Ethidium bromide transport across *Mycobacterium smegmatis* cell-wall: correlation with antibiotic resistance

Liliana Rodrigues¹², Jorge Ramos¹, Isabel Couto¹³, Leonard Amaral¹²⁴, Miguel Viveiros¹⁴*

**Abstract**

**Background:** Active efflux systems and reduced cell-wall permeability are considered to be the main causes of mycobacterial intrinsic resistance to many antimicrobials. In this study, we have compared the *Mycobacterium smegmatis* wild-type strain mc²¹⁵⁵ with knockout mutants for porins MspA (the main porin of *M. smegmatis*) and MspC, the efflux pump LfrA (the main efflux pump system of *M. smegmatis*) and its repressor LfrR for their ability to transport ethidium bromide (EtBr) on a real-time basis. This information was then correlated with minimum inhibitory concentrations (MICs) of several antibiotics in the presence or absence of the efflux inhibitors chlorpromazine, thioridazine and verapamil.

**Results:** In the absence of porins MspA and MspC, accumulation of ethidium bromide decreased and the cells became more resistant to several antibiotics, whereas the knockout mutant for the LfrA pump showed increased accumulation of EtBr and increased susceptibility to EtBr, rifampicin, ethambutol and ciprofloxacin. Moreover, the efflux inhibitors caused a reduction of the MICs of streptomycin, rifampicin, amikacin, ciprofloxacin, clarithromycin and erythromycin in most of the strains tested.

**Conclusions:** The methodology used in this study demonstrated that porin MspA plays an important role in the influx of quaternary ammonium compounds and antibiotics and that efflux via the LfrA pump is involved in low-level resistance to several antimicrobial drugs in *M. smegmatis*. The results obtained with this non-pathogenic mycobacterium will be used in future studies as a model for the evaluation of the activity of the same efflux inhibitors on the susceptibility of multidrug resistant strains of *Mycobacterium tuberculosis* to isoniazid and rifampicin.

**Background**

The intrinsic resistance of mycobacteria to most antimicrobial agents is generally attributed to their relatively impermeable cell-wall, which provides a barrier to noxious compounds and limits drug uptake [1]. This low permeability is due to the structure and lipid-rich composition of the mycobacterial cell-wall that comprises long-chain fatty acids, the mycolic acids, covalently bound to a peptidoglycan-arabinogalactan polymer, and extractable lipids not covalently linked to the peptidoglycan-arabinogalactan [1-3]. Diffusion of hydrophilic nutrients is mediated by pore-forming proteins like the MspA porin of *M. smegmatis*, which is described as the major diffusion pathway for hydrophilic solutes in these mycobacteria [4,5]. Along with the controlled permeability by the cell-wall, active efflux systems can also provide resistance by extruding noxious compounds prior to their reaching their intended targets. Intracellular concentration of a given compound is therefore a result of interplay between permeability and efflux [6]. In order to develop effective antimycobacterial therapeutic strategies at a time when multidrug resistant and extensively drug resistant tuberculosis continue to escalate [7], the contributions made by alterations of permeability due to down regulation of porins and increased expression of efflux pumps that render these infections problematic for therapy, must be understood.

¹ Correspondence: mviveiros@ihmt.unl.pt
² Unit of Mycobacteriology, Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa, Rua da Junqueira 100, 1349-008 Lisboa, Portugal
Full list of author information is available at the end of the article

© 2011 Rodrigues et al; licensee BioMed Central Ltd. This is an Open Access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/2.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.
Several mycobacterial efflux pumps have been identified and characterized to date [8-14]. However, their role in intrinsic and acquired drug resistance in mycobacteria is not completely understood. LfrA, a transporter protein of the major facilitator superfamily of \textit{M. smegmatis}, was the first efflux pump to be genetically described in mycobacteria and it has been associated with resistance to ethidium bromide (EtBr), acriflavine, doxorubicin, rhodamine 123 and fluoroquinolones [14-17]. The regulation of LfrA is controlled by the upstream region of \textit{lfrA} that contains a gene coding for LfrR, a putative transcriptional repressor of the TetR family, which represses the transcription of the \textit{lfrRA} operon by directly binding to the promoter region [18,19].

The efflux pump substrate EtBr is widely used as a probe to detect and quantify efflux activity by bacteria [20-23]. EtBr emits weak fluorescence in aqueous solution (outside cells) and becomes strongly fluorescent when concentrated in the periplasm of Gram-negative bacteria and in the cytoplasm of Gram-positive bacteria. As long as EtBr is not intercalated between nucleic bases of DNA, it is subject to extrusion. When it is intercalated, the binding constant is sufficiently strong to keep EtBr from access to the efflux pump system of the bacterium [24]. Recently, a semi-automated fluorometric method was developed using EtBr as substrate for the real-time assessment of efflux pump activity in bacteria [25-27]. The method was developed considering that EtBr accumulation inside the cell is the result of the interplay between cell-wall permeability and efflux activity. The fluorescence that results from the overall intracellular EtBr content is monitored by real-time fluorometry.

In the study to be described, we used this semi-automated fluorometric method to study EtBr transport in \textit{M. smegmatis}, using the wild-type strain \textit{mc} \textsuperscript{155} and mutant strains carrying in-frame deletions of genes coding for porins MspA and MspC, the efflux pump LfrA and its repressor LfrR, and correlated this information with the corresponding antibiotic profile. Since many efflux pumps of \textit{M. smegmatis} have their homologues in \textit{Mycobacterium tuberculosis}, the use of \textit{M. smegmatis} as a model mycobacterium may provide data that will help to understand efflux-mediated drug resistance in \textit{M. tuberculosis} and other mycobacteria that infect the human [15].

\textbf{Results and Discussion}

\textbf{MspA as a major pathway for EtBr in \textit{M. smegmatis}}

The \textit{M. smegmatis} strains used in this study are described in Table 1. The accumulation of increasing concentrations of EtBr by strains SMR5, MN01 (\textit{ΔmspA}) and ML10 (\textit{ΔmspAΔmspC}) is presented by Figure 1. Accumulation of EtBr under conditions that maximize efflux (presence of glucose and incubation at 37°C) begins to take place at a concentration of 1 mg/L in the case of \textit{M. smegmatis} SMR5. This concentration of EtBr marginally exceeds the ability of the intrinsic efflux system of SMR5 to extrude the substrate. In the case of the SMR5 derived porin mutants MN01 (\textit{ΔmspA}) and ML10 (\textit{ΔmspAΔmspC}), the marginal concentration that results in accumulation of EtBr is increased to 2 and 4 mg/L, respectively (Figure 1) and considered to be the result of a decreased influx rate of EtBr due to the deletion of porins in these strains [3,5]. These concentrations were selected to test the effect of the efflux inhibitors chlorpromazine, thioridazine and verapamil in the accumulation of EtBr by these strains. This is to ensure that the increase of accumulation of EtBr is due to inhibition of efflux pumps and not to the use of an EtBr concentration that the cell’s efflux system cannot extrude. As shown by Figure 2, the efflux inhibitors chlorpromazine, thioridazine and verapamil, used at ½ the minimum inhibitory concentration (MIC; see Table 1), increased accumulation of EtBr, although only marginally in strain ML10. We interpret these results as indicating that because of the absence of both porins in ML10, little EtBr enters the cell, accumulation does not take place, and hence, there is no EtBr subject for extrusion.

\textbf{LfrA is the main efflux system involved in EtBr extrusion in \textit{M. smegmatis}}

The accumulation of increasing concentrations of EtBr by strains \textit{mc} \textsuperscript{155}, XZL1675 (\textit{ΔlfrA}) and XZL1720

\begin{table}[h]
\centering
\caption{Description of \textit{M. smegmatis} strains used in this study and corresponding MICs determined for EtBr and efflux inhibitors}
\begin{tabular}{lllrrrr}
\hline
\textbf{\textit{M. smegmatis} strain} & \textbf{Description [Reference]} & \multicolumn{4}{l}{\textbf{MICs (mg/L)}} \\
 & & \textbf{EtBr} & \textbf{CPZ} & \textbf{TZ} & \textbf{VP} \\
\hline
\textit{mc} \textsuperscript{155} & Wild-type [34] & 6.25 & 25 & 125 & 200 \\
SMR5 & \textit{mc} \textsuperscript{155} derivative; resistant to streptomycin due to a mutation in ribosomal protein S12 (\textit{rpsL}) [29] & 6.25 & 25 & 125 & 400 \\
MN01 & SMR5 \textit{ΔmspA} [5] & 6.25 & 25 & 125 & 400 \\
ML10 & SMR5 \textit{ΔmspAΔmspC} [28] & 6.25 & 25 & 25 & 250 \\
XZL1675 & \textit{mc} \textsuperscript{155} \textit{ΔlfrA} [15] & 6.25 & 25 & 125 & 200 \\
XZL1720 & \textit{mc} \textsuperscript{155} \textit{ΔlfrR} [15] & 6.25 & 25 & 125 & 200 \\
\hline
\end{tabular}
\end{table}

CPZ, chlorpromazine; EtBr, ethidium bromide; TZ, thioridazine; VP, verapamil.
The deletion (ΔlfrR) is presented by Figure 3. Concerning the knockout mutant for the efflux pump LfrA (strain XZL1675), EtBr started to accumulate at a concentration of 0.25 mg/L. Since in the wild-type strain M. smegmatis mc²155, accumulation took place at a concentration of 1 mg/L of EtBr, these results demonstrate an increased susceptibility of the mutant strain to EtBr due to the inactivation of efflux pump LfrA. In the case of the IfrR knockout mutant XZL1720, EtBr accumulation started at a concentration of 2 mg/L, a higher concentration than the observed for the wild-type. This could be due to the constitutive expression of LfrA in this strain as a consequence of the deletion of its repressor, LfrR. These results are in agreement to what has been previously reported regarding LfrA as the main efflux system involved in EtBr extrusion [15-17]. In order to determine the effect of the efflux inhibitors chlorpromazine, thioridazine and verapamil on EtBr efflux activity, efflux assays were performed for M. smegmatis mc²155, XZL1675 and XZL1720. As shown by Figure 4, all strains presented efflux of EtBr at 37°C in the presence of glucose. Moreover, this efflux activity was inhibited by chlorpromazine, thioridazine and verapamil. However, the concentration of EtBr used for the IfrR mutant was 15-fold lower than the concentration used for the wild-type and IfrR deleted strains (0.2 mg/L for XZL1675 vs 3 mg/L for mc²155 and XZL1720, ½ MIC for each strain - see Table 1). This further demonstrates that deletion of IfrA hinders the cell’s ability to efflux EtBr, resulting in a low MIC for this fluorochrome and a decreased EtBr efflux activity when compared to mc²155 and XZL1720.

**Effect of efflux inhibitors on the antibiotic resistance of M. smegmatis**

In order to correlate the data obtained from the fluorometric method with a drug susceptibility profile, the MICs of several antibiotics were determined for each strain (Table 2). Moreover, the effect of the efflux inhibitors on the reduction of MICs of the same antibiotics was also tested (Table 2). M. smegmatis SMR5, MN01 and ML10 present an MIC for streptomycin above 256 mg/L due to the presence of a mutation in the rpsL gene that confers resistance to this antibiotic [5,28,29]. Deletion of porins MspA (MN01) and MspC (ML10) caused a decreased susceptibility to clarithromycin, erythromycin and rifampicin. Deletion of IfrA (XZL1675) increased the susceptibility to ciprofloxacin and ethambutol (Table 2), which suggests that LfrA might contribute to the intrinsic resistance of M. smegmatis to these drugs, as already reported by other studies [15]. Moreover, the LfrA mutant also showed increased susceptibility to EtBr, thioridazine and verapamil (Table 1).

Relatively to the effect of the efflux inhibitors on the MICs of the tested antibiotics, there is an overall reduction of the MICs, with the exception of ethambutol, in all of the studied strains. The fact that the effect of these inhibitors is not dependent of a given genotype suggests that these compounds have a wide range of activity against efflux and are not specific of a particular efflux pump.

Some of the results obtained in this study are at variance with those reported by others. Firstly, the previous characterization of the IfrA and IfrR knockout mutant strains by Li and Nikaido [15] showed that there is no difference between the mutant strains and the wild-type concerning the MIC for rifampicin (authors reported an MIC of 1 mg/L for each strain). In our study, we observed a decrease of the MIC against the IfrA and IfrR deleted mutants. Secondly, whereas deletion of IfrR is reported to increase the ciprofloxacin MIC from 0.25 mg/L (wild-type) to 4 mg/L (XZL1720) [15], our results show that the MIC for ciprofloxacin against the IfrR mutant is the same observed for the IfrR mutant. The variance between our results and those of others may be due to the use of different methods for the determination of the MICs: microdilution method in Middlebrook 7H9 medium supplemented with oleic acid albumin dextrose catalase (OADC) (this study) or microdilution method in Middlebrook 7H9 medium supplemented with OADC and Tween 80 in combination with drug gradient plates [15].

**Conclusions**

The detection of EtBr influx and efflux can be used to anticipate transport-mediated antibiotic resistance in bacteria, since some of these compounds use similar channels to enter and leave the cell. In this study, we have compared the wild-type M. smegmatis mc²155 with knockout mutants for LfrA and MspA for their ability to transport EtBr. It was observed that in the absence of MspA, the major porin of M. smegmatis, accumulation of EtBr decreased and the mycobacteria became more resistant to several antibiotics. This is in accordance with previous studies that demonstrated MspA as the major diffusion pathway for hydrophilic solutes in M. smegmatis, mediating the uptake of small and hydrophilic nutrients such as sugars and phosphates across the outer membrane [4,28,30]. Permeability of the cell to EtBr is, in our opinion, dependent for the most part on the presence of the major porin MspA. If this were not so, we would then expect little difference in the accumulation between intact and MspA deficient strains. This conclusion is supported by others that demonstrated that deletion of the mspa gene increased the resistance of M. smegmatis not only to hydrophilic molecules, but also to hydrophobic...
antibiotics, such as erythromycin [31]. However, deletion of \textit{mspA} causes the alteration in the organisation of lipids of the mycobacterial outer membrane, resulting in a decreased rate of uptake of hydrophobic agents such as chenodeoxycholate [31,32]. In fact, it has been previously demonstrated that a \textit{M. tuberculosis} mutant lacking oxygenated mycolic acids also presents altered lipid organisation within its outer membrane, and the permeability to various agents is also altered [31,32]. Undoubtedly, the lipid

\includegraphics{figure1.png}

\textbf{Figure 1} Accumulation of increasing concentrations of EtBr (0.5-8 mg/L) by \textit{M. smegmatis} SMR5, MN01 (Δ\textit{mspA}) and ML10 (Δ\textit{mspAΔmspC}).
organisation and lipid composition of the outer membrane of mycobacteria significantly affects the permeability of agents into the cell.

The mutant for the LfrA pump showed increased accumulation of EtBr and increased susceptibility to EtBr, ethambutol and ciprofloxacin. This is in agreement with other studies that showed that disruption of the LfrA gene decreased the MIC of EtBr, acriflavine, ciprofloxacin, doxorubicin and rhodamine [13,16]. Moreover, it was shown that resistance to the tested antibiotics

Figure 2 Effect of efflux inhibitors on the accumulation of EtBr at 1, 2 and 4 mg/L by M. smegmatis SMR5, MN01 (ΔmspA) and ML10 (ΔmspAΔmspC), respectively. CPZ, chlorpromazine; EPI, efflux pump inhibitor; TZ, thioridazine; VP, verapamil.
decreased in the presence of efflux inhibitors in the studied strains, demonstrating that these inhibitors have a broad range of activity that is not specific to a given genotype.

In conclusion, the methodology used in this study demonstrates that porin MspA plays an important role in the entrance of quaternary ammonium compounds and antibiotics into the cell. Whether its absence is the main cause for decreased permeability, or that its absence has resulted in altered lipid structure of the outer membrane that is less permeable remains to be elucidated. The same methodology used to assess permeability also assessed the

---

**Figure 3** Accumulation of increasing concentrations of EtBr (0.25-8 mg/L) by *M. smegmatis mc^2^155, XZL1675 (ΔlfrA) and XZL1720 (ΔlfrR).
activity of the main efflux pump LfrA of the wild-type strain and of LfrA and LfrR depleted mutants and correlated the degree of activity with low-level resistance to several antimicrobial drugs.

The methodology used and the results obtained in this work will be used in future studies as a working model for the evaluation of influx and efflux of substrates by multidrug resistant *M. tuberculosis* clinical isolates and, therefore, determine the cause for the multidrug resistant phenotype beyond simple mutation of relevant targets.

**Methods**

**Materials**

EtBr, glucose, phosphate buffered solution (PBS), chlorpromazine, thioridazine, verapamil, amikacin,
ciprofloxacin, ethambutol, erythromycin, rifampicin and streptomycin were purchased from Sigma Aldrich Química SA (Madrid, Spain). Clarithromycin was obtained from Abbott Laboratories (Abbott Park, IL, USA). Middlebrook 7H9 broth and OADC supplement were purchased from Difco (Detroit, MI, USA). All solutions were prepared on the day of the experiment.

### Bacteria

The *M. smegmatis* strains used in this work are described in Table 1. *M. smegmatis* strains SMR5, MN01 and ML10 were kindly provided by Michael Niederweis (Department of Microbiology, University of Alabama at Birmingham, Birmingham, U.S.A); strains XZL1675 and XZL1720 were kindly provided by Hiroshi Nikaido (Department of Molecular and Cell Biology,

### Table 2 Effect of efflux inhibitors on the MICs of antibiotics for wild-type and mutant strains of *M. smegmatis*

| Antibiotic | mcs155 (wild-type) | SM55 (mcs155 STR) | MN01 (mcs155 ΔmspA) | ML10 (mcs155 ΔmspA ΔmSpC) | XZL1675 (mcs155 ΔlfrA) | XZL1720 (mcs155 ΔlfrR) |
|------------|-------------------|------------------|---------------------|---------------------------|------------------------|------------------------|
| AMK        | No EPI            |                  | 0.5                 | 0.5                       | 0.5                    | 0.5                    |
|            | CPZ               | 0.125            | 0.125               | 0.125                     | 0.063                  | 0.063                  |
|            |                   |                  |                     |                           |                        |                        |
|            | TZ                | 0.063            | 0.063               | 0.25                      | 0.25                   | 0.25                   |
|            |                   |                  |                     |                           |                        |                        |
|            | VP                | 0.125            | 0.125               | 0.125                     | 0.125                  | 0.125                  |
|            |                   |                  |                     |                           |                        |                        |
| CIP        | No EPI            | 0.25             | 0.25                | 0.25                      | 0.25                   | 0.25                   |
|            | CPZ               | 0.063            | 0.063               | 0.063                     | 0.032                  | 0.032                  |
|            |                   |                  |                     |                           |                        |                        |
|            | TZ                | 0.063            | 0.063               | 0.063                     | 0.063                  | 0.063                  |
|            |                   |                  |                     |                           |                        |                        |
|            | VP                | 0.063            | 0.063               | 0.063                     | 0.063                  | 0.063                  |
|            |                   |                  |                     |                           |                        |                        |
| CLT        | No EPI            | 0.25             | 0.25                | 0.5                       | 1                      | 0.25                   |
|            | CPZ               | 0.25             | 0.25                | 0.5                       | 1                      | 0.25                   |
|            |                   |                  |                     |                           |                        |                        |
|            | TZ                | 0.25             | 0.25                | 0.5                       | 1                      | 0.25                   |
|            |                   |                  |                     |                           |                        |                        |
|            | VP                | 0.5              | 0.5                 | 0.5                       | 1                      | 0.5                    |
|            |                   |                  |                     |                           |                        |                        |
| EMB        | No EPI            | 1                | 1                   | 1                         | 0.5                    | 1                      |
|            | CPZ               | 1                | 1                   | 1                         | 0.5                    | 1                      |
|            |                   |                  |                     |                           |                        |                        |
|            | TZ                | 1                | 1                   | 1                         | 0.5                    | 1                      |
|            |                   |                  |                     |                           |                        |                        |
|            | VP                | 1                | 1                   | 1                         | 0.5                    | 1                      |
|            |                   |                  |                     |                           |                        |                        |
| ERY        | No EPI            | 32               | 32                  | 64                        | 64                     | 32                     |
|            | CPZ               | 4                | 4                   | 8                         | 8                      | 4                      |
|            |                   |                  |                     |                           |                        |                        |
|            | TZ                | 4                | 4                   | 16                        | 16                     | 4                      |
|            |                   |                  |                     |                           |                        |                        |
|            | VP                | 8                | 8                   | 8                         | 8                      | 8                      |
|            |                   |                  |                     |                           |                        |                        |
| RIF        | No EPI            | 4                | 4                   | 8                         | 8                      | 0.5                    |
|            | CPZ               | 1                | 1                   | 2                         | 2                      | 0.125                  |
|            |                   |                  |                     |                           |                        |                        |
|            | TZ                | 2                | 2                   | 4                         | 4                      | 0.125                  |
|            |                   |                  |                     |                           |                        |                        |
|            | VP                | 2                | 2                   | 4                         | 4                      | 0.125                  |
|            |                   |                  |                     |                           |                        |                        |
| STR        | No EPI            | 0.125            | >256                | >256                       | >256                   | 0.032                  |
|            | CPZ               | 0.125            | >256                | >256                       | >256                   | 0.125                  |
|            |                   |                  |                     |                           |                        |                        |
|            | TZ                | 0.25             | >256                | >256                       | >256                   | 0.25                   |
|            |                   |                  |                     |                           |                        |                        |
|            | VP                | 0.25             | >256                | >256                       | >256                   | 0.25                   |

AMK, amikacin; CIP, ciprofloxacin; CLT, clarithromycin; CPZ, chlorpromazine; EMB, ethambutol; EPI, efflux pump inhibitor; ERY, erythromycin; RIF, rifampicin; STR, streptomycin; TZ, thioridazine; VP, verapamil. Data in bold type represents significant (at least 4-fold) reduction of the MIC produced by the presence of an efflux inhibitor.

---

Rodrigues et al. BMC Microbiology 2011, 11:35
http://www.biomedcentral.com/1471-2180/11/35
Page 8 of 10
University of California, Berkeley, California, U.S.A). Mycobacteria were grown at 37°C in Middlebrook 7H9 broth or Middlebrook 7H11 solid medium, supplemented with 10% (v/v) of OADC.

**Determination of Minimum Inhibitory Concentrations**

The determination of MICs of EtBr, the efflux inhibitors chlorpromazine, thioridazine and verapamil and of antibiotics studied alone and in the presence of an efflux inhibitor, was performed by the broth microdilution method according to the CLSI guidelines [33]. Briefly, mycobacterial strains were grown at 37°C in Middlebrook 7H9 broth supplemented with 10% OADC until an optical density (O.D.) of 0.8 at a wavelength of 600 nm. The number of colony-forming units (cfu) corresponding to aliquots of the inoculum was routinely calculated in order to ensure a constant number of bacterial cells from experiment to experiment. Bacterial cultures were diluted in PBS to equal the McFarland No. 0.5 standard and the final inoculum was prepared by diluting the bacterial suspension at 1:100. Aliquots of 0.1 mL were transferred to each well of a 96-well plate that contained 0.1 mL of each compound at concentrations prepared from 2-fold serial dilutions in 7H9/OADC medium. The inoculated plates were incubated at 37°C until growth in the agent-free control-well was evident (2-3 days). The MIC was defined as the lowest concentration of compound that inhibited visible growth.

**Semi-automated fluorometric method**

The assessment of accumulation and extrusion of EtBr on a real-time basis by *M. smegmatis* strains wild-type mc²155, SMR5, porin mutants, MN01 and ML10 and efflux mutants XZL1675 and XZL1720 (Table 1) was performed using the semi-automated fluorometric method, as previously described [25-27].

(i) **Accumulation assay**

*M. smegmatis* strains were grown in 5 mL of 7H9/OADC medium at 37°C until an O.D.₆₀₀ of 0.8. Cultures were centrifuged at 13000 rpm for 3 minutes, the supernatant discarded and the pellet washed in PBS (pH 7.4). The O. D.₆₀₀ was adjusted to 0.4 with PBS and glucose was added at final concentration of 0.4%. Aliquots of 0.095 mL of bacterial suspension were distributed to 0.2 mL PCR microtubes and EtBr was added at concentrations that ranged from 0.25 to 8 mg/L. Fluorescence was measured in the Rotor-Gene™ 3000 (Corbett Research, Sydney, Australia), using the 530 nm band-pass and the 585 nm high-pass filters as the excitation and detection wavelengths, respectively. Fluorescence data was acquired every 60 seconds for 60 minutes at 37°C.

The effect of chlorpromazine, thioridazine and verapamil on the accumulation of EtBr was determined by adding 0.005 mL of each compound to aliquots of 0.095 mL of EtBr-containing bacterial suspension previously distributed to 0.2 mL PCR microtubes. Fluorescence was measured every 60 seconds for 60 minutes at 37°C in the Rotor-Gene™ 3000. Each inhibitor was used at ½ the MIC in order to not compromise the cellular viability (as confirmed by CFUs counting).

(ii) **Efflux assay**

Mycobacteria were exposed to conditions that promote maximum accumulation of EtBr: EtBr at ½ MIC for each strain; no glucose; presence of the efflux inhibitor that caused maximum accumulation, in this case verapamil; and incubation at 25°C [25-27]. The EtBr loaded cells were centrifuged at 13000 rpm for 3 minutes and resuspended in EtBr-free PBS containing 0.4% glucose. After adjusting the O.D.₆₀₀ to 0.4, aliquots of 0.095 mL were transferred to 0.2 mL microtubes. Fluorescence was measured in the Rotor-Gene™ 3000 as described for the accumulation assay. Efflux activity was quantified by comparing the fluorescence data obtained under conditions that promote efflux (presence of glucose and absence of efflux inhibitor) with the data from the control in which the mycobacteria are under conditions of no efflux (presence of an inhibitor and no energy source). Thus, the relative fluorescence corresponds to the ratio of fluorescence that remains per unit of time, relatively to the EtBr-loaded cells.

**Acknowledgements**

The authors wish to thank Prof. Hiroshi Nikaido (Department of Molecular and Cell Biology, University of California, Berkeley, California, U.S.A) and Prof. Michael Niederweis for kindly providing the *M. smegmatis* mutant strains used in this work and to Prof. Winfried V. Kern (Center for Infectious Diseases and Travel Medicine, University Hospital, Freiburg, Germany) for valuable suggestions and scientific discussions. This work was supported by grants EU-FSE/FEDER-PTDC/BA-MIC/71280/2006, EU-FSE/FEDER-PTDC/BA-MIC/105509/2008 and EU-FSE/FEDER-PTDC/SAU-FCF/102807/2008 provided by Fundação para a Ciência e a Tecnologia (FCT) of Portugal. L. Rodrigues was supported by grant SFRH/BD/24931/2005 (FCT, Portugal).

**Author details**

1. Unit of Mycobacteriology, Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa, Rua da Junqueira 100, 1349-008 Lisboa, Portugal.
2. UPWM, Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa, Rua da Junqueira 100, 1349-008 Lisboa, Portugal.
3. Centro de Recursos Microbiológicos (CRIEM), Faculdade de Ciências e Tecnologia, UNL, 2829-516 Caparica, Portugal.
4. Cost Action BM0701 (ATENS).

**Authors’ contributions**

LR designed the experiments, carried out the EtBr accumulation and efflux assays and drafted the manuscript. JR performed the MIC determination assays and participated in the EtBr efflux assays. IC participated in the study design and coordination and helped to draft the manuscript. LA participated in the study design and revised the manuscript. MV conceived of the study, participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

**Received**: 22 September 2010 **Accepted**: 18 February 2011

**Published**: 18 February 2011

**References**

1. Brennan PJ, Nikaido H. The envelope of mycobacteria. *Annu Rev Biochem* 1995, 64:29-63.
2. Brennan PJ: Structure, function, and biogenesis of the cell wall of Mycobacterium tuberculosis. *Tuberculosis* 2003, 83:91-97.

3. Niederweis M: Mycobacterial porins - new channel proteins in unique outer membranes. *Mol Microbiol* 2003, 49:1167-1177.

4. Niederweis M, Ehrn S, Heinz C, Klöcker U, Karosi S, Schwerek KM, Riley LW, Benz R: Cloning of the mpqA gene encoding a porin from Mycobacterium smegmatis. *Mol Microbiol* 1999, 33:933-945.

5. Stahl C, Kubetzko S, Kaps I, Seeber S, Engelhardt H, Niederweis M: MsPa provides the main hydrophilic pathway through the cell wall of Mycobacterium smegmatis. *Mol Microbiol* 2001, 40:651-664.

6. Nikaio H: Preventing drug access to targets: cell surface permeability barriers and active efflux in bacteria. *Semin Cell Dev Biol* 2001, 12:215-23.

7. World Health Organization: *MMWR* 2001, 50

8. Altmann JA, Blakopoel MC, Otal J, Young DB, De Smet KA, Martin C: Molecular cloning and characterization of Tap, a putative multidrug efflux pump present in *Mycobacterium fortuitum* and *Mycobacterium tuberculosis*. *J Bacteriol* 1998, 180:5836-5843.

9. Choudhuri BS, Bhakta S, Banik R, Basu J, Chakrabarti P: Role of the *mycobacterium tuberculosis* gene encoding a porin from *Mycobacterium smegmatis* in drug resistance: an unresolved question. *FEMS Microbiol Rev* 2006, 30:36-52.

10. Siddiqi N, Das R, Pathak N, Banerjee S, Ahmed N, Katoch VM, Hasnain SE: Mycobacterium tuberculosis isolate with a distinct genomic identity overexpresses a top-like efflux pump. *Infection* 2004, 32:109-111.

11. Ramón-García S, Martin C, Thompson CJ, Almiras JA: Role of the *Mycobacterium tuberculosis* PSS efflux pump in intrinsic drug resistance, oxidative stress responses, and growth. *Antimicrob Agents Chemother* 2009, 53:3675-3682.

12. Takek HE, Cimino M, Musso MC, Weisbrod T, Martinez R, Delgado MB, Salazar L, Bloom BR, Jacobs WR Jr: Efflux pump of the proton antipporter family confers low-level fluoroquinolone resistance in *Mycobacterium smegmatis*. *Pac Natl Acad Sci USA* 1996, 93:362-366.

13. Viveiros M, Leandro C, Amaral L: Mycobacterial efflux pumps and chemotherapeutic implications. *Int J Antimicrob Agents* 2003, 22:274-278.

14. Li XZ, Zhang L, Nikaio H: Efflux pump-mediated intrinsic drug resistance in *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother* 2004, 48:2415-2423.

15. Liu J, Takek HE, Nikaio H: Active efflux of fluoroquinolones in *Mycobacterium smegmatis* mediated by LfrA, a multidrug efflux pump. *J Bacteriol* 1996, 178:3791-3795.

16. Sander P, De Rossi E, Rodríguez J, Martín C, Thompson C, Almiras JA: Role of the *Mycobacterium tuberculosis* PSS efflux pump LfrA to innate *mycobacterial* drug resistance. *Mol Microbiol* 2009, 72:279-285.

17. De Rossi E, Almiras JA, Riccardi G: Role of mycobacterial efflux transporters in drug resistance: an unresolved question. *FEMS Microbiol Rev* 2000, 3:36-52.

18. Siddiqi N, Das R, Pathak N, Banerjee S, Ahmed N, Katoch VM, Hasnain SE: Mycobacterium tuberculosis isolate with a distinct genomic identity overexpresses a top-like efflux pump. *Infection* 2004, 32:109-111.

19. Ramón-García S, Martin C, Thompson CJ, Almiras JA: Role of the *Mycobacterium tuberculosis* PSS efflux pump in intrinsic drug resistance, oxidative stress responses, and growth. *Antimicrob Agents Chemother* 2009, 53:3675-3682.

20. Takek HE, Cimino M, Musso MC, Weisbrod T, Martinez R, Delgado MB, Salazar L, Bloom BR, Jacobs WR Jr: Efflux pump of the proton antipporter family confers low-level fluoroquinolone resistance in *Mycobacterium smegmatis*. *Pac Natl Acad Sci USA* 1996, 93:362-366.

21. Viveiros M, Leandro C, Amaral L: Mycobacterial efflux pumps and chemotherapeutic implications. *Int J Antimicrob Agents* 2003, 22:274-278.

22. Liu J, Takek HE, Nikaio H: Efflux pump-mediated intrinsic drug resistance in *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother* 2004, 48:2415-2423.

23. Li XZ, Zhang L, Nikaio H: Efflux pump-mediated intrinsic drug resistance in *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother* 2004, 48:2415-2423.

24. Jernaes MW, Steen HB: Staining of *Escherichia coli* for flow cytometry: influx and efflux of ethidium bromide. *Cytometry* 1994, 17:302-309.

25. Greulich KD: Single molecule techniques for biomedicine and pharmacology. *Curr Pharm Biotechnol* 2004, 5:243-259.

26. Martinis M, Santos B, Martins A, Vivieiros M, Couto I, Cruz A, Pagés JM, Molnar J, Fanning S, Amaral L, Management Committee Members of Cost B16 European Commission/European Science Foundation: An instrument-free method for the determination of efflux pump activity of bacteria. *In Vivo* 2006, 20:657-664.

27. Schumacher A, Tittler R, Rohner JA, Kümmerer K, Pagés JM, Kern WW: Intracellular accumulation of linezolid in *Escherichia coli*, *Citrobacter freundii* and *Enterobacter aerogenes*: role of enhanced efflux pump activity and inactivation. *J Antimicrob Chemother* 2007, 59:1261-1264.

28. Sharpes B, Brown JR: Correlation of the base specificity of DNA-intercalating ligands with their physico-chemical properties. *FEBS Lett* 1976, 69:37-40.

29. Rodrigues L, Wagner D, Viveiros M, Sampalo D, Couto I, Vavva M, Kern WW, Amaral L: Thiadizone and chlorpromazine inhibition of ethidium bromide efflux in *Mycobacterium avium* and *Mycobacterium smegmatis*. *J Antimicrob Chemother* 2008, 61:1076-1082.

30. Viveiros M, Martins A, Paixão L, Rodrigues L, Martins M, Couto I, Fahnhric E, Kern WW, Amaral L: Demonstration of intrinsic efflux activity of *Escherichia coli* K-12 AG100 by an automated ethidium bromide method. *Int J Antimicrob Agents* 2008, 31:458-462.

31. Viveiros M, Rodrigues L, Martins M, Couto I, Spengler G, Martins A, Amaral L: Evaluation of efflux activity of bacteria by a semi-automated fluorometric system. In *Antibiotic Resistance Methods and Protocols (Methods in Molecular Medicine).* Volume 642. 2 edition. Edited by: S. H. Gillespie. New York: Humana Press, 2010.159-172.

32. Stephan J, Bender J, Wolschendorf F, Hoffmann C, Roth E, Mailänder C, Engelhardt H, Niederweis M: The growth rate of *Mycobacterium smegmatis* depends on sufficient porin-mediated influx of nutrients. *Mol Microbiol* 2005, 58:714-730.

33. Sander P, Meier A, Böttger EC: *pmlA* - a dominant selectable marker for gene replacement in *mycobacteria*. *Mol Microbiol* 1995, 16:691-1000.

34. Wolschendorf F, Mailhoud M, Niederweis M: Porins are required for uptake of phosphates by *Mycobacterium smegmatis*. *J Bacteriol* 2007, 189:2435-2442.

35. Stephan J, Mailänder C, Ennne G, Daffé M, Niederweis M: Multidrug resistance of a porin deletion mutant of *Mycobacterium smegmatis*. *Antimicrob Agents Chemother* 2004, 48:4163-4170.

36. Dubnau E, Chan J, Raynaud C, Mohan VP, Laneelle YA, Yu K, Quemard A, Smith I, Daffé M: Oxynolated mycolic acids are necessary for virulence of *Mycobacterium tuberculosis* in mice. *Mol Microbiol* 2000, 36:630-637.

37. Clinical and Laboratory Standards Institute (CLSI): *Susceptibility Testing of *Mycobacteria, Nocardiae, and Other Aerobic Actinomycetes; Approved Standard.* CLSI M44-A Wayne, PA, 2003.

38. Snapper SB, Melton RE, Mustafa S, Kiser T, Jacobs WR Jr: Isolation and characterization of efficient plasmid transformation mutants of *Mycobacterium smegmatis*. *Mol Microbiol* 1990, 4:1911-1919.

Cite this article as: Rodrigues et al.: Ethidium bromide transport across *Mycobacterium smegmatis* cell-wall: correlation with antibiotic resistance. *BMC Microbiology* 2011 11:35.

Submit your next manuscript to BioMed Central and take full advantage of:

- Convenient online submission
- Thorough peer review
- No space constraints or color figure charges
- Immediate publication on acceptance
- Inclusion in PubMed, CAS, Scopus and Google Scholar
- Research which is freely available for redistribution

Submit your manuscript at www.biomedcentral.com/submit