly, a recent study has reported promising therapeutic activity of thioridazine in a mouse model of MDR-TB (Van Soolingen *et al.*, 2010). Another study has shown that the addition of verapamil, a known EP inhibitor, to first-line anti-tuberculosis drugs significantly reduced CFU in the lungs of mice after 1 and 2 months of treatment (Louw *et al.*, 2011). Taking these results into account, further clinical trials might be warranted to assess whether these compounds may be safely and efficiently used as an adjunct therapy in tuberculosis.

## Putative transcriptional regulator of EP genes

Several examples of the transcriptional regulation of EP genes have been described in the literature. The TetR-like transcriptional repressor LfrR regulates the expression of *lfrA* and it has been shown that some compounds such as

acriflavine, ethidium bromide or rhodamine 123 enhance the expression of this local regulator. Gene disruption in the native host rendered the mutant more susceptible to multiple drugs such as fluoroquinolones, ethidium bromide and acriflavine (Buroni *et al.*, 2006; Bellinzoni *et al.*, 2009).

In *M. tuberculosis*, an antibiotic tolerance system similar to the MDR system present in *Actinobacter* sp has been described. This system is dependent on *whiB7*, which is induced by subinhibitory concentrations of antibiotics. Expression of *whiB7* was found to be increased fivefold in the late exponential and early stationary phases, with a subsequent decline to control levels in the late stationary phase of growth. Gene expression analysis showed that transcription of *whiB7* determined drug resistance by activating the expression of a regulon that includes Rv1258c and Rv1473 (Morris *et al.*, 2005; Geiman *et al.*, 2006).

MarA is another transcriptional regulator that, in *E. coli*, is related to an increase of drug efflux and, when overexpressed in *M. smegmatis*, resulted in an increased resistance to rifampicin, isoniazid, ethambutol, tetracycline and chloramphenicol. MarA was mainly associated with a positive regulation of an EP of the RND family of transporters (McDermott *et al.*, 1998). In *M. tuberculosis*, Schaller *et al.* (2002) observed that low concentrations of salicylate induced resistance to different drugs such as rifampicin, isoniazid, ethambutol and streptomycin. Although salicylate can also induce antibiotic resistance through a Mar-independent pathway, it is possible to infer the presence of a transcriptional activator like MarA in *M. tuberculosis* (Cohen *et al.*, 1993). In fact, Rv1931c, Rv3736 and Rv3833 present in the *M. tuberculosis* genome show 30% identity to MarA and could be related to an EP gene overexpression (http://genolist.pasteur.fr/TubercuList/).

It remains to be seen and investigated how these mechanisms of transcriptional regulation occur in real life and whether they are responsible for clinically relevant drug resistance in *M. tuberculosis*.

## EPs and virulence

It has been recently shown that there is a relationship between EPs and virulence. In some bacteria, EPs can also export virulence determinants such as adhesins, toxins or other proteins that are important for colonization and persistence in human and animal cells (Piddock, 2006b).

The survival of *M. tuberculosis* in the macrophage depends on its capacity to obstruct the normal maturation of the phagosome. Isolation of defective mutants unable to arrest phagosome maturation showed that some affected genes were homologues of putative EPs and lipid synthesis enzymes. One such mutant had a knockout in Rv1819c, which is characterized as a putative EP of the ABC family. It is interesting to note that an increasing number of MDR ABC transporters have been shown to transport lipids. Thus, it is feasible that Rv1819c might also be responsible for the transport of lipids to the exterior of the bacterium (Borst *et al.*, 2000; Pethe *et al.*, 2004).

In *M. tuberculosis lprG* (Rv1411) and *P55* (Rv1410) form an operon. Mutants ΔRv1411 of *M. tuberculosis* H37Rv do not produce LprG or P55 and result in attenuated strains in a mouse model of infection, confirming that *lprG* is required for the growth of *M. tuberculosis* in immunocompetent mice (Bigi *et al.*, 2004). However, conservation of the operon in the nonpathogenic *M. smegmatis* suggests that the protein is at least partially necessary in environmental mycobacteria. It has also been shown in *M. smegmatis* that the lprG-Rv1410c operon is required for resistance to ethidium bromide and for maintaining a normal cell surface composition. (Farrow & Rubin, 2008). More recently, the role of P55 in the oxidative stress response and normal growth *in vitro* has been reported (Ramón-García *et al.*, 2009).

Recently, Domenech *et al.* (2009) showed that the ABC transporter encoded by Rv1819c, which shares 39% similarity to the BacA protein of *Brucella abortus*, plays a significant role in the maintenance of extended chronic tuberculosis infection in mice. *Mycobacterium tuberculosis* strains deficient in BacA showed no compromise of the membrane integrity, but had increased resistance to bleomycin. Expression of this BacA homologue in *E. coli* conferred resistance to antimicrobial peptides (Domenech *et al.*, 2009). It is not known by which mechanism the BacA deficiency causes this attenuation of infection in mice; however, it has been reported that in *B. abortus bacA* mutants induce larger amounts of proinflammatory cytokines compared with the parental strain (Parent *et al.*, 2007). While BacA does not appear to contribute directly to drug efflux or efflux of components of the *M. tuberculosis* cell wall, it may be involved in the transport of certain antimicrobial peptides important in determining the progression of infection in the host.

A recent interesting study has been reported by Dutta *et al.* (2010) analyzing the effects of thioridazine in *M. tuberculosis*. On analyzing gene expression profiles after the treatment of *M. tuberculosis* with thioridazine, it was found that the drug modulated the expression of genes coding for membrane proteins, EPs, oxidoreductases and enzymes of fatty acid metabolism and aerobic respiration. The authors hypothesize that thioridazine also damages the cell envelope of the bacteria and turns on the expression of the sigmaB regulon that has been shown to be responsible for the protection of *M. tuberculosis* from envelope damage (Fontán *et al.*, 2009).

## Concluding remarks

Bioinformatics and experimental data showing the relationship between efflux mechanisms and drug resistance in

*M. tuberculosis* have stressed the need to further our knowledge on the mechanisms of drug resistance in tuberculosis.

It is necessary to better identify the correlation between EP gene expression and their inducers, both under environmental conditions and in the presence of drugs. Although the EPs described in *M. tuberculosis* show a narrow spectrum of substrates, point mutations could radically alter the spectrum of substrates as has been described in other microorganisms (Klyachko & Neyfakh, 1998).

Molecular analysis using multicopy plasmids or gene knockouts has the advantage of identifying the precise *locus* involved in efflux-mediated resistance; however, its limitation is the limited approach on the complex interaction between the EP, the regulatory proteins and several inducers. Phenotypic methods, on the other hand, have the advantage of identifying integral efflux mechanisms as a result of the broad interaction of several genes responsible for efflux, but they are unable to determine the specific EP involved. While the role of efflux in acquired and intrinsic drug resistance is not completely elucidated, it is possible to consider this mechanism as a factor contributing to clinical drug resistance working in synergy with other mechanisms of resistance such as impermeability of the cell wall and drug resistance mutations.

Regarding clinical practice, the detection of drug efflux remains an important goal because it allows identifying the mechanisms that could be related to an increase in drug resistance. For this reason, it would be important to develop accurate and simple diagnostic methods that could identify and characterize efflux events. Along this line, although not yet used routinely, MIC determination in the presence or absence of known efflux inhibitors or evaluation of substrate accumulation using radiolabeled substrates or instrument-free methods have been proposed (Martins *et al.*, 2006; Viveiros *et al.*, 2008).

Our current knowledge on EPs indicates that efflux inhibitors could become candidate tools to treat infectious diseases (Lomovskaya & Bostian, 2006; Piddock, 2006a). Certain compounds already in use in clinical practice for other purposes, such as verapamil, reserpine and omeprazole, are capable of inhibiting efflux mechanisms in several eukaryotic and prokaryotic cells. However, they have been mostly active at concentrations higher than those used clinically (Kaatz, 2002).

In conclusion, several compounds that inhibit efflux activity have been synthesized or obtained from natural sources, but none of them are currently being used to treat infectious diseases (Yamada *et al.*, 1997; Lomovskaya *et al.*, 2001; Kaatz, 2005). Considering the limited number of antimicrobials available for the treatment of tuberculosis and the unquestionable relationship between efflux and drug resistance in *M. tuberculosis*, it is urgent to deepen our knowledge on drug efflux as well as to develop compounds that could offset this resistance mechanism.

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