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

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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.

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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 *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 *lfrA* that contains a gene coding for LfrR, a putative transcriptional repressor of the TetR family, which represses the transcription of the *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 *M. smegmatis*, using the wild-type strain mc²155 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 *M. smegmatis* have their homologues in *Mycobacterium tuberculosis*, the use of *M. smegmatis* as a model

mycobacterium may provide data that will help to understand efflux-mediated drug resistance in *M. tuberculosis* and other mycobacteria that infect the human [15].

Results and Discussion

MspA as a major pathway for EtBr in *M. smegmatis*

The *M. smegmatis* strains used in this study are described in Table 1. The accumulation of increasing concentrations of EtBr by strains SMR5, MN01 ($\Delta mspA$) and ML10 ($\Delta mspA \Delta mspC$) 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 *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 ($\Delta mspA$) and ML10 ($\Delta mspA \Delta mspC$), 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.

LfrA is the main efflux system involved in EtBr extrusion in *M. smegmatis*

The accumulation of increasing concentrations of EtBr by strains mc²155, XZL1675 ($\Delta lfrA$) and XZL1720

Table 1 Description of *M. smegmatis* strains used in this study and corresponding MICs determined for EtBr and efflux inhibitors

<i>M. smegmatis</i> strain	Description [Reference]	MICs (mg/L)			
		EtBr	CPZ	TZ	VP
mc ² 155	Wild-type [34]	6.25	25	12.5	200
SMR5	mc ² 155 derivative; resistant to streptomycin due to a mutation in ribosomal protein S12 (<i>rpsL</i>) [29]	6.25	25	12.5	400
MN01	SMR5 $\Delta mspA$ [5]	6.25	25	25	400
ML10	SMR5 $\Delta mspA \Delta mspC$ [28]	12.5	25	25	250
XZL1675	mc ² 155 $\Delta lfrA$ [15]	0.4	25	6.25	125
XZL1720	mc ² 155 $\Delta lfrR$ [15]	6.25	25	12.5	200

CPZ, chlorpromazine; EtBr, ethidium bromide; TZ, thioridazine; VP, verapamil.

($\Delta lfrR$) is presented by Figure 3. Concerning the knock-out 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 *lfrR* 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 *lfrA* mutant was 15-fold lower than the concentration used for the wild-type and *lfrR* 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 *lfrA* 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 *lfrA* (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 *lfrA* and *lfrR* 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 *lfrA* and *lfrR* deleted mutants. Secondly, whereas deletion of *lfrR* 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 *lfrR* mutant is the same observed for the *lfrA* 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 *mshA* gene increased the resistance of *M. smegmatis* not only to hydrophilic molecules, but also to hydrophobic

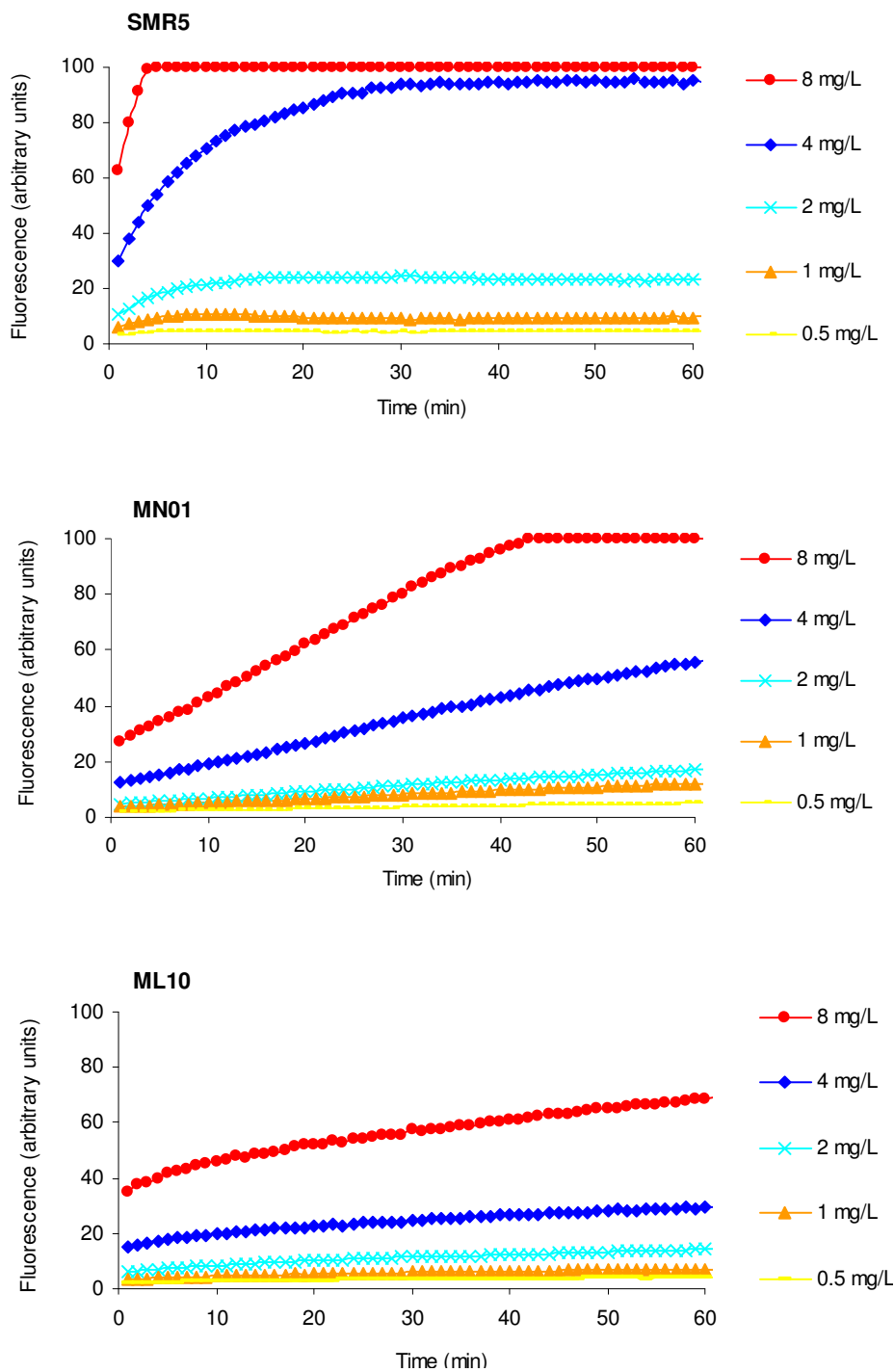


Figure 1 Accumulation of increasing concentrations of EtBr (0.5-8 mg/L) by *M. smegmatis* SMR5, MN01 ($\Delta mspA$) and ML10 ($\Delta mspA\Delta mspC$).

antibiotics, such as erythromycin [31]. However, deletion of *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 *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

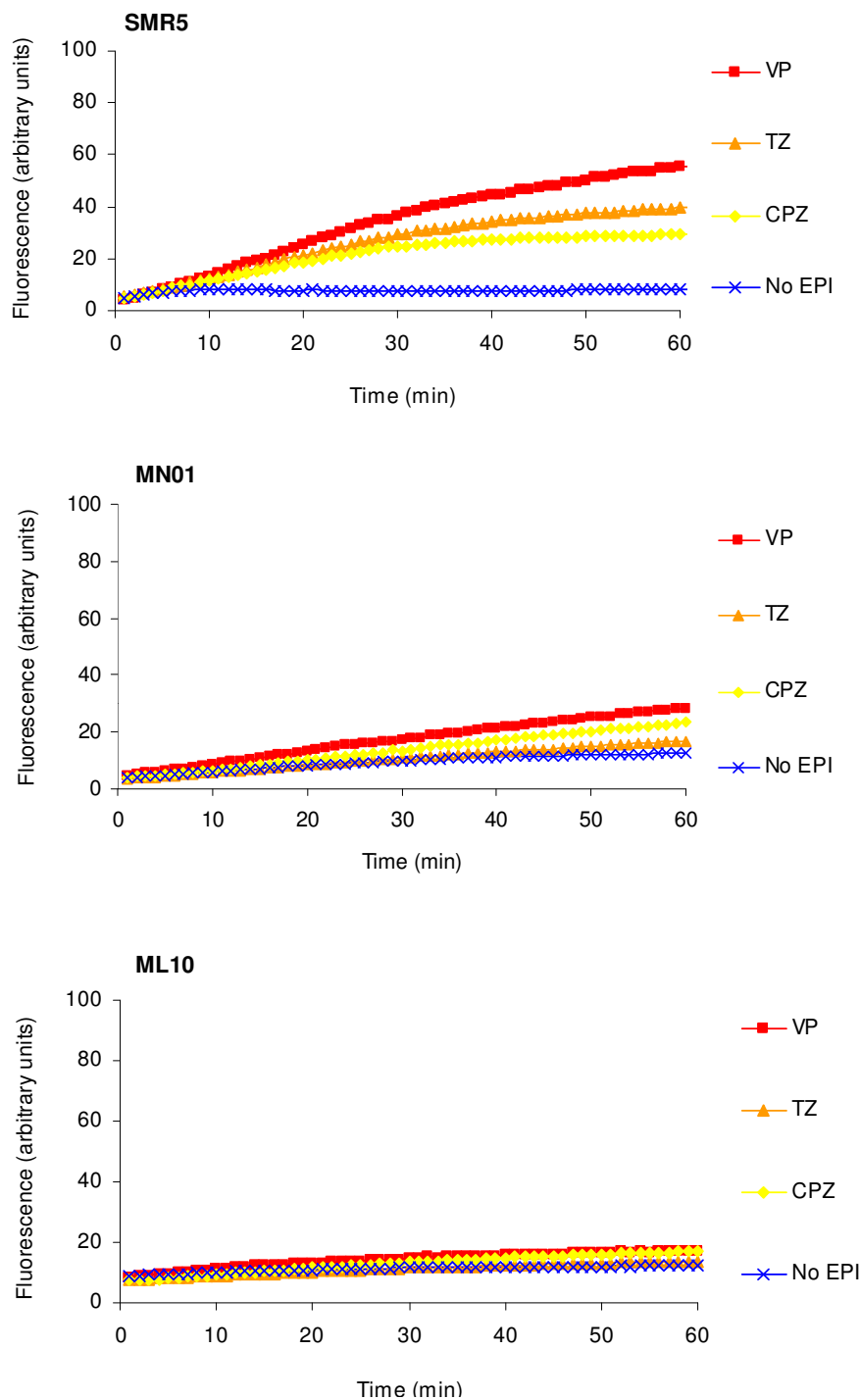


Figure 2 Effect of efflux inhibitors on the accumulation of EtBr at 1, 2 and 4 mg/L by *M. smegmatis* SMR5, MN01 ($\Delta mspA$) and ML10 ($\Delta mspA\Delta mspC$), respectively. CPZ, chlorpromazine; EPI, efflux pump inhibitor; TZ, thioridazine; VP, verapamil.

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

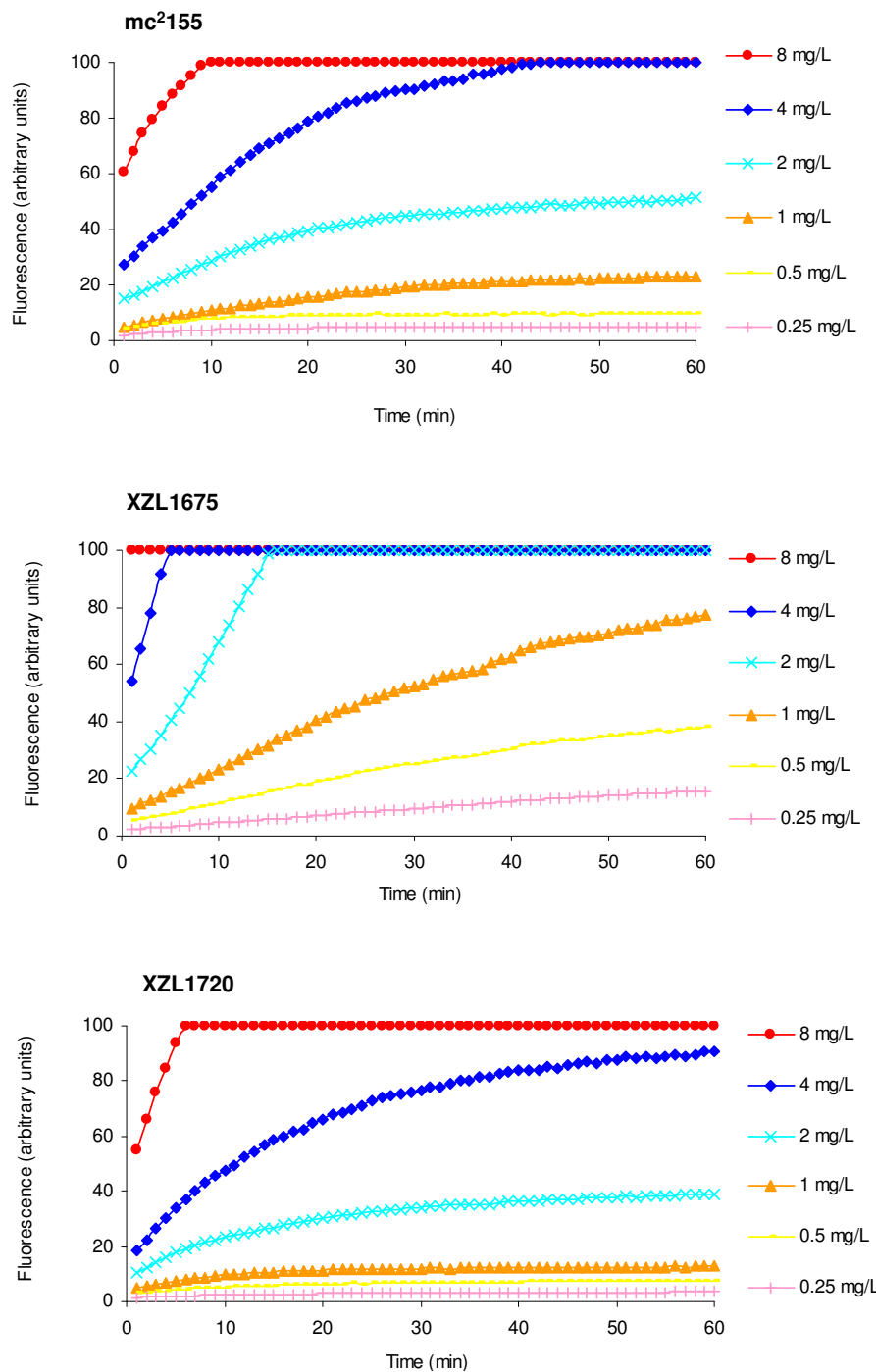


Figure 3 Accumulation of increasing concentrations of EtBr (0.25-8 mg/L) by *M. smegmatis* mc²155, XZL1675 ($\Delta IfrA$) and XZL1720 ($\Delta IfrR$).

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

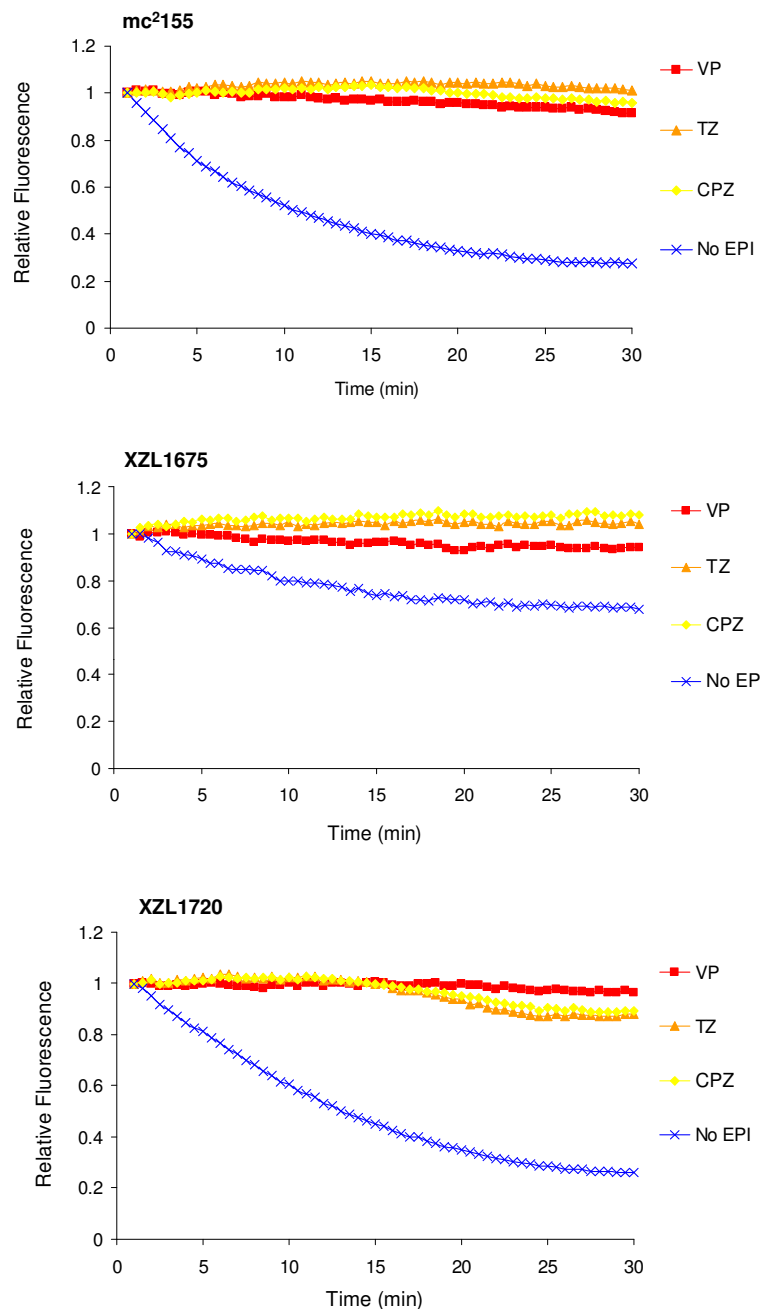


Figure 4 Efflux of EtBr by *M. smegmatis* mc²155, XZL1675 (Δ *lfrA*) and XZL1720 (Δ *lfrR*). Efflux takes place at 37°C in the presence of glucose and is inhibited by the efflux inhibitors thioridazine and verapamil. EtBr was used at ½ MIC for each strain in order to ensure maximum EtBr-loading of the bacteria, without compromising cellular viability. CPZ, chlorpromazine; EPI, efflux pump inhibitor; TZ, thioridazine; VP, verapamil.

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,

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

		MICs (mg/L)					
		<i>M. smegmatis</i> strains					
Antibiotic/ EPI	mc ² 155 (wild-type)	SMR5 (mc ² 155 STR ^r)	MN01 (SMR5 Δ <i>mspA</i>)	ML10 (SMR5 Δ <i>mspA</i> Δ <i>mspC</i>)	XZL1675 (mc ² 155 Δ <i>lfrA</i>)	XZL1720 (mc ² 155 Δ <i>lfrR</i>)	
	No EPI	0.5	0.5	0.5	0.5	0.5	0.5
AMK	CPZ	0.125	0.125	0.125	0.25	0.063	0.063
	TZ	0.063	0.063	0.125	0.25	0.063	0.063
	VP	0.125	0.125	0.125	0.25	0.125	0.125
	No EPI	0.25	0.25	0.25	0.25	0.125	0.125
CIP	CPZ	0.063	0.063	0.063	0.063	0.063	0.063
	TZ	0.063	0.063	0.063	0.063	0.032	0.032
	VP	0.063	0.063	0.063	0.063	0.063	0.063
	No EPI	2	2	8	8	2	2
CLT	CPZ	0.25	0.25	0.5	1	0.25	0.25
	TZ	0.25	0.25	1	1	0.25	0.25
	VP	0.5	0.5	0.5	1	0.5	0.5
	No EPI	1	1	1	1	0.5	1
EMB	CPZ	1	1	1	1	0.5	1
	TZ	1	1	1	1	0.5	1
	VP	1	1	1	1	0.5	1
	No EPI	32	32	64	64	32	32
ERY	CPZ	4	4	8	8	4	4
	TZ	4	4	16	16	4	4
	VP	8	8	8	8	8	8
	No EPI	4	4	8	8	0.5	0.5
RIF	CPZ	1	1	2	2	0.125	0.125
	TZ	2	2	4	4	0.125	0.125
	VP	2	2	4	4	0.125	0.25
	No EPI	0.5	>256	>256	>256	0.5	0.5
STR	CPZ	0.125	>256	>256	>256	0.032	0.063
	TZ	0.125	>256	>256	>256	0.125	0.25
	VP	0.25	>256	>256	>256	0.25	0.125

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.

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,

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.

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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.

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