Universidade Nova de Lisboa

Instituto de Higiene e Medicina Tropical

The role of the efflux mechanisms in multidrug resistance in *Mycobacterium tuberculosis*

Liliana Isabel Dias Rodrigues

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Lisboa, Junho de 2010

_____

Liliana Isabel Dias Rodrigues

To my parents

''Rich countries thought TB was a disease of the past ... a problem of the poorest. But it kills twice as many people as malaria and almost as many as AIDS. If you think this doesn't affect you, you are dead wrong''

Mario Raviglione

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Abstract

The emergence of multi and extensively drug resistant tuberculosis (MDRTB and XDRTB) has increased the concern of public health authorities around the world. The World Health Organization has defined MDRTB as tuberculosis (TB) caused by organisms resistant to at least isoniazid and rifampicin, the main first-line drugs used in TB therapy, whereas XDRTB refers to TB resistant not only to isoniazid and rifampicin, but also to a fluoroquinolone and to at least one of the three injectable second-line drugs, kanamycin, amikacin and capreomycin. Resistance in *Mycobacterium tuberculosis* is mainly due to the occurrence of spontaneous mutations and followed by selection of mutants by subsequent treatment. However, some resistant clinical isolates do not present mutations in any genes associated with resistance to a given antibiotic, which suggests that other mechanism(s) are involved in the development of drug resistance, namely the presence of efflux pump systems that extrude the drug to the exterior of the cell, preventing access to its target. Increased efflux activity can occur in response to prolonged exposure to subinhibitory concentrations of anti-TB drugs, a situation that may result from inadequate TB therapy. The inhibition of efflux activity with a non-antibiotic inhibitor may restore activity of an antibiotic subject to efflux and thus provide a way to enhance the activity of current anti-TB drugs.

The work described in this thesis foccus on the study of efflux mechanisms in the development of multidrug resistance in *M. tuberculosis* and how phenotypic resistance, mediated by efflux pumps, correlates with genetic resistance. In order to accomplish this goal, several experimental protocols were developed using biological models such as *Escherichia coli*, the fast growing mycobacteria *Mycobacterium smegmatis*, and *Mycobacterium avium*, before their application to *M. tuberculosis*. This approach allowed the study of the mechanisms that result in the physiological adaptation of *E. coli* to subinhibitory concentrations of tetracycline (Chapter II), the development of a fluorometric method that allows the detection and quantification of efflux of ethidium

bromide (Chapter III), the characterization of the ethidium bromide transport in *M. smegmatis* (Chapter IV) and the contribution of efflux activity to macrolide resistance in *Mycobacterium avium* complex (Chapter V). Finally, the methods developed allowed the study of the role of efflux pumps in *M. tuberculosis* strains induced to isoniazid resistance (Chapter VI).

By this manner, in Chapter II it was possible to observe that the physiological adaptation of *E. coli* to tetracycline results from an interplay between events at the genetic level and protein folding that decrease permeability of the cell envelope and increase efflux pump activity. Furthermore, Chapter III describes the development of a semi-automated fluorometric method that allowed the correlation of this efflux activity with the transport kinetics of ethidium bromide (a known efflux pump substrate) in *E. coli* and the identification of efflux inhibitors.

Concerning *M. smegmatis*, we have compared the wild-type *M. smegmatis* mc^2155 with knockout mutants for LfrA and MspA for their ability to transport ethidium bromide. The results presented in Chapter IV showed that MspA, the major porin in *M. smegmatis*, plays an important role in the entrance of ethidium bromide and antibiotics into the cell and that efflux *via* the LfrA pump is involved in low-level resistance to these compounds in *M. smegmatis*.

Chapter V describes the study of the contribution of efflux pumps to macrolide resistance in clinical *M. avium* complex isolates. It was demonstrated that resistance to clarithromycin was significantly reduced in the presence of efflux inhibitors such as thioridazine, chlorpromazine and verapamil. These same inhibitors decreased efflux of ethidium bromide and increased the retention of [^{14}C]-erythromycin in these isolates.

Finaly, the methods developed with the experimental models mentioned above allowed the study of the role of efflux pumps on *M. tuberculosis* strains induced to isoniazid resistance. This is described in Chapter VI of this Thesis, where it is demonstrated that induced resistance to isoniazid does not involve mutations in any of the genes known

to be associated with isoniazid resistance, but an efflux system that is sensitive to efflux inhibitors. These inhibitors decreased the efflux of ethidium bromide and also reduced the minimum inhibitory concentration of isoniazid in these strains. Moreover, expression analysis showed overexpression of genes that code for efflux pumps in the induced strains relatively to the non-induced parental strains.

In conclusion, the work described in this thesis demonstrates that efflux pumps play an important role in the development of drug resistance, namely in mycobacteria. A strategy to overcome efflux-mediated resistance may consist on the use of compounds that inhibit efflux activity, restoring the activity of antimicrobials that are efflux pump substrates, a useful approach particularly in TB where the most effective treatment regimens are becoming uneffective due to the increase of MDRTB/XDRTB.

Resumo

O aumento da tuberculose multirresistente e extensivamente resistente (TBMR e TBXDR) gerou um agravamento das preocupações por parte das autoridades de Saúde Pública em todo o mundo. A Organização Mundial de Saúde (OMS) define TBMR como tuberculose (TB) resistente pelo menos à isoniazida e à rifampicina, os principais fármacos de primeira-linha utilizados no tratamento da TB, enquanto que a TBXDR refere-se a casos de TB resistente não só à isoniazida e à rifampicina, mas também a uma fluoroquinolona e a pelo menos a um dos três fármacos de segunda-linha injectáveis canamicina, amicacina e capreomicina. A resistência em *Mycobacterium tuberculosis* deve-se principalmente à ocorrência de mutações espontâneas, à qual se segue a selecção de mutantes resistentes durante o tratamento. No entanto, algumas estirpes clínicas de *M. tuberculosis* resistentes não apresentam mutação em qualquer um dos genes que se sabe estarem associados à aquisição de resistência a um determinado fármaco, o que sugere que outro(s) mecanismo(s) deverão estar envolvidos no desenvolvimento de resistência, nomeadamente a presença de sistemas de bombas de efluxo que efectuam a extrusão do composto para o exterior da célula, evitando que o mesmo atinja o seu alvo. Um aumento da actividade de efluxo pode ocorrer devido a uma exposição prolongada a concentrações subinibitórias dos antibacilares, uma situação que pode resultar de uma terapia inadequada. A inibição da actividade de efluxo com um inibidor que não seja um antibiótico poderá restaurar a actividade de um antibiótico que seja substrato de bombas de efluxo e desta forma consistir uma forma de aumentar a actividade dos actuais fármacos utilizados no tratamento da TB.

O trabalho descrito nesta dissertação tem como objectivo o estudo dos mecanismos de efluxo no desenvolvimento de multirresistência em *M. tuberculosis* e de como a resistência fenotípica mediada por bombas de efluxo se correlaciona com a resistência genética. De forma a alcançar este objectivo, foram desenvolvidos vários protocolos

experimentais utilizando modelos biológicos, tais como *Escherichia coli*, *Mycobacterium smegmatis* uma micobactéria de crescimento rápido e *Mycobacterium avium*, antes da sua aplicação ao estudo de *M. tuberculosis*. Esta abordagem permitiu o estudo dos mecanismos que resultam na adaptação fisiológica de *E. coli* à tetraciclina por exposição a concentrações subinibitórias deste antibiótico (Capítulo II), o desenvolvimento de um método fluorimétrico que permite a detecção e quantificação da actividade de efluxo (Capítulo III), a caracterização do transporte de brometo de etídeo em *M. smegmatis* (Capítulo IV) e a contribuição da actividade de efluxo para a resistência aos macrólidos no complexo *Mycobacterium avium* (Capítulo V). Por fim, os métodos desenvolvidos permitiram o estudo do papel das bombas de efluxo em estirpes de *M. tuberculosis* induzidas a resistência à isoniazida.

Assim, como descrito no Capítulo II, foi possível observar que a adaptação fisiológica de *E. coli* à presença de tetraciclina resulta de uma interacção entre mecanismos a nível genético e modificações pós-traducionais a nível da conformação de proteínas que diminui a permeabilidade da parede celular e aumenta a actividade das bombas de efluxo. Para além disso, o Capítulo III descreve o desenvolvimento de um método fluorimétrico semi-automático que permitiu correlacionar esta actividade de fluxo com a cinética de transporte do brometo de etídeo (um conhecido substrato de bombas de efluxo) em *E. coli* e também a identificação de inibidores do efluxo.

Relativamente a *M. smegmatis*, comparou-se a estirpe selvagem *M. smegmatis* mc^2155 com mutantes "knockout" para LfrA e MspA, no que respeita à sua capacidade de transportar brometo de etídeo. Os resultados apresentados no Capítulo IV demonstraram que MspA, a principal porina de *M. smegmatis*, desempenha um papel importante na entrada de brometo de etídeo e antibióticos na célula e que o efluxo através da bomba LfrA está envolvido na resistência de baixo nível a estes compostos em *M. smegmatis*.

O Capítulo V descreve o estudo da contribuição de bombas de efluxo na resistência aos macrólidos em estirpes clínicas do complexo *M. avium*. Demonstrou-se que a resistência à claritromicina sofreu uma redução significativa na presença dos inibidores de efluxo tioridazina, clorpromazina e verapamil. Estes inibidores também diminuíram o efluxo de brometo de etideo e aumentaram a retenção de eritromicina marcada com ^{14}C nestas estirpes.

Por fim, os métodos desenvolvidos com os modelos experimentais referidos acima permitiram o estudo do papel das bombas de efluxo em estirpes de *M. tuberculosis* induzidas à resistência à isoniazida. Este trabalho encontra-se descrito no Capítulo VI desta dissertação, onde se demonstra que a indução de resistência à isoniazida não resultou da ocorrência de mutações em qualquer um dos genes associados com a resistência a este fármaco, mas de um sistema de efluxo que é sensível a inibidores do efluxo. Estes inibidores provocaram a diminuição do efluxo de brometo de etídeo e também a redução da concentração mínima inibitória da isoniazida nestas estirpes. Para além disso, a análise de expressão genética demonstrou a sobre-expressão de genes que codificam para bombas de efluxo nas estirpes induzidas comparativamente com as estirpes originais não induzidas.

Concluindo, o trabalho descrito nesta dissertação demonstra que as bombas de efluxo desempenham um papel importante no desenvolvimento de resistência, em particular nas micobactérias. Uma estratégia para ultrapassar a resistência mediada por mecanismos de efluxo poderia passar pela utilização de compostos que inibem a actividade de efluxo, restaurando a actividade de antimicrobianos que são substratos de bombas de efluxo, uma abordagem útil particularmente em TB em que os regimes de tratamento mais eficazes se estão a tornar ineficazes face ao aumento da TBMR/TBXR.

Publications

Chapters in books

- Amaral L., Fanning S., Spengler G., **Rodrigues L.**, Iversen C., Martins M., Martins A., Viveiros M., Couto I. 2010. Genetic regulation, physiology, assessment and inhibition of efflux pumps responsible for multi-drug resistant phenotypes of bacterial pathogens. NovaScience Publishers.
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List of abbreviations

ABC – ATP-Binding Cassette

ACP – Acyl Carrier Protein

AIDS – Acquired Immunodeficiency Syndrome

AMK – Amikacin

ATP – Adenosine Triphosphate

CCCP – Carbonyl Cyanide *m*-Chlorophenylhydrazone

CLSI – Clinical and Laboratory Standards Institute

CLT – Clarithromycin

CIP – Ciprofloxacin

CPZ – Chlorpromazine

C_T – Comparative Threshold Cycle

DCCD – Dicyclohexylcarbodiimide

DGS – Direcção Geral de Saúde

DHPS – Dihydropteroate Synthase

DIM – Dimycocerosate

DNA – Deoxyribonucleic Acid

DOTS – Directly Observed Treatment Short Course

dTMP – Deoxythymidine Monophosphate

dTTP – Deoxythymidine Triphosphate

dUMP – Deoxyuracil Monophosphate

EDTA – Ethylenediaminetetraacetic Acid

EMB – Ethambutol

EPI – Efflux Pump Inhibitor

ERDR – Ethambutol Resistance-Determining Region

ERY – Erythromycin

EtBr – Ethidium Bromide

FASII – Fatty Acid Synthase II

GAPDH – D-glyceraldehyde-3-phosphate-dehydrogenase

GI – Growth Index

HIV – Human Immunodeficiency Virus

INH – Isoniazid

IUATLD – International Union Against Tuberculosis and Lung Disease

KAN – Kanamycin

LAM – Lipoarabinomycolate

LB – Luria Bertani

LM – Lipomannans

MAC – *Mycobacterium avium* complex

MATE – Multidrug and Toxic Extrusion

MDRTB – Multidrug Resistant Tuberculosis

MFS – Major Facilitator Superfamily

MIC – Minimum Inhibitory Concentration

MIRU-VNTR – Mycobacterial Interspersed Repetitive Units - Variable Number of Tandem Repeats

MDR – Multidrug Resistant

MRSA – Methicillin Resistant *Staphylococcus aureus*

NAD – Nicotinamide Adenine Dinucleotide

OADC – Oleic Acid/Albumin/Dextrose/Catalase

OD – Optical Density

OFL – Ofloxacin

PABA – ρ-Amino Benzoic Acid

PBS – Phosphate Buffered Solution

PCR – Polymerase Chain Reaction

PDIM – Phthiocerol Dimycocerosate

PIM – Phosphatidylinositol Mannosides

POA – Pyrazinoic Acid

PZA – Pyrazinamide

QRDR – Quinolone Resistance Determining Region

qRT-PCR – quantitative Reverse Transcriptase-Polymerase Chain Reaction

RES – Reserpine

RIF – Rifampicin

RND – Resistance Nodulation Division

RNA – Ribonucleic Acid

rRNA – ribossomal Ribonucleic Acid

SMR – Small Multidrug Resistance

STR – Streptomycin

TB – Tuberculosis

TET – Tetracycline

TPP – Tetraphenylphosphonium

TZ – Thioridazine

USA – United States of America

VP – Verapamil

WHO – World Health Organization

XDRTB – Extensively Drug Resistant Tuberculosis

List of Units

ºC - degrees Celsius

µm - micrometers

cpm - counts per minute

mg/L - milligrams per litre

min - minutes

mL - millilitres

mM - millimolar

ng - nanograms

nm - nanometers

nM - nanomolar

rpm - revolutions per minute

The organization of this thesis is in accordance to the Bologna Agreement guidelines, which employs as a basis the publications that have resulted from thesis research. The sections of the thesis have been organized so that each section can stand on its own.

Chapter I

Introduction

Introduction

I.1 Tuberculosis

I.1.1 The global burden of Tuberculosis

Tuberculosis (TB) has been part of human history for thousands of years and signs of this disease have been found since antiquity with the identification of mycobacterial deoxyribonucleic acid (DNA) in Ancient Egypt mummies dated between 3000 and 2400 years B.C. (Crubezy E. *et al*, 1998; Donoghue H.D. *et al*, 2004; Rothschild B. *et al*, 2001). In Ancient Greece, Hippocrates described TB as phthysis or consumption, a term by which this disease was known until the early 20th century. At the beginning of the 17th century and for the next 200 years, the TB epidemic (known as the Great White Plague) started in Europe and became the major cause of mortality (Bates J.H. *et al*, 1993). However, despite the long co-existence of TB and mankind, *Mycobacterium tuberculosis*, the agent responsible for this infection in Man, was described only in 1882 by Robert Koch (Koch, R. 1882).

In the early 20th century, the general improvement in public health in Europe and in the United States of America (U.S.A) helped to reduce the burden of TB. The establishment of TB control programmes, along with the introduction of a successful anti-TB treatment, resulted in an evident decrease of infection and mortality. In the middle of the 20th century, the fact that the disease was considered so close to elimination caused a decreased interest of the industrialized countries and, consequently, the abandonment of the TB control programmes. In fact, it was considered that the recent developments in anti-mycobacterial chemotherapy had determined the disappearance of the disease as a public health problem. However, despite the decrease of TB cases, the disease never disappeared completely and

around 1985, TB began to increase again in industrialized countries (Brudney K. *et al*, 1991; Daniel T.M., 2006). The other major factor that contributed for this resurgence, along with the decline of the TB control programmes, was the advent of the human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS) (Brudney K. *et al*, 1991). Today, TB gained once more the attention of the international community and is still without doubt one of the major threats to public health. In 1993, the World Health Organization (WHO) declared TB a global health emergency and in 1998, together with the International Union Against Tuberculosis and Lung Disease (IUATLD) and other international partners, formed the Stop TB Initiative, a new global strategy to control TB that has recently evolved into a global partnership, the Stop TB Partnership (Stop TB Partnership, 2006). However, despite of these global efforts, TB is still a major threat. In fact, the WHO estimates that one third of the world population (approximately two billion people) is infected with *M. tuberculosis*, with eight million new cases of TB every year and two million deaths from TB each year (WHO, 2009). The higher rates of TB (≥ 300/100 000 inhabitants/year) occur in Sub-Saharan Africa, Indonesia and Philippines (Figure I.1). If effective TB control programmes are not created, WHO estimates that in 2020 *M. tuberculosis* will infect approximately 1000 million people, 150 million will develop the disease and 36 million will die of TB (WHO, 2009).

The global resurgence of TB is not only related with the higher rates of co-infection with HIV, but also with the emergence of *M. tuberculosis* strains resistant to anti-TB drugs. An example was the extensive outbreak of TB in New York City in the early 1990's. This form of TB was resistant to the most effective anti-TB drugs and occurred mainly in patients co-infected with HIV. In fact, one in three new cases were found resistant to one drug and one in five to more than one drug (Frieden T.R. *et al*, 1996).

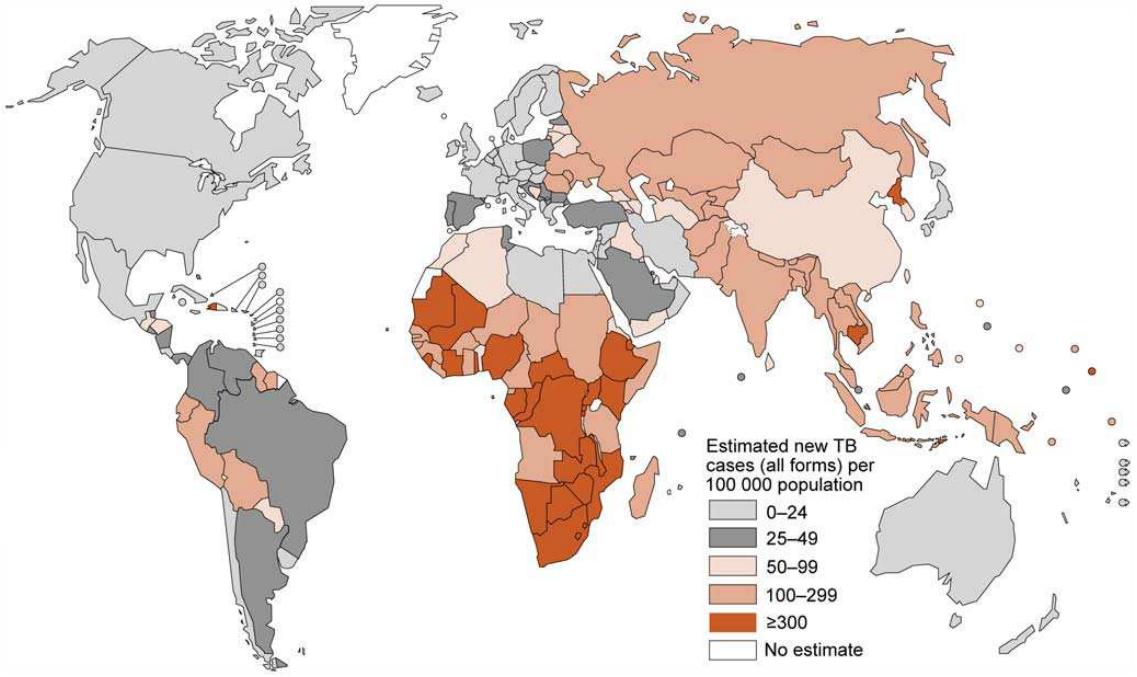

Figure I.1. Global incidence of TB. Estimated number of new TB cases per 100 000 population for 2007 (Comas I. *et al*, 2009; WHO, 2009).

WHO defined multidrug resistant TB (MDRTB) as TB cases caused by *M. tuberculosis* strains resistant, simultaneously, to isoniazid (INH) and rifampicin (RIF), two of the first-line drugs used to treat TB. It is estimated that 50 million people are infected with *M. tuberculosis* strains resistant to anti-TB drugs, with 300 000 news cases of MDRTB each year (WHO/ IUATLD, 2008). Recently, a new form of TB has been reported that presents resistance not only to first-line drugs, but also to second-line drugs (Figure I.2). The WHO has defined this form of TB as extensively drug resistant TB (XDRTB) and refers to *M. tuberculosis* strains that present resistance not only to INH and RIF (MDRTB), but also to any fluoroquinolone and at least to one of the three injectable second-line drugs kanamycin, amikacin and capreomycin (WHO, 2006). XDRTB can develop when these second-line drugs are misused or mismanaged and, consequently, become ineffective (Migliori G.B. *et al*, 2007).

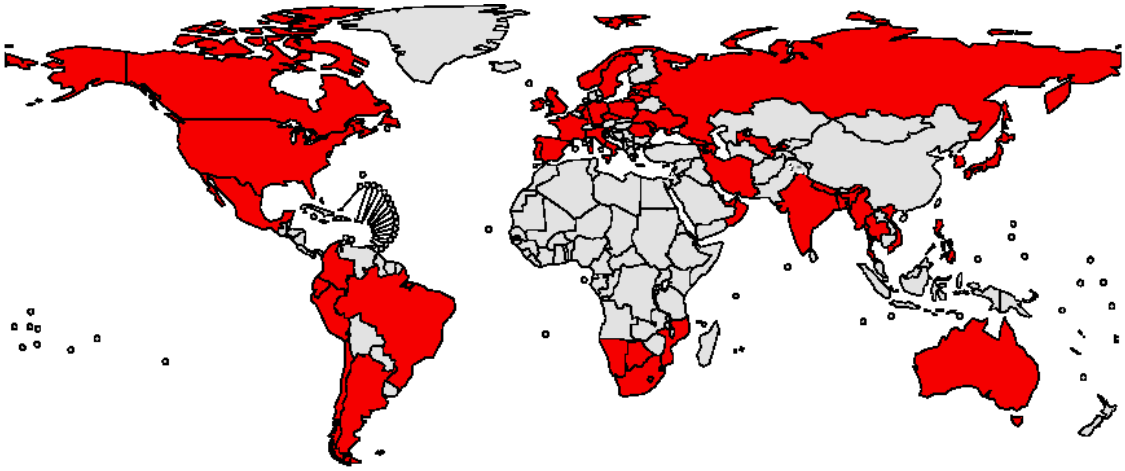

Figure I.2. Countries that reported at least one XDRTB case by the end of 2008 (WHO, 2009).

I.1.2 Tuberculosis in Portugal

Concerning the situation of TB in Portugal, the rates of new TB cases are the highest for Western Europe, although small gradual declines were evident in recent years. In 2003, the Portuguese Health Authorities reported 34.4 cases of TB per 100 000 inhabitants, with 1.8% cases of MDRTB (DGS, 2008). In Lisbon, the rates of MDRTB were as high as 28% in 2003 and declined to less than 8% by 2006 (new cases plus re-treatments) (DGS, 2003; DGS, 2007). However, in Porto, the second largest city of Portugal, the rates of MDRTB continue to escalate (DGS, 2003; DGS, 2007).

In Portugal, the majority of the MDRTB strains circulating in the Lisbon Health Region belong to family Lisboa, a particular family of strains genetically related. This family was involved in an outbreak of MDRTB, mainly in HIV patients, 10 years ago (Portugal I. *et al*, 1999). Over the past years, this family of strains has been associated almost exclusively to MDRTB, with a prevalence of about 50% in 2001-2002, 56% in 2003 and 88% in 2004-2006 (Perdigão J. *et al*, 2008). Recently, MDRTB has progressed to XDRTB, representing in Lisbon about 50% of the MDRTB strains (Perdigão J. *et al*, 2008). The use of the mycobacterial inter-spersed repetitive units–variable number of tandem repeats (MIRU–VNTR) technique showed that XDRTB strains are clustered in

two different, but related profiles that belong to family Lisboa, and are probably a result of more than 10 years of family Lisboa MDRTB strains circulating in Portugal. These strains while mainly detected in the Lisbon Region, disseminated to other regions of the country, turning family Lisboa into a threat to the control of TB in Portugal.

I.2 *Mycobacterium* sp - General Characteristics

Mycobacterium is the only genus of the Family Mycobacteriaceae, included in Order Actinomycetales, Phylum Actinobacteria and it is presumed to have originated more than 150 million years ago (Daniel T.M., 2006; Gutierrez C. *et al*, 2005). Mycobacteria are straight or slightly curved rods between 0.2-0.6 µm wide by 1.0-10 µm long, aerobic with optimum growth temperatures that vary according to the species and range from 25ºC to 37ºC. Mycobacteria also have a genomic DNA with a high guanine plus cytosine (G+C) content (61-71%) and a characteristic cell-wall that will be described in Section I.2.2.

I.2.1 Classification of mycobacteria

Mycobacteria can be separated into different groups according to their phenotypic characteristics. The most used classification system was created by Runion in 1959 and establishes four groups based on culture characteristic, the incubation period required for growth and the development of pigmentation in the presence/absence of light (Runyon E.H. 1959; Eisenstadt J. *et al*, 1995). The first three groups include slow growth mycobacteria that require longer periods of incubation (*e.g. M. tuberculosis*) and the fourth group comprises rapid growth mycobacteria that form colonies within seven days of incubation (*e.g. M. smegmatis*):

- Group 1 (Photochromogens) – mycobacteria that produce nonpigmented colonies when grown in the dark and pigmented colonies only after exposure to light (*e.g.*: *M. kansasii*, *M. marinum);*

- Group 2 (Scotochromogens) – mycobacteria that produce yellow to orange colonies when grown in the dark (*e.g.*: *M. gordonae*, *M. xenopi*);
- Group 3 (Non-chromogens) – mycobacteria nonpigmented in the light or dark. However, older cultures can develop a yellow pigmentation (*e.g*. *M. tuberculosis*, *M. avium, M. bovis*, *M. ulcerans*);
- Group 4 – rapid growth mycobacteria that have a slight yellow pigment that does not intensify after light exposure (*e.g*. *M. fortuitum*, *M. chelonae*).

The majority of mycobacteria identified until now are environmental and/or commensal to Man (non-pathogenic mycobacteria). However, there are some species that are strictly pathogenic to Man and/or animals (strictly pathogenic mycobacteria). There is also a group of mycobactera usually commensal to Man, but they can become pathogenic in certain circumstances (potentially pathogenic mycobacteria) (table I.1). The number of cases of infection by this group of mycobacteria has increased in recent years due to opportunistic infections in HIV patients.

The several species that comprise the *Mycobacterium* genus can be grouped in different complexes whose members have similar genetic and phenotypic characteristics. The *M. tuberculosis* complex, which includes *M. tuberculosis*, *Mycobacterium bovis*, *M. bovis* BCG, *Mycobacterium africanum, Mycobacterium microti* and the three recent newcomers *Mycobacterium canetti*, *Mycobacterium caprae* and *Mycobacterium pinnipedii*, comprises slow growth mycobacteria with generation periods of approximately 24 hours (Brosch R. *et al*, 2001). All of the species included in this complex are known to cause TB in humans.

Table I.1.Classification of mycobacteria according to the risk of infection (Brosch R. *et al*, 2001; David H.L.,1989)

Rarely pathogenic		Potentially pathogenic	Strictly pathogenic
M. smegmatis	*M. aurum*	*M. avium*	*M. tuberculosis*
M. phlei	*M. chitae*	*M. intracellulare*	*M. bovis*
M. fallax	*M. duvalii*	*M. chelonae*	*M. africanum*
M. thermoresistibile	*M. gadium*	*M. fortuitum*	*M. ulcerans*
M. parafortuitum	*M. gilvum*	*M. kansasii*	*M. microti*
M. gastri	*M. komossense*	*M. malmoense*	*M. canetti*
M. triviale	*M. lepraemurium*	*M. marinum*	*M. caprae*
M. nonchromogenicum	*M. neoaurum*	*M. scrofulaceum*	*M. pinnipedii*
M. gordonae	*M. terrae*	*M. simiae*	*M. leprae*
M. flavescens	*M. vaccae*	*M. szulgai*	
M. farcinogenes	*M. agri*	*M. xenopi*	
M. senegalense	*M. aichiense*	*M. asiaticum*	
M. paratuberculosis	*M. austroafricanum*	*M. haemophilum*	
M. porcinum	*M. chubuense*	*M. shimoidei*	
M. diernhoferi	*M. obuense*		
M. pulveris	*M. rhodesiae*		
M. tokaiense	*M. moriokaense*		
M. poriferae			

The *M. avium* complex (MAC) comprises, among other environmental species, *M. avium* subsp. *avium* (responsible for disease in birds, but also for disseminated disease in patients with AIDS, cervical lymphadenitis, and chronic lung disease), *M. avium* subsp. *paratuberculosis* (the etiologic agent of Johne's disease or paratuberculosis, a chronic granulomatous enteric disease of ruminant livestock and wildlife), *M. avium* subsp. *silvaticum* (causes TB-like lesions in wood pigeons) and *M. intracellulare*

(associated with MAC-associated pulmonary infections in immunocompetent or non-HIV patients) (Turenne C.Y. *et al*, 2007).

The *Mycobacterium fortuitum* complex includes the rapid growth mycobacteria *Mycobacterium fortuitum*, *Mycobacterium peregrinum*, *Mycobacterium abcessus* and *Mycobacterium chelonae*. These organisms have generation periods of 20-30 minutes and are frequently responsible for abscess formation in local injection or surgical wounds. They can also be associated with pulmonary disease, usually *M. abcessus,* particularly in the case of imunossupression of the host, and are resistant to the anti-TB drugs currently in use (Griffith D.E. *et al*, 2007).

I.2.2 The mycobacterial cell-wall

The mycobacterial cell-wall, shown schematically in Figure I.3, is a complex structure, rich in high molecular weight lipids (Brennan P. *et al*, 1995; Christensen H. *et al*, 1999; Daffé M. *et al*, 1998; Draper P., 1998; Jarlier V. *et al*, 1994). The basic structural skeleton of the cell-wall is formed by peptidoglycan linked to a second polysaccharide polymer, the arabinogalactan. The mycolic acids are covalently linked to the arabinogalactan polymer, forming the inner region of a thick waxy coat surrounding the peptidoglycan-arabinogalactan skeleton. These long chain length branched fatty acids (70–90 carbon) are characteristic of mycobacteria and account for up to 60% of the whole cell dry weight (Brennan P. *et al*, 1995; Daffé M. *et al*, 1998; Draper P., 1998). The outer surface of this layer contains other complex lipids, including glycopeptidolipids, trehalose containing lipooligosaccharides, sulpholipids, phthiocerol dimycocerosate and phenolic glycolipids. Spanning the outer layer are porin proteins which have similar properties to those found in the Gram-negative outer membrane, providing a pathway for low molecular weight hydrophilic nutrients (Niederweis M., 2003). Lipoarabinomycolate (LAM) is thought to be anchored *via* phosphatidylinositol to

the outer region of the cytoplasmic membrane and protrude through the wall to the outer cell surface (Brennan P. *et al*, 1995; Daffé M. *et al*, 1998; Draper P., 1998).

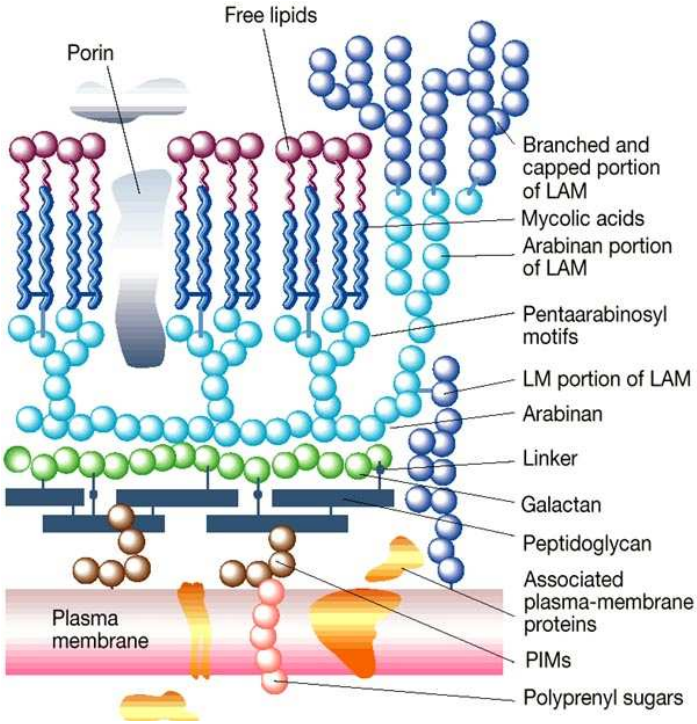

Figure I.3. Schematic representation of the mycobacterial cell-wall (Park S.H. *et al*, 2000; Lee R.E. *et al*, 1996). LAM, lipoarabinomannans; LM, lipomannans; PIM, phosphatidylinositol mannosides.

Recently, several studies have suggested the presence of a periplasmic space and an outer membrane in mycobacteria (Niederweis M. *et al*, 2010; Hoffmann C. *et al*, 2008; Zuber B. *et al*, 2008). The periplasm would include the peptidoglycan-arabinogalactan polymer that may correspond to an unidentified structure that has been observed by cryo-electron microscopy (Hoffmann C. *et al*, 2008). Relatively to the outer membrane organization, it can be represented by two models that differ in the conformation of the mycolic acids. One model suggests that the mycolic acids have an elongated conformation that would span the complete hydrophobic matrix, leaving space for free lipids to intercalate (Nikaido H. *et al*, 1993; Hong, X. *et al*, 2004), whereas a second

model, based on results of monolayer experiments and simulation data, suggests a folded conformation for the mycolic acids. This folded conformation may be stabilized by keto or methoxy groups that were recently proposed to interact with lipid head groups (Zuber B. *et al*, 2008).

The composition of the cell-wall confers a highly impermeable character to mycobacteria. In fact, they are resistant to a wide variety of disinfectants and dyes used in most staining techniques like Gram and Giemsa. The expression “acid-fastness” describes the resistance of these microorganisms to stain removal with acid-alcohol solutions after staining with alkaline dyes such as carbol fuchsin. This feature became the basis for the staining technique developed by Paul Ehrlich that evolved into the protocol used today to detect acid-fast bacillus bacillus by microscopy (Ziehl-Neelsen staining) (Brennan P., 1995; Nikaido H., 2001). The cell-wall also provides an important contribution to the resistance of these microrganisms to most antimicrobial drugs and this subject will be addressed in detail in section I.5.1 "Intrinsic Resistance".

I.2.3 The cord factor

In general, *M. tuberculosis* bacilli produce rough textured colonies on solid media and are arranged in serpentine cords on microscopic smears. The recognition of these two characteristics allows an experienced microbiologist to separately identify *M. tuberculosis* from other mycobacteria in cultured specimens. These characteristics have been attributed to the glycolipid trehalose 6, 6’-dimycolate, also known as cord factor, which is present in the mycobacterial cell-wall (Hunter N. *et al*, 2006a). It is responsible for: (i) inducing animal granulomas similar to those characteristic of TB infection (Hunter N. *et al*, 2006b); (ii) increasing cytokine production (Ryll R. *et al*, 2001); (iii) inhibiting the transfer of phagocytosed bacteria to acidic compartments in macrophages (Indrigo J. *et al*, 2003.); and (iv) influencing the morphology of

mycobacterial colonies (Hunter N. *et al*, 2006a). In liposomes, trehalose dimycolate inhibits vesicle fusion, which could explain its role in preventing the phagosome-lysosome fusion *in vivo* (Spargo B.J. *et al*, 1991). However, the role that trehalose dimycolate may play outside the host environment remains to be completely clarified.

I.2.4 The mycobacterial genome

The complete sequencing of the *M. tuberculosis* H37Rv genome has provided more information concerning the characteristics of this organism, such as its slow growth, cell-wall complexity and adaptation to environmental conditions (Cole S.T. *et al*, 1998). The genome of *M. tuberculosis* H37Rv presents a sequence of 4411529 bp and a characteristically high guanine plus cytosine (G+C) content (65.5 %). Genome analysis revealed an efficient DNA repair system with nearly 45 genes related to DNA repair mechanisms, including three copies of the *mutT* gene, which encodes the enzyme responsible for removing oxidized guanines whose incorporation during replication causes base-pair mismatching (Mizrahi V. *et al*, 1998). Concerning transcriptional regulation, *M. tuberculosis* codifies for 13 putative sigma factors and more than 100 regulatory proteins. The presence of a single ribosomal ribonucleic acid (rRNA) operon (*rrn*), contrary to most eubacteria that have more than one *rrn* operon, has been pointed to be a factor contributing to the slow growth of *M. tuberculosis* (Brosch R. et al, 2000).

From the genome sequence it is clear that *M. tuberculosis* has the potential to switch from one metabolic route to another, including aerobic (*e.g.* oxidative phosphorylation) and anaerobic respiration (*e.g.* nitrate reduction). This flexibility is useful for survival within the human host environment that can range from high oxygen tension in the lung alveolus to microaerophilic/anaerobic conditions within the tuberculous granuloma.

There are also genes encoding for 250 distinct enzymes involved in fatty acid metabolism that allow the synthesis and degradation of several lipids from simple fatty

acids to complex molecules such as mycolic acids (Cole S.T. *et al*, 1998). Moreover, the genome of *M. tuberculosis* H37Rv codes for multiple putative efflux proteins, of which the majority have not yet been characterized (Cole S.T. *et al*, 1998; Louw G.E. *et al*, 2009).

Altogether, the presence of a permeability barrier established by the outer membrane, metabolic versatility, high fidelity replication machinery and multidrug efflux compensate for the slow growth of these organisms by allowing *M. tuberculosis* to successfully adapt to challenging environments, namely host defense mechanisms and antibiotic pressure.

I.3 Pathogenesis of tuberculosis

M. tuberculosis is a mainly intracellular pathogen that enters the body *via* the respiratory pathway. However, most people infected by *M. tuberculosis* do not develop the disease during their lifetime. The risk of developing the disease increases when an imunossupression takes place, such as co-infection with HIV. This shows that the immune response plays a crucial role in the outcome of *M. tuberculosis* infection (Flynn J.L. *et al*, 2001). The first event in the host-pathogen relationship is the phagocytosis of *M. tuberculosis* by alveolar macrophages. This is followed by cell-mediated immunity, with a flow of lymphocytes and activated macrophages into the lesion resulting in granuloma formation. This controls the exponential growth of the bacilli and the dead macrophages form a caseum. The bacilli are then contained in the caseous centers of the granuloma. Then, one of the following scenarios can take place: (i) the bacilli may remain forever within the granuloma (latent TB); (ii) the bacilli can be re-activated later; and (iii) the bacilli can be discharged into the airways after enormous increase in number, necrosis of bronchi and cavitation. Finally, fibrosis develops around the area of necrosis in order to contain the infection. This occurs as a final defence mechanism of the host when all other mechanisms have failed (Flynn J.L. *et al*, 2001).

HIV infection is a risk factor for TB. Not only does HIV increase the risk of reactivating latent *M. tuberculosis* infection, it also increases the risk of developing TB after infection or reinfection. In patients infected only with *M. tuberculosis*, the risk of developing TB during their lifetime ranges between 10% and 20%. However, patients co-infected with HIV have their annual risk increase in 10% (Goletti D. *et al*, 1996; Nakata K. *et al*, 1997). In the co-infected patient, a complex interaction occurs between *M. tuberculosis* and HIV that results in the aggravation of both pathologies. In fact, HIV promotes the progression of *M. tuberculosis* latent infection to disease, whereas *M. tuberculosis* enhances HIV replication, accelerating the progress of HIV infection (Goletti D. *et al*, 1996; Mariani F. *et al*, 2001; Nakata K. *et al*, 1997; Rosas-Taraco A.G. *et al*, 2006). HIV infection impairs *M. tuberculosis*-specific IFN-γ production, which is not reversed by anti-retroviral treatment (Sutherland R. *et al*, 2006). The clinical presentation of TB in HIV/AIDS patients is related to the blood level of $CD4^+$ T lymphocytes, a measure of the degree of immunossupression of the patient (Jones B.E. *et al*, 1993). A level of 200 $CD4^+$ T cells/μL was established as a threshold for severe immunossupression. Above this level, the TB granuloma is produced in response to *M. tuberculosis* infection, including multinucleated giant cells, macrophages, $CD4^+$ and $CD8^+$ T lymphocytes and a central caseous necrosis. $CD4^+$ T cell counts below 200/μL, cause the progressive impairment of the granuloma. Even in the presence of severe immunossupression, pulmonary TB is still the most common presentation of the disease, although extrapulmonary and disseminated TB is also very frequent in these patients (approximately 50% of cases).

I.4 Treatment

Treatment of TB is used not only to cure the disease but also to interrupt the transmission and to prevent relapse (most relapses occur within 6-12 months after the end of therapy) (Böttger E.C. *et al*, 2008). In 1994, the WHO introduced the Directly

Observed Treatment Short Course (DOTS), a strategy for the detection and treatment of TB, in which patients are observed to take each dose of anti-TB medication, until the end of therapy (WHO, 2001). Monthly sputum specimens are then taken until 2 consecutive specimens are negative. However, the effectiveness of DOTS is facing new challenges due to the increase of MDRTB and the emergence of XDRTB. This lead to a new strategy called DOTS-plus, a comprehensive management initiative built upon the DOTS strategy with the goal of preventing further development and spread of MDRTB (WHO, 2006; WHO/ IUATLD, 2008).

Treatment can be divided into first-line and second-line drugs: the first-line drugs used are INH, RIF, pyrazinamide (PZA), ethambutol (EMB) and streptomycin (STR) and the second-line drugs include fluoroquinolones, aminoglycosides such as kanamycin and amikacin, cyclic peptides like capreomycin, D-cycloserine, ethionamide, and ρ-amino salicylic acid. Each treatment regimen for pulmonary TB caused by susceptible organisms has an initial 2 months intensive phase with INH, RIF, PZA and EMB, followed by a continuation phase with INH and RIF for 4 to 7 months. STR can be used as an interchangeable drug with EMB in the initial phase of treatment. However, STR is only recommended to be interchangeable with EMB when the organism is known to be susceptible to the drug or the patient is from a community in which STR resistance is unlikely (Blumberg H.M. *et al*, 2003; WHO, 2001). The mechanisms of action of these drugs will be discussed in subchapter I.5.2 "Genetic Resistance".

I.5 Resistance to anti-mycobacterial drugs

In TB, clinical drug resistance is classified as acquired resistance, when drug resistant mutants are selected as a result of ineffective treatment, or as primary resistance, when a patient is infected with a resistant *M. tuberculosis* strain (Figure I.4). The selection of drug resistant mutants has to do with the long generation time of *M. tuberculosis*, its low metabolic activity and capacity for dormancy (Wayne, L.G. 1994).

Furthermore, the compartmentalization of the infection increases the probability of exposure to monotherapy, since *M. tuberculosis* may be located inside pulmonary cavities, with difficult antibiotic access (Elliott A.M. *et al*, 1995). This effect is enhanced in the presence of an inadequate dosage of anti-TB drugs, due to inadequate prescription by the physician or non-adherence by the patient. In order to prevent drug resistance it is necessary to understand the mechanisms by which *M. tuberculosis* becomes resistant. Over the years, several studies have described the mechanisms of action of most of the anti-TB agents used in clinical practice.

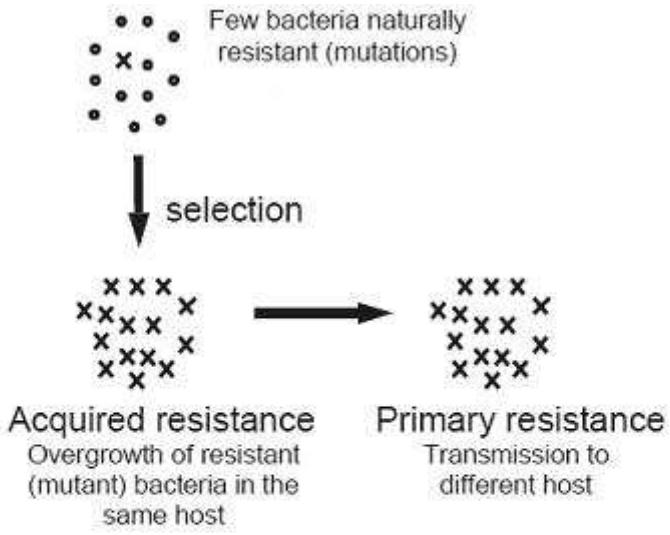

Figure I.4. Development of resistance in TB. Acquired resistance develops due to the selection of resistant mutants as a consequence of ineffective treatment and non-compliance. Primary resistance occurs through the transmission of the resistant bacteria to a new host (adapted from Johnson R. *et al*, 2006).

I.5.1 Intrinsic resistance

The intrinsic resistance of mycobacteria to many antimicrobial drugs is mainly attributed to the permeability barrier provided by the mycobacterial cell-wall (Jarlier V. *et al*, 1994; Nguyen L. *et al*, 2006, Niederweiss M. *et al*, 2010). However, this does not fully explain intrinsic drug resistance in mycobacteria, suggesting that there must be

other systems, such as enzymatic inactivation (*e.g.* β-lactamases) or active efflux of drugs that work in synergy with the permeability barrier (Jarlier V. *et al*, 1994).

o Cell-wall impermeability

The lipid-rich mycobacterial cell-wall establishes a barrier to the entry of antimicrobials protecting the cell from toxic compounds (Brennan P.J. *et al*, 1995; Draper P. *et al*, 1998). The peptidoglycan and arabinogalactan layers limit the entry of hydrophobic molecules, whereas the mycolic acid layer limits the access of both hydrophobic and hydrophilic molecules (Brennan P.J. *et al*, 1995). Relatively hydrophobic antibiotics such as RIF and fluoroquinolones may enter the cell by diffusion through the hydrophobic bilayer. However, hydrophilic antibiotics and nutrients that cannot diffuse through the cell-wall may use porin channels (Lambert P.A., 2002; Niederweis M., 2008; Niederweiss M. *et al*, 2010). The presence of porins in a lipid bilayer supports the hypothesis that the mycobacterial cell-wall has an outer-membrane analogous to that of Gram-negative bacteria. However, mycobacterial porins are much less abundant than in the Gram-negative outer membrane and only allow low rates of uptake for small hydrophilic nutrients and antibiotics (Niederweis M., 2008; Trias J. *et al*, 1992).

o Porins

Porins are defined as non-specific protein channels in bacterial outer membranes that allow the entry of hydrophilic solutes (Nikaido M., 2003; Niederweiss M. *et al*, 2010). Channel-forming proteins that are functionally similar to porins of Gram-negative bacteria have been described in many mycobacteria (Niederweis M., 2003). MspA was the first porin of *M. smegmatis* to be described and deletion of *mspA* reduced the outer-membrane permeability to cephaloridine and glucose, suggesting that MspA was the major diffusion pathway for hydrophilic solutes in *M. smegmatis* (Niederweis M. *et al*, 1999; Stahl C. *et al*, 2001). Moreover, consecutive deletions of *mspA* and another porin

gene, *mspC* lowered the permeability for glucose 75-fold and the growth rate of *M. smegmatis* also decreased (Stephan J. *et al*, 2005). It was demonstrated that deletion of MspA and MspC increased resistance of *M. smegmatis* to β-lactam antibiotics, chloramphenicol and norfloxacin. Furthermore, expression of MspA in *M. tuberculosis* and *M. bovis* BCG promoted glucose uptake, and increased the growth rate and the susceptibility to hydrophilic antibiotics such as β-lactams, INH, EMB and STR (Mailaender C. *et al*, 2004). These studies demonstrated that small and hydrophilic antibiotics use the Msp porins to enter the cell.

Other studies demonstrated the presence of channel-forming proteins in *M. tuberculosis* and *M. bovis* BCG (Kartmann B. *et al*, 1999; Lichtinger T. *et al*, 1999; Senaratne R.H. *et al*, 1998). An example is the OmpATb of *M. tuberculosis*. A study has shown that uptake of serine, but not of glycine, was reduced in an *ompATb* mutant compared to wild-type *M. tuberculosis*. This suggested that OmpATb was a porin, which was consistent with its apparent channel-forming activity *in vitro* (Raynaud C. *et al*, 2002). However, the uptake of glicine was higher in the mutant, which is not consistent with OmpATb being a major porin. Therefore, it is not clear that OmpATb has a significant porin function in *M. tuberculosis*. Nevertheless, the failure of the *ompATb* mutant to grow at low pH, and the induction of *ompATb* transcription at low pH and in macrophages, suggests that this protein may play a role in the adaptation to low pH and in *M. tuberculosis* survival inside the host's macrophages (Niederweis M., 2003; Raynaud C. *et al*, 2002).

A recent study used a bioinformatic approach to predict outer membrane proteins of *M. tuberculosis* (Song H. *et al*, 2008). The porin MspA of *M. smegmatis* provided the basis for a secondary structure analysis performed for 587 proteins of *M. tuberculosis* predicted to be exported to the outer membrane. Using this approach, Rv1698 and

Rv1973 were identified as outer membrane proteins. Nevertheless, more studies are needed to identify porin-channels that can provide a pathway for anti-TB drugs across *M. tuberculosis* cell-wall.

o Efflux pumps

Along with cell-wall impermeability, active efflux systems can also provide resistance by extruding drug molecules that enter the cell. Knowing that the intracellular concentration of a given drug depends on the balance between its influx and efflux, it is therefore of great importance to increase our understanding of the processes of drug influx through porins and drug efflux *via* efflux pumps, in order to prevent drug resistance (Niederweis M., 2003; Nikaido H. 2001.). The *M. tuberculosis* genome encodes many putative drug exporters, and several studies have demonstrated the association of protein transporters with resistance to tetracycline (TET), aminoglycosides, fluoroquinolones, RIF, INH and chloramphenicol in several mycobacteria (Ainsa J. *et al*, 1998; Banerjee S.K. *et al*, 2000; De Rossi E. *et al*, 1998a; Li X.Z. *et al*, 2004; Pasca M.R. *et al*, 2004; Silva P.E. *et al*, 2001; Viveiros M. *et al*, 2003). This topic will be further adressed in section 1.6 "Efflux Pumps", and in particular in 1.6.2 "Efflux Pumps in Mycobacteria".

I.5.2 Genetic resistance

Genetic resistance to antibiotics in most bacteria can be due to transmissible genetic elements, like transposons, integrons and plasmids, acquired by transduction or transformation. In *M. tuberculosis*, genetic resistance occurs mainly through chromosomal mutations, although mobile genetic elements, like the insertion sequence IS6110, have been associated with resistance through the inactivation of critical genes (Gillespie S. 2002; Böttger E.C. *et al*, 2008; Dale J. W. *et al*, 1995). This section will

address the genetic mechanisms of resistance of each of the anti-TB drugs used in the first- and second-lines of treatment.

I.5.2.1 First-line drugs

I.5.2.1.1 Isoniazid

In the 1950', it was discovered that isonicotinic acid hydrazide (latter known as INH) had a high activity against TB, higher than any other compound used at the time (Bernstein J.W. *et al*, 1952; Vilchèze C., 2007). Soon INH became one of the major first-line anti-TB drugs and its mechanisms of action and resistance have been studied for more than 50 years.

- **Mechanism of action**

INH is particularly active against slowly growing mycobacteria (Pansy F. *et al*, 1952). The minimum inhibitory concentration (MIC) of INH against *M. tuberculosis* is 0.05 mg/L, but is 100-fold higher against the fast-growing *M. smegmatis*. It is thought that INH enters the mycobacteria through passive diffusion through the cell-wall and that it is active only against dividing bacteria (Bardou F. *et al*, 1998; Mitchison D.A. *et al*, 1956). The first study regarding the mechanism of action of INH was published in 1970 by Winder and Collins (Winder F.G. *et al*, 1970). In this study, the authors use the following approach to demonstrate that mycolic acid biosynthesis was inhibited by INH: *M. bovis* BCG and *M. tuberculosis* H37Ra were exposed to INH and [^{14}C]-glycerol for six hours and the mycolic acids were extracted and analyzed by thin layer chromatography, showing that inhibition of mycolic acid synthesis occurred after this treatment. This effect was absent in INH resistant strains, which suggested that INH inhibited an enzyme involved in mycolic acid biosynthesis. Later studies confirmed these findings, with the demonstration that inhibition of mycolic acid biosynthesis was

correlated with cell death and accumulation of long-chain fatty acids. However, the unavailability of genetic tools at the time did not allow further analyses. (Davidson L.A. *et al*, 1979; Takayama K. *et al*, 1975).

- **INH activation by KatG**

INH is a prodrug that needs activation by the catalase-peroxidase KatG and it is the activated form of INH that can inhibit mycolic acid biosynthesis. The idea that KatG might play a role in activation of INH rose with the first isolation of INH resistant mutants, which were catalase-negative (Middlebrook G., 1954). Zhang *et al.* demonstrated that transforming INH resistant *M. tuberculosis* strains with wild-type *M. tuberculosis katG* restored INH sensitivity (Zhang Y. *et al*, 1993). Subsequent studies demonstrated that KatG coverts INH into a range of activated species, such as an isonicotinoyl radical, that can acylate numerous compounds (Johnsson K. *et al*, 1994; Lei B. *et al*, 2000; Wilming M. *et al*, 1999).

- **INH inhibition of the enoyl-ACP reductase InhA**

InhA is a NADH-dependent enoyl-acyl carrier protein (enoyl-ACP) reductase of the fatty acid synthase type II (FASII) system (Figure I.5), involved in mycolic acid biosynthesis (Dessen A. *et al*, 1995; Marrakchi H. *et al*, 2000).

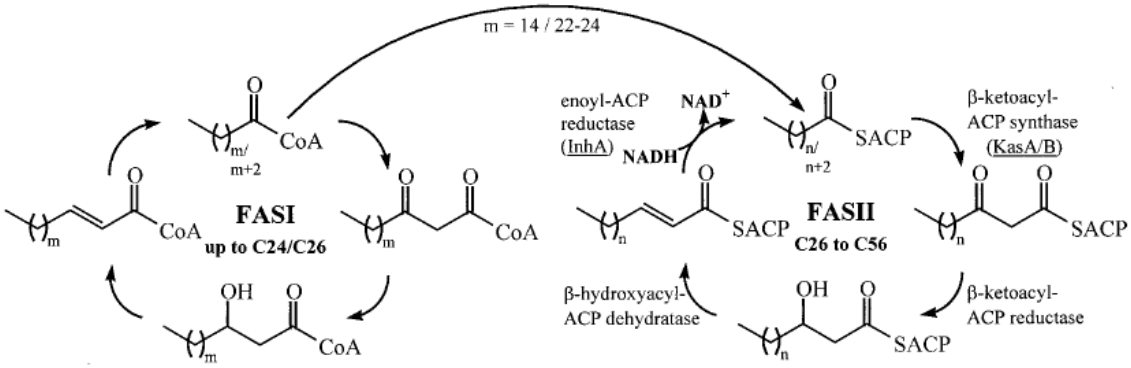

Figure I.5. The fatty acid synthase type I (FASI) and II (FASII) systems (Vilchèze C. *et al*, 2000).

A proposed mechanism of action is that INH is activated by the catalase-peroxidase KatG to form an isonicotinoyl radical that binds to nicotinamide adenine dinucleotide (NAD^+). This INH-NAD adduct inhibits the enoyl-ACP reductase InhA, which causes accumulation of long-chain fatty acids, inhibition of mycolic acid biosynthesis and, ultimately, cell death (Figure I.6) (Lei B. *et al*, 2000; Vilchèze C. *et al*, 2006; Vilcheze C. *et al*, 2007).

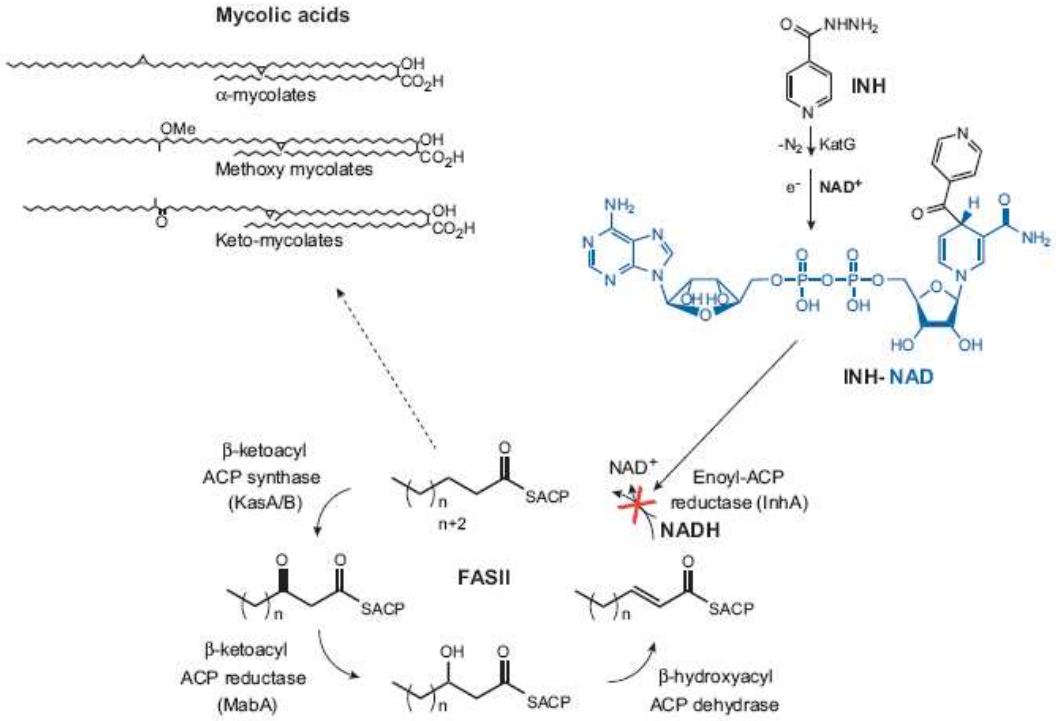

Figure I.6. Proposed model for the mechanism of action of INH (Vilchèze C. *et al*, 2007).

o **KasA, a hypothetical INH target**

The β-ketoacyl ACP synthase KasA, another enzyme of the FASII system, has also been proposed as the main target of INH (Mdluli K. *et al*, 1998; Slayden R.A. *et al*, 2000). This enzyme was isolated in an 80 kDa complex that also contained INH and the mycobacterial acyl-carrier protein AcpM, after exposure of *M. tuberculosis* to [^{14}C]-INH. These results and the presence of mutations in *kasA* in INH resistant *M.*

tuberculosis clinical isolates suggested that KasA was the main target of INH (Mdluli K. *et al*, 1998). However, later studies have identified mutations in *kasA* in INH susceptible isolates and the transfer of *kasA* mutations into *M. tuberculosis* H37Rv and *M. bovis* BCG using specialized transduction did not result in INH resistance (Lee A.S. *et al*, 1999; Piatek A.S. *et al*, 2000; Ramaswamy S.V. *et al*, 2003; Vilcheze C. *et al*, 2006). Furthermore, the 80 kDa complex described above could also be obtained when *M. tuberculosis* was treated with other InhA inhibitors, but not with KasA inhibitors. This demonstrated that INH was not a part of this complex and only inhibition of InhA, but not of KasA, induced the formation of this complex (Kremer L. *et al*, 2003). In conclusion, the hypothesis of KasA as a target for INH is still controversial.

- **Mechanisms of INH resistance**

(i) **Loss of catalase-peroxidase (KatG) activity**

Resistance to INH in *M. tuberculosis* clinical isolates is most frequently associated with mutations in *katG* that result in a decrease or loss of catalase-peroxidase activity and, consequently, in a reduced ability to form the INH-NAD adduct that inhibits InhA (Heym B. *et al*, 1995; Zhang Y. *et al*, 1992). At least 130 mutations in *katG* (2223 bp) have been reported from amino acids 1 to 735. Missense, nonsense mutations, insertions, deletions, truncation and full gene deletion have been observed (Ramaswamy S.V. *et al*, 2003; Zhang M. *et al*, 2005; Zhang Y. *et al*, 1992). The most common mutation is S315T, which results in a high deficiency in forming the INH-NAD adduct (Guo H. *et al*, 2006; Hazbon M.H. *et al*, 2006; Zhang Y. *et al*, 1992).

(ii) **Over-expression or alteration of InhA**

Other mutations associated with INH resistance occur in *inhA* the gene coding for the INH target, InhA. The most common mutation in *inhA* is mutation S94A, located in the

NADH binding site of InhA (Quemard A. *et al*, 1995). This mutation reduces the affinity of the enzyme for its cofactor NADH, resulting in a lower affinity for the INH-NAD adduct, leading to resistance to the inhibition by the INH-NAD adduct (Vilcheze C. *et al*, 2006). However, mutations in the *inhA* gene usually co-exist with mutations in the promoter region of *inhA* or *katG* mutations (Basso L.A. *et al*, 1998; Guo H. *et al*, 2006; Hazbon M.H. *et al*, 2006; Zhang M. *et al*, 2005). Overexpression of *inhA* is also associated with resistance to INH by titration of the drug. Mutation C-15T in the *inhA* promoter is the second most common mutation in INH resistant clinical isolates (Guo H. *et al*, 2006; Hazbon M.H. *et al*, 2006; Zhang M. *et al*, 2005). This mutation increases the *inhA* mRNA level, resulting in InhA overexpression and an increase in resistance to INH (Vilcheze C. *et al*, 2006).

(iii) **Other mutations associated with INH resistance**

Mutations in the *ndh* gene coding for NADH dehydrogenase NdhII were identified in INH resistant *M. tuberculosis* clinical isolates (Hazbon M.H. *et al*, 2006; Lee A.S. *et al*, 2001). These mutations result in low NdhII activity, which causes an accumulation of NADH, the substrate for NdhII. This NADH accumulation causes a competition between NADH and INH-NAD for binding to InhA and protects this enzyme against the inhibitory effect of the INH-NAD adduct (Figure I.7) (Vilcheze C. *et al*, 2005).

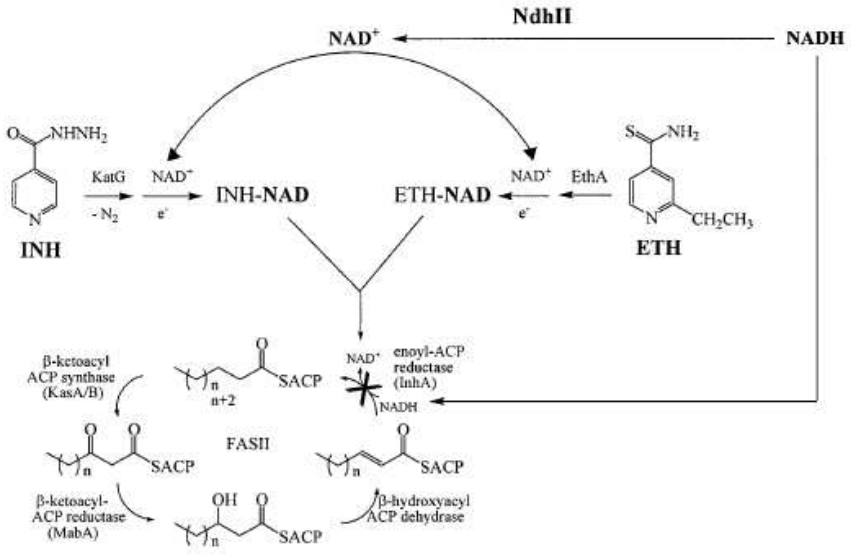

Figure I.7. Proposed mechanism of resistance to INH, and structurally similar drug ethionamide, by mutations in *ndh*. These mutations increase the NADH intracellular concentration and cause resistance by competing with the INH-NAD adduct for the binding to InhA (Vilchèze C. *et al*, 2005).

Other mutations involved in resistance to INH are located in the *ahpC-oxyR* intergenic region. OxyR is a regulatory protein that is an oxidative-stress sensor and activator of gene transcription (Farr S.B. *et al*, 1991). OxyR controls the expression of *katG* and *ahpC* genes coding for the catalase-peroxidase (KatG) and alkyl hydroperoxidase (AhpC), respectively (Farr S.B. *et al*, 1991). In *M. tuberculosis* complex, *oxyR* is naturally inactive due to several frameshift mutations and deletions. This inactivation confers increased susceptibility to INH, providing an explanation to the high susceptibility of *M. tuberculosis* to this drug (MIC of 0.05 mg/L) (Pagan-Ramos E. *et al*, 2006). Mutations in the *ahpC* promoter sequence were found in INH resistant *M. tuberculosis* with a deficient KatG activity, but not in INH susceptible isolates (Figure I.8) (Dhandayuthapani S. *et al*, 1996; Sherman D.R. *et al*, 1996; Springer B. *et al*, 2001).

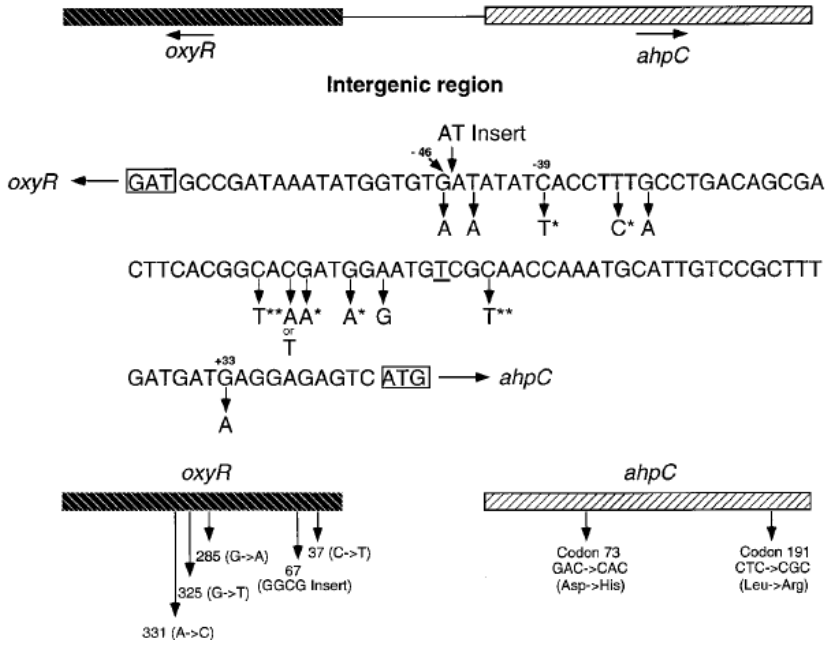

Figure I.8. Mutations identified in the *ahpC-oxyR* intergenic region. Sequence polymorphisms are designated with one asterisk and sequence variants with two asterisks (Sreevatsan S. *et al*. 1997a).

These mutations consist in compensatory alterations that occur as a result of loss of catalase-peroxidase activity. This results in a mechanism of resistance since organisms INH resistant due to decreased KatG activity require a compensatory mutation that results in overexpression of AhpC in order to survive the toxic effects of organic peroxides generated by the host macrophage (Sherman D.R. *et al*, 1996; Springer B. *et al*, 2001).

I.5.2.1.2 Rifampicin

RIF is one of the most important anti-TB drugs since it has an early bactericidal effect on metabolically active *M tuberculosis*, but also exhibits late sterilizing action on semi-dormant organisms with short bursts of metabolic activity. This late effect of RIF, coupled with the effectiveness of PZA, has allowed the reduction of standard TB treatment from 1 year to 6 months (Grosset J., 1978). The mechanism of action of RIF is the inhibition of transcription by targeting the DNA-dependent RNA polymerase.

Resistance to RIF occurs most often in strains that are also resistant to INH and, whereas monoresistance to INH is common, monoresistance to RIF is rare. This makes RIF resistance a surrogate marker for MDRTB. More than 96% of the RIF resistant strains contain a mutation in a 81 bp (27 codons) central region of the gene *rpoB* that codes for the β-subunit of RNA polymerase (Ramaswamy S. *et al*, 1998; Somoskovi A. *et al*, 2001; Telenti A. *et al*, 1993). The most common mutations (65-86%) are located at codons 526 and 531 and result in high-level resistance to RIF (MIC > 32 mg/L). However, alterations in codons 511, 516, 518, and 522 result in low-level resistance to RIF (Moghazeh S.L. *et al*, 1996; Ohno H. *et al*, 1996). The location of the most comon mutations in the 81 bp region of *rpoB* allows a straightforward approach to rapidly detect RIF resistance and/or MDRTB using molecular methods. However, in some isolates no mutation was found in the 81 bp region, but rare mutations in the amino-terminal region of *rpoB* have been found instead (Heep M. *et al*, 2000).

I.5.2.1.3 Pyrazinamide

PZA is another important first-line drug for the treatment of TB and plays a unique role in anti-TB treatment. It has a sterilizing effect on semi-dormant bacilli and, when used in combination with INH and RIF, reduces the duration of treatment from 1 year to 6 months (Grosset J., 1978).

- **Mechanism of action**

PZA is a prodrug that is converted to its active form pyrazinoic acid (POA) by the mycobacterial enzyme pyrazinamidase. In *M tuberculosis*, accumulation of intracellular POA occurs when the extracellular pH is acidic. Studies suggest that PZA enters *M. tuberculosis* by passive diffusion, is converted into POA by pyrazinamidase and, due to a inefficient efflux system, POA accumulates in the bacterial cytoplasm (Salfinger M. *et al*, 1990; Somoskovi A. *et al*, 2001; Zhang Y. *et al*, 1999;). This inefficient efflux system

is thought to be the cause for *M. tuberculosis* unique susceptibility to PZA, contrary to other mycobacteria, such as *M. smegmatis*, that have an active efflux mechanism that rapidly extrudes POA (Zhang Y. *et al*, 1999). The accumulation of POA lowers the intracellular pH to a suboptimal level that can result in inactivation of target enzymes, such as the fatty acid synthase I (Zimhony O. *et al*, 2000). However, other studies show that PZA may not have a specific target, but instead the accumulation of POA causes the disruption of membrane potential, inhibiting ATP production and membrane transporters in *M. tuberculosis* (Zhang Y. *et al*, 2003). Moreover, it was also observed that inhibition of enzymes responsible for the generation of membrane potential, such as proton-ATPase, NADH dehydrogenase and cytochrome c oxidase by N,N'-dicyclohexylcarbodiimide (DCCD), rotenone and azide, synergizes with PZA activity. This further supports the idea that PZA or POA disrupt membrane energetics as a mechanism of action (Zhang Y. *et al*, 2003).

- **Mechanism(s) of PZA resistance**

The isolation of the first PZA resistant *M. tuberculosis* isolates showed the absence of pyrazinamidase activity in these organisms (Konno K. *et al*, 1967). It was demonstrated that 72–97% of all PZA resistant clinical isolates tested carried a mutation in the structural gene or in the promoter region of *pncA* coding for the pyrazinamidase (Somoskovi A. *et al*, 2001; Scorpio A. *et al*, 1996). However, isolates with high-level resistance to PZA, but without mutations in the *pncA* gene have been described, which suggests that other mechanisms are also involved in the development of PZA resistance (such as deficient PZA uptake, *pncA* regulation, or POA efflux) (Raynaud C. *et al*, 1999).

I.5.2.1.4 Ethambutol

- **Mechanism of action**

EMB is especially important when used in drug combinations to prevent the emergence of drug resistance or to treat single drug resistant TB. Several studies have suggested that EMB interferes with the synthesis of the cell-wall core polymers arabinogalactan and lipoarabinomannan, components of the mycobacterial cell-wall, leading to a lack of arabinan receptors for the mycolic acids. The targets of EMB are thought to be the arabinosyl transferases, encoded by the *embCAB* operon, which are involved in polymerizing arabinose into the arabinan components of arabinogalactan and lipoarabinomannan (Lee R.E. *et al*, 2005.; Telenti A. *et al*, 1997; Wolucka B.A., 1994).

- **Mechanism(s) of EMB resistance**

Resistance to EMB in *M. tuberculosis* has been associated with mutations in the *embCAB* operon, particularly in the *embB* gene. Mutations at codon 306 of the *embB* gene (*embB*306), located in the EMB resistance-determining region (ERDR), are found in 28–68% of all EMB resistant clinical isolates (Plinke C. *et al*, 2006; Rinder H. *et al*, 2001; Sreevatsan S. *et al*, 1997b). However, there is still some controversy about the role of these mutations in mediating EMB resistance, since *embB*306 mutations have recently been identified in EMB susceptible *M. tuberculosis* clinical isolates (Lee A.S. *et al*, 2004; Mokrousov I. *et al*, 2002; Perdigão J. *et al*, 2009). Recent studies showed that *embB306* mutants have an altered EMB susceptibility and that the acquisition of mutations in this codon may not necessarily cause clinical EMB resistance. Instead, these mutations may represent a first step in the development of resistance and other genes may also be involved (Safi H. *et al*, 2008; Perdigão J. *et al*, 2009). On the other hand, a recent study has demonstrated that EmbC is a more suitable target for EMB in *M. tuberculosis* (Goude R. *et al*, 2009). In this study, mutations in *embC* that reduced

the arabinosyltransferase activity resulted in increased EMB susceptibility in *M. tuberculosis* and, although overexpression of EmbC was not possible in *M. tuberculosis*, overexpression of EmbC led to EMB resistance in *M. smegmatis*. Moreover, overexpression of *embB* in *M. tuberculosis* also resulted in EMB resistance, but at a lower level than that caused by EmbC. Overexpression of *embA* in *M. tuberculosis* had no effect on EMB resistance. These results suggest that, more than EmbB or EmbA, EmbC is a direct target of EMB (Goude R. *et al*, 2009).

I.5.2.1.5 Streptomycin

- **Mechanism of action**

STR is an aminoglycoside antibiotic and is one of the first-line drugs used to treat TB. Early studies in *E. coli* demonstrated that STR binds to the 16S rRNA, inhibiting protein translation (Moazed D. *et al*, 1987; Ramaswamy S. *et al*, 1998). Contrary to other bacteria that have multiple copies of rRNA genes, members of *M. tuberculosis* complex have only one copy, which means that single nucleotide changes can result in antibiotic resistance (Bercovier H. *et al*, 1986).

- **Mechanism(s) of STR resistance**

Mutations associated with STR resistance in *M. tuberculosis* have been identified in the *rrs* and *rpsL* genes coding for the 16S rRNA and ribosomal protein S12, respectively (Douglass J. *et al*, 1993; Meier A. *et al*, 1996a). Mutations in *rrs* are most frequently found in two regions around nucleotides 530 and 915. In secondary structure models, the 530 loop of 16S rRNA is highly conserved and is located adjacent to the 915 region. Several nucleotides in both regions interact with the ribosomal S12 protein (Stern S. *et al*, 1988) and previous studies in *E. coli* showed that STR interacts directly with 530 loop and 915 region of 16S rRNA, providing further evidence that mutations at

these sites result in STR resistance (Moazed D. *et al*, 1987). In the 530 loop, the most common mutations are C→T transitions at positions 491, 512, and 516, and transversions (A→C/T) at position 513. In the 915 region, the most frequent mutations are C→A/G alteration at position 903 and A→G at position 904. However, the majority of mutations associated with STR resistance occur in *rpsL* and the most common mutation is an AAG→AGG substitution in codon 43 (Ramaswamy S. *et al*, 1998).

I.5.2.2 Second-line drugs

When the recommended short-course treatment with first-line drugs fails due the emergence of MDRTB, management of TB in these cases is based on the use of second-line drugs (WHO, 2001). However, these drugs are more toxic and less effective than first-line drugs and treatment is more prolonged and expensive (Figure I.9).

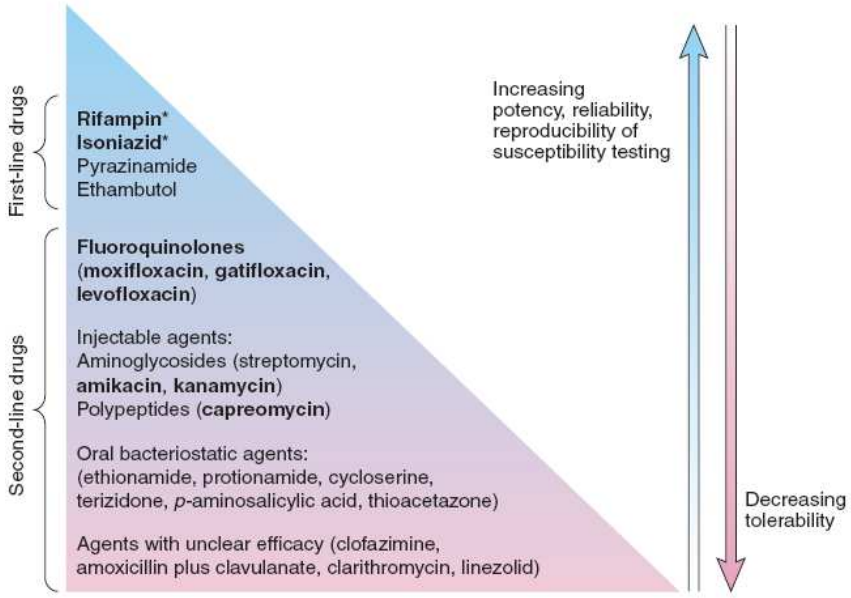

Figure I.9. Effectiveness and tolerability relation of first- and second-line drugs used in TB treatment (Dorman S.E. *et al*, 2004).

There are six classes of second-line drugs used for the treatment of TB: aminoglycosides, such as amikacin and kanamycin; cyclic peptides, such as capreomycin; fluoroquinolones, such as ciprofloxacin, levofloxacin and moxifloxacin; thioamides, like ethionamide; D-cycloserine; and *p*-aminosalicylic acid. The increased use of these drugs has contributed to the emergence of XDRTB strains and, therefore, it is important to understand the mechanisms by which *M. tuberculosis* becomes resistant to these drugs.

I.5.2.2.1 Kanamycin, amikacin and capreomycin

The aminoglycosides kanamycin and amikacin and the cyclic peptide capreomycin are important second-line drugs used to treat patients with MDRTB. Despite not belonging to the same group of antibiotics, aminoglycosides and cyclic peptides have the same mechanism of action, which consists in the inhibition of protein synthesis. These drugs interfere with several ribosomal functions including the formation of the 30S subunit initiation complex, by blocking tRNA translocation from the A to the P site (Harms J.M. *et al*, 2003). Unlike STR resistance, which is associated with alterations of the ribosomal target site resulting from mutations in the *rpsL* gene (coding for S12 ribosomal protein) or in a specific region of the *rrs* gene of the 16S rRNA, kanamycin and amikacin resistance is associated with mutations in the 1400 region of the *rrs* gene (Alangaden G.J. *et al*, 1998; Douglass J. *et al*, 1993; Meier A. *et al*, 1996a). Mutations in the *rrs* gene associated with resistance to kanamycin and amikacin in *M. tuberculosis* include A1401G (MIC > 128 mg/L), C1402T and G1484T (Jugheli L. *et al*, 2009; Suzuki Y. *et al*, 1998).

In the case of capreomycin, several studies suggest that it interacts at the aminoglycoside site within helix 44 of 16S rRNA and with 23S rRNA, although the site of action on the larger rRNA remains unclear (Maus C.E. *et al*, 2005a). Resistance to capreomycin is associated not only to mutations in the 16S rRNA gene (*rrs*) like the

A1401G alteration, but also to mutations in the *tlyA* gene, encoding a putative rRNA methyltransferase (Johansen S.K. *et al*, 2006; Maus C.E. *et al*, 2005b). Thus, capreomycin resistance in mycobacteria is due to the loss of the ability of the drug to bind to and inhibit ribosomes, because of the lack of methylation of rRNA (*tlyA* mutation) or changes in the 16S rRNA (*rrs* mutation).

I.5.2.2.2 Fluoroquinolones

Fluoroquinolones are part of the drug regimens recommended for treatment of MDRTB (Blumberg H.M. *et al*, 2003). Fluoroquinolones inhibit the bacterial type II topoisomerase DNA gyrase and topoisomerase IV. These ATP-dependent enzymes act by a transient double-stranded DNA break and cooperate to facilitate DNA replication (Levine C. *et al*, 1998). In particular, DNA gyrase catalyzes the negative supercoiling of DNA and is essential for efficient DNA replication, transcription and recombination, whereas topoisomerase IV has a specialized role in chromosome segregation. DNA gyrase is a tetrameric A2B2 protein. The A subunit carries the breakage-reunion active site, whereas the B subunit promotes ATP hydrolysis. Genes coding for DNA gyrase were identified in the *M. tuberculosis* genome as *gyrA* and *gyrB* that code for the A and B subunits, respectively (Madhusudan K. *et al*, 1994). However, there is no evidence of the topoisomerase IV *parC* and *parE* gene homologs in the genome of *M. tuberculosis* (Cole S.T. *et al*, 1998). Thus, it appears that DNA gyrase is the only topoisomerase target for fluoroquinolones in *M. tuberculosis*.

Resistance to fluoroquinolones is thought to occur mainly by mutations in the quinolone resistance-determining region (QRDR) of the *gyrA* gene. However, resistance associated with mutations in the *gyrB* gene was only observed in *in vitro* selected strains and to date only mutations in the QRDR of *gyrA* were described in clinical isolates of *M. tuberculosis* (Guillemin V. *et al*, 1998; Kocagoz T. *et al*, 1996; Takiff H.E. *et al*, 1994). The association between mutations in this region and fluoroquinolone

resistance is attributed to the conservation of the region and its involvement in the interaction between fluoroquinolones and DNA gyrase (Alangaden G.J. *et al*, 1995; Ginsburg A.S. *et al*, 2003; Williams K.J. *et al*, 1996).

I.5.2.2.3 Ethionamide

Ethionamide is structurally similar to INH and also inhibits mycolic acid biosynthesis (Winder F.G. *et al*, 1971). It was demonstrated that a single aminoacid mutation of *inhA*, S94A, as well as overexpression of *inhA* confered resistance to both ethionamide and INH in *M. smegmatis*, *M. bovis* and *M. tuberculosis* (Banerjee A. *et al*, 1994; Larsen M.H. *et al*, 2002; Vilcheze C. *et al*, 2006; Wilson T.M. *et al*, 1995). Several *M. tuberculosis* clinical isolates resistant to INH contain mutations in the *inhA* gene, and have been found to be cross-resistant to ethionamide (Morlock G.P. *et al*, 2003). This demonstrated that the primary target of INH and ethionamide was InhA, the enoyl-acyl ACP reductase involved in mycolic acid biosynthesis. Like INH, ethionamide is also a prodrug that requires activation in order to have anti-TB activity. However, KatG mutant strains resistant to INH are suscepible to ethionamide, suggesting that ethionamide has a different activator (Morlock G.P. *et al*, 2003). Mutations in the *ethA* gene were found in clinical isolates resistant to ethionamide (De Barber A.E. *et al*, 2000; Morlock G.P. *et al*, 2003). Like *katG* and INH, the overexpression of *ethA* in *M. smegmatis* resulted in increased susceptibility to ethionamide (Baulard A.R. *et al*, 2000). This suggested that the product of *ethA,* a membrane associated flavin mono-oxygenase enzyme, plays a crucial role in the activation of ethionamide (Vannelli T.A. *et al*, 2002). The active form of ethionamide has never been isolated *in vitro*, although some inactive metabolites produced by the catalytic oxidation of ethionamide by EthA have been studied by thin layer chromatography and high performance liquid chromatography (De Barber A.E. *et al*, 2000).

I.5.2.2.4 D-Cycloserine

D-Cycloserine is an analog of D-alanine and inhibits peptidoglycan biosynthesis in *M. tuberculosis*. Peptidoglycan contains the aminoacids D-alanine, D-glutamate and diaminopimelate. D-Alanine is one of the central molecules of the cross-linking step of peptidoglycan assembly and three enzymes are involved in the D-alanine branch of peptidoglycan biosynthesis: the pyridoxal phosphate-dependent D-alanine racemase (Alr); the ATP-dependent D-alanine:D-alanine ligase (Ddl); and the ATP-dependent D-alanine:D-alanine-adding enzyme (MurF) (Feng Z. *et al*, 2003; Walsh C.T., *et al* 1989). Previous studies demonstrated that D-cycloserine inhibits *M. tuberculosis* Alr and Ddl enzymes (David H. *et al*, 1969; Strych U. *et al*, 2001). It was suggested that D-cycloserine resistance in *M. tuberculosis* is primarily due to mutations in the *ddl* gene and that inhibition of Alr plays only a minor role in the mechanism of action of D-cycloserine (David H. *et al*, 1969; David H., 1971). However, another study showed that a strain overexpressing both the *alr* and *ddl* genes presented an increased level of resistance to D-cycloserine and that Ddl was not significantly affected by D-cycloserine at the concentration that inhibits Alr (Feng Z. *et al*, 2003). The authors suggested that Alr overproduction contributed to the maintenance of the internal D-alanine pool, antagonizing the inhibition of Ddl by D-cycloserine. In fact, D-cycloserine treatment in *M. smegmatis* decreased the intracellular level of D-alanine in strains with wild-type Alr activity, and overproduction of Alr maintained a relatively abundant level of D-alanine. It was also demonstrated that overexpression of the *ddl* gene confered D-cycloserine resistance, but at lower levels than those for strains overproducing Alr (Feng Z. *et al*, 2003).

However, *M. smegmatis alr* null mutants are not dependent on D-alanine for growth, indicating that there is another pathway for D-alanine biosynthesis (Chacon O. *et al*, 2002). A hypothesis could be that Alr is a high-affinity D-cycloserine binding target that would protect Ddl, a low-affinity target from drug inhibition. According to this

hypothesis, the major mechanism of resistance to D-cycloserine is the overproduction of the high-affinity but otherwise non essential target, while the bactericidal effect is due to the inhibition of the low-affinity lethal target (Feng Z. *et al*, 2003). However, one can not exclude the possibility that the bactericidal effect of D-cycloserine may result from the inhibition of a different target(s). In conclusion, more studies are needed in order to identify the exact target(s) of D-cycloserine.

I.5.2.2.6 ρ-aminosalicylic acid

ρ-aminosalicylic acid was one of the first antibiotics found to be effective in the treatment of TB in the 1940's and was used later with INH and STR in combination chemotherapy against *M. tuberculosis* (Lehmann J., 1946; Murray J.F., 2004.). However, ρ-aminosalicylic acid caused gastrointestinal toxicity leading to poor patient compliance (Pugh D.L. *et al*, 1952). Consequently, the use of ρ-aminosalicylic acid decreased as more easily tolerated antibiotics became available. Recently, ρ-aminosalicylic acid has been reintroduced in anti-TB therapy due to a new formulation of the drug that has fewer side effects and the emergence of MDRTB that required alternatives to the first-line anti-TB agents (WHO, 2000). However, despite the long history of ρ-aminosalicylic acid use in therapy, its mechanism of action remains to be clarified. ρ-aminosalicylic acid has structural similarities to sulphonamides, structural analogues of ρ-amino benzoic acid (PABA), which suggests that it might compete with PABA for dihydropteroate synthase (DHPS), an enzyme involved in folate biosynthesis. However, unlike sulphonamides, ρ-aminosalicylic acid appears to be a poor inhibitor of DHPS *in vitro*, raising the possibility that it may have a different target (Nopponpunth V. *et al*, 1999). However, the lack of inhibition *in vitro* does not prove that DHPS is not the *in vivo* target. One possibility is that ρ-aminosalicylic acid accumulates in the bacterial cells, leading to inhibition of DHPS activity *in vivo*. Other hypothesis is that ρ-aminosalicylic acid might be converted to an active form inside bacterial cells, like INH

and PZA (Somoskovi A. *et al*, 2001). It is known that mutations on *thyA* that codes for thymidylate synthase A, an enzyme required for thymine biosynthesis, are associated with resistance to folate antagonists (Rengarajan J. *et al*, 2004). Thymidylate synthase A catalyzes the reductive methylation of dUMP to dTMP, required for *de novo* dTTP synthesis (Kunz B.A. *et al*, 1991). Since thymidylate synthase A is a major consumer of reduced folate in bacteria, when enzyme activity decreases or is inhibited, utilization of THF derivatives is also diminished. Thus, more reduced folate becomes available for other essential reactions allowing the bacteria to survive in the presence of a folate antagonist, such as ρ-aminosalicylic acid (Rengarajan J. *et al*, 2004).

A recent study investigated the mutations associated with ρ-aminosalicylic acid resistance in *M. tuberculosis* clinical isolates and ρ-aminosalicylic acid resistant spontaneous mutants (Mathys V. *et al*, 2009). The following genes were analyzed: *thyA*, *dfrA*, *folC*, *folP1*, and *folP2* that code for enzymes of the folate pathway; *thyX*, encoding an alternative thymine biosynthetic enzyme; and three N-acetyltransferase genes (*nhoA*, *aac*(1), and *aac*(2)) possibly associated with the modification of ρ-aminosalicylic acid. Sixty-three percent of the ρ-aminosalicylic acid resistant isolates had no mutations in the studied genes, suggesting that ρ-aminosalicylic acid resistance mechanism in *M. tuberculosis* is associated with others than those involved in the biosynthesis of thymine nucleotides.

I.5.3 The relation between intrinsic resistance and genetic resistance

It has now become generally accepted that the overall bacterial resistance to antimicrobial agents is due not just to one resistance mechanism, but to a synergy between intrinsic resistance and genetic resistance (Nikaido H., 2009). As an example, it has been shown that efflux mechanisms appear prior to acquisition of target gene mutations and that overproduction of efflux pumps results in an increase in antibiotic

resistance (Baucheron S. *et al*, 2004; Quinn T. *et al*, 2006). Moreover, it has been suggested that the combination of a diminished outer membrane permeability, due to porin alterations, and an increased activity of efflux pumps, decrease the antibiotic concentration in the cell (Davin-Regli A *et al*, 2008; Mallea M. *et al*, 1998; Viveiros M. *et al*, 2007). This allows the bacteria to survive for a longer period of time than what may have been expected according to the minimum inhibitory concentration for that organism in the presence of that antibiotic, conferring a low-level resistance phenotype. It is possible that this prolonged survival under antibiotic pressure may increase the probability of spontaneous mutants, which contain mutations in genes encoding the target protein, to emerge from this subpopulation of bacteria presenting a high-level resistance phenotype (Pagès J.M. *et al*, 2009; Piddock L., 2006a; Quinn T. *et al*, 2006). This fact may be particularly relevant in the case of the long-term therapy such as that used in TB treatment, where a sustained pressure of sub-inhibitory concentrations of an antibiotic can result in an increased efflux activity and allow the selection of spontaneous mutants, thus rendering the organism resistant to that antibiotic.

A way to prevent these events from occurring could be the inhibition of efflux pumps, which would restore the activity of antibiotics that are subject to efflux. If efflux pumps play, indeed, a role on the selection/stabilization of mutants, these should appear with decreased frequency in the presence of an efflux inhibitor than in its absence. By this manner, it is necessary to understand the organization of efflux pumps and the mechanisms behind efflux-mediated resistance.

I.6 Efflux Pumps

Bacterial efflux pumps are known for their association with antimicrobial resistance. However, the presence of efflux pumps in bacteria is previous to the development of antibiotics. Therefore, their natural physiological role is not related with the use of antibiotics, but instead consists in the extrusion of noxious agents from the cell,

allowing the bacteria to survive in a hostile environment (Piddock L.J., 2006; Poole K., 2007). An example of a natural function of efflux pumps is the secretion of intracellular metabolites and protection against bile salts and fatty acids in enteric bateria as a response to their natural environment.

Antimicrobial resistance due to an increased efflux activity can be caused by the genetic overexpression of the efflux pump, or by aminoacid substitutions in the protein itself that can render the pump more efficient. Both mechanisms cause the reduction of the intracellular concentration of the antimicrobial and, consequently, the organism becomes less susceptible to that agent.

Efflux pumps may be substrate-specific or transport a broad range of structurally dissimilar compounds (including antibiotics of multiple classes), the latter of which may be associated with multiple drug resistance. Genes coding for efflux pumps can be found on the bacterial chromosome or on transmissible elements such as plasmids (Piddock L.J., 2006; Poole K., 2007). The following sections will address the characteristics of efflux pumps, their role on drug resistance and strategies to prevent efflux-mediated multidrug resistance.

I.6.1 Classes and Organization of Efflux Pump Systems

Efflux pump systems can be organized into five different families according to their energetic and structural characteristics (Figure I.10): the ATP-binding cassette (ABC) superfamily; the major facilitator superfamily (MFS); the multidrug and toxic compound extrusion (MATE) family; the small multidrug resistance (SMR) family; and the resistance nodulation division (RND) family. Efflux pumps that are included in the ABC superfamily are considered primary transporters because they hydrolyze ATP as a source of energy, whereas the other families of efflux pumps use the proton (or sodium

in the case of MATE family) gradient as an energy source and are thus called secondary transporters (Lomovskaya O. *et al*, 2007; Marquez B., 2005; Piddock L.J, 2006; Poole K., 2007).

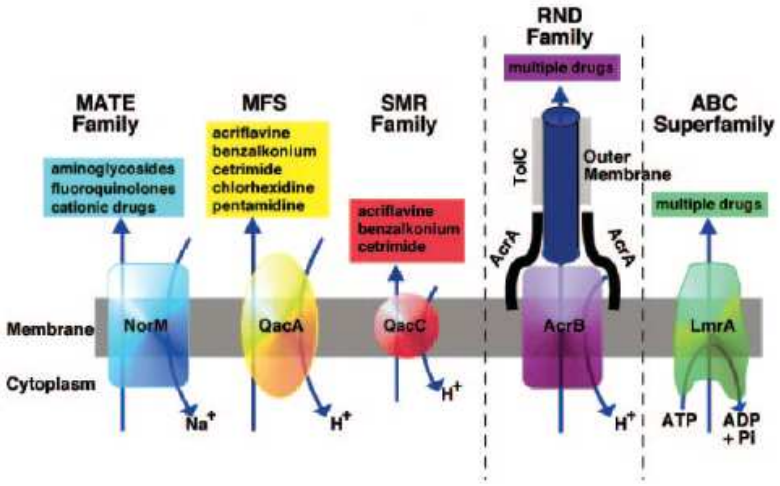

Figure I.10. Schematic representation of the different classes of efflux pump systems (Piddock L.J., 2006).

o ATP-binding cassette (ABC) superfamily

ABC transporters are involved in different transport functions such as the efflux of toxins, metabolites and drugs. These systems consist of two cytoplasmic domains that bind ATP and two hydrophobic transmembrane domains (Davidson A.L. *et al*, 2008; Higgins C.F., 2001). The nucleotide binding domains are highly homologous and possess the Walker A and B motifs, common to all ATP-binding proteins, and a Signature Motif specific to ABC transporters (Davidson A.L. *et al*, 2008; Kerr I.A., 2002). One of the most studied ABC transporters is the mammalian P-glycoprotein (P-gp) that, when overexpressed, confers resistance to compounds used in cancer chemotherapy (Lage H., 2003). Other ABC transporters have been described in Gram-positive and Gram-negative bacteria (Davidson A.L. *et al*, 2008; Lubelski J. *et al*, 2007). An example is LmrA, a well studied ABC transporter from *Lactococcus lactis* that confers multidrug resistance and presents structural and functional homologies

with the human P-gp (Poelarends G.P. *et al*, 2002; Lubelski J. *et al*, 2007). Genome sequencing revealed putative LmrA homologues in other bacteria such as *E. coli*, *Bacillus subtilis*, *Helycobacter pylori*, *Haemophillus influenzae*, *Mycoplasma genitalium* and *Staphylococcus aureus* (Davidson A.L. *et al*, 2008; Lubelski J. *et al*, 2007).

o Major facilitator superfamily (MFS)

MFS is a large superfamily of transporters involved in symport, uniport or antiport of various substrates. Most proteins are 400-600 aminoacid residues in size and possess either 12 or 14 putative transmembrane domains (Fluman N. *et al*, 2009; Law C.J. *et al*, 2008; Saidijam M. *et al*, 2006). An example of an MFS drug efflux protein in Gram-negative bateria is the MdfA from *E. coli*, which can extrude compounds like chloramphenicol and various cationic compounds (Bibi E. *et al*, 2001). MdfA is a typical MFS 12 transmembrane helix protein with a large and complex multidrug recognition pocket. In addition to its function as a multidrug transporter, other studies revealed that MdfA plays a physiological role in alkaline pH homeostasis, possibly through its K^+/proton antiporter activity (Lewinson O. *et al*, 2004). EmrD is another MFS drug/proton antiporter from *E. coli* and was first identified as an efflux pump for uncouplers of oxidative phosphorylation, which can rapidly inhibit bacterial growth by depleting the proton gradient (Naroditskaya V. *et al*, 1993). It was later discovered that EmrD could also transport detergents such as benzalkonium and sodium dodecylsulfate (Nishino K. *et al*, 2001). EmrD is a close homologue to other MFS transporters, including MdfA from *E. coli*, NorA from *S. aureus*, LmrP from *L. lactis* and Bmr *from B. subtilis* (Paulsen I.T. *et al*, 1996a).

o Multidrug and toxic compound extrusion (MATE) family

Efflux pumps of the MATE family have been described for various bacteria, including *Vibrio parahaemolyticus* (NorM), *Vibrio cholerae* (VcrM; VcmA), *Bacteroides*

thetaiotaomicron (BexA), *Haemophilus influenzae* (HmrM), *Pseudomonas aeruginosa* (PmpM), *Clostridium difficile* (CdeA), and *S. aureus* (MepA) (McAleese F. *et al*, 2005; Omote H. *et al*, 2006; Otsuka M. *et al*, 2005). Most MATE members consist of 400–550 residues with 12 transmembrane helices. Although no apparent consensus sequence is conserved in all MATE proteins, all proteins share 40% sequence similarity. Two energy sources have been identified for MATE transporters: the proton motive force and the sodium ion gradient. Some MATE transporters, such as NorM of *V. parahaemolyticus*, have been shown to be energetically coupled to the sodium ion gradient force across the plasma membrane. This force is established by either a primary Na^+ pump or a Na^+-proton antiporter coupled with respiration. However, in the case of AbeM (*Acinetobacter baumannii*) and PmpM *(P. aeruginosa*) activity is coupled to the proton motive force across the plasma membrane (Omote H. *et al*, 2006). It was demonstrated that these transporters confer resistance against cationic drugs such as ethidium bromide (EtBr), tetraphenylphosphonium (TPP), berberine, acriflavine and norfloxacin (Omote H. *et al*, 2006). In particular, MepA from *S. aureus* has been associated with resistance to tigecycline, an antibiotic that shows antimicrobial activity against methicillin-resistant *S. aureus* (MRSA) (McAleese F. *et al*, 2005).

o Small multidrug resistance (SMR) family

The smallest secondary transporters belong to the SMR family. These proteins are typically around 110 aminoacid residues in length with 4 predicted transmembrane helices. SMR transporters can confer resistance to several compounds, such as methyl viologen, TPP, benzalkonium, cetyltrimethylammonium bromide, cetylpyridinium chloride, EtBr, acriflavine, proflavin, crystal violet, pyronine Y and safranin O (Bay D.C. *et al*, 2008). The most characterized members of this family are the plasmid encoded Smr from *S. aureus*, which confers resistance to EtBr and other quartenary compounds, and chromosomal encoded EmrE from *E. coli* that is now considered the

structural archetype of all SMR proteins (Paulsen I.T. *et al*, 1996b; Schuldiner S., 2007; Tate C.G., 2006). EmrE confers resistance to monovalent cations, such as ethidium, proflavine, pyronin Y and safranin O, as well as to erythromycin, sulfadiazine, TPP and TET.

o Resistance nodulation division (RND) family

RND transporters have been mostly studied in Gram-negative bacteria, because they are involved in intrinsic antibiotic resistance in these microorganisms. However, in Gram-positive bacteria their function is still mostly unknown. These transporters present a broad range of substrates and can extrude positive, negative or neutral charged molecules, hydrophobic and hydrophilic compounds (Piddock L.J., 2006; Poole K., 2007). Most RND transporters are composed of a polypeptide chain with 700-1300 aminoacid residues and are predicted to span the membrane 12 times with two large periplasmic domains located between transmembrane helices 1 and 2 and between 7 and 8 (Seeger M.A. *et al*, 2008). Examples of the RND superfamily include AcrB and AcrF from *E. coli*, MexB from *P. aeruginosa* and MtrD from *Neisseria gonorrhoeae*. Two other putative *E. coli* proteins, AcrD and YhiV, may also be RND multidrug efflux proteins (Piddock L.J., 2006; Poole K., 2007). These transporters combine with membrane fusion proteins and outer membrane proteins (also called outer membrane factor) to form a tripartite efflux pump system. The most studied example of such structure is the AcrAB-TolC system of *E. coli* (Figure I.11). This tripartite efflux system comprises the following components: a RND transporter protein in the inner membrane (AcrB), a periplasmic membrane fusion protein (AcrA) and an outer membrane protein channel (TolC) (Piddock L.J., 2006; Poole K., 2007). AcrB binds the substrate within either the phospholipid bilayer of the inner membrane of the bacterial cell-wall or the cytoplasm and transports them to the exterior of the cell using TolC. AcrA mediates the cooperation between AcrB and TolC (Piddock L.J., 2006;

Seeger M.A. *et al*, 2008). The AcrAB-TolC system transports a wide range of antibiotics such as β-lactams (*e.g.* oxacillin), macrolides (*e.g.* erythromycin), fluoroquinolones (*e.g.* ciprofloxacin) and tetracyclines, RIF, novobiocin, fusidic acid and nalidixic acid, and also other compounds such as EtBr, acriflavine, bile salts and short-chain fatty acids (Piddock L.J., 2006; Poole K., 2007).

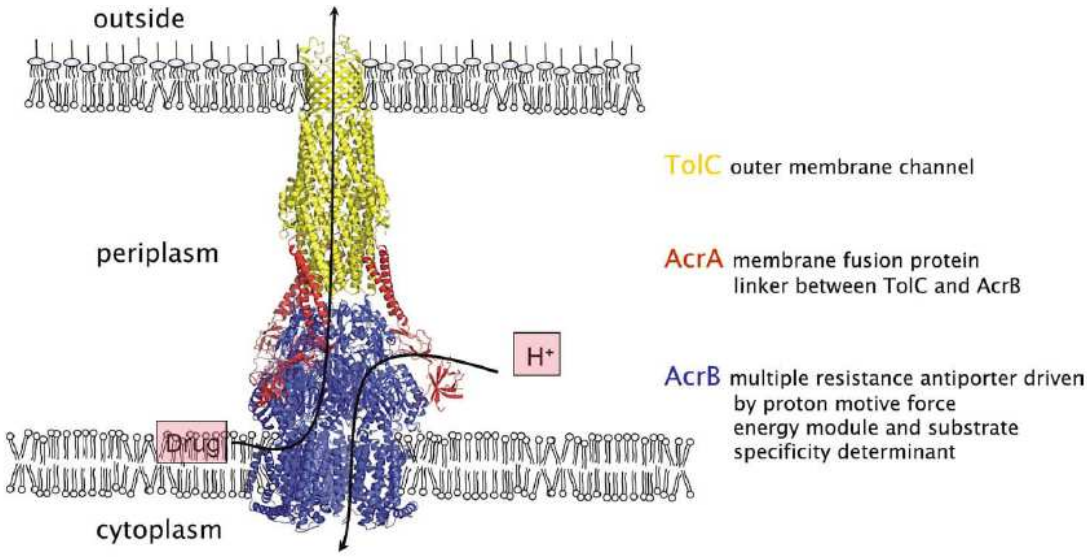

Figure I.11.Schematic representation of AcrAB–TolC of *E. coli*. AcrB is responsible for substrate recognition and energy transduction. Drugs are extruded in exchange with protons. TolC forms a pore in the outer membrane, which is extended by a long periplasmic channel. AcrA mediates the interaction between AcrB and TolC (Seeger M.A. *et al*, 2008).

I.6.2 Efflux pumps in mycobacteria

Several mycobacterial drug efflux pumps have been identified and characterized to the present day (Louw G.E. *et al*, 2009; Viveiros M. *et al*, 2003). Table I.2 summarizes most of these pumps and the most characterized transporters are discussed bellow:

o Genes encoding ABC transporters comprise approximately about 2.5% of the *M. tuberculosis* genome and at least 37 complete and incomplete ABC transporters have been identified (Braibant M. *et al*, 2000). However, only a few of these transporters have been characterized and shown to be involved in drug resistance. *M. tuberculosis* contains a putative doxorubicin-resistance operon, *drrABC* (Choudhuri B.S. *et al*, 2002). The DrrAB genes expressed in *M. smegmatis* confer resistance to a broad range of antibiotics, including TET, erythromycin, ethambutol, norfloxacin, STR and chloramphenicol. The resistant phenotype is reversed by treatment with reserpine or verapamil, compounds known to inhibit efflux (Choudhuri B.S. *et al*, 2002). Studies have suggested that the main role of the Drr proteins of *M. tuberculosis* may be the export of lipids to the exterior of the cell and, in particular, DrrC seems to be involved in the transport of phthiocerol dimycocerosates (Camacho L.R. *et al*, 2001). The *M. tuberculosis* Rv2686c-Rv2687c-Rv2688c operon encodes an ABC transporter responsible for fluoroquinolone efflux when produced from a multicopy plasmid. When overexpressed in *M. smegmatis,* this operon increases 8-fold the MIC of ciprofloxacin and 2-fold the MIC of norfloxacin. The level of resistance decreases in the presence of reserpine, carbonyl cyanide m-chlorophenylhydrazone (CCCP) and verapamil (Pasca M.R. *et al*, 2004).

o The plasmid-encoded TET efflux pump Tet(V) was isolated from *M. smegmatis* and, when overexpressed, increases the MIC of TET from 2- to 4-fold. The distribution of the *tet(V)* gene among the genus *Mycobacterium* has been investigated by polymerase chain reaction (PCR). However, *M. smegmatis* and *M. fortuitum* were the only species tested that revealed a *tet(V)* gene (De Rossi E. *et al*, 1998a).

Table I.2. Efflux pump genes and transporters associated with drug resistance in mycobacteria (adapted from Louw G.E. *et al*, 2009).

Efflux pump	Microorganism	Possible substrates	Family	Ref.
PstB	*M. smegmatis* *M. tuberculosis*	INH, RIF, EMB, CIP	ABC	Bhatt K. *et al*, 2000
Rv2686c-Rv2687c-Rv2688c	*M. tuberculosis*	CIP	ABC	Pasca M.R. *et al*, 2004
Rv1747	*M. tuberculosis*	INH	ABC	Braibant M. *et al*, 2000
DrrA-DrrB-DrrC	*M. tuberculosis*	TET, STR, EMB	ABC	Choudhuri B.S. *et al*, 2002
Tet(V)	*M. smegmatis*	TET	MFS	De Rossi E. *et al*, 1998a
LfrA	*M. smegmatis*	Fluoroquinolones, EtBr, acriflavine	MFS	Takiff H.E. *et al*, 1996
Rv1258c (Tap homologue)	*M. tuberculosis*	INH, RIF, EMB, OFL	MFS	Siddiqi N. *et al*, 2004
Rv1877	*M. tuberculosis*	TET, KAN, ERY	MFS	Li X.Z. *et al*, 2004
Rv1634	*M. tuberculosis*	Fluoroquinolones	MFS	De Rossi E. *et al*, 2002
EfpA	*M. tuberculosis*	Possibly INH	MFS	Doran J.L. *et al*, 1997
P55	*M. tuberculosis* *M. bovis*	Aminoglycosides, TET, RIF	MFS	Silva P.E. *et al*, 2001
Tap	*M. fortuitum*	Aminoglycosides, TET	MFS	Ainsa J.A. *et al*, 1998
MmpL7	*M. tuberculosis*	INH	RND	Pasca M.R. *et al*, 2005
Mmr	*M. tuberculosis*	TPP, EtBr, ERY, acriflavine	SMR	De Rossi E. *et al*, 1998b
IniA-IniB-IniC	*M. tuberculosis*	INH	Membrane protein	Colangeli, R. *et al*, 2005

ABC, ATP-binding cassette; CIP, ciprofloxacin; INH, isoniazid; EMB, ethambutol; EtBr, ethidium bromide; ERY, erythromycin; KAN, kanamycin; MFS, major facilitator superfamily; OFL, ofloxacin; RIF, rifampicin; RND, resistance nodulation division; SMR, small multidrug resistance; STR, streptomycin; TET, tetracycline; TPP, tetraphenyl phosphonium.

o The LfrA protein of *M. smegmatis* was the first efflux pump to be described in the genus *Mycobacterium* and may be responsible for low-level resistance to fluoroquinolones, acridine and quaternary ammonium compounds (Liu J. *et al*, 1996; Sander P. *et al*, 2000; Takiff H.E. *et al*, 1996). When overexpressed, LfrA seems to play an important role in the resistance to ciprofloxacin in *M. smegmatis*. However, in the absence of overexpression, LfrA is thought to have no effect on the susceptibility to fluoroquinolones. In fact, disruption of the *lfrA* gene decreased 8-fold the MIC of EtBr and acriflavine and decreased only 2-fold the MICs of ciprofloxacin, doxorubicin and rhodamine (Li X.Z. *et al*, 2004). This discrepancy of results between EtBr and ciprofloxacin can be explained if EtBr is a better substrate for LfrA (Li X.Z. *et al*, 2004). Other explanation resides in the fact that efflux pumps other than LfrA may extrude ciprofloxacin in *M. smegmatis*. The region upstream from the *lfrA* gene revealed the presence of an open reading frame encoding a putative polypeptide of 195 amino acids, LfrR, homologous to several transcriptional regulators of the TetR family (Li X.Z. *et al*, 2004). The *lfrR* and *lfrA* genes are organized into an operon probably controlled by LfrR. It has been demonstrated that deletion of the *lfrR* gene enhances *lfrA* expression, increasing the resistance to ciprofloxacin, norfloxacin, EtBr and acriflavine (Li X.Z. *et al*, 2004). No known homologue of the *lfrA* gene as been described in the *M. tuberculosis* genome (De Rossi E. *et al*, 2002).

o The *M. fortuitum* Tap efflux pump and its *M. tuberculosis* homologue Rv1258c confer resistance to TET and aminoglycosides, including STR (Ainsa J. *et al*, 1998). When cloned on a plasmid, Tap increased the resistance of *M. smegmatis* mc^2155 to gentamicin, STR and TET (Ainsa J.A. *et al*, 1998). In the case of a clinical strain of *M. tuberculosis,* the expression of *rv1258c* increased in the presence of RIF and ofloxacin (Siddiqi N. *et al*, 2004). Furthermore, TET accumulation experiments showed that the efflux activity of Tap from *M. fortuitum* is inhibited by CCCP and reserpine, a result

consistent with the decrease of the MIC of this antibiotic in the presence of these compounds. In addition, CCCP, reserpine and also chlorpromazine reduced the MIC of TET in a *M. smegmatis* strain expressing the Tap protein (Ramon-Garcia S. *et al*, 2006).

o Rv1634 is a MFS efflux pump that has been suggested as a new fluoroquinolone efflux transporter in *M. tuberculosis* (De Rossi E. *et al*, 2002). It was demonstrated that this pump decreased susceptibility to various fluoroquinolones when overexpressed in *M. smegmatis*. Furhermore, accumulation assays suggest that Rv1634 is also involved in norfloxacin and ciprofloxacin efflux (De Rossi E. *et al*, 2002).

o The P55 efflux pump from *M. bovis* and *M. tuberculosis* has been associated with low-level resistance to several drugs including TET, aminoglycosides and RIF (Ramon-Garcia S. *et al*, 2009; Silva P.E. *et al*, 2001). Rv1410c, the gene for P55 in *M. tuberculosis*, forms an operon with Rv1411c, encoding the lipoprotein LprG. It is thought that both genes support *in vivo* growth of *M. tuberculosis* and studies performed in *M. smegmatis* have shown that this operon is required for survival in the presence of EtBr and for normal cell surface composition (Bigi F. *et al*, 2004; Farrow M.F. *et al*, 2008). A recent study has demonstrated that P55 plays a role in at least three important processes: (i) it extrudes and provides resistance to several drugs (including rifampicin); (ii) it is part of the oxidative stress response; and (iii) it is needed to maintain normal growth characteristics on solid and in liquid media (Ramon-Garcia S. *et al*, 2009).

o The *M. tuberculosis* putative efflux protein EfpA presents the transporter motifs characteristic of QacA of *S. aureus*, including those associated with proton antiporter function and those specific to drug transporters. It was shown that expression of *efpA*

increases in the presence of INH, which could suggest that the protein encoded by this gene transports molecules involved in mycolic acid synthesis (Wilson M. *et al*, 1999). The deletion of the *efpA* homologue in *M. smegmatis* resulted in a 2-fold increased susceptibility to EtBr, gentamicin and fluoroquinolones and an 8-fold increased susceptibility to acriflavine. However, it also resulted in a 4-fold decreased susceptibility to rifamycins and chloramphenicol and a 2-fold decreased susceptibility to INH and erythromycin (Li X.Z. *et al*, 2004). Moreover, this *efpA* deleted mutant grew more slowly than the wild-type strain, which could mean that its higher susceptibility may be because of impaired growth (Li X.Z. *et al*, 2004). Thus, the role of EfpA in drug resistance remains unclear. However, since the deletion of this gene increases susceptibility to EtBr, it is pssible that EtBr is a substrate of this pump (Li X.Z. *et al*, 2004).

o The genome of *M. tuberculosis* contains several genes that code for putative transport proteins of the RND superfamily. These proteins have been designated MmpL (mycobacterial membrane proteins, large) and are thought to be involved in the transport of fatty acids (Tekaia F. *et al*, 1999). In *M. tuberculosis*, MmpL7 exports phthiocerol dimycocerosate (PDIM), a lipid component of the outer membrane (Camacho L.R. *et al*, 2001). Upstream from the *mmpL7* gene is the *fadD28* gene, which encodes an acyl-CoA synthase probably involved in the release and transfer of mycocerosic acid from mycocerosic acid synthase to diols. A strain with an insertion in *mmpL7* produces a dimycocerosate (DIM) molecule that is retained in the cytoplasm or the cytoplasmic membrane. The production of MmpL7 in *M. smegmatis* promotes a 32-fold increase of the MIC of INH. However, this phenotype is reversed if *fadD28* and *mmpL7* are expressed simultaneously, which suggests that DIM and INH compete for the same MmpL7 transporter (Pasca M.R. *et al*, 2005).

o Mmr is the only protein of the SMR family that has been described in *M. tuberculosis* (De Rossi E. *et al*, 1998b). The chromosomal gene *mmr*, when inserted into a multicopy plasmid, decreases the susceptibility of *M. smegmatis* to TPP, EtBr, erythromycin, acriflavine, safranin O and pyronin Y. Accumulation assays have shown that Mmr extrudes TPP, in a process dependent of the proton motive force. The presence of *mmr*-like genes in other *Mycobacterium* species (*M. simiae*, *M. gordonae*, *M. marinum* and *M. bovis*) has been demonstrated by Southern hybridization (De Rossi E. *et al*, 1998b).

o The operon Rv0341-Rv0342-Rv0343 was demonstrated to be induced by treatment with INH (Alland D. *et al*, 1998). The three genes that form this operon were designated as *iniB*, *iniA* and *iniC* (for isoniazid inducible gene) in the order that they appeared in the operon. From the three genes the most studied is *iniA* and it has been shown that it may be involved in the development of tolerance to INH and EMB (Colangeli R. *et al*, 2005). In fact, deletion of *iniA* from *M. tuberculosis* increased the susceptibility to INH, whereas the overexpression of this gene in *M. bovis* allowed the survival of the organism for a longer period of time in the presence of INH and EMB and also resulted in resistance to EtBr. The exposure of the *iniA* overexpressing *M. bovis* BCG strain to reserpine reversed both tolerance to INH and resistance to EtBr. The fact that IniA forms multimeric structures containing a central pore suggests that this protein could be a pump component. By this manner, IniA may function through an efflux pump like mechanism, although it does not seem to directly transport INH from the bacterial cell (Colangeli R. *et al*, 2005).

I.6.3 Efflux pump inhibitors and the “helper compound” concept

The contribution of efflux pumps to the increase of multidrug resistance has made these pumps new drug targets for the development of new antimicrobial drugs that can

be used in combination with an antibiotic (Martins M. *et al*, 2008; Pagès J.M. *et al*, 2009). Strategies to combat efflux mediated resistance involve the modification of antibiotics into derivatives that are minimally affected by efflux and the development of therapeutic agents that inhibit the efflux activity (efflux pump inhibitors, EPIs) and can be used as adjuvants (also called 'helper compounds') to existing antibiotics. However, to be strictly definied as an EPI, a compound has to satisfy several criteria: 1) it must enhance the activity of multiple substrates of the pump; 2) it must not potentiate antibiotics that are not efflux substrates; 3) it must not have activity in strains lacking efflux pumps; 4) it must increase accumulation and decrease extrusion of efflux pump substrates; and 5) it must not affect the proton gradient across the inner membrane (Lomovskaya O. *et al*, 2001). Although until now no EPI has fully answered these criteria nor reached or passed clinical trials for the treatment of bacterial infections, several compunds have been used to inhibit efflux activity *in vitro*. A classic example is CCCP, which affects the energy level of the bacterial membrane and is used to dissipate the proton motive force and inhibit the efflux of several drugs. However, this compound also reduces the viability of the bacterium and cause cell death and, therefore, the observed effect on the efflux activity may be due to causes other than efflux inhibition. Moreover, this compound is described as highly noxious and cytotoxic and is also a substrate of efflux pumps (Krulwich T.A. *et al*, 1990; Pagès J.M. *et al*, 2009).

Phe-Arg-β-naphthylamine (PAβN) is a peptidomimetic that was described to inhibit the efflux of quinolones in *Pseudomonas aeruginosa* (Renau T.E. *et al*, 2002). This molecule has showed an activity against other efflux systems of *E. coli*, *E. aerogenes*, *Klebsiella pneumoniae* and *Salmonella enterica* (Lomovskaya O. *et al*, 2006; Lomovskaya O. *et al*, 2007; Pagès JM *et al*, 2005). However, the MexB efflux pump of *P. aeruginosa* recognizes PAβN as a pump substrate (Lomovskaya O. *et al*, 2007).

PAβN acts by competition with an antibiotic that is substrate of an efflux pump system. This results in the extrusion of PAβN, while the antibiotic remains in the cell until it reaches its target. By this manner, PAβN reduces/reverses resistance to a certain antibiotic to which the bacteria were initially resistant (Pagès J.M. *et al*, 2009).

It has been demonstrated that phenothiazines, such as thioridazine and chlorpromazine, reduce or reverse multidrug resistance in bacteria (Amaral L. *et al*, 2004; Kristiansen M.M. *et al*, 2003; Michalak K. *et al*, 2006; Molnar J. *et al*, 1997). These compounds inhibit the transport of calcium (Ca^{2+}) by preventing its binding to Ca^{2+}-binding proteins, such as calmodulin (Martins M. *et al*, 2008; Weiss B. *et al*, 1980). As a consequence, enzyme systems dependent of Ca^{2+}, such as those involved in generating cellular energy from hydrolysis of ATP, are inhibited (Garcia J.J. *et al*, 1995). Considering that overexpression of efflux pumps is associated with multidrug resistance and that most of these systems are driven by the proton-motive force, which is dependent of Ca^{2+}-dependent enzyme systems, the inhibition of Ca^{2+}-dependent enzymes will render the bacteria susceptible to an antibiotic to which they were initially resistant (Bhatnagar K. *et al*, 2003; Martins M. *et al*, 2008). Furthermore, it has been shown that phenothiazines also enhance the killing of intracellular bacteria. This can be due to a concentration effect, as the agent reaches lethal concentrations inside the macrophage, or to the inhibition of K^+ and Ca^{2+} transport processes (Wittekindt O.H. *et al*, 2006). Although the concentration effect cannot be ruled out, recent experiments have shown that inhibitors of K^+ and Ca^{2+} transport enhance the killing of intracellular bacteria, further suggesting that phenothiazines enhance killing by the same mechanism (Ahluwalia J. *et al*, 2004; Martins M. *et al*, 2008).

Reserpine is a plant alkaloid known to inhibit P-gp in eukaryotic cells (Marquez B., 2005; Stavri M. *et al*, 2007). Other studies demonstrated that reserpine increases the

activity of fluoroquinolones on multidrug resistant (MDR) Gram-positive bacteria, decreases the emergence of resistance in *S. aureus* and *S. pneumoniae in vitro* and inhibits LmrA, an ABC efflux system of *L. lactis* (Brenwald N. *et al*, 1997; Markham P.N. *et al*, 1996; Markham P.N., 1999; Marquez B., 2005). However, bacterial resistance to reserpine has already been described (Ahmed M. *et al*, 1993).

The calcium channel antagonist verapamil, another known inhibitor of P-gp, also inhibits several bacterial ABC efflux pumps, including EfrAB of *Enterococcus faecalis*, and Rv2686c-Rv2687c-Rv2688c and DrrAB of *M. tuberculosis* (Choudhuri B.S. et al, 2002; Lee E.W. *et al*, 2003; Pasca M.R. *et al*, 2004).

In summary, various compounds have been used to inhibit efflux activity *in vitro*, but none of them is used in clinical practice for this purpose. These inhibitors could be used as ‘helper compounds’, administered in combination with conventional antibiotics to which the organism was initially resistant. However, clinical trials are needed to demonstrate the importance of efflux inhibitors in the therapy of drug resistant bacterial infections. There is also a concern regarding the selectivity of these compounds, since some of them have been shown to inhibit both eukaryotic and bacterial efflux systems (Marquez B., 2005; Martins M. *et al*, 2008). In conclusion, the search for EPIs has proven to be a challenge and more studies are required until effective and specific inhibitors are found.

I.7 Objectives of this thesis

The main objective is to study the role of efflux mechanisms in the development of multidrug resistance in *M. tuberculosis* and how phenotypic resistance, mediated by efflux pumps, correlates with genetic resistance. The concept behind this work consists in the fact that increased activity of efflux pumps can occur in response to prolonged exposure to subinhibitory concentrations of anti-TB compounds, a common situation that results from inadequate TB therapy. This may render an organism increasingly resistant to the compound and be the basis for the maintenance of an increased mycobacterial population in the patient undergoing therapy. The inhibition of mycobacterial efflux would restore activity of an antibiotic subject to efflux and provide a way to enhance the activity of current anti-TB drugs.

In order to evaluate the contribution of efflux to drug resistance in *M. tuberculosis*, several experimental protocols had to be developed and optimized before their application to *M. tuberculosis*. This was performed using biological models such as *E. coli* and the fast growing mycobacteria *M. smegmatis*, since *M. tuberculosis* is a slow-growth mycobacteria and a level III pathogen. By using other microorganisms as experimental models, one can thus overcome the biosafety difficulties and time consuming aspect associated with this pathogen, and obtain reliable methods that are easily applied to *M. tuberculosis*. By this manner, the following approach has been used in this work:

- **Development of a protocol that would allow the quantification of the expression level of genes coding for efflux pumps (Chapter II)**

In order to correlate the bacterial phenotypic resistance provided by efflux pumps with the genetic expression of genes encoding those pumps, it was necessary to develop and optimize a protocol based on the real-time quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) methodology. This was performed using *E. coli*

K-12 as a conceptual model, comparing the mRNA levels extracted from *E. coli* strains induced to resistance to TET with the mRNA extracted from the susceptible non-induced counterparts. The use of *E. coli* as a model allowed not only the development of a protocol that would be applied to *M. tuberculosis*, but also demonstrated a sequence of events concerning the regulation of efflux pumps and porins during the TET adaptation process, thus providing more insight to the complex mechanisms of regulation of efflux activity (Viveiros M. *et al*, 2007).

- **Development of a fluorometric method that allows the real-time detection of efflux pump activity (Chapter III)**

An efflux quantification protocol was developed using real-time fluorometry to measure the accumulation and extrusion of EtBr. Using *E. coli* K-12 AG100 as a model, it was possible to study the accumulation of EtBr under conditions that limit energy (*i. e.* absence of glucose, low temperature) and its extrusion under optimum conditions. It also allowed the determination of the activity of efflux inhibitors, namely chlorpromazine, in the transport of EtBr across the cell-wall. Furthermore, the application of this method allowed the development of a mathematical model that quantifies EtBr transport across *E. coli* cell wall (Paixão L. *et al*, 2009; Viveiros M. *et al*, IJAA, 2008a; Viveiros M. *et al*, 2010a).

- **Application of the fluorometric method to mycobacteria: assessment of EtBr transport across the *M. smegmatis* cell-wall (Chapter IV)**

After the optimization of the main parameters and experimental conditions using *E. coli* K-12 AG100 as a model, the real-time fluorometric method was used for the assessment of efflux pump activity in mycobacteria, starting with the adjustment of parameters using *M. smegmatis* mc^2155 (a frequently used model in mycobacterial research) and mutant strains carrying a deletion of genes coding for an efflux pump or

for porins (Li X.Z. *et al*, 2004; Niederweiss M. *et al*, 1999) and correlated this information with an antibiotic profile. Since many homologous pumps are present in both *M. tuberculosis* and *M. smegmatis*, studies that use *M. smegmatis* as a model organism can provide data that will help to understand efflux-mediated drug resistance mechanisms in other mycobacteria, including *M. tuberculosis* and *M. avium* (Li X.Z. *et al*, 2004; Rodrigues L. *et al*, 2008; Rodrigues L. *et al*, 2010).

- **Study of the role of efflux pumps in antimicrobial resistance: the case of *M. avium* complex and macrolide resistance (Chapter V)**

The study of efflux activity of *M. avium* complex strains is of interest due to their clinical prevalence among HIV-infected patients and will provide data for the selection of the most effective EPIs to be used in *M. tuberculosis* strains. The real-time fluorometric method previously developed was used to detect the efflux activity of reference strains and clinical isolates of *M. avium* complex. This efflux activity was correlated with an antibiotic profile obtained by the determination of MICs of macrolides in the presence and absence of agents such as chlorpromazine, thioridazine and verapamil (Rodrigues L. *et al*, 2008; Rodrigues L. *et al,* 2009).

- **Efflux pumps and isoniazid resistance in *M. tuberculosis* complex (Chapter VI)**

M. bovis BCG strain Pasteur, *M. tuberculosis* H37Rv (ATCC27294) and *M. tuberculosis* clinical strains, susceptible to INH, were induced to resistance by a gradual, step-wise increase of antibiotic concentration over a prolonged period of time (Viveiros M. *et al*, 2002). Evaluation of efflux pump activity as the cause of INH induced resistance was performed using the real-time fluorometric method and the expression of genes coding for efflux pumps was conducted by qRT-PCR. This data coupled with the determination of the MIC of INH in the presence and absence of efflux inhibitors, such as

chlorpromazine, thioridazine, verapamil and reserpine, provided information concerning the role of efflux pumps in the development of INH resistance in *M. tuberculosis* complex.

In summary, this thesis aimed to increase the understanding of the mechanisms of efflux-mediated bacterial resistance, using a working algorithm based on the development of techniques that allow the detection and quantificaton of efflux activity on a real-time basis. The ultimate objective consists in the application of these techniques, and data obtained with experimental bacterial models, to *M. tuberculosis* in order to clarify the role of efflux pumps in the mechanism(s) by which this organism becomes resistant to INH and one step closer to multidrug resistance. Moreover, the clarification of how intrinsic resistance, provided by the increased activity of efflux pumps, correlates with the emergence of genetic resistance, by the occurrence of mutations, will provide further insight on the mechanims behind the emergence of MDR/XDRTB. By this manner, a possible strategy to prevent the emergence of such resistant strains could be the use of efflux inhibitors that would restore the activity of the antibiotic(s) subject to efflux by inhibiting the efflux pump. Furthermore, it has been shown that inhibitors of K^+ and Ca^{2+} efflux pumps enhance the killing of intracellular *M. tuberculosis* by nonkilling macrophages. This double effect wielded by these inhibitors (inhibition of bacterial efflux activity and enhancement of killing activity by the macrophage) may be a potential strategy to be used in the control of MDR/XDRTB (Viveiros M. *et al*, 2010b).

Chapter II

Antibiotic stress, genetic response and altered permeability of *Escherichia coli*

This chapter contains data published in:

- Viveiros M., Dupont M., **Rodrigues L**., Couto I., Davin-Regli A., Martins M., Pagès J.M., Amaral L. 2007. Antibiotic stress, genetic response and altered permeability of *E. coli*. *PLoS One* **2**(4):e365.
- Viveiros M., Jesus A., Brito M., Leandro C., Martins M., Ordway D., Molnar A.M., Molnar J., Amaral L. 2005. Inducement and reversal of tetracycline resistance in *Escherichia coli* K-12 and expression of proton gradient-dependent multidrug efflux pump genes. *Antimicrob. Agents Chemother.* **49**: 3578–3582.

Antibiotic Stress, Genetic Response and Altered Permeability of *Escherichia coli*

II.1 Summary

Membrane permeability is the first step involved in resistance of bacteria to an antibiotic. The number and activity of efflux pumps and outer membrane proteins that constitute porins play major roles in the definition of intrinsic resistance in bacteria that is altered under antibiotic exposure.

In this work we describe, with the aid of real-time qRT-PCR and western blot detection, the genetic regulation of porins and efflux pumps of *E. coli,* used as a working model for the mycobacteria work in the forthcoming chapters, during prolonged exposure to increasing concentrations of TET. This study demonstrates that, in addition to the transcriptional regulation of genes coding for membrane proteins, the post-translational regulation of proteins involved in the permeability of bacteria also plays a major role in the physiological adaptation to antibiotic exposure. A model is presented that summarizes the events that occur during the physiological adaptation of *E. coli* to TET. This work also allowed for the development of an qRT-PCR protocol that would be latter adapted to *M. tuberculosis* (Chapter VI).

II.2 Introduction

Intrinsic antibiotic resistance in Gram-negative bacteria (without chromosomal mutation or acquisition of mobile genetic elements encoding resistance determinants) can be increased by preventing the antibiotic from entering the cell. This can be achieved by the control of the outer membrane permeability and by the efflux of antibiotics (Gootz T.D., 2006; Nikaido H., 2001; Piddock L.J., 2006). Intrinsic resistance to antibiotic agents is mainly attributed to the outer membrane of Gram-negative bacteria that acts as a barrier and delays the influx of various antibiotics, detergents and dyes, and to

efflux pumps, which extrude the drug to the exterior of the cell, enabling the bacteria to survive in the presence of these noxious agents (Davin-Regli A. *et al*, 2006; Nikaido H., 2001). Additional resistance is afforded by overexpressed efflux pumps that extrude a wide variety of unrelated antibiotics. Overexpressed efflux pumps of Gram-negative bacteria result in a MDR phenotype known to be a prevalent form of clinical resistance (Piddock L.J., 2006; Viveiros *et al*, 2007). It was previously demonstrated that it is possible to induce high-level resistance to TET in susceptible *E. coli* K-12 by a gradual, step-wise increase in the exposure to the antibiotic (Viveiros M. *et al*, 2005). The induction process took about 110 days and this resistance was reversed by transfer to drug free medium or by the use of PAβN, an inhibitor of the AcrAB efflux pump system (Davin-Regli A. *et al*, 2006; Piddock L.J., 2006; Viveiros M. *et al*, 2005). The major inner membrane transporter genes of *E. coli* K-12 were overexpressed after prolonged exposure to TET, with *acrB* being the most expressed transporter gene and a clear connection between the induced activity of the AcrAB system and TET induced resistance was demonstrated (Viveiros M. *et al*, 2005). Besides becoming resistant to TET, the induced strain became resistant to a variety of other antibiotics, detergents and dyes that are not substrates of the AcrAB system (Davin-Regli A. *et al*, 2006; Piddock L.J., 2006; Viveiros M. *et al*, 2005). The development of this MDR phenotype led us to explore and analyse the interplay between the major efflux pump systems present in *E. coli* and the control of the outer membrane permeability through the regulation of porin channels. In *E. coli*, outer membrane permeability is regulated by the balance of porin proteins, the diffusion channels that are the major route for passage of small hydrophilic compounds (Nikaido H., 2001; Nikaido H., 2003; Pagès J.M. *et al*, 2005). The two major outer membrane proteins in *E. coli* are OmpC and OmpF, consisting of three 16-stranded β-barrels defining a transmembrane pore in the outer membrane (Basle A. *et al*, 2006; Cowan S.W. *et al*, 1992). Highly expressed under optimal environmental conditions, their level of expression is adjusted when it is

necessary to minimize penetration of noxious compounds or maximize access to nutrients (Ferenci T., 2005; Liu X. *et al*, 2001; Nikaido H., 2003). It has been demonstrated that the level of expression of porins OmpC and OmpF not only controls the permeability of the outer membrane to glucose and nitrogen uptake under nutrient limitation (Ferenci T., 2005; Liu X. *et al*, 2001), but may also be differentially regulated by the concentration of certain antibiotics in the environment (Castillo-Keller M. *et al*, 2006; Davin-Regli A. *et al*, 2006; Randall L.P. *et al*, 2002;). The OmpC and OmpF coding genes are transcriptionally regulated by a two-component signal transduction regulatory system consisting of the OmpR and EnvZ proteins (Hall M.N. *et al*, 1981). Recently, it has been shown that the overexpression of OmpX, structurally related to the eight β-strand OmpA (a major outer membrane protein involved in the stabilization of the bacterial membrane), leads to a decrease in the expression of OmpC and OmpF porins and a decreased susceptibility to β-lactams and other antibiotics in *E. coli* (Dupont M. *et al*, 2004).

Besides porins, efflux pumps also play an important role in the control of bacterial permeability that contributes to the development of an MDR phenotype.

E. coli has been shown to have at least nine distinct proton dependent efflux pump systems that bestow resistance to two or more antibiotics (MDR). The genes coding for each of these efflux pumps are *emrE*, *acrEF* (formerly envCD), *emrAB*, *emrD*, *acrAB-tolC*, *mdfABC*, *tehA*, *acrD* (an *acrB* homologue) and *yhiUV* (Edgar R. *et al*, 1995; Kawamura-Sato K. *et al*, 1999; Lomovskaya O. *et al*, 1992; Ma D. *et al*, 1995; Naroditskaya V. *et al*, 1993; Nishino K. *et al*, 2001; Purewal A.S., 1991; Rosenberg E.Y. *et al*, 2000; Turner R.J. *et al*, 1997). These transporters belong to one of three efflux pump families: MFS (*emrD*, *mdfA*, *emrB*), RND (*acrB*, *acrF*, *acrD*, *yhiV*) and SMR (*emrE*, *tehA*) (Davin-Regli A. *et al*, 2006; Piddock L.J., 2006). The tripartite AcrAB-TolC system is the most well-studied MDR pump system consisting of an inner membrane efflux transporter (AcrB) that removes antibiotics from the cytoplasm to the periplasm,

where the linker protein (AcrA) directs the inter-membrane transport of the antibiotic through the outer membrane channel (TolC) to the environment (Nikaido H., 2001; Pagès J.M *et al*., 2005; Piddock L.J., 2006). The major efflux pump systems in *E. coli* are from the RND family and have broad substrate specificity. Their expression is controlled by transcriptional activators like the MarA, encoded by the multiple antibiotic resistance operon *marRAB*, and homologs like SoxS and Rob (Cohen S.P. *et al*, 1993; Davin-Regli A. *et al*, 2006; Martin R.G. *et al*, 2001). MarA not only controls the expression of efflux systems, but is also involved in the control of porin expression (by decreasing it) through the activation of *micF*, a small antisense RNA that binds with *ompF* mRNA preventing its translation, and activates the expression of the porin expression down-regulator OmpX (Dupont M. *et al*, 2004; Barbosa T.M. *et al*, 2000). These global activators, when induced by oxidative stress or the presence of noxious compounds in the environment, enhance resistance of enterobactereaceae to a variety of antibiotics, hence an MDR phenotype (Davin-Regli A. *et al*, 2006; Delihas N. *et al*, 2001; Nakajima H. *et al*, 1997). Moreover, they control the degree of intrinsic resistance of enterobactereaceae and increase the level of efflux pump expression. The regulation of porin level and expression of multidrug resistance efflux pumps has been suggested to occur by a common pathway and/or a cascade of events (Davin-Regli A. *et al*, 2006; Li X.Z. *et al*, 2004). Studies of step-wise induction of resistance, like the gradual exposure of *E. coli* K-12 to TET, may afford an understanding of the genetic regulation of multidrug resistance efflux pumps, their interplay and relationship to the permeability barrier (Viveiros M. *et al*, 2005). Therefore, with the aid of real-time qRT-PCR and western blot detection we have analysed and correlated the activity of: (i) regulatory genes that affect the MDR phenotype, (ii) genes that code for efflux pumps; (iii) genes that encode outer membrane proteins; and (iv) the level of outer membrane proteins during the process of induced resistance of *E. coli* K-12 to increasing concentrations of TET.

II.3 Results

II.3.1 Inducement of resistance to tetracycline

The TET susceptible *E. coli* K-12 AG100 parent strain (MIC of 2.0 mg/L) was induced by gradual stepwise increase of TET to significant levels of resistance to the antibiotic (Figure II.1) (Viveiros M. *et al,* 2005). Repeated serial transfer of the TET induced strain to drug-free medium restored the level of susceptibility to that initially present in *E. coli* K-12 AG100 after 40 days (Figure II.1).

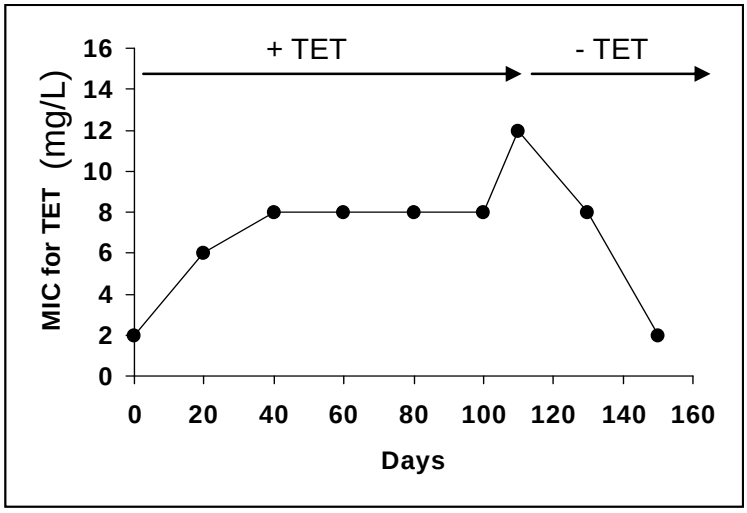

Figure II.1. Time course of inducement of TET resistance in *E. coli* K-12 AG100 and reversal of induced resistance by transfer to drug-free medium. The strain was induced to TET resistance by serial transfer of the bacteria to media containing increasing concentrations of the antibiotic (+ TET). Reversal of resistance was achieved by serial transfer to drug-free medium (- TET). MICs were periodically determined and confirmed in solid media by the TET E-test (AB Biodisk, VIVA Diagnostica, Huerth, Germany) according to the manufacturer's instructions (Viveiros M. *et al*, 2005).

The MICs of a panel of antibiotics were determined for *E. coli* K-12 AG100 and for the TET induced strain, designated as *E. coli* $AG100_{TET}$. As shown by the data presented in Table II.1, susceptibility of $AG100_{TET}$ is significantly decreased, with the exception of kanamycin and erythromycin. The MICs of EtBr and efflux inhibitors against the above mentioned strains are presented in Table II.2

Concerning the effect of known efflux inhibitors, used at ½ of their MIC, to prevent any direct effect on the growth of *E. coli* K-12 AG100 and $AG100_{TET}$ (Viveiros M. *et al*, 2005), chlorpromazine, CCCP and PAβN reduced the MIC of TET against AG100 from 2.0 to 0.5 mg/L (Table II.3). With respect to the effects of the above inhibitors on the susceptibility of $AG100_{TET}$ to TET, CPZ and PAβN reduced the susceptibility of this from 12 to 0.5 mg/L.

Table II.1. MIC of antibiotics against *E. coli* K-12 AG100 and $AG100_{TET}$.

Antibiotic	**MIC (mg/L)**	
	E. coli AG100	*E. coli* $AG100_{TET}$
Tetracycline	2.0	12
Kanamycin	15	10
Erythromycin	100	100
Ofloxacin	0.12	0.48
Ciprofloxacin	0.03	0.12
Chloramphenicol	8.0	>16
Penicillin	16	64
Oxacillin	256	>512

Table II.2. MICs of EtBr and efflux inhibitors against *E. coli* AG100 and AG100$_{TET}$.

Compound	MIC (mg/L)	
	E. coli AG100	*E. coli* AG100$_{TET}$
EtBr	150	300
CPZ	60	140
CCCP	10	20
PAβN	>200	>200

EtBr, ethidium bromide; CCCP, carbonyl cyanide m-chlorophenylhydrazone; CPZ, chlorpromazine; PβAN, Phe-Arg-β-naphtylamide.

Table II.3. MICs of TET in the presence and absence of and efflux inhibitors against *E. coli* AG100 and AG100$_{TET}$.

Compound	MIC (mg/L)	
	E. coli AG100	*E. coli* AG100$_{TET}$
TET	2	12
TET + CPZ	**0.5**	**3**
TET + CCCP	**0.5**	12
TET + PAβN	**0.5**	**0.5**

CCCP, carbonyl cyanide m-chlorophenylhydrazone; CPZ, chlorpromazine; PβAN, Phe-Arg-β-naphtylamide; TET, tetracycline.

II.3.2 Evaluation of gene expression by qRT-PCR

The step-wise induction of TET resistance in *E. coli* K-12 afforded an understanding of the genetic regulation of multidrug efflux pumps and their interplay with the permeability barrier (Viveiros M. *et al*, 2005). By this manner, real-time qRT-PCR was used to analyse the activity of genes that code for transcriptional regulators, efflux pumps and outer membrane proteins that may be involved in this process of induced resistance by prolonged exposure to increasing concentrations of TET. This analysis was performed at the end of four stages of the induction process; control culture (no TET added); the

initial stage where the cells are first exposed to 1.5 mg/L of TET (MIC 2 mg/L); half-way of the induction process where they grew at 4.0 mg/L of TET (MIC 6 mg/L) and at the end of the induction process where they grew at 10 mg/L of TET (MIC 12 mg/L). The results obtained are presented by Figure II.2 and discussed bellow in terms of relationships that have been established for regulatory and responding genes.

II.3.2.1 Genes responding to stress:

(i) *soxS* and *rob* - After resistance to TET has been established at the highest initial concentration of the antibiotic (*i.e.* 1.5 mg/L), the response of the *soxS* gene is 2.8 times more active than that of the unexposed control. This response is further increased to 3.5 times after resistance to 4 mg/L has been achieved. However, by the time the strain has become resistant to 10 mg/L of TET, the response of the gene has been reduced to a level below that initially observed, suggesting that the stress gene *soxS* performs its functions quite early under conditions of antibiotic pressure. The activity of the *rob* gene during the process of TET induced resistance is significantly increased after the bacteria become resistant to 4 mg/L of TET. As was the case of the other stress-response gene *soxS*, the increased activity noted is apparently not required for higher levels of resistance (*i.e.* 10 mg/L). Although *rob* has been reported to respond to exposure to solvents, detergents and metals (Ariza R.R. *et al*, 1995; Nakajima H. *et al*, 1995), an antibiotic response is included in this study. Moreover, the parallel response of both stress-responding genes noted in the current study supports the conclusions of Michan *et al.* (Michan C. *et al*, 2002).

(ii) *marA*, *marB* and *marR* - The regulatory product of gene *marR* is known to down-regulate the activity of genes *marA* and *marB* by binding to the promoter region of the operator *marO*. TET is known to bind to the product of *marR*, causing a MDR

phenotype (Martin R.G. *et al*, 1995). Once the repressor activity is inhibited, there is an increase of the activity of the universal regulator *marA*. This is confirmed by the data in Figure II.2. In fact, of all of the regulator genes, it is *marA* which is increased to the highest level (9.7 fold) at the time that the organism has developed resistance to 10 mg/L of TET.

Although nothing is known of the role of *marB* during MDR phenotypic expression, its expression is increased along with *marA*. However, their precise relationship during the development of induced resistance to TET remains to be clarified.

(iii) *micF* - *micF* codes for an antisense small RNA that has been shown to down-regulate outer membrane proteins (Pratt L.A. *et al*, 1996) and is activated by *marA*, *rob* and *sox* (Guillier M. *et al*, 2006). In this study, the increase of activity of *micF* reaches its maximum level when the organism has become resistant to 10 mg/L of TET and parallels the rise of activity of *marA*. This behaviour of *micF* was also found in other studies (Barbosa T.M. *et al*, 2000). The overproduction of MicF has been previously reported to decrease the amount of *ompF* mRNA (Guillier M. *et al*, 2006). In fact, a 3-fold decrease of porin mRNA was observed after resistance to 4 and 10 mg/L of TET had been induced (Figure II.2). This variation may be caused by the MicF effect on *ompF* mRNA. Similarly MicC may have the same effect on *ompC* mRNA stability. In contrast, it is important to note that the level of *ompF* mRNA and *ompC* mRNA of TET exposed cells remained, at least, similar to that observed for the untreated control (ratio of 1 at 10 mg/L of TET).

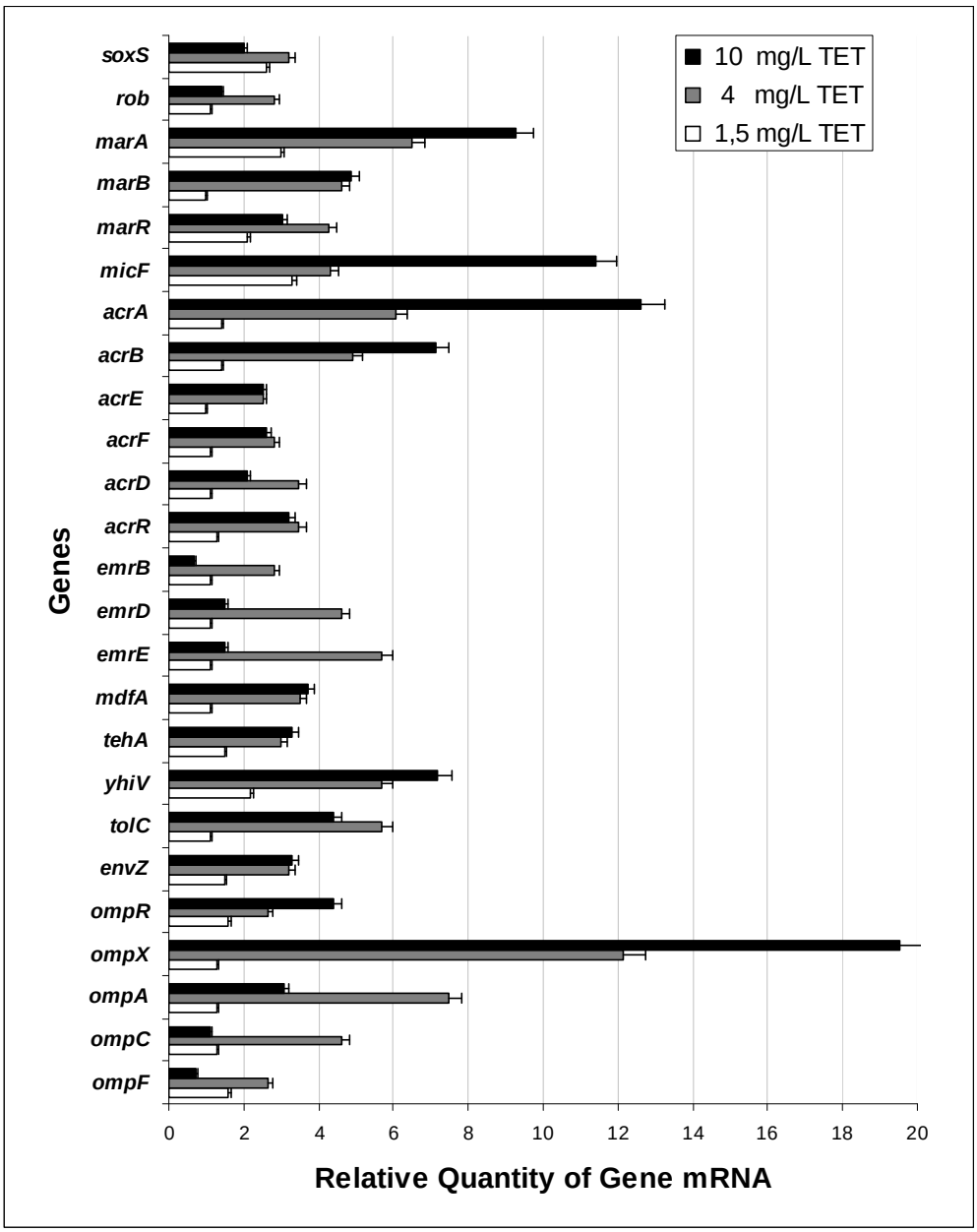

Figure II.2. Relative expression of outer membrane proteins, transcriptional regulators and inner membrane transporter genes. Data from three independent total mRNA extractions of *E. coli* $AG100_{TET}$ (physiologically adapted to increasing concentrations of TET) compared to its parental non-induced strain (*E. coli* K-12 AG100) grown in the absence of antibiotic. A ratio of 1 corresponds to no alterations in expression compared with the untreated control cells. Values were corrected for standard deviation range.

II.3.2.2 Efflux pump genes

***acrAB* and the other efflux pump transporter genes** - The response of the *acrAB-tolC* when the organism is initially exposed to a concentration of TET of 1.5 mg/L (MIC of 2 mg/L) is marginal. This suggests that the activity of this system under initial conditions of stress imposed by the exposure to low levels of TET is sufficient for the organism to escape from harm. With further exposure to increasing concentrations of TET, the expression of *acrAB-tolC* increases as the organism becomes more and more resistant to the antibiotic.

The response of the other efflux pump transporter genes is one of increased activity. However, with the exception of the *yhiV* efflux transporter gene, an RND type efflux pump in *E. coli* with significant homology to AcrB (Bohnert J.A. *et al*, 2007), the level of activity expressed by the other efflux systems is much less than that evident for the *acrB* and *yhiV*. In addition, the other efflux pump genes express the highest level of activity when the organism has reached a level of resistance of 4 mg/L and with further increase of resistance, their level decreases. This suggests that the stress imposed during the early stages of exposure to TET requires the cooperation of all of the efflux pumps and, as the level of activity of the two main efflux pumps of the bacterium is increased, there is a reduction in activity of the other pumps. The increased activity of *acrAB-tolC* parallels the increased activity of the regulator *marA*, a relationship that has been proposed in other studies (Barbosa T.M. *et al*, 2000).

II.3.2.3 Stress regulator genes of outer membrane proteins

ompR* and *envZ - The *ompR* and *envZ* genes are regulators of outer membrane proteins that permit hydrophilic compounds to enter the cell. These regulators belong to the two-component signalling family and modulate gene activities of *ompF* and *ompC*,

the two major outer membrane protein genes that code for tri-barrel porins (Nikaido H., 2003). When *E. coli* is under stress, a cascade of gene activities is initiated, involving several global regulators such as MarA and MicF, which result in the down-regulation of porins (Ferenci T., 2005; Martin R.G. *et al*, 2001; Pratt L.A. *et al*, 1996). This down-regulation results in decreased activity of *ompF* and *ompC*. As shown in Figure II.2, whereas the increase in the expression of the *ompR* and *envZ* genes is maintained for the duration of exposure to increasing concentrations of TET, the response of the *ompA*, *C* and *F* genes is transiently increased and subsequently reduced to levels comparable to those of the *E. coli* cells that were not exposed to TET.

Since *micF* is considered to be a post-transcriptional regulator of porins, the activity of *ompF* and *ompC* may be a result of the expression of this gene. A high increase in the *micF* expression was observed in TET induced cells (Figure II.2). MicF binds the *ompF* mRNA generating an RNA duplex that alters the translation and mRNA stability. Since over-production of MicF is observed in TET induced cells, a decrease of *ompF* mRNA would be expected as described with OmpC (Chen S. *et al*, 2004). In contrast, the porin mRNA level is similar to that produced in untreated cells.

The analyses of genes involved in the increased resistance to TET suggest that the up-regulation of efflux pump genes is accompanied by a decrease of OmpF and OmpC synthesis. As shown by an immunoblot analysis presented in Figure II.3, OmpC is reduced and OmpF is significantly reduced in the strain that has become resistant to 10 mg/L of TET. The observed alterations in porin content were confirmed by the use of the antibody that recognizes the specific internal loop domain of general porins (Figure II.3). These results are consistent with the notion that when the bacterium is placed under antibiotic stress, in conditions that permit it to adjust (namely slow exposure to sub-lethal concentrations of the antibiotic and nutrient availability), resistance is increased by the upregulation of efflux pumps and down-regulation of porins.

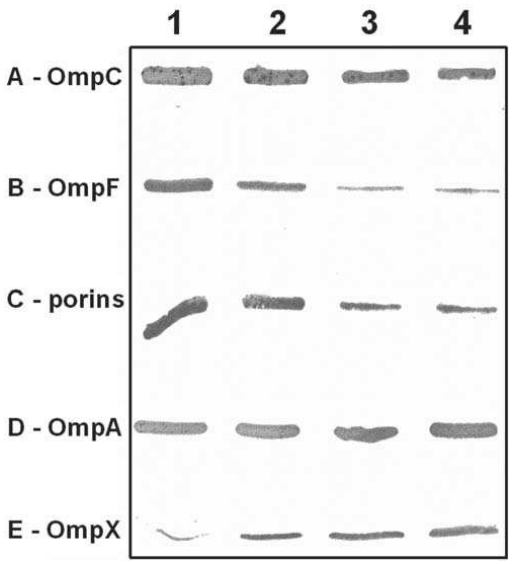

Figure II.3. Immunodetection of outer membrane proteins. The detections were carried out using antisera prepared against OmpC porin (A), OmpF porin (B), antigenic peptide located inside the internal loop 3 porin (C), OmpA (D) and OmpX (E), respectively. Immunodetection were carried out with total cell extracts from non-induced *E. coli* K-12 AG100 (1, 2) and *E. coli* $AG100_{TET}$ induced to 10 mg/L of TET (3, 4). Strains were grown in LB and MH media (odd and even lanes, respectively).

OmpA and OmpX - OmpA is considered to be a structural outer membrane protein that contributes to the integrity of the cell envelope as a tri-barrel structure (Ruiz N. *et al*, 2006). In this study, there was a transient increase of *ompA* expression when the organism became resistant to 4 mg/L of TET (Figure II.2). An explanation could be that the exposure to increasing concentrations of TET reduces protein synthesis and thus the need for structural strengthening of the cell envelope takes place. However, with the increased activity of efflux pumps and down-regulation of OmpC and F, fewer molecules of antibiotic reach their ribosomal targets even when resistance to TET has increased to 10 mg/L, and the extra need for OmpA is obviated.

OmpX is an outer membrane protein whose over-production is described to induce a reduction of the porin level in *E. aerogenes* (Dupont M. *et al*, 2004). In the results

presented in Figure II.2, the activity of *ompX* is the highest of all of the genes evaluated. Since the level of OmpX detected is also increased in TET induced strains (Figure II.3), the regulatory role of this outer membrane protein may involve a direct effect on porin assembly. Two hypotheses may be proposed: (i) OmpX alters the normal synthesis of outer membrane proteins; and (ii) a component such as a chaperone is required for the construction of nascent porin (Ruiz N. *et al*, 2006). Concerning the first hypothesis, no modification of OmpA synthesis was noted in TET induced strains (Figure II.3) suggesting a more specific effect of OmpX on the porin expression. In this respect, the overproduction of OmpX and TolC in the strain induced to 10 mg/L of TET may induce a saturation of outer membrane protein chaperones, such as YaeT and YfiO, that are necessary for the insertion of stably folded proteins into the outer membrane and subsequent construction of the tri-barrel porin (Ruiz N. *et al*, 2006; Wu T. *et al*, 2005). The increase of OmpX may then impair the normal assembly of porins. The unstable unfolded porin monomers will then be degraded by Deg proteases, serine-type proteases that play an important role in the proteolysis of misfolded and damaged proteins, to avoid toxic accumulation of abortive membrane protein (Castillo-Keller M. *et al*, 2006), leading to drastic decrease of porin content as has been observed in the TET induced cells. This hypothesis is supported by recent data showing competition between TolC and porins during assembly and by the role of DegP protease that removes the misfolded membrane proteins accumulated within the periplasm (Castillo-Keller M. *et al*, 2006; Charlson E.S. *et al*, 2006). In addition, the degradation of misassembled unfolded forms of porin occurs very rapidly due to their unstable conformation (Bolla J.M. *et al*, 1988). Moreover, an increased activity of genes that code for proteases in *E. coli* (Charlson E.S. *et al*, 2006; Douchin V. *et al*, 2006) was noted in this study (Figure II.4). The activity of *degP*, *clpP*, *rseP* and *degS* was increased from 2 to 4-fold after the organism has become resistant to 10 mg/L of TET

and may account for the large reduction of porins due to the degradation of unfolded forms of OmpC and OmpF (Figure II.4).

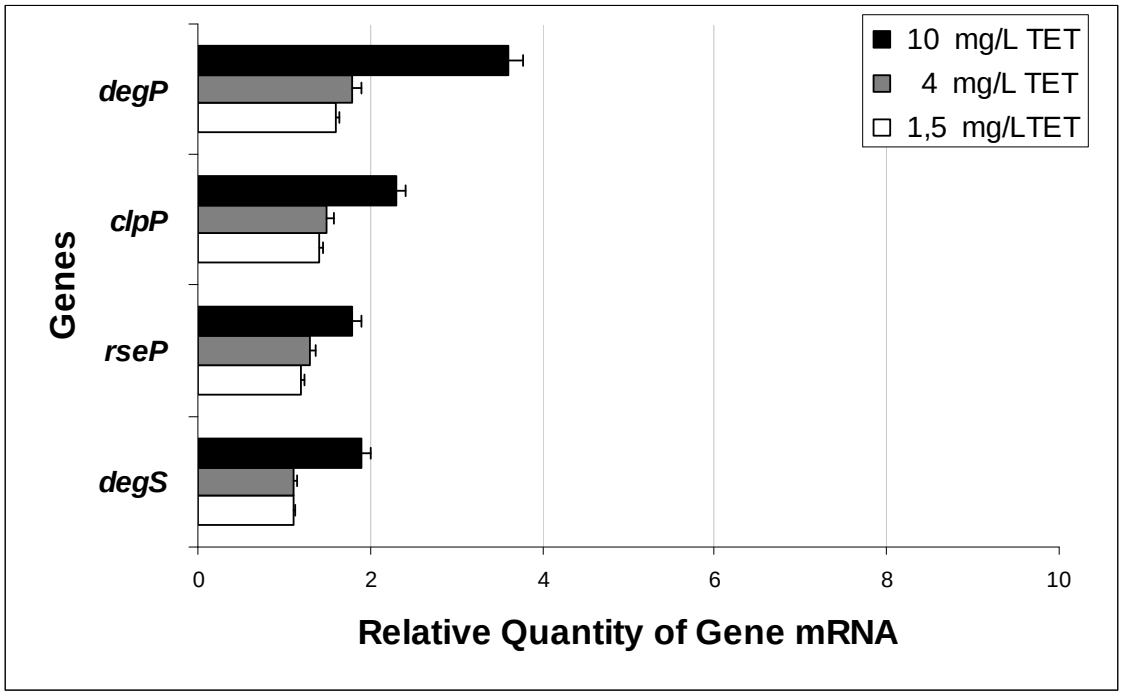

Figure II.4. Relative quantification of the expression level of the protease genes. Data from three independent total mRNA extractions of *E. coli* AG100 physiologically adapted to increasing concentrations of TET compared to its parental non-induced strain grown in absence of TET. A ratio of 1 corresponds to no alterations in expression compared with untreated control cells. Values were corrected for standard deviation range.

II.4 Conclusions

In this study, *E. coli* K-12 AG100 becomes increasingly resistant to TET when exposure to TET is gradually increased. The increased expression of efflux pumps is not the only mechanism involved in the physiological adaptation processes to TET pressure, there is a well-regulated and coordinated interplay between events at the genetic level and protein folding that decrease permeability of the cell envelope and increase efflux pump activity. In the presence of initial non-lethal concentrations of TET, the wild-type *E. coli* reacts through the activation of early stress responses as seen by

the immediate increase of the global regulators like MarA, SoxS, Rob and the activation of membrane and periplasmic proteases that release sigma factors in order to regulate the two major outer membrane proteins OmpC and OmpF. Following this initial stress response, a long-term adaptative response becomes noticeable with a sustainable increase of MarA that is not followed by the other two global regulators (SoxS and Rob) and, instead, is followed by two specific down-regulators of OmpC and OmpF expression, MicF and OmpX. Concomitantly, the over-expression of MarA leads to the transcriptional activation of AcrAB-TolC expression, the major efflux pump system of *E. coli* along with an increased expression of the other efflux systems. This is the basis for the development of an MDR phenotype (Baquero F. *et al*, 2001; Gambino L. *et al*, 1993). The gradual step-wise physiological adaptation of *E. coli* to TET forces the cell to answer to a constant stressful environment by the activation of a cascade of long-term events that are summarized in Figure II.5.

The results and conclusions obtained in this chapter were summarized in the publication Viveiros M. *et al*, 2007. The results and conclusions of this chapter were the basis upon which the scientific rational was designed and implemented for the evaluation of the role of mycobacterial porins and efflux pumps in the development of MDR phenotypes, and will be presented in chapters IV, V and VI of this thesis.

Although we have been able to demonstrate in this chapter that increased antibiotic pressure induces permeability changes in bacteria that promotes an MDR phenotype through the decrease of the porin level and overexpression of efflux pumps, which was put in evidence by the use of immuneblot and a qRT-PCR, there was still the need to develop an efficient technique to monitor and quantify the transport of porin and efflux pump substrates across the cell-wall of bacteria that could be correlated with multidrug resistance.

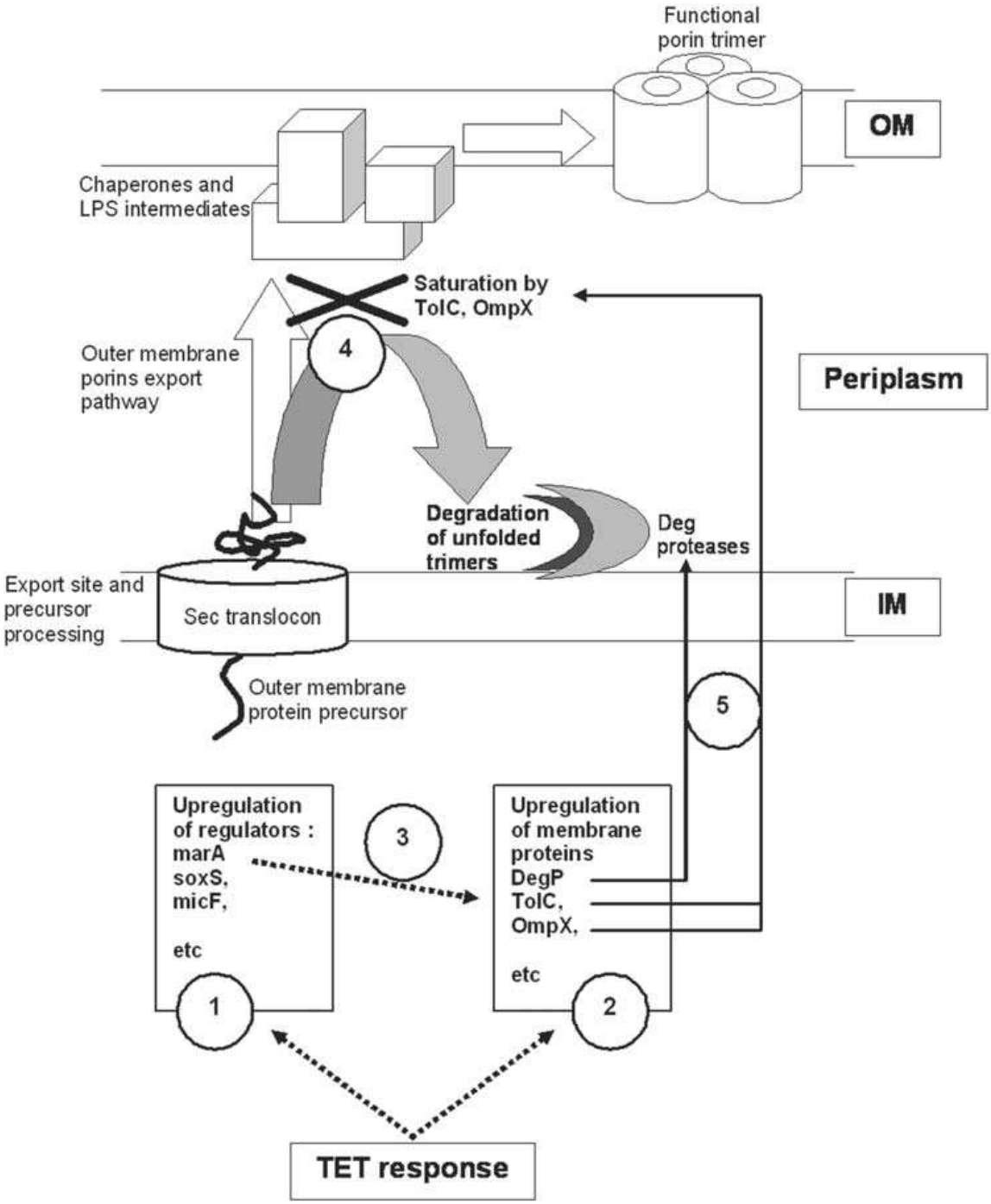

Figure II.5. TET activation cascade of *E. coli* resistance physiological adaptation. Broken arrows indicate the activation in 1 and 2 over-expression of specific gene (direct TET pressure effect), in 3, the regulation by induced regulators (second level of control), in 4 the effect of activated genes coding for membrane proteins (third level of effect). Thick arrows (5) illustrate the effect of over-production of outer membrane proteins and proteases.

II.5 Materials and Methods

Materials

The antibiotics TET, kanamycin, erythromycin, ofloxacin, ciprofloxacin, chloramphenicol, penicillin and oxacillin and the following compounds, EtBr, CCCP, chlorpromazine, and PAβN were purchased from Sigma Aldrich Quimica SA, Madrid, Spain. All solutions, except TET and CCCP, were prepared in distilled sterile water, and filtered with 0.2 µm syringe filters. TET solutions were prepared in methanol whereas CCCP was prepared in 50% methanol. All solutions were prepared on the day of the experiment. *E. coli* cultures were grown in solid (2% agar) or liquid Luria Bertani (LB) medium, purchased from Difco, Detroit, Mi, USA, which was supplemented when necessary at the given concentrations of the tested compounds. Mueller-Hinton (Oxoid, Hampshire, UK) was employed for the determination of the TET MIC by the E-test strip (0.016–256 mg/L), purchased from AB Biodisk (VIVA Diagnostica, Huerth, Germany).

Bacterial Strains

Wild-type *E. coli* K-12 AG100 strain (*argE3 thi-1 rpsL xyl mtl delta (gal-uvrB) supE44*) (Okusu H. *et al*, 1996), was kindly offered by Hiroshi Nikaido, Department of Molecular and Cell Biology and Chemistry, University of California, Berkely, California, USA. Growth conditions, preparation of inoculum and determination of the MIC by the broth macrodilution method in LB for each compound employed, and TET MIC by the E-test have been previously described (Eliopoulos G.M. *et al*, 1991; Okusu H. *et al*, 1996; Viveiros M. *et al*, 2005). The process by which the resistance of *E. coli* K-12 AG100 to TET was increased from 2.0 to 12.0 mg/L has been previously described (Viveiros M. *et al*, 2005). Briefly, the MICs of TET for the parental AG100 strain was initially determined as 2.0 mg/L (Okusu H. *et al*, 1996). The tubes employed for the determination of susceptibility to TET that would normally be discarded after a

maximum of 18 h were retained in the incubator. By the end of additional 24–48 h the tubes corresponding to concentration just above the MIC yielded evidence of growth. These cultures were tested for purity and TET susceptibility by the broth macrodilution method in LB and E-test (Eliopoulos G.M. *et al*, 1991; Viveiros M. *et al*, 2005). These new cultures were used to inoculate media containing increasing concentrations of TET that ranged from that from which the inoculae were prepared to higher concentrations and incubated at 37ºC until evidence of full growth was present. New inoculae were prepared from the cultures that contained the highest concentration under which the strains grew. This cycle was repeated until significant increase in the resistance of the strain to TET was evident and yielded *E. coli* AG100 that were capable of growing in LB broth containing a concentration of TET as high as 10 mg/L (MIC of 12 mg/L) (Viveiros M. *et al*, 2005).

Expression analyses of the membrane efflux transporter genes, outer membrane proteins and regulators, by the use of real-time qRT-PCR

The TET sensitive *E. coli* K-12 AG100 parent strain (MIC 2.0 mg/L) was induced to significant levels of resistance to TET by gradual step-wise exposure to the antibiotic. Transcript levels of the inner membrane efflux transporter genes of the major *E. coli* efflux pump systems proton dependent genes (*acrB*, *acrF*, *acrD, mdfA*, *tehA*, *yhiV*, *emrB*, *emrD* and *emrE*), the linker proteins AcrA and AcrE, the outer membrane channel TolC, the outer membrane proteins OmpC, OmpF, OmpA, the transcriptional regulators encoded by the multiple antibiotic resistance operon, *marRAB*, and homologs SoxS and Rob, porin transcription regulators *ompR* and *envZ*, the regulators *micF* and *ompX*, as well as the protease genes *degP*, *clpP*, *rseP* and *degS* were determined by real-time qRT-PCR at the end of four stages of the induction process; control culture (no TET added); the initial stage where the cells are first exposed to 1.5 mg/L of TET (MIC 2 mg/L); half-way of the induction process where they grew at 4.0

mg/L of TET (MIC 6 mg/L) and at the end of the induction process where they grew at 10 mg/L of TET (MIC 12 mg/L). Gene transcript levels were normalized against the *E. coli* house-keeping gene D-glyceraldehyde-3-phosphate-dehydrogenase (GAPDH). The change of the expression levels of these transporter genes, membrane proteins and regulators is presented by Figure II.2 as the relative quantification of the expression level in the TET induced strains relative to wild-type *E. coli* K-12 AG100 grown in the absence of TET at each stage of the induction process. Each result represents the average of three independent cultures grown at its respective TET resistance induction level. A ratio of 1.00 corresponds to no change of expression of the transcript levels to the parental strain. To prevent the degradation of extracted RNA after cell lysis that might alter the expression profile of each sample at the time of harvesting, required for assuring reliable gene expression analyses, total RNA was isolated in an RNase-free environment with the aid of the RNeasy Protect Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The integrity, purity and concentration of the extracted RNA templates were assessed by spectrophotometery at 260 nm and agarose gel (1.5%). Purified RNA was stored in RNase-free water in microtubes and maintained at -20ºC until quantification was performed. The real-time quantification of the RNA templates by quantitative real-time one-step RT-PCR was performed in a Rotor-Gene^{TM} 3000 thermocycler (Corbett Research, Sydney, Australia) strictly adhering to manufacturer recommendations of the QuantiTect SYBR Green RT-PCR Kit (QIAGEN, Hilden, Germany). Briefly, each 0.2 ml standard microtube contained, in a final volume of 25 ml, 12.5 ml of the 2× QuantiTect SYBR Green RT-PCR master mix, 0.25 ml of 10× QuantiTect RT mix, 900 nM of each primer and approximately 20 ng of total RNA in RNase free water. The primers used for real-time RT-PCR quantification of expression of each gene are described in Table II.3. These were designed using Primer Express 1.5 Software (Applied Biosystems, CA, USA) based on the sequence entries in the GenBank for *E. coli* K-12 complete genome

(accession number NC_000913). Primer design and PCR experimental conditions were optimised to minimise amplification of contaminating *E. coli* genomic DNA potentially present in the total RNA sample, as well as for the prevention of nonspecific primer annealing. The house-keeping GAPDH gene (Branlant G. *et al*, 1985) was chosen as the endogenous reference RNA for relative quantification since it revealed consistent expression levels under the experimental conditions with the primers presented in Table II.4.

Table II.4. Primers used in this study

Efflux transporter gene and house-keeping gene	Primer sequence (5'-3')	Length of amplicon
marA	CATAGCATTTTGGACTGGAT TACTTTCCTTCAGCTTTTGC	187 bp
marB	ATAGCAGCTGCGCTTATTC ACTTATCACTGCCAGTACCC	154 bp
marR	AGCGATCTGTTCAATGAAAT TTCAGTTCAACCGGAGTAAT	170 bp
acrA	CTTAGCCCTAACAGGATGTG TTGAAATTACGCTTCAGGAT	189 bp
acrB	CGTACACAGAAAGTGCTCAA CGCTTCAACTTTGTTTTCTT	183 bp
acrD	GATTATCTTAGCCGCTTCAA CAATGGAGGCTTTAACAAAC	187 bp
acrE	GCCCTCCTTTATTCTGATCT GGCTATACGATAAGCATTGG	166 bp
acrF	TAGCAATTTCCTTTGTGGTT CCTTTACCCTCTTTCTCCAT	247 bp
micF	TCATCATTAACTTTATTTATTACCG GCATCCGGTTGAAATAGG	70 bp
soxS	CCATTGCGATATCAAAAATC ATCTTATCGCATGGATTGAC	210 bp
rob	GTCGTCTTTATCCTGACTCG TTTGTCACCCTGGAAGATAC	189 bp
envZ	CGTTGAGGTCAACAAAAGTT GTCGGTTCTGGATACGAATA	185 bp

Table II.4. (cont.)

emrB	ATTATGTATGCCGTCTGCTT TTCGCGTAAAGTTAGAGAGG	196 bp
emrD	TGTTAAACATGGGGATTCTC TCAGCATCAGCAAATAACAG	243 bp
emrE	GGATTGCTTATGCTATCTGG GTGTGCTTCGTGACAATAAA	156 bp
mdfA	TTTATGCTTTCGGTATTGGT GAGATTAAACAGTCCGTTGC	182 bp
tehA	TGCTTCATTCTGGAGTTTCT TCATTCTTTGTCCTCTGCTT	232 bp
yhiV	GCACTCTATGAGAGCTGGTC CCTTCTTTCTGCATCATCTC	203 bp
ompF	GAACTTCGCTGTTCAGTACC CGTACTTCAGACCAGTAGCC	209 bp
ompC	CTTCAAAGGTGAAACTCAGG GTTGTCAGAACCGTAGGTGT	241 bp
ompA	ACGACTGGTTAGGTCGTATG ACGTTGGATTTAGTGTCTGC	166 bp
ompX	ACCTGAAATACCGCTATGAA TCAGTGGTCTGGAATTTACC	208 bp
ompR	GACGTCTTCGTAGTCAGAGC TTGAACTTACCGAAAGCAAT	229 bp
degP	TGGTAGTGAACAACGTGAAA AACAGGTAGATGGTGCTGTC	184 bp
clpP	CAAAAGGTAAACGTTTTTGC AATGATTGACCCGTATGAAG	163 bp
rseP	TTGTTTATTACCTGCCGTTT ATTAACAGCACCAGCAGAAT	190 bp
degS	TTCCAGTTAGCAACCAAAAT TGACACTTCATTAACCACGA	153 bp
GAPDH	ACTTACGAGCAGATCAAAGC AGTTTCACGAAGTTGTCGTT	170 bp

Amplification efficiencies of the target genes and reference gene were determined through the amplification of one-step RT-PCR template dilution series and PCR conditions were optimised until comparable amplification efficiencies were obtained for identical amounts of template RNA (absolute slope values less then 0.1) from calibration curve plots (Langmann T. *et al*, 2003). Amplification of GAPDH and other genes of interest were then run in separate tubes using the same amount of total RNA retrieved from the same sample. Thermal cycling conditions consisted of an initial reverse transcription step at 50ºC during 30 minutes, an initial PCR activation step at 95ºC for 15 minutes followed by 35 cycles of denaturation (94ºC, 60 seconds), annealing (51ºC–53ºC for 60 seconds, depending on optimised conditions for the primers used) and extension (72ºC for 60 seconds). The relative quantities of the mRNA of each gene of interest were determined by the use of the comparative threshold cycle (C_T) method. Taking advantage of the fact that samples with higher initial mRNA template concentration reach the significant threshold level for real-time detection at lower PCR cycle numbers than samples containing lower initial template concentrations, it is possible to obtain a quantitative measure of the expression magnitude (ΔCT) of each gene of interest, normalized by the house-keeping gene (GAPDH) expression in each sample to correct variation in RNA content and amplification efficiencies between samples. The equation $2^{-\Delta\Delta CT}$ allows the relative quantification of differences of each gene expression level between two samples, the sample of interest (the TET induced AG100 strain) and a calibrator or reference strain (the parental AG100 strain). Briefly, from three independent total mRNA extractions form *E. coli* AG100 and $AG100_{TET}$ strains, grown under the described conditions, ΔC_T of the reference and samples for each gene tested was obtained by subtracting the C_T value of the GAPDH gene from the C_T value obtained for each gene. $\Delta\Delta C_T$ was calculated by subtracting the average ΔC_T values of the reference strain (AG100) from the corresponding TET induced ΔC_T for each gene tested. The relative quantifications

were then calculated by the equation $2^{-\Delta\Delta CT}$ as the number of fold of mRNA quantity differences relative to the calibrator or reference strain (Langmann T. *et al*, 2003; Livak K.J *et al*, 2001). All data was collected and analysed with the aid of the Rotor-Gene™ 3000 real-time analysis software.

SDS-PAGE analyses and immunoblotting

Bacteria in exponential growth phase were pelleted and solubilised as previously described (Malléa M. *et al*, 1998). Proteins were analysed on 10% SDS polyacrylamide gel system for OmpC, OmpF, OmpA and AcrA detection and 12% SDS-PAGE gel for OmpX, (Dupont M. *et al*, 2004; Malléa M. *et al*, 1998). Gels were stained with Coomassie Brillant Blue R-250 to standardized protein samples. For western blots, proteins were electrotransfered onto nitrocellulose membranes (Schleicher & Schlull, Keene, NH, USA) in transfer buffer (20 mM Tris, 150 mM glycine, 20% isopropanol, 0.05% SDS). An initial saturating step was performed overnight at 4ºC with Tris-buffered sodium (TBS: 50 mM Tris- HCl, 150 mM NaCl, pH 8) containing skimmed milk powder (10%). The nitrocellulose membranes were then incubated in TBS containing skimmed milk powder (10%) and Triton X-100 (0.2%) for 2 hours at room temperature in the presence of polyclonal antibodies directed against denatured OmpC, OmpF, OmpA and OmpX, or with F4 polyclonal antibody directed against the L3 internal loop of *E. coli* porins (Dé E. *et al*, 2001). The detection of antigenantibody complexes was performed with alkaline phosphatase conjugated AffinitiPure goat anti-rabbit IgG antibodies (Jackson ImmunoResearch, West Grove PA, USA).

Chapter III

Fluorometric determination of ethidium bromide efflux kinetics in *E. coli*

This chapter contains data published in:

- Paixão L., **Rodrigues L.**, Couto I., Martins M., Fernandes P., de Carvalho C.C., Monteiro G.A., Sansonetty F., Amaral L., Viveiros M. 2009. Fluorometric determination of ethidium bromide efflux kinetics in *Escherichia coli*. *J. Biol. Eng.* **3**:18.
- Viveiros M., Martins A., Paixão L., **Rodrigues L**., Martins M., Couto I., Fähnrich E., Kern W.V., Amaral L. 2008. Demonstration of intrinsic efflux activity of *Escherichia coli* K-12 AG100 by an automated ethidium bromide method. *Int. J. Antimicrob. Agents* **31**: 458–462.

Fluorometric determination of ethidium bromide efflux kinetics in *E. coli*

III.1 Summary

In the previous chapter, a protocol to analyze the expression levels of efflux pumps was developed by qRT-PCR using *E. coli* as a model. The methodology employed allowed the demonstration of events involved in the regulation of porins and efflux pumps during the adaptation of *E. coli* to increased concentrations of TET. However, there is still the need to correlate this genetic information with the physiological detection and quantification of efflux pump activity. The development of methods for the early detection and quantification of drug transport across the bacterial cell-wall is a tool essential to understand and overcome efflux-mediated drug resistance. This chapter describes the development of a protocol for the quantification of efflux developed by the use of real-time fluorometry to assess the accumulation and extrusion of fluorescent compounds by efflux pumps. *E. coli* was once again used as a model in the development of the protocol that will be applied latter to mycobacteria (chapters IV, V and VI).

EtBr transport across the cell envelope of *E. coli* K-12 and derivatives was analysed by a real-time fluorometric method. Accumulation and efflux of EtBr was studied under limiting energy supply (absence of glucose and low temperature) and in the presence and absence of the efflux inhibitor, chlorpromazine. The bulk fluorescence variations were also observed by flow cytometry analysis, revealing that once inside the cells, leakage of EtBr does not occur and its efflux is mediated by active transport. The importance of AcrAB-TolC, the main efflux system of *E. coli*, in the extrusion of EtBr was put in evidence by comparing strains with different levels of AcrAB expression. An experimental model was developed to describe the transport kinetics in the three strains. The model integrates passive entry (influx) and active efflux of EtBr, and discriminates different degrees of efflux between the studied strains that vary in the

activity of their efflux systems (AG100 – wild-type, AG100A – AcrAB deleted, $AG100_{TET}$ – AcrAB overexpressed), as evident from the calculated efflux rates: k^{-}_{AG100} = 0.0173 ± 0.0057 min^{-1}; k^{-}_{AG100A} = 0.0106 ± 0.0033 min^{-1}; and $k^{-}_{AG100TET}$ = 0.0230 ± 0.0075 min^{-1}.

The combined use of a semi-automated fluorometric method and an experimental model allowed quantifying EtBr transport in *E. coli* strains that differ in their overall efflux activity. This methodology can be used for the early detection of differences in the drug efflux capacity in bacteria accounting for antibiotic resistance, as well as for expedite screening of new drug efflux inhibitors libraries and transport studies across the bacterial cell wall.

III.2 Introduction

Efflux pumps are major defensive components of the bacterial cell wall that actively extrude noxious compounds from the periplasm and/or cytoplasm, thereby decreasing their intracellular concentration (Li X.Z. *et al*, 2004; Piddock L.J., 2006). Active efflux of antibiotics by bacteria was first described in 1978 in *E. coli* resistant to TET (Levy S.B. *et al*, 1978) and, since then, it has been proven that the constitutive or inductive expression of these systems is responsible for the intrinsic and acquired resistance of many bacterial species to antimicrobials (Bambeke F.V. *et al*, 2006; Pagès J.M. *et al*, 2005).

Several methods have been used to detect and quantify the activity of bacterial efflux pump systems using radio-labeled, metal-labeled or fluorescent substrates to monitor their efflux in bacterial cells (Greulich K.O., 2004; Jernaes M.W. *et al*, 1994; Joux F. *et al*, 2000; Kyriacou S.V. *et al*, 2004; Lomovskaya O. *et al*, 2006; Martins M. *et al*, 2006; Mortimer P.G. *et al*, 1991). Bulk measurement techniques that use fluorescence spectroscopy yield a general understanding that represents the balance between entry

and extrusion of a given substrate, which may result from the efflux activities of one or several pumps (Joux F. *et al*, 2000; Lomovskaya O. *et al*, 2006), and where cell membrane permeability also plays an important role (Davin-Regli A. *et al*, 2008; Viveiros M. *et al*, 2007). Because the permeability to the substrate may be regulated by a decreased number of porins or by an increase of the lipopolysaccharide layer of the cell envelope (Davin-Regli A. *et al*, 2008; Viveiros M. *et al*, 2007), the demonstration of efflux activity invariably involves the use of an agent that promotes a significant increase of substrate accumulated (Lomovskaya O. *et al*, 2006; Viveiros M. *et al*, 2008a; Viveiros M. *et al*, 2008b). Unless the demonstration of the role of a specific efflux pump is readily made, i.e. by the quantification of the expression level of a specific transporter (Viveiros M. *et al*, 2005; Viveiros M. *et al*, 2007), or by the use of mutants whose specific efflux pump has been deleted or inactivated (Okuso H. *et al*, 1996), very little can be said about the role that any efflux pump system may play in the MDR phenotype of bacteria.

A semi-automated fluorometric method was developed for the assessment of efflux pump activity in bulk bacterial cells (Viveiros M. *et al*, 2008a; Viveiros M. *et al*, 2010a). The method uses the common efflux pump substrate EtBr that has been shown to be a particularly suitable probe for these studies. In fact it emits weak fluorescence in aqueous solution (external to the cell) and becomes strongly fluorescent when its concentration within the periplasm exceeds that of the aqueous solution due to its binding to cellular components (Jernaes M.W. *et al*, 1994). Such binding must be of a weak type if the agent is to serve as a useful probe for efflux, since later intercalation into DNA results in strong binding that precludes any dissociation of the substrate for efflux (Jernaes M.W. *et al*, 1994; Joux F. *et al*, 2000). This method can distinguish accumulation, which reflects the balance between influx and efflux, from efflux itself (Viveiros M. *et al*, 2008a; Viveiros M. *et al*, 2010a) and, therefore, has the potential for

the study of the kinetics of EtBr transport. The method was developed using the wild-type *E. coli* K-12 AG100 as the bacterial model and the accumulation and efflux of EtBr was monitored by real-time fluorometry using the Rotor-Gene^{TM} 3000 from Corbett Research (Sydney, Australia) (Viveiros M. *et al*, 2008a; Viveiros M. *et al*, 2008b; Viveiros M. *et al*, 2010a). The major innovation of this method is the simultaneous evaluation of efflux pump activity *in vivo* and on-line of a large number of samples, with the possibility to expose the same cell preparation to different environmental conditions in a single assay.

For the objectives of this thesis, the semi-automated fluorometric method has been used to define and parameterize a mechanistic experimental model that demonstrates the kinetics of EtBr influx and efflux by *E. coli* strains that differ in their capacity to extrude this molecule. The determination of the influx and efflux rates (k^+ and k^-, respectively) of a fluorescent substrate, by the approach proposed, allows the quantification of the cell overall efflux capacity. This information can be useful to interpret different phenotypes resulting from this efflux activity, including multidrug resistance in clinical bacterial strains.

III.3 Results

III.3.1 Detecting EtBr efflux mediated by *E. coli* efflux pump systems

Experiments were carried out with EtBr concentrations that did not affect in any way the cell viability, *i.e.*, that did not exceed ½ the MIC for EtBr (Table III.1). In this case and as previously demonstrated, accumulation of EtBr and its extrusion from *E. coli* are the result of the balance between EtBr entry by passive diffusion (influx) and the extrusion activity of efflux pump systems, primarily the AcrAB-TolC (Viveiros M. *et al*, 2005; Viveiros M. *et al*, 2007; Viveiros M. *et al*, 2010a). As shown by Figure III.1, the

accumulation of EtBr under conditions that are considered to maximize efflux, *i.e.*, presence of glucose and 37ºC, begins to take place at a concentration of EtBr that exceeds 1.0 mg/L for the wild-type *E. coli* K-12 AG100 (Figure III.1A), 0.250 mg/L for AG100A (*acrAB* inactivated; Figure III.1B), and 1.0 mg/L for $AG100_{TET}$ (*acrAB* and other efflux pump genes overexpressed; Figure III.1C). As expected, the strain that over-express efflux systems ($AG100_{TET}$) accumulates the least amount of EtBr even when the maximum concentration of EtBr to which it is exposed is 6 mg/L. On the contrary, the AG100A, whose AcrAB efflux pump has been inactivated, accumulates approximately 25 times more EtBr when exposed to a far lower concentration of 1.5 mg/L of EtBr. The importance of the AcrAB-TolC efflux system is further illustrated by the comparison of accumulation of EtBr by the wild-type AG100 to that by the AG100A, when both strains are exposed to 1 mg/L of EtBr.

Table III.1. MIC values of EtBr and several antibiotics for *E. coli* AG100, AG100A and $AG100_{TET}$.

***E. coli* strains**	**MIC (mg/L)**							
	EtBr	**KAN**	**CIP**	**OFX**	**CHL**	**ERY**	**TET**	**TET + CPZ**
AG100	150	15	0.03	0.12	8	100	2.0	0.5
AG100A	5	>200	0.004	0.015	2	6.25	0.5	0.5
$AG100_{TET}$	300	10	0.12	0.48	>16	100	12	3

CHL, chloramphenicol; CIP, ciprofloxacin; CPZ, chlorpromazine; EtBr, ethidium bromide; ERY, erythromycin; KAN, kanamycin; OFX, ofloxacin; TET, tetracycline. Kanamycin was included as control for the presence of Tn*903* in *E. coli* AG100A (Δ*acrAB*::Tn*903* Kan^r). The TET MICs were also calculated in the presence of 30 mg/L (AG100), 10 mg/L (AG100A) and 70 mg/L ($AG100_{TET}$) of CPZ (½ MIC).

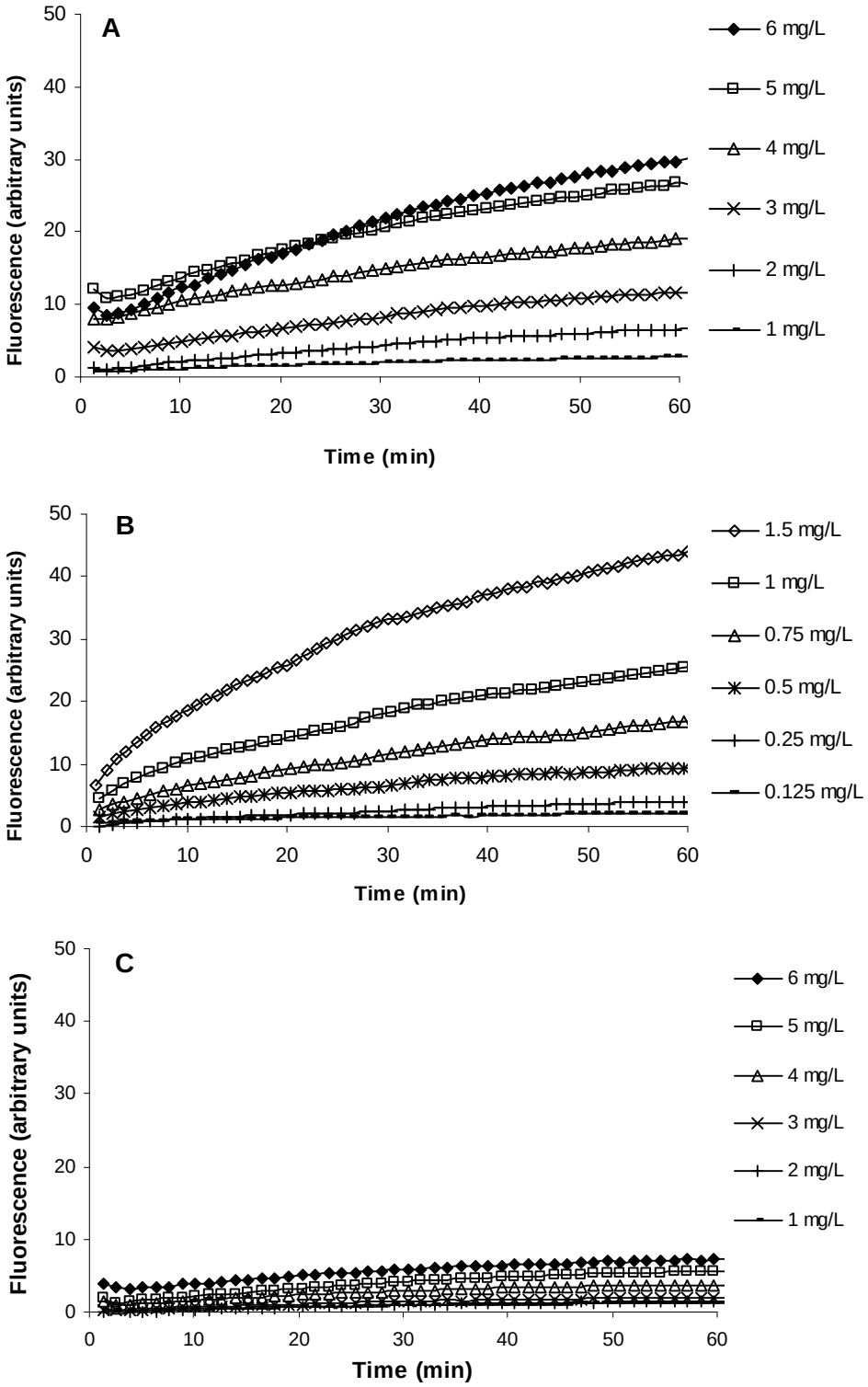

Figure III.1. Accumulation of EtBr at increasing concentrations by *E. coli* K-12 AG100(A), AG100A(B) and $AG100_{TET}$ (C). The bacteria were exposed to increasing concentrations of EtBr at 37ºC in the presence of glucose. Note: assays for AG100A were carried out with lowest EtBr concentrations (0.125 - 1.5 mg/L) – see text.

Accumulation of EtBr inside the bacterial cells can be increased in the presence of an efflux inhibitor such as phenothiazines (*e.g.* chlorpromazine), compounds that have been shown to inhibit efflux activity of Gram-negative bacteria (Bambeke F.V. *et al*, 2006; Pagès J.M. *et al*, 2005; Viveiros M. *et al*, 2008a). Thus, these compounds are usually employed for the demonstration of overexpressed efflux pumps of bacteria (Lomovskaya O. *et al*, 2006; Pagès J.M. *et al*, 2005; Viveiros M. *et al*, 2008a; Viveiros M. *et al*, 2008b). Because of the real-time capacity and sensitivity of the semi-automated fluorometric method, the demonstration of the effects of an efflux inhibitor, such as chlorpromazine, on the intrinsic efflux system of the wild-type *E. coli* K-12 AG100 is readily made. As shown by Figure III.2A (A1 and A2), high concentrations of chlorpromazine maximize the amount of EtBr accumulated only when glucose is absent from the medium. Similar results were obtained for the strains $AG100_{TET}$ and AG100A (data not shown). The effect of chlorpromazine on the accumulation of EtBr by wild-type *E. coli* K-12 AG100 is similarly demonstrated and confirmed with the aid of flow cytometry (Figure III.2B).

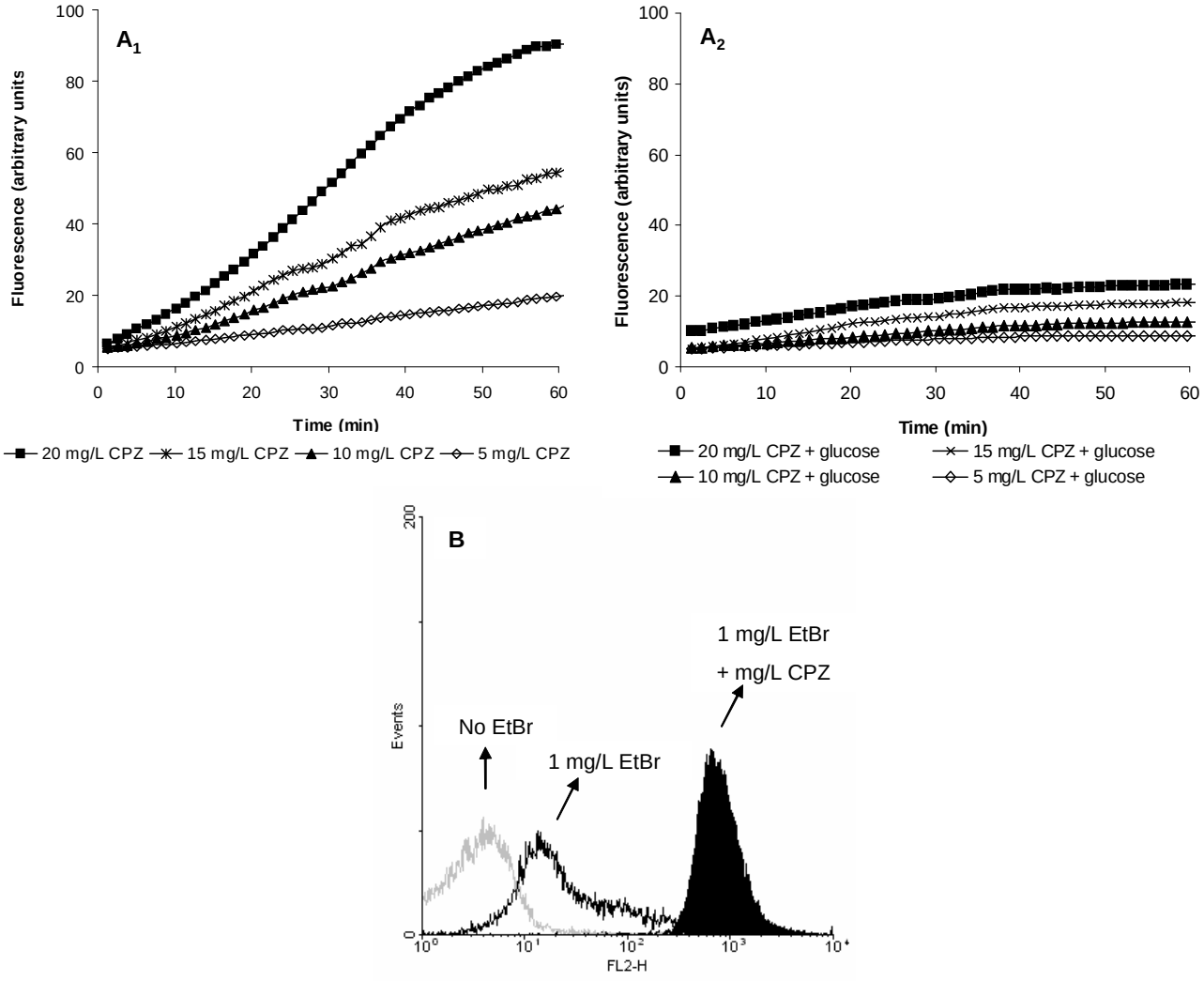

Figure III.2. Effect of chlorpromazine (CPZ) on EtBr accumulation by *E. coli* K-12 AG100. The bacteria were loaded with EtBr at 1 mg/L in the presence of increasing concentrations of chlorpromazine for a period of 60 minutes at 37ºC in the presence and absence of glucose and accumulation assessed by: (A1 and A2) the semi-automated fluorometric method; (B) flow cytometry, represented by an histogram overlay corresponding to the data selected for analysis (2nd gate) for *E. coli* K-12 AG100 (i) without EtBr or chlorpromazine, (ii) after 60 minutes of exposure to EtBr (1 mg/L) and (iii) after 60 minutes of exposure to EtBr (1 mg/L) and chlorpromazine (20 mg/L) at 37ºC without glucose. Data in graphics A1 and A2 correspond to the same assay and were separated for the sake of figure clarity.

The on-line visualization of efflux activity by the EtBr loaded cells requires that accumulation of EtBr has previously taken place. Therefore, before performing the efflux assays, bacteria are exposed to conditions that promote significant accumulation of EtBr (Viveiros M. *et al*, 2008a; Viveiros M. *et al*, 2008b). To this extent, the following conditions were chosen: a temperature of 25ºC, absence of glucose and presence of chlorpromazine (20 mg/L), to promote maximum accumulation, within 60 minutes. When maximum accumulation has taken place under these conditions, the bacteria are washed free of EtBr and chlorpromazine and resuspended in fresh buffer with and without glucose. Fluorescence readings were conducted over a period of 15 minutes at 37ºC. As shown by Figure III.3, restoration of optimum conditions of glucose, noted to prevent accumulation, are required for the extrusion of EtBr. This assay shows that *E. coli* cells need an energy source for efflux to take place.

Therefore, this methodological approach proved its usefulness for the demonstration of active efflux of EtBr in *E. coli* and the strategy developed allowed to differentiate influx (passive diffusion into the cell) from efflux activity (active efflux through pumps).

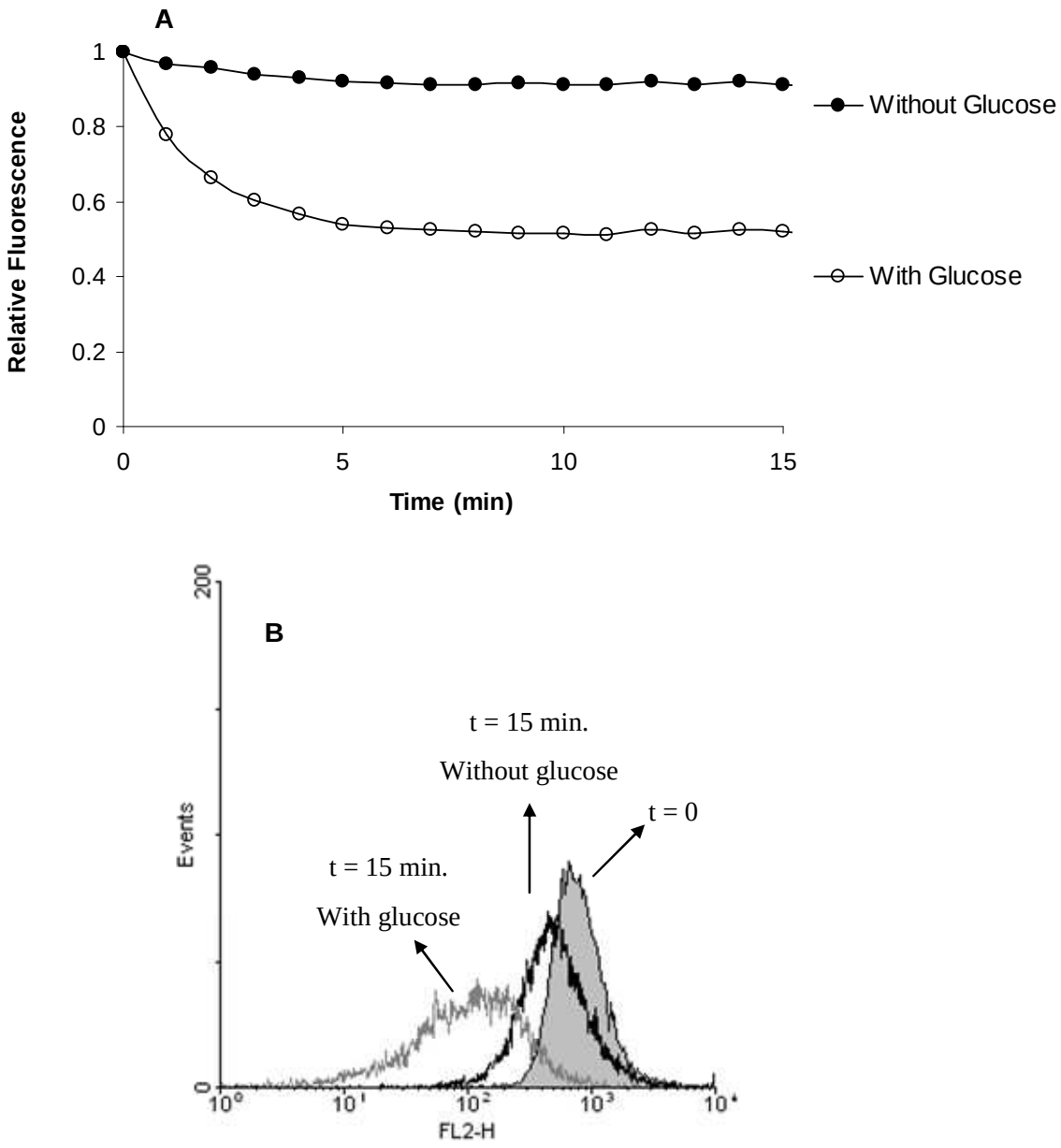

Figure III.3. Detection of EtBr efflux in *E. coli* K-12 AG100. The bacteria were loaded with EtBr at 1 mg/L in the presence of chlorpromazine (20 mg/L) for a period of 60 minutes at 25ºC. After replacing the buffer with EtBr-free PBS with or without glucose, the efflux was assessed by: (A) the semi-automated fluorometric method, performed at 37ºC for 15 minutes; and (B) flow cytometry, represented by an histogram overlay corresponding to the data selected for analysis (2^{nd} gate) for *E. coli* K-12 AG100 immediately after replacing the buffer with EtBr-free PBS (t=0) and 15 minutes after incubation at 37ºC with or without glucose.

III.3.2 Modeling EtBr transport across *E. coli* cell wall

In order to quantify the efflux activity, a mechanistic experimental model that establishes a balance related to the intracellular EtBr concentration was developed, as described below:

$$\frac{d[EtBr]_{int}}{dt} = k^{+}[EtBr]_{ext} - k^{-}[EtBr]_{int} \qquad \text{(eq. 1.1.)}$$

where $[EtBr]_{int}$ is the intracellular EtBr concentration (mg/L); $[EtBr]_{ext}$ is the external EtBr concentration (mg/L); k^{+} is the rate of EtBr entry into the cells (min^{-1}) and k^{-} corresponds to the rate of EtBr efflux from the cells (min^{-1}). Therefore,

$$[EtBr]_{Tot} = [EtBr]_{ext} + [EtBr]_{int} \qquad \text{(eq. 1.2.)}$$

where $[EtBr]_{Tot}$ is the total EtBr concentration (mg/L). Replacing equation (1.2) in equation (1.1) and solving it for $[EtBr]_{int}$, it is possible to obtain the following equation, which describes the EtBr transport across the cell wall of *E. coli*:

$$[EtBr]_{int}(t) = \frac{k^{+}}{k^{+}+k^{-}}[EtBr]_{Tot} + \left([EtBr]_{int}(t=0) - \frac{k^{+}}{k^{+}+k^{-}}[EtBr]_{Tot} \right) e^{-(k^{+}+k^{-})t} \qquad \text{(eq. 1.3.)}$$

The equation developed herein (eq. 1.3) allied to the technical approach used to quantify the intracellular EtBr concentration (Methods) allowed to quantify the degree of EtBr efflux activity in the three *E. coli* strains (AG100, AG100A and $AG100_{TET}$). This strategy is based on the distinct levels of EtBr fluorescence in solution and inside the cells. EtBr emits fluorescence when it is free in solution (in this case, PBS) in a concentration dependent manner, accurately captured by the photomultiplier detector with the 585 nm high-pass filter of the Rotor-Gene™ 3000 that differentiates mg/L differences (Viveiros M. *et al*, 2008a; Viveiros M. *et al*, 2010a). The high sensitivity of

this detector ensue the definition of a calibration curve, which correlates fluorescence (FL_0) and the initial EtBr concentration in solution, before the cells are added to the medium, varying from 0 mg/L to 6 mg/L:

$$[\mathrm{EtBr}]_{ini} = 0.1201 \times FL_0 + 0.0373 \quad (R = 0.99)$$

where $[EtBr]_{initial} = [EtBr]_{Total}$. When the *E. coli* cells are exposed to EtBr containing buffer (1 to 6 mg/L for AG100 and $AG100_{TET}$; 0.125 to 1.5 mg/L for AG100A), the cells start to accumulate EtBr that enters by passive diffusion, with the efflux pump systems trying to balance this entry according to the kinetics presented in Figure III.1. At these low concentrations, EtBr does not reach the cellular components in the cytoplasm to which it could bind irreversibly, being effluxed from the cytoplasm and periplasm by either single- or multi-component efflux systems (Lee A. *et al*, 2000; Seeger M.A. *et al*, 2008). When the concentration of EtBr within the periplasmic space exceeds significantly its extrusion, EtBr is translocated beyond the inner plasma membrane where the medial sites of the cell contain nucleic acids to which the EtBr can intercalate almost irreversibly, after which extrusion is not possible (Jernaes M.W. *et al*, 1994; Joux F. *et al*, 2000; Lee A. *et al*, 2000; Seeger M.A. *et al*, 2008).

Using the rationale described in Methods, it is possible to correlate the fluorescence readings by the semi-automated fluorometric method in bulk cells to the EtBr concentration that remains inside the cells; $[\mathrm{EtBr}]_{int} = 0.022 \times FL_{int\ exp\ corr} + 0.06$ (R=0.99). With the assistance of the software *Table Curve*™ 2D from Jandel Scientific - AISN Software STATISTICA, it is then possible to adjust the model (eq. 1.3.) to the experimental data and determine the values of the constants k^+ and k^-, which represent a measure of the rate of entry and the rate of efflux of EtBr into and out of the cell, respectively (Figure III.4). Table III.2 summarizes the values of k^+ and k^- obtained for the three strains tested. The values obtained show similar influx rates for the three *E.*

coli strains studied, as expected, since this parameter reflects the entry of EtBr by passive diffusion. Both the passive influx absolute values and their variation among the three strains are negligible when compared to the values obtained for the efflux rates.

The model proposed accurately describes the behavior of the three strains, showing a lower efflux rate for the AG100A strain when compared to the wild-type AG100 (P value = 0.0023), as a result of the inactivation of the AcrAB-TolC system in the mutant strain. For the $AG100_{TET}$ strain the model revealed a significantly increased efflux rate compared to the wild-type strain (P value = 0.0057). This result reflects the higher efflux activity in this TET adapted strain due to the overexpression of efflux pumps, particularly of the AcrAB-TolC system (Viveiros M. *et al*, 2005; Viveiros M. *et al*, 2007).

Table III.2. Influx (k^+) and efflux (k^-) rates for *E. coli* K-12 AG100, AG100A and $AG100_{TET}$.

***E. coli* strains**	$\mathbf{k^+}$ (min^{-1})	$\mathbf{k^-}$ (min^{-1})
AG100	0.0019 ± 0.0009	0.0173 ± 0.0057
AG100A	0.0035 ± 0.0012	0.0106 ± 0.0033
$AG100_{TET}$	0.0025 ± 0.0009	0.0230 ± 0.0075

k^+ and k^- were determined using the software Table Curve™ 2D from Jandel Scientific - AISN Software STATISTICA to adjust the model (eq. 1.3.) to the experimental data obtained with the semi-automated fluorometric method (Figure III.1). The results represent an average of three independent assays with the correspondent standard deviation.

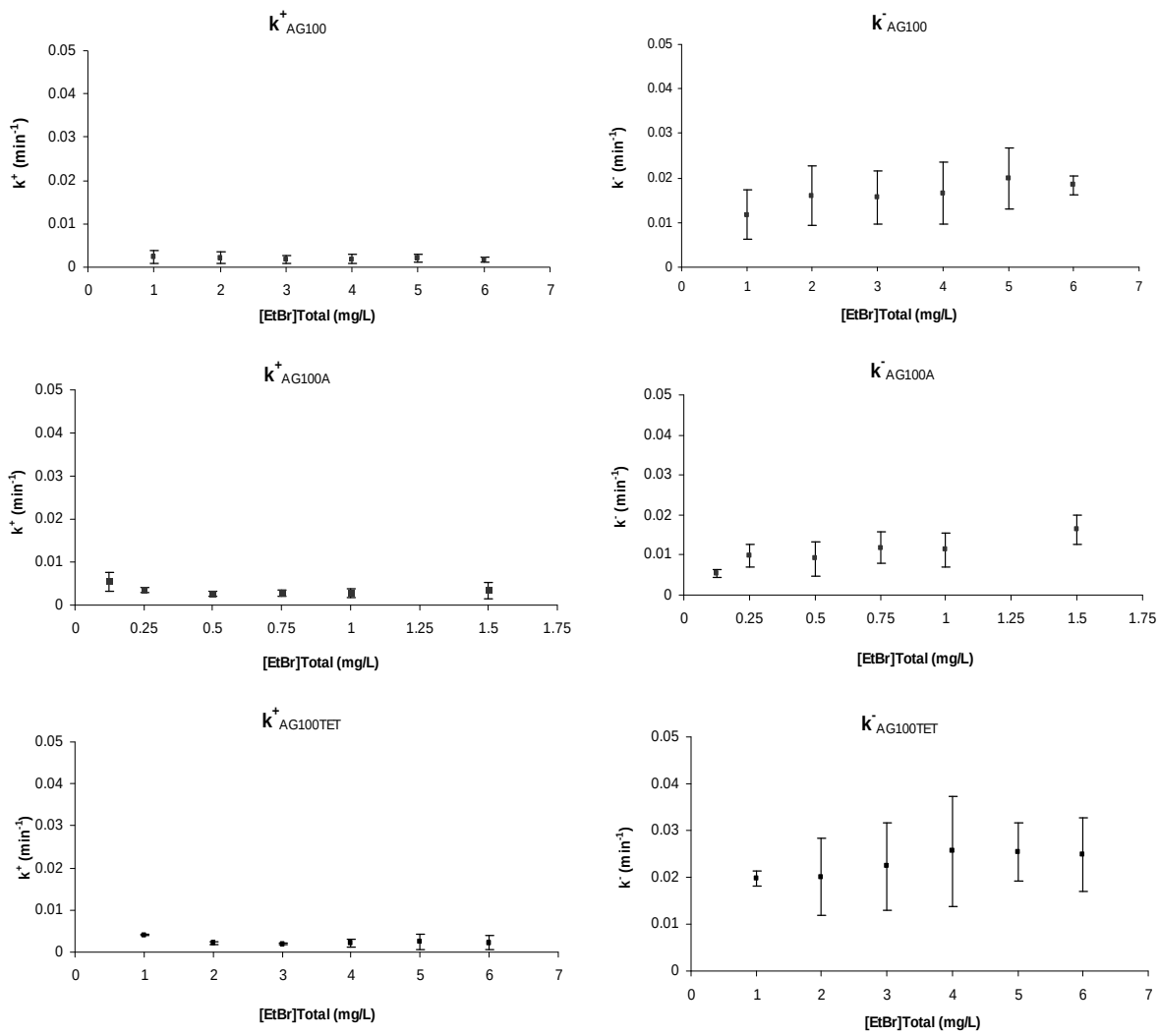

Figure III.4. Influx (k^+) and efflux (k^-) rates of EtBr in the *E. coli* strains tested. The values shown represent averages of three independent experiments and respective standard deviations for the three *E. coli* strains tested: AG100, AG100A and $AG100_{TET}$.

Moreover, the results provided by the model, in terms of EtBr efflux rates, correlate with the MIC for EtBr for each of the strains studied (Table III.1). While the wild-type strain, *E. coli* AG100 (MIC_{EtBr} = 150 mg/L), showed an EtBr efflux rate of $k^-_{AG100} = 0.0173 \pm 0.0057$ min^{-1}, the AcrAB deleted strain, *E.coli* AG100A (MIC_{EtBr} = 5 mg/L), had the lowest EtBr efflux rate $k^-_{AG100A} = 0.0106 \pm 0.0033$ min^{-1}. Conversely, the TET induced strain, *E. coli* $AG100_{TET}$, overexpressing the AcrAB system, shows a much higher efflux

rate $k^{-}_{AG100TET}$ = 0.0230 ± 0.0075 min^{-1}, which corresponds to a MIC for EtBr of 300 mg/L. MICs of each strain towards several antibiotics known to be efflux pump substrates also showed a correlation between efflux activity and resistance level to these drugs (Table III.1). The determination of the MICs for TET in the presence of the efflux inhibitor chlorpromazine reinforced the role played by efflux on such resistance, as the MICs values decreased in the presence of subinhibitory concentrations of this efflux inhibitor, according to the efflux activity previously determined for each strain (Table III.1).

This approach allowed to calculate the values of EtBr influx/efflux rates (k^+ and k^-) for *E. coli* and to construct a predictive model for the accumulation curve of this molecule at any concentration by a given *E. coli* strain, provided that EtBr concentration does not affect the cellular viability. An example of such calculation is provided in Figure III.5, for three different concentrations of EtBr for the *E. coli* strains used. When these predictive curves are compared to the experimental data a close fit is obtained (Figure III.5), thus validating the proposed model.

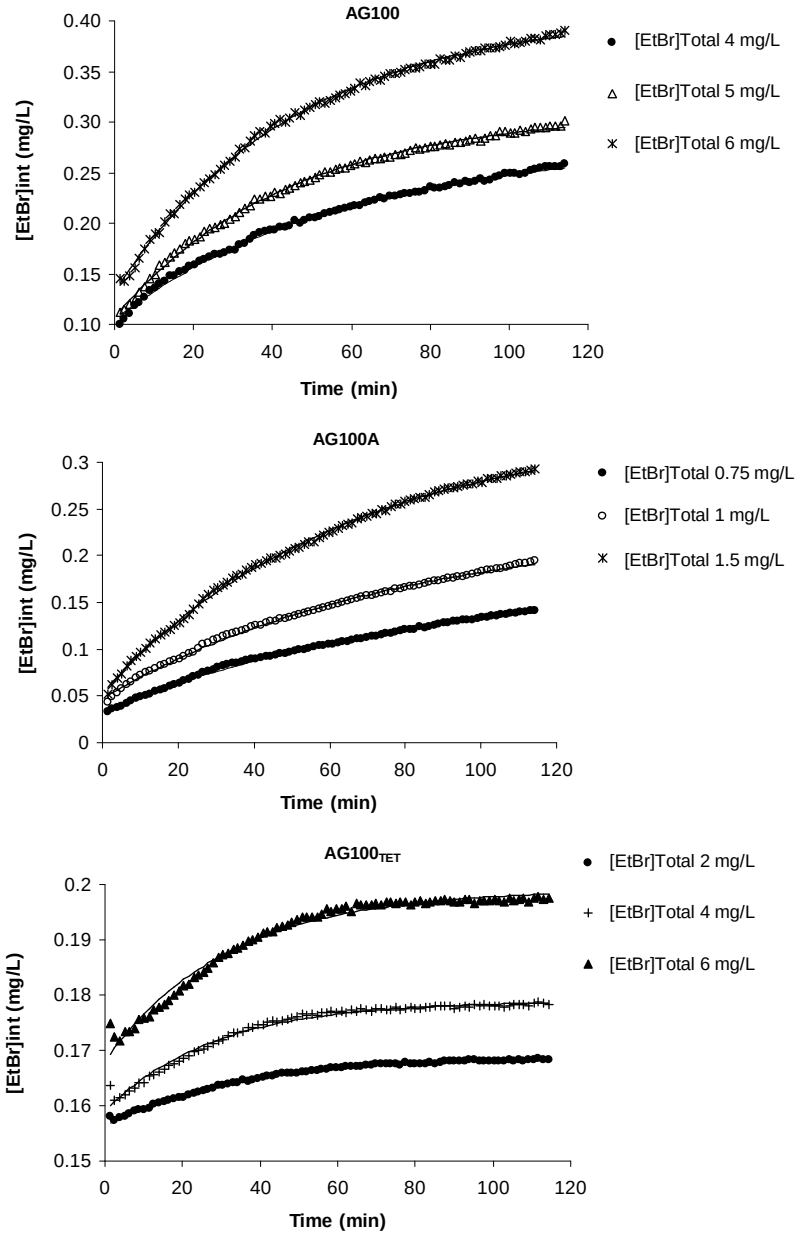

Figure III.5. Examples of model fitting to experimental data. The figures represent the fitting of the model to the experimental data for the time course of intracellular EtBr concentration in *E. coli* strains AG100, AG100A and $AG100_{TET}$, incubated in the presence of different EtBr concentrations. In each case, the solid line represents the model fit.

III.4 Conclusions

In this work, the technical advantages of the semi-automated fluorometric method (Viveiros M. *et al*, 2008a; Viveiros M. *et al*, 2010a) were applied for the development and application of an experimental model to describe and quantify the efflux pump activity in *E. coli*. The semi-automated fluorometric method affords the *in vivo* and on-line evaluation of the efflux pump activity by real-time fluorometry using the Rotor-Gene™ 3000, and represents an innovative technique when compared to other technologies (Viveiros M. *et al*, 2008b). Based upon the differences of fluorescence that reflect differences on the EtBr concentrations outside and inside the cells, the technique provides a powerful tool for the real-time monitoring of efflux kinetics in bacterial cells. Moreover, it has potential for the identification of new efflux pump substrates, as well as new bacterial EPIs (Viveiros M. *et al*, 2008b). A more fundamental application is the extension of this methodology to understand the transport kinetics of EtBr or other fluorescent substrates in bacterial cells.

This approach was used to differentiate and quantify EtBr transport in *E. coli,* and an experimental model was developed which parameterizes passive entry (influx) and active efflux of EtBr. The methodology developed allowed to detect differences in the EtBr efflux activity among strains that differ in their efflux pump expression, highlighting the importance of these systems in the extrusion from the cell of toxic compounds such as EtBr (Lomovskaya O. *et al*, 2007; Okuso H. *et al*, 1996; Poole K., 2007).

Although the experimental model presented was designed based upon a set of three canonic, isogenic strains, as a way to assess the method´s sensitivity to differentiate their singular efflux activities, it can be applied to other *E. coli* strains or bacteria, since it is based on the overall transport of EtBr across the bacterial cell wall, which is common among bacteria (Jernaes MW. *et al*, 1994; Piddock L.J., 2006; Poole K., 2007; Viveiros M. *et al*, 2008a; Viveiros M. *et al*, 2008b). It should be stressed however that it

allows measuring the overall efflux activity rather than efflux related to a specific pump. For evaluation of the role played by a pump, a mutant specific for that pump must be used. The determination of influx and efflux rates for each of the strains to be analyzed opens new insights and opportunities for exploration of cell wall permeability, as well as for the control of the efflux activity in bacterial cells, representing a quantitative method to determine efflux activity. This approach may be used to elaborate a standardized data base for different reference strains relative to efflux activity (efflux rates), providing the means by which one can evaluate the resistance due to efflux pump activity in standardized conditions between laboratories. It represents a particularly suitable procedure for the assessment of efflux pump activity in MDR clinical isolates and screening for potential inhibitors of such activity. Such approaches may contribute to a better understanding of the bacterial efflux systems as a resistance mechanism and to design new therapeutic strategies against MDR bacterial infections (Martins M. *et al*, 2008).

The results and conclusions obtained in this chapter were summarized in the publications Viveiros M. *et al*, 2008a and Paixão L. *et al*, 2009. It was demonstrated in this chapter that the semi-automated fluorometric method is an efficient technique to monitor and quantify the transport of substrates across the cell-wall of bacteria, which could be correlated with multidrug resistance in any bacteria and will be applied to mycobacteria in the following chapters.

III. 5 Materials and methods

Bacteria

The following bacteria were employed in this study: *E. coli* K-12 AG100 wild-type strain (*argE3 thi-l rpsL xyl mtl* Δ(*gal-uvrB*) *supE44*), containing a fully functional AcrAB-TolC efflux pump system; *E. coli* AG100A (Δ*acrAB*::Tn*903* Kan^r) with the AcrAB-TolC efflux

pump system inactivated due to the insertion of the transposon Tn*903* in the *acrAB* operon (Okuso H. *et al*, 1996); and *E. coli* $AG100_{TET}$, a AG100 progeny strain, induced to high level of resistance to TET (able to survive in 10 mg/L of tetracycline, with an MIC of 12 mg/L), as previously described, and over-expressing several efflux pumps, among which *acrAB* shows the highest expression level when exposed to high levels of TET (Viveiros M. *et al*, 2005; Viveiros M. *et al*, 2007). *E. coli* K-12 AG100 and AG100A have been previously characterized and were kindly offered by Hiroshi Nikaido (University of California, Berkeley, California, USA) (Okuso H. *et al*, 1996). Bacterial cultures were grown in Luria-Bertani (LB) medium at 37ºC with agitation (220 rpm). Cultures of AG100A and $AG100_{TET}$ were supplemented with 100 mg/L of kanamycin and 10 mg/L of TET, respectively.

Reagents

PBS, glucose, EtBr, kanamycin, ciprofloxacin, ofloxacin, chloramphenicol, erythromycin, TET and chlorpromazine were purchased from Sigma-Aldrich Química SA (Madrid, Spain). EtBr solutions were stored at 4ºC and protected from light. TET stock solution was prepared in methanol, whereas kanamycin, ciprofloxacin, ofloxacin, erythromycin and chlorpromazine were prepared in distilled water and filtered with 0.22 µm syringe filters (Millipore Corporation, Bedford, USA). Chloramphenicol stock solution was prepared in ethanol. All working solutions were prepared in distilled water on the day of the experiment. LB medium was purchased from Difco (Detroit, Michigan, USA). Mueller-Hinton (MH) broth medium was purchased from Oxoid (Basingstoke, Hampshire, UK).

Determination of minimum inhibitory concentrations

The MICs for EtBr, the efflux inhibitor chlorpromazine, kanamycin, ciprofloxacin, ofloxacin, chloramphenicol, erythromycin and TET were determined by the broth

microdilution method in 96-well microtitre plates according to the CLSI guidelines (CLSI, 2005). Briefly, bacterial strains were incubated overnight in 5 mL of MH broth at 37ºC with shaking at 220 rpm. Bacterial cultures were then diluted in PBS to a McFarland 0.5 turbidity standard. Aliquots of 0.05 mL were transferred to each well of the 96-well plate that contained 0.15 mL of each compound at concentrations prepared at 2-fold serial dilutions in MH broth medium. The plates were incubated at 37ºC and the MIC results registered after 16-18 hours. The MIC was defined as the lowest concentration of compound for which no growth was observed. In order to assure that the EPI did not compromise the cellular viability, the concentrations used in the following work did not exceed ½ of the MIC. The MIC for TET was also calculated in the presence of chlorpromazine at ½ MIC.

The semi-automated fluorometric method

The method was carried out using the real-time thermocycler Rotor-Gene™ 3000 (Corbett Research, Sidney, Australia) to monitor the accumulation and extrusion of EtBr on a real-time basis (Viveiros M. *et al*, 2008a; Viveiros M. *et al*, 2008b). The instrument allows the selection of the excitation and emission wavelengths that for EtBr are 530 nm band-pass and 585 nm high-pass filters, respectively. The fluorescence was acquired in cycles of 60 seconds, during the desired time interval, at the required temperature (37ºC or 25ºC). An explanatory diagram is provided in Figure III.6.

(i) Accumulation assay. The accumulation of EtBr was carried out as previously described (Viveiros M. *et al*, 2008a). Briefly, the *E. coli* strains were grown in 10 mL of LB medium until they reached a mid-log phase, which corresponded to an optical density at 600 nm (OD_{600}) of 0.6. The bacteria were then centrifuged at 13000 rpm for 3 minutes, the pellet washed twice with the same volume of PBS and the OD_{600} of the cellular suspension adjusted to 0.3. Glucose was added to the cellular suspension to a

final concentration of 0.4% (v/v) and aliquots of 0.095 mL were transferred to 0.2 mL microtubes. EtBr was added in aliquots of 0.005 mL to obtain final concentrations that ranged from 1.0 to 6.0 mg/L for *E. coli* K-12 AG100 and $AG100_{TET}$ and from 0.125 to 1.5 mg/L for AG100A. The fluorescence was monitored in the Rotor-Gene^{TM} 3000 at 37ºC under the conditions described above. The effect of chlorpromazine in the accumulation of EtBr was determined under conditions that optimize efflux (presence of glucose and incubation at 37ºC). Chlorpromazine was used at concentrations that did not exceed ½ the MIC and the fluorescence was measured over a period of 60 minutes.

(ii) Efflux assay. The assessment of the efflux of EtBr was conducted as previously described (Viveiros M. *et al*, 2008a). Briefly, the *E. coli* strains were loaded with EtBr under conditions that favor accumulation (no glucose, 25ºC and presence of chlorpromazine). When the maximum level of EtBr accumulation was reached (approximately 60 minutes), the bacteria were centrifuged (13000 rpm for 3 minutes) and the medium was replaced by: i) PBS without glucose; ii) PBS containing glucose; and iii) PBS without glucose and with chlorpromazine (control of minimum efflux). Aliquots of 0.1 mL were transferred to 0.2 mL microtubes and the assay was performed at 37ºC with continuous measurement of fluorescence as described above. The efflux of EtBr is presented in terms of relative fluorescence, which is obtained from the comparison between the fluorescence observed for the bacteria in the presence or absence of glucose and the control in which the cells are exposed to conditions of minimum efflux (*i.e.*, absence of glucose and presence of chlorpromazine). Each experiment was conducted in triplicate and the results obtained did not vary.

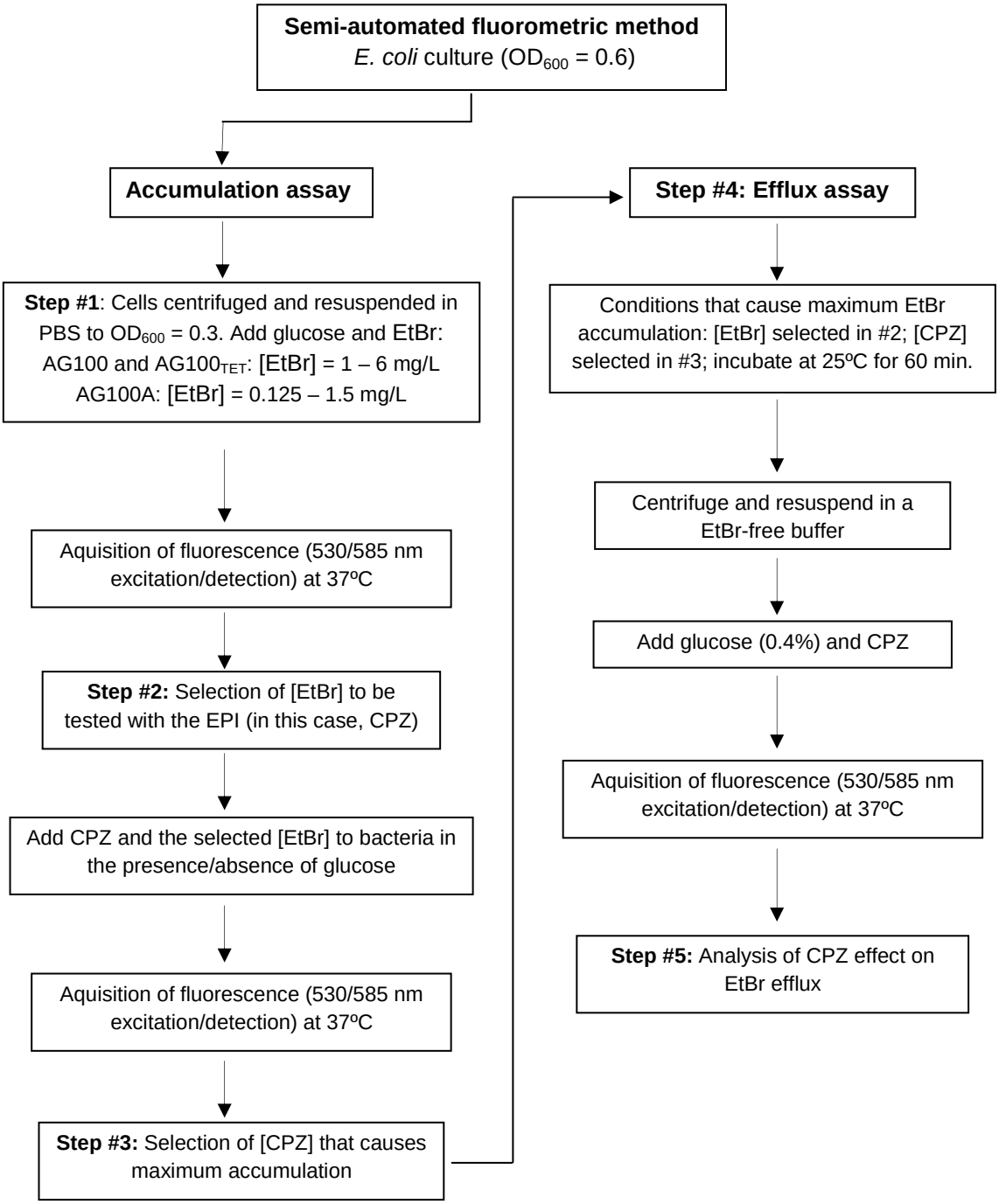

Figure III.6. Experimental flowchart of the semi-automated fluorometric method. EPI, efflux pump inhibitor; EtBr, ethidium bromide; CPZ, chlorpromazine; PBS, phosphate buffered solution.

Model to quantify EtBr efflux in *E. coli*

(i) Quantification of intracellular EtBr concentration. In order to assess the amount of EtBr extruded from the cells, one has to quantify the amount retained inside the cells. First, a calibration curve is determined that correlates the initial fluorescence of the buffer containing EtBr (FL_o) with the corresponding EtBr concentration (varying from 0 to 6 mg/L):

$$[EtBr]_{initial} = 0.1201 \times FL_0 + 0.0373 \; (R = 0.99).$$

Then, for each concentration tested, an EtBr accumulation assay was performed, as described above. To quantify the intracellular EtBr concentration, at the end of the accumulation assay, 0.6 mL of each EtBr containing buffer were separated from the cells by centrifugation at 13000 rpm for 10 minutes and the supernatant filtered with 0.22 µm syringe filters from Millipore Corporation (Bedford, USA). The fluorescence of the cell-free buffer was measured in the Rotor-Gene™ 3000 ($FL(PBS+EtBr)_{\text{filt non corrected}}$).

Since some EtBr is retained by the filter itself, a correction factor must be used to account for this difference. Thus, the corrected fluorescence of the filtered buffer corresponds to:

$$FL(PBS+EtBr)_{\text{filt corrected}} = 1.2289 \times FL(PBS+EtBr)_{\text{filt non corrected}} + 0.7032$$

(R=0.99)

The difference between the initial fluorescence of the EtBr containing buffer (FL_o) and $FL(PBS+EtBr)_{\text{filt corrected}}$ gives the fluorescence corresponding to the intracellular EtBr concentration. However, the fluorescence data from the transport assays measured experimentally still contains a component from the EtBr that remains in solution. Thus, each value of the experimental accumulation curve can then be corrected by subtracting the background corresponding to the fluorescence of the initial

EtBr concentration, originating $FL_{int\ exp\ corr}$. By plotting the previously determined EtBr intracellular concentration for the final value of $FL_{int\ exp\ corr}$, for each initial EtBr concentration tested, a new relation is achieved:

$$[\mathrm{EtBr}]_{int} = 0.022 \times \mathrm{FL}_{int\ exp\ corr} + 0.06 \text{ (R=0.99)},$$

which allows the conversion of the experimental fluorescence data over time into intracellular EtBr concentration over time. The correlations shown here were determined for the wild-type *E. coli* K-12 AG100. Similar relations were determined specifically for the other two strains used (AG100A and $AG100_{TET}$).

(ii) Mathematical and statistical analyses. The model was fitted to the experimental values ($[\mathrm{EtBr}]_{int}$ *vs* t) using the software *Table Curve*™ 2D from Jandel Scientific - AISN Software STATISTICA, which allowed the determination of the values of the parameters describing the influx and efflux rates, k^+ *and* k^-, respectively. Three independent assays were conducted and the results shown are the average ± standard deviation. P-value from the ANOVA statistical test was calculated.

(iii) Model validation. The predictive model proposed was validated by comparing the experimental data obtained using *E. coli* K-12 AG100, AG100A and $AG100_{TET}$ cell suspensions incubated in the presence of different concentrations of EtBr and the curves generated by the model, using the k^+ *and* k^- constants determined as described above.

Flow cytometry

This methodology was used to corroborate the analysis of the data obtained with the semi-automated fluorometric method. Therefore, the samples from the accumulation and efflux assays, used in the semi-automated fluorometric method, were also

analyzed by flow cytometry, in order to compare the results obtained with these two methodologies. Data acquisition and analysis were performed using a FACSCalibur™ (BD Biosciences, San Jose, CA, USA). EtBr was excited at 488 nm and the fluorescence detected through a 585 nm filter (FL-2 channel).

(i) Accumulation assay. *E. coli* strains were cultured in 10 mL of LB medium at 37ºC and 220 rpm until an OD_{600} of 0.6. Aliquots of 1.0 mL were centrifuged at 13000 rpm for 3 minutes, the supernatant discarded and the pellet washed twice with PBS. The OD_{600} of the bacterial suspension was adjusted to 0.3 using PBS without glucose. EtBr was added at a final concentration of 1 mg/L and chlorpromazine was added to a final concentration of 20 mg/L. Following incubation at 25ºC for 60 minutes, aliquots of 0.5 mL were taken for fluorescence measurement in the flow cytometer FACSCalibur™. Data was collected for at least 10,000 events per sample.

(ii) Efflux assay. After loading the bacteria with EtBr (1 mg/L) in the presence of chlorpromazine (20 mg/L), the bacterial suspension was centrifuged at 13000 rpm for 3 minutes. The supernatant was removed and the pellet resuspended in EtBr-free PBS, adjusting the OD_{600} to 0.3. Efflux was assessed in the presence and absence of glucose at 0.4% (v/v). Aliquots of 0.5 mL were taken after 2.5, 5, 15, 30 and 60 minutes after incubation at 37ºC, for fluorescence measurement in the flow cytometer FACSCalibur™. Analyses were performed with an acquisition of at least 10,000 events per sample.

Chapter IV

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

This chapter contains data in preparation for publication:

o **Rodrigues L.**, Ramos J., Couto I., Amaral L., Viveiros M. 2010 Ethidium bromide transport across *Mycobacterium smegmatis* cell-wall: correlation with antibiotic resistance. Submitted to *BMC Microbiology*.

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

IV.1 Summary

This chapter will focus on the application of the semi-automated method, which was developed using *E. coli* as a model, on the assessment of efflux pump activity in mycobacteria. In particular, we have compared the *M. smegmatis* wild-type strain mc^2155 with knockout mutants for LfrA (the main efflux pump system of *M. smegmatis*) and MspA (the main porin of *M. smegmatis*) for their ability to transport EtBr. This information was then correlated with the determination of MICs of several antibiotics in the presence or absence of the efflux inhibitors chlorpromazine, thioridazine and verapamil. The results obtained show that, in the absence of MspA, the accumulation of EtBr decreased and the cells became more resistant to several antibiotics, whereas the mutant for LfrA showed increased accumulation of EtBr and increased susceptibility to EtBr, INH, EMB and CIP. Moreover, reduction of the MIC for STR, RIF, amikacin, ciprofloxacin, clarithromycin and erythromycin was observed in the presence of the tested inhibitors. These results put in evidence that MspA plays an important role in the entrance of quaternary ammonium compounds and antibiotics into the cells and that active efflux *via* the LfrA pump is involved in low-level resistance to several antimicrobial drugs in *M. smegmatis*. The methodology described in this study and the results obtained with this non-pathogenic mycobacterium will be used in further studies as a model for the evaluation of the activity of the same efflux inhibitors on the susceptibility of *M. tuberculosis* to INH (Chapter VI).

IV.2 Introduction

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 (Brennan P.J. *et al*, 1995). This low permeability is due to the structure and lipid-rich composition of the mycobacterial cell-wall that comprises mycolic acids, long-chain fatty acids covalently bound to a peptidoglycan-arabinogalactan polymer, and extractable lipids not covalently linked to the peptidoglycan-arabinogalactan (Brennan P.J. *et al*, 1995; Brennan P.J., 2003; Niederweis M., 2003). 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 (Niederweis M. *et al*, 1999; Stahl C. *et al*, 2001). Along with the cell-wall impermeability, active efflux systems can also provide resistance by extruding compounds that have entered the cell, making the intracellular concentration of a given compound a result of the interplay between permeability and efflux (Nikaido H., 2001). Therefore, it is crucial to clarify the mechanisms of drug efflux *via* efflux pumps, in order to develop new antimycobacterial therapeutic strategies. Several mycobacterial efflux pumps have been identified and characterized to date (Ainsa J.A. *et al*, 1998; Choudhuri *B. S. et al*, 2002; De Rossi E. *et al*, 2006; Siddiqi N. *et al*, 2004; Silva P.E. *et al*, 2001; Takiff H.E. *et al*, 1996; Viveiros M. *et al*, 2003). However, the role played by these pumps in drug resistance in mycobacteria is not completely understood. LfrA, a transporter protein of the major facilitator superfamily (MFS) of *M. smegmatis*, was the first functional efflux pump to be described in mycobacteria and it has been associated with resistance to EtBr, acriflavine, doxorubicin, rhodamine 123 and fluoroquinolones (Li X.Z. *et al*, 2004; Liu J. *et al*, 1996; Sander P. *et al*, 2000; Takiff H.E. *et al*, 1996). Moreover, it was demonstrated that the upstream region of *lfrA* 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 (Bellinzoni M. *et al*, 2009; Buroni S. *et al*, 2006).

In the study to be described, we used the semi-automated fluorometric method to study EtBr transport in *M. smegmatis*, using the wild-type strain mc^2155 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 homologous pumps are present in *M. tuberculosis* and *M. smegmatis*, the use of *M. smegmatis* as a model organism can provide data that will help to understand efflux-mediated drug resistance in *M. tuberculosis* and other mycobacteria causing infections in humans (Li X.Z. *et al*, 2004).

IV.3 Results

IV.3.1 MspA plays an important role in EtBr influx in *M. smegmatis*

The *M. smegmatis* strains used in this study are described in Table IV.1. The accumulation of EtBr under optimum efflux conditions (presence of glucose and incubation at 37ºC) begins to take place at a concentration of 1 mg/L in the case of *M. smegmatis* SMR5 (a mc^2155 derivative streptomycin resistant strain, see Table IV.1) (Figure IV.1). This concentration of EtBr corresponds to what the intrinsic efflux systems of these organisms are capable of extruding. In the case of the SMR5 derivatives porin mutants MN01 (Δ*mspA*) and ML10 (Δ*mspA* Δ*mspC*), this concentration increased to 2 and 4 mg/L, respectively (Figure IV.1).

This could be the consequence of a decreased influx rate of EtBr due to the deletion of porins in these strains, since mycobacterial porins, namely MspA, are the major pathways for hydrophilic molecules (Niederweis M. *et al*, 2003; Stahl C. *et al*, 2001). Moreover, the efflux inhibitors thioridazine, chlorpromazine and verapamil (used at ½ MIC, see Table IV.2) failed to increase accumulation of EtBr at 1 mg/L in strains MN01

and ML10, in contrast to what is observed in the parental strain SMR5 (Figure IV.2). This excluded the possibility that the decreased accumulation of EtBr in MN01 and ML10 was caused by an active efflux system. Altogether, these results suggest a decreased permeability of the cell-wall of the mutant strains towards EtBr and demonstrate that the presence of MspA is required for EtBr to enter the cell.

Table IV.1: Description of *M. smegmatis* strains used in this study.

***M. smegmatis* strains**	**Description**	**Ref.**
mc^2155	Wild-type	Snapper S.B. *et al*, 1990
SMR5	mc^2155 derivative; resistant to streptomycin due to a mutation in ribosomal protein S12 (*rpsL*)	Sander P. *et al*, 1995
MN01	SMR5 Δ*mspA*	Stahl C. *et al*, 2001
ML10	SMR5 Δ*mspA* Δ*mspC*	Stephan J. *et al*, 2005
XZL1675	mc^2155 Δ*lfrA*	Li X.Z. *et al*, 2004
XZL1720	mc^2155 Δ*lfrR*	Li X.Z. *et al*, 2004

Table IV.2. MICs of EtBr and efflux inhibitors against *M. smegmatis* mc^2155 and mutant strains.

***M. smegmatis* strain**	**MICs (mg/L)**			
	TZ	**CPZ**	**VP**	**EtBr**
mc^2155	12.5	25	200	6.25
SMR5	12.5	25	400	6.25
MN01	25	25	400	6.25
ML10	25	25	250	12.5
XZL1675	6.25	25	125	0.4
XZL1720	12.5	25	200	6.25

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

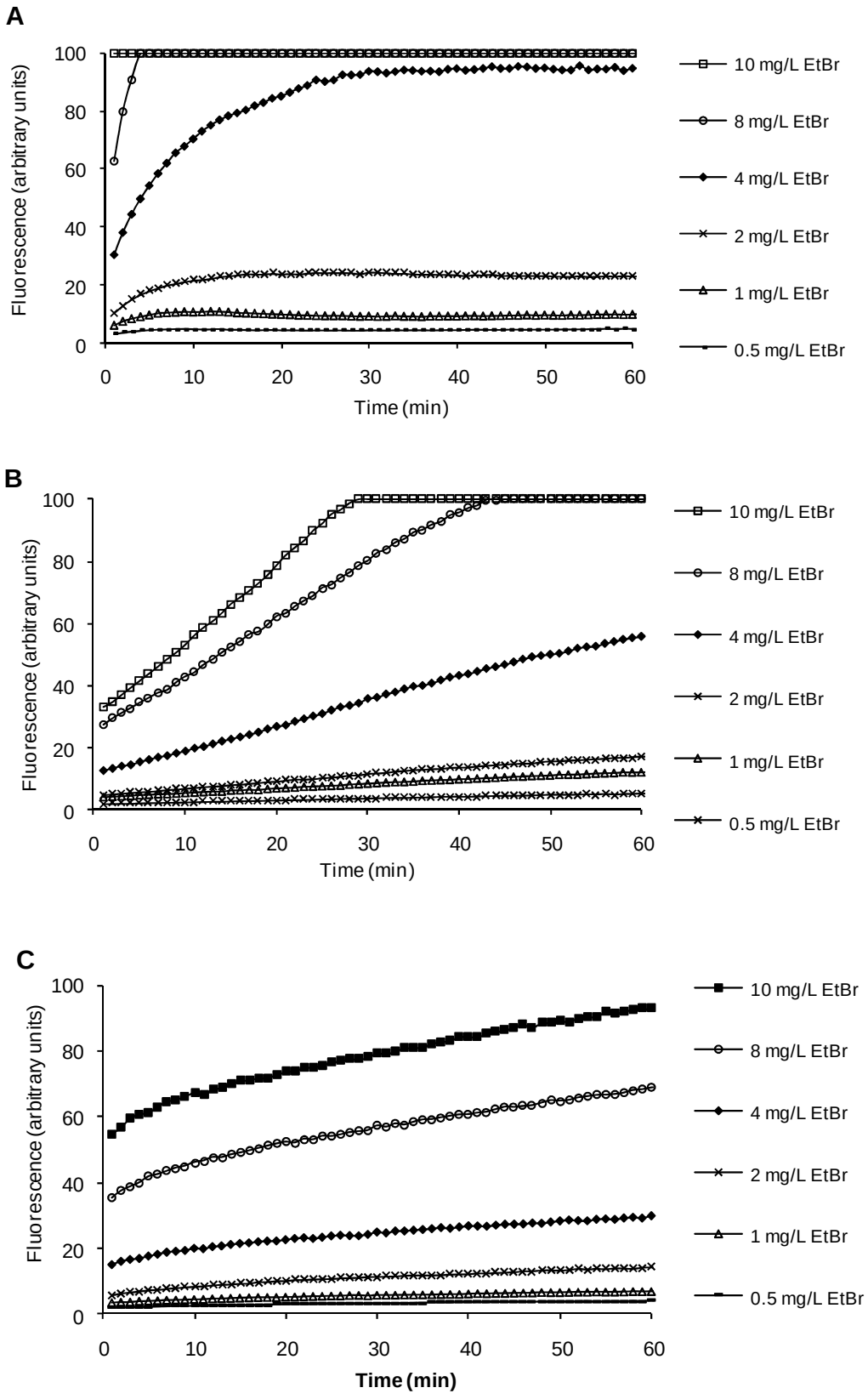

Figure IV.1. Accumulation of EtBr at increasing concentrations (0.5-10 mg/L) by *M. smegmatis* SMR5 (A), MN01 (B) and ML10 (C).

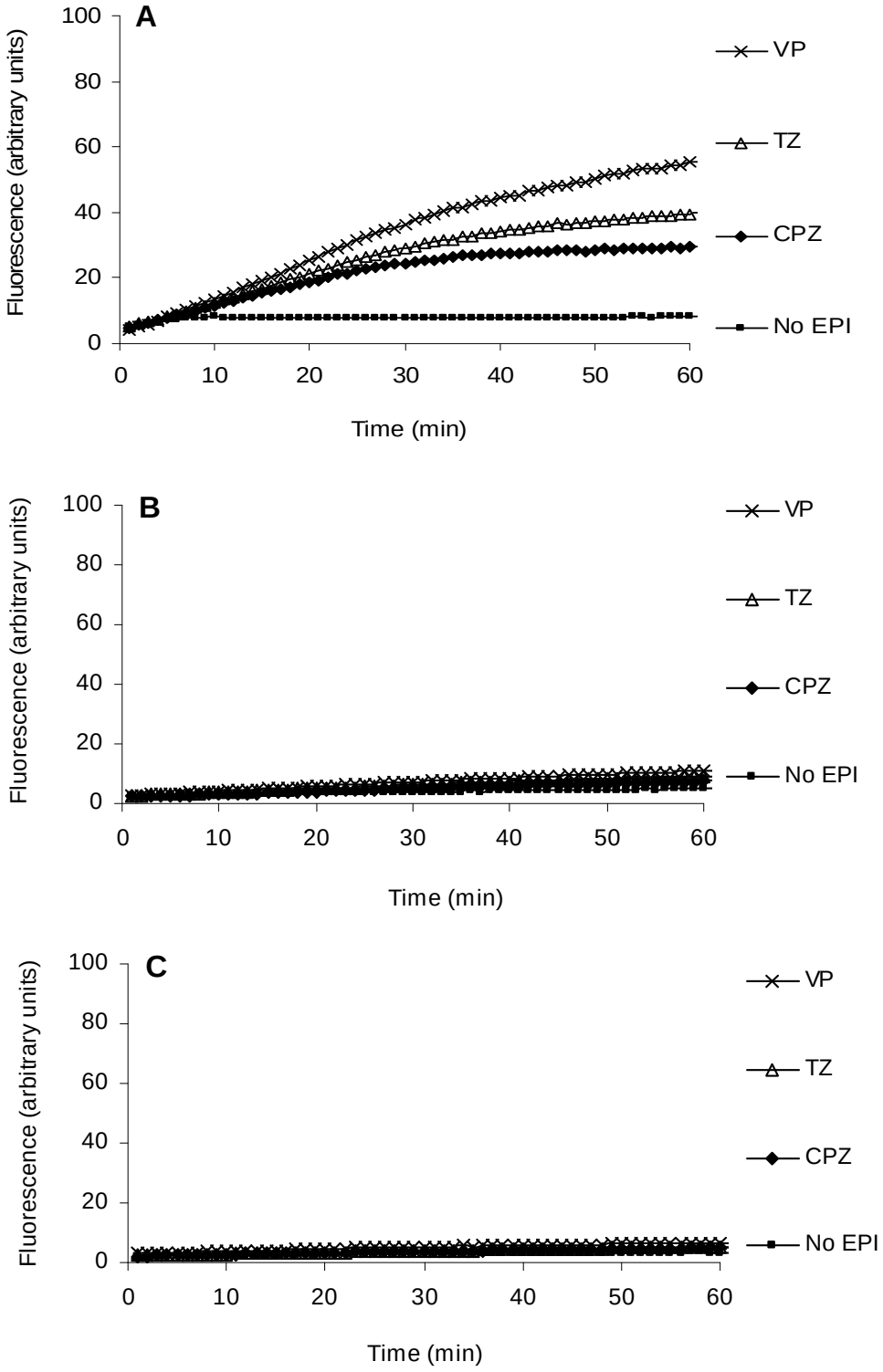

Figure IV.2.Effect of efflux inhibitors on the accumulation of EtBr (1 mg/L) in *M. smegmatis* SMR5 (A), MN01 (B) and ML10 (C). CPZ, chlorpromazine; TZ, thioridazine; VP, verapamil.

III.3.2 LfrA is the main efflux system involved in EtBr extrusion in *M. smegmatis*

Concerning the knockout mutant for the efflux pump LfrA (strain XZL1675), EtBr started to accumulate at a concentration of 0.25 mg/L (Figure IV.3). Since in the wild-type *M. smegmatis* mc^2155, accumulation took place at a concentration of 1 mg/L of EtBr (Figure IV.3), these results demonstrate an increased susceptibility of the mutant strains to EtBr due to a decreased efflux activity. In the case of the *lfrR* mutant XZL1720, EtBr accumulation started at a concentration of 2 mg/L, a higher concentration than the observed for the wild-type *M. smegmatis* mc^2155. This could be due to the constitutive expression of LfrA in this strain as a consequence of the deletion of the repressor LfrR (Buroni S. *et al*, 2006; Li X.Z *et al*, 2004). These results are in agreement to what has been previously reported regarding LfrA as the main efflux system involved in EtBr extrusion (Li X.Z. *et al*, 2004; Liu J. *et al*, 1996; Sander P. *et al*, 2000; Takiff H.E. *et al*, 1996). In order to determine the effect of the inhibitors thioridazine, chlorpromazine and verapamil on EtBr efflux activity, efflux assays were performed for *M. smegmatis* mc^2155, XZL1675 and XZL1720. As shown by Figure IV.4, all strains presented efflux of EtBr at 37ºC in the presence of glucose and this efflux activity was inhibited in the presence of thioridazine, chlorpromazine and verapamil. However, it must be noted that 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 mc^2155 and XZL1720 – ½ MIC for each strain, see Table IV.2). This further demonstrates that the 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 mc^2155 and XZL1720.

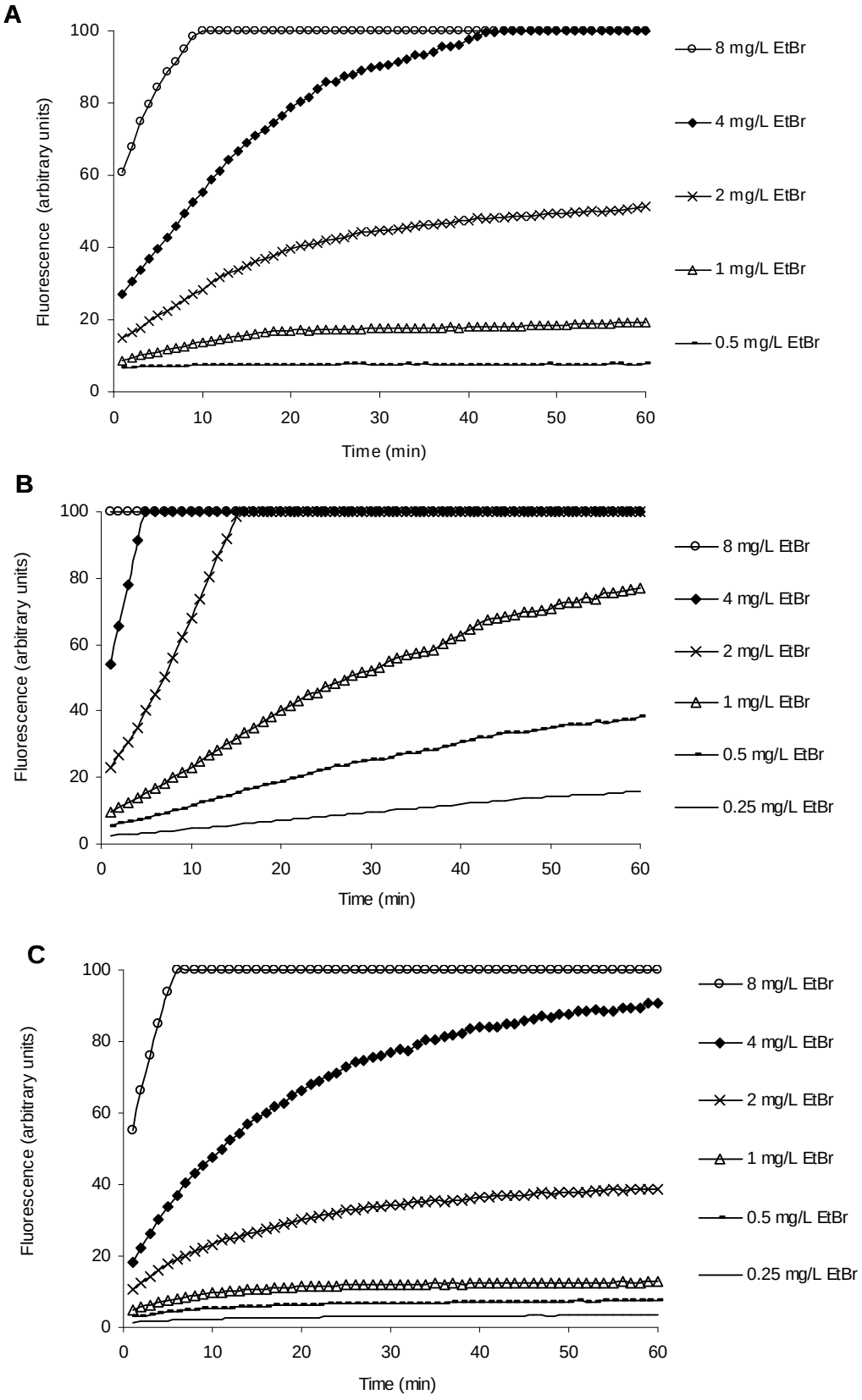

Figure IV.3. Accumulation of EtBr at increasing concentrations (0.25-8 mg/L) by *M. smegmatis* mc^2155 (A), XZL1675 (B) and XZL 1720 (C).

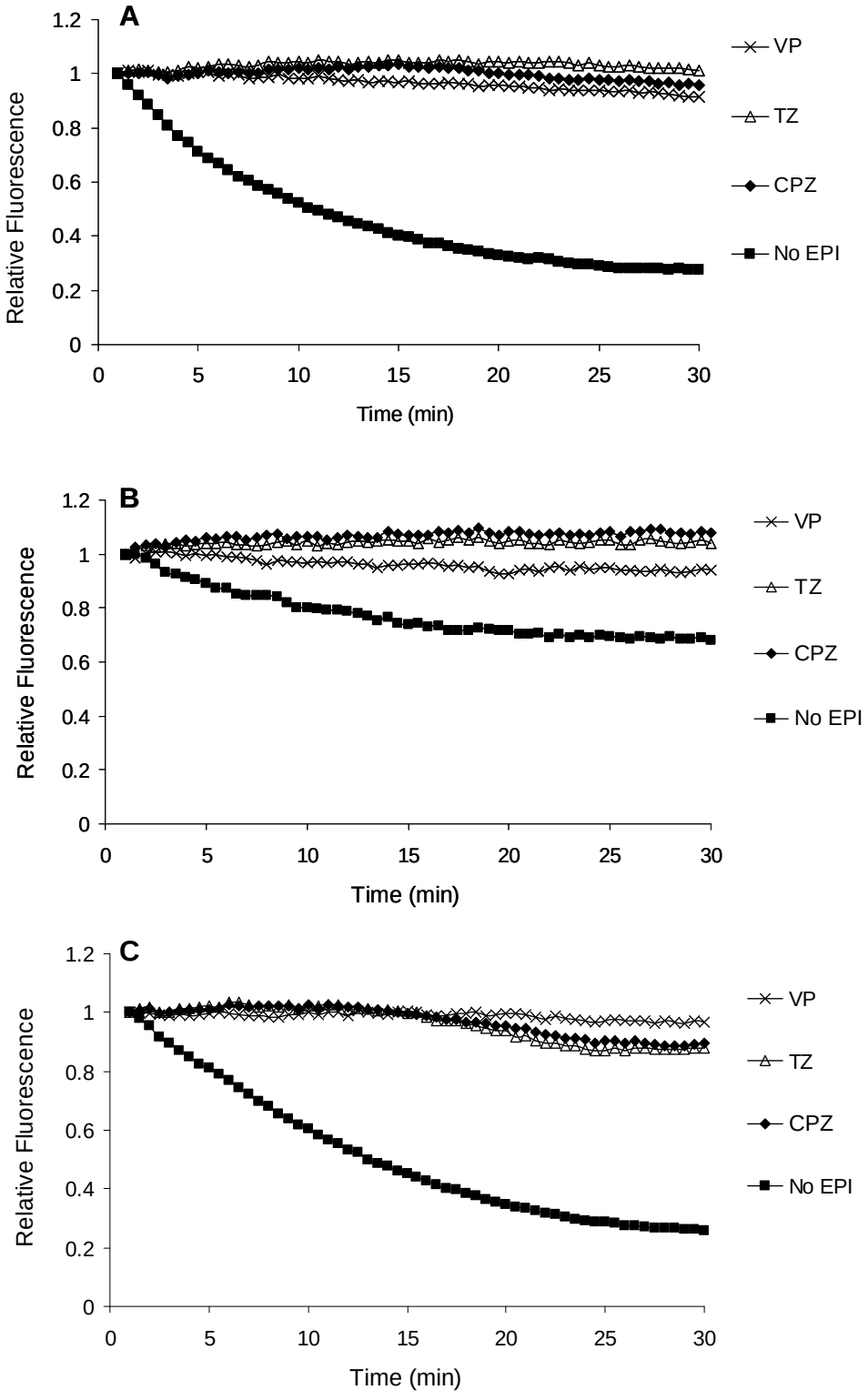

Figure IV.4. Efflux of EtBr by *M. smegmatis* mc^{2}155 (A), XZL1675 (B) and XZL1720 (C). Efflux takes place at 37ºC in the presence of glucose and is inhibited by thioridazine, chlorpromazine and verapamil. CPZ, chlorpromazine; EPIs, efflux pump inhibitors; TZ, thioridazine; VP, verapamil.

III.3.3 Effect of efflux inhibitors on the antibiotic resistance of *M. smegmatis*

The effect of the efflux inhibitors on the reduction of MICs of antibiotics against *M. smegmatis* mc^2155 and mutant strains is presented in Table IV.3. In the case of wild-type *M. smegmatis* mc^2155, thioridazine, chlorpromazine and verapamil promoted a significant (at least 4-fold) reduction of the MIC of STR, RIF, amikacin, ciprofloxacin, clarithromycin and erythromycin. The mutants 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. Deletion of porins MspA (MN01) and MspC (ML10) caused a decreased susceptibility to INH, RIF, clarithromycin and erythromycin, and MIC reduction was observed for amikacin (SMR5 and MN01), RIF, ciprofloxacin, clarithromycin and erythromycin (SMR5, MN01 and ML10). Deletion of *lfrA* (XZL1675) increased the susceptibility to INH, EMB, and ciprofloxacin, which suggests that LfrA might contribute to the intrinsic resistance of *M. smegmatis* to these drugs, as already reported by other studies (Li X.Z. *et al*, 2004). In the LfrA mutant XZL1675, a significant reduction of the MIC was observed for STR, RIF, amikacin, clarithromycin and erythromycin, which suggests that other efflux pumps are at work in this strain. The same efflux inhibitors also had an effect in increasing the susceptibility of the *lfrR* mutant (XZL1720) to the tested antibiotics, with the exception of INH and EMB.

However, some of the results obtained were discrepant with the ones found in the literature. In the particular case of RIF, there is a decrease of the MIC in this strain that also occurs in the *lfrR* deleted mutant. Thus, the observed decrease of the MIC may not be a result of the deletion of the pump, but could be due to other causes. The previous characterization of these strains by Li and Nikaido (Li X.Z. *et al*, 2004) showed that there is no difference between the mutant strains and the wild-type concerning the MIC for RIF (authors reported an MIC of 1 mg/L for each strain). Also, our results show that the MIC for ciprofloxacin in the case of the *lfrR* mutant is the same observed for

the *lfrA* mutant, whereas in the literature the deletion of *lfR* is reported to increase the ciprofloxacin MIC from 0.25 mg/L (wild-type *M. smegmatis* mc^2155) to 4 mg/L (*M. smegmatis* XZL1720). The discrepancy between our results and the ones reported in the literature could be due to the different methods used for the determination of theMICs: microdilution method in Middlebrook 7H9 medium supplemented with oleic acid-albumine-dextrose-catalase (OADC) (this study) or microdilution method in 7H9 medium supplemented with OADC and Tween 80 in combination with drug gradient plates (Li X.Z. *et al*, 2004).

Table IV.3. Effect of efflux inhibitors on the MICs of antibiotics for wild-type and mutant strains of *M. smegmatis*.

MICs (mg/L)							
Antibiotic/EPI		***M. smegmatis* strains**					
		mc^2155	**SMR5**	**MN01**	**ML10**	**XZL1675**	**XZL1720**
STR	No EPI	0.5	>256	>256	>256	0.5	0.5
	TZ	**0.125**	>256	>256	>256	**0.125**	0.25
	CPZ	**0.125**	>256	>256	>256	**0.032**	**0.063**
	VP	0.25	>256	>256	>256	0.25	**0.125**
INH	No EPI	8	128	128	128	4	8
	TZ	8	128	128	128	4	4
	CPZ	8	128	128	128	4	4
	VP	8	128	128	128	4	4
RIF	No EPI	4	4	8	8	0.5	0.5
	TZ	2	2	4	4	**0.125**	**0.125**
	CPZ	**1**	**1**	**2**	**2**	**0.125**	**0.125**
	VP	2	2	4	4	**0.125**	0.25

Table IV.3. (cont.)

EMB	No EPI	1	1	1	1	0.5	1
	TZ	1	1	1	1	0.5	1
	CPZ	1	1	1	1	0.5	1
	VP	1	1	1	1	0.5	1
AMK	No EPI	0.5	0.5	0.5	0.5	0.5	0.5
	TZ	**0.063**	**0.063**	**0.125**	0.25	**0.063**	**0.063**
	CPZ	**0.125**	**0.125**	**0.125**	0.25	**0.063**	**0.063**
	VP	**0.125**	**0.125**	**0.125**	0.25	**0.125**	**0.125**
CIP	No EPI	0.25	0.25	0.25	0.25	0.125	0.125
	TZ	**0.063**	**0.063**	**0.063**	**0.063**	**0.032**	**0.032**
	CPZ	**0.063**	**0.063**	**0.063**	**0.063**	0.063	0.063
	VP	**0.063**	**0.063**	**0.063**	**0.063**	0.063	0.063
CLT	No EPI	2	2	8	8	2	2
	TZ	**0.25**	**0.25**	**1**	**1**	**0.25**	**0.25**
	CPZ	**0.25**	**0.25**	**0.5**	**1**	**0.25**	**0.25**
	VP	**0.5**	**0.5**	**0.5**	**1**	**0.5**	**0.5**
ERY	No EPI	32	32	64	64	32	32
	TZ	**4**	**4**	**16**	**16**	**4**	**4**
	CPZ	**4**	**4**	**8**	**8**	**4**	**4**
	VP	**8**	**8**	**8**	**8**	**8**	**8**

AMK, amikacin; CIP, ciprofloxacin; CLT, clarithromycin; CPZ, chlorpromazine; EPI, eflux pump inhibitor; ERY, erythromycin; ETB, ethambutol; INH, isoniazid; 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 EPI.

IV.4 Conclusions

The detection of EtBr influx and efflux can be used to anticipate transport-mediated antibiotic resistance in bacteria since these compounds use similar channels to enter and leave the cell. In this study, we have compared the wild-type *M. smegmatis* mc^2155 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 general 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 (Niederweis M. *et al*, 1999, Stephan J. *et al*, 2005 Wolschendorf F. *et al*, 2007). However, this does not necessarily mean that EtBr uses MspA as a direct pathway into the cell. In fact, one cannot rule out the possibility that the deletion of porins alters the structure and composition of the cell-wall, thus making it more impermeable. Studies have 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 (Stephan J. *et al*, 2004). It was suggested that *mspA* deletion causes the alteration of lipid arrangement in the mycobacterial outer membrane. This was supported by the finding that the *mspA* mutant presented a decreased rate of uptake of the hydrophobic agent chenodeoxycholate compared to that by the wild-type. Decreased permeability to this agent was also observed in a mutant of *M. tuberculosis* lacking oxygenated mycolic acids, which shows that the lipid arrangement is important for the permeability of the mycobacterial outer membrane (Dubnau E. *et al*, 2000; Stephan J. *et al*, 2004).

The mutant for the LfrA pump showed increased accumulation of EtBr and also an increased susceptibility to INH, EMB and ciprofloxacin. This is also in agreement to

what has been demonstrated in other studies that showed that disruption of the *lfrA* gene decreased the MIC of EtBr, acriflavine ciprofloxacin, doxorubicin and rhodamine (Liu J. *et al*, 1996; Takiff H.E. *et al*, 1996). Moreover, resistance to these antibiotics decreased in the presence of the efflux inhibitors in the wild-type strain and the mutant strain for the LfrA repressor, further demonstrating that active efflux by LfrA plays an important role in the extrusion of a broad range of substrates. Altogether these results show that MspA plays an important role in the entrance of quaternary ammonium compounds and antibiotics into the cells and that active efflux *via* the LfrA pump is involved in low-level resistance to several antibiotics and quaternary ammonium compounds in *M. smegmatis*.

The results and conclusions obtained in this chapter were summarized in the publication Rodrigues L. *et al*, 2010. We have been able to demonstrate in this chapter that in the absence of the porin MspA, *M. smegmatis* became more resistant to several antibiotics and EtBr, whereas the mutant for the efflux pump LfrA showed increased accumulation of EtBr and increased susceptibility to EtBr, INH, EMB and ciprofloxacin. In addition, MIC reductions for STR, RIF, amikacin, ciprofloxacin, clarithromycin and erythromycin were observed in the presence of efflux inhibitors. Therefore, the porin MspA plays an important role in the entrance of quaternary ammonium compounds and antibiotics into the cells and the LfrA pump is involved in the extrusion and, consequently, in low-level resistance to several antimicrobial drugs. The rational and methodology applied in this chapter to non-pathogenic mycobacteria will be used for the evaluation of the activity of the same efflux inhibitors on the susceptibility of *M. avium* complex reference and clinical strains to macrolides (Chapter V) and to *M. tuberculosis* complex strains to INH (Chapter VI).

IV.5 Materials and Methods

Materials

EtBr, glucose, PBS, chlorpromazine, thioridazine, verapamil, STR, INH, RIF, EMB, amikacin and ciprofloxacin 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 IV.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 37ºC 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 thioridazine, chlorpromazine 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 Clinical Laboratory Standards Institute (CLSI) guidelines (CLSI, 2003). Briefly, mycobacterial strains were grown at 37ºC in Middlebrook 7H9 broth supplemented with 10% OADC until an $O.D._{600}$ of 0.8. The 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 37ºC 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 in the studied *M. smegmatis* strains was performed using the semi-automated fluorometric method, previously developed as described in Chapter III.

(i) Accumulation assay. *M. smegmatis* strains were grown in 5 mL of 7H9/OADC medium at 37ºC until an $O.D._{600}$ of 0.8. The culture was 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 10 mg/L. The 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 37ºC.

The effect of thioridazine, chlorpromazine 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. The fluorescence was measured every 60 seconds for 60 minutes at 37ºC in the Rotor-Gene™ 3000.

(ii) Efflux assay. Mycobacteria were exposed to conditions that promote accumulation of EtBr: no glucose; presence of the efflux inhibitor that caused maximum accumulation, in this case verapamil; and incubation at 25ºC (Viveiros M. *et al*, 2008;

Rodrigues L. *et al*, 2008). 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. The 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 efflux 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.

Chapter V

The role of efflux pumps in macrolide resistance in *Mycobacterium avium* complex

This chapter contains data published in:

- **Rodrigues L**., Sampaio D., Couto I., Machado D., Kern W.V., Amaral L., Viveiros M. 2009. The role of efflux pumps in macrolide resistance in *Mycobacterium avium* complex. *Int. J. Antimicrob. Agents* **34**(6):529-533.
- **Rodrigues L.**, Wagner D., Viveiros M., Sampaio D., Couto I., Vavra M., Kern W.V., Amaral L. 2008. Thioridazine and chlorpromazine inhibition of ethidium bromide efflux in *Mycobacterium avium* and *Mycobacterium smegmatis*. *J. Antimicrob. Chemother.* **61**: 1076–1082.

The role of efflux pumps in macrolide resistance in *Mycobacterium avium* complex

V.1 Summary

M. avium complex (MAC) is clinically important since it can cause severe infections in immunocompromised individuals such as patients with AIDS. The use of the macrolides clarithromycin and azithromycin has improved the outcome of MAC infections, but therapeutic failure is still a major problem. Along with the objectives of this thesis and following the evidences previously gathered, we have studied, in this chapter, the contribution of efflux pump activity in macrolide resistance in MAC clinical strains. Eighteen clinical strains isolated from AIDS patients were evaluated for macrolide resistance in the presence and absence of the efflux inhibitors thioridazine, chlorpromazine and verapamil. The efflux activity of these strains was then assessed by the semi-automated fluorometric method that detects extrusion of EtBr, as demonstrated in Chapter III and IV. Resistance to clarithromycin was significantly reduced in the presence of thioridazine, chlorpromazine and verapamil. The same efflux inhibitors were effective in decreasing the efflux of EtBr from MAC cells. Moreover, increased retention of [^{14}C]-erythromycin in the presence of these efflux inhibitors further demonstrated that active efflux contributes to MAC resistance to macrolides. This study demonstrates that efflux pumps play an important role in MAC resistance to antibiotics and can now be extended to highly pathogenic mycobacteria.

V.2 Introduction

MAC comprises a heterogeneous group of environmental mycobacteria whose clinical relevance has acquired greater significance in recent decades because it causes severe infections in AIDS patients and other immunocompromised individuals (Field S.K. *et al*, 2004; Griffith D.E. *et al*, 2007; Guthertz L.S. *et al*, 1989; Primm T.P. *et al*,

2004). The classic definition of MAC includes the genetically distinct species *Mycobacterium avium* and *Mycobacterium intracellulare* (Inderlied C.B., 1993). *M. avium* is more prevalent in clinical and environmental samples and is mainly responsible for disseminated MAC disease in HIV patients, whereas *M. intracellulare* is an important contributor to MAC-associated pulmonary infections in immunocompetent or non-HIV patients (Guthertz L.S. *et al*, 1989; Tsang A.Y. *et al*, 1992; Turenne C.Y. *et al*, 2007; Yakrus M.A. *et al*, 1990). MAC infections are difficult to treat owing to the intrinsic multidrug resistance of the organism, which is assumed to be a consequence of decreased permeability of the cell wall, mainly due to the composition of the thick arabinogalactan–peptidoglycan–mycolic and, in MAC strains, this layer is surrounded by a second electrondense layer of serovar-specific glycopeptidolipids (Inderlied C.B. *et al*, 1993). Thus, the wide range of resistance shown by MAC isolates to most antimicrobial drugs has been ascribed to the composition of its unique cell wall, the integrity of which, when affected, results in increased susceptibility to a variety of drugs (Griffith D.E.*l*, 2007; Guthertz L.S. *et al*, 1989; Jarlier V. *et al*, 1994; Tomioka H., 2004). The only antimicrobial agents for which a correlation has been demonstrated between in vitro susceptibility results and clinical outcome in controlled clinical trials are the macrolides clarithromycin and azithromycin. Because susceptibility to clarithromycin predicts susceptibility to azithromycin, CLSI guidelines state that clarithromycin should be the only drug routinely evaluated for activity against MAC (CLSI, 2003). There are no CLSI interpretive guidelines for other drugs since in vitro results are not predictive of clinical outcome and may be misleading (CLSI, 2003). Nevertheless, other drugs such as rifabutin, EMB, amikacin, clofazimine, linezolid and the fluoroquinolones are relatively effective in controlling MAC bacteraemia as long as they are able to reach their internal target and the target has not mutated (Griffith D.E., 2007; Tomioka H., 2004). It is now known that efflux pumps contribute to intrinsic resistance of mycobacteria to antibiotics. Moreover, when these efflux pumps are overexpressed

they extrude a wide variety of chemically and structurally unrelated noxious compounds from the cell, thereby preventing them from reaching their intended targets (Amaral L. *et al*, 2007; De Rossi E. *et al*, 2006; Doucet-Populaire F. *et al*, 2002; Silva P.E. *et al*, 2001; Viveiros M. *et al*, 2003). Although overexpressed activity of efflux pumps may account for MDR phenotypes of some MAC and *M. tuberculosis* clinical strains, evidence in support of this possibility is essentially absent. It has been previously demonstrated that the activity of efflux pumps in *M. tuberculosis* responsible for induced high-level resistance to isoniazid can be inhibited by efflux inhibitors such as reserpine (De Rossi E. *et al*, 2006; Viveiros M. *et al*, 2001; Viveiros M. *et al*, 2003). In this chapter, we intend to demonstrate the presence of an intrinsic efflux system in *M. avium* reference strain ATCC25291^T and to correlate efflux pump activity with macrolide resistance in MAC clinical strains (Rodrigues L. *et al*, 2008, Rodrigues L. *et al*, 2009).

V.3 Results

V.3.1 Effect of efflux inhibitors in macrolide resistance in reference and clinical isolates of MAC

The MICs for clarithromycin and erythromycin against the identified strains are summarized in Table V.1. Among the clinical strains tested, *M. avium* 47/07, *M. avium* 386/08, *M. intracellulare* 253/07 and *M. intracellulare* AL2 presented clinically significant resistance to clarithromycin (breakpoints according to CLSI guidelines: S ≤ 16 mg/L; I = 32 mg/L; R ≥ 64 mg/L) and a high MIC for erythromycin. This high-level clarithromycin/erythromycin resistance is invariably associated with a mutation in adenine 2058 or 2059 in domain V of the 23S rRNA gene and is the main mechanism of resistance accounting for this phenotype (Griffith D.E. *et al*, 2006; Meier A. *et al*, 1996b; Nash K.A. *et al*, 1995).

In order to determine if the drug susceptibility phenotype of these strains was affected by the presence of an efflux inhibitor, the MIC for each antibiotic was determined in the absence and presence of ½ of the MICs of thioridazine, chlorpromazine and verapamil (Table V.1).

Table V.1. Effect of thioridazine, chlorpromazine and verapamil on the MICs of clarithromycin and erythromycin against MAC strains.

Strain	MICs (mg/L)							
	Clarithromycin				Erythromycin			
	No EPI	TZ	CPZ	VP	No EPI	TZ	CPZ	VP
M. avium ATCC25291^T	4	2	2	2	16	**0.5**	**0.5**	**4**
M. avium 104	8	8	8	8	256	256	256	256
M. avium HSB1	8	**2**	**2**	4	256	256	256	256
M. avium HSB2	8	**2**	**2**	8	256	256	256	256
M. avium HSB3	8	**2**	**2**	8	256	**64**	**16**	128
M. avium HSB4	8	**1**	**1**	8	256	128	128	256
M. avium HSB5	8	**2**	**2**	**2**	256	256	256	256
M. avium HSB6	8	**2**	**2**	8	256	256	256	256
M. avium AL1	8	**2**	**1**	**1**	128	64	**16**	**16**
M. avium 47/07	1024	1024	1024	512	512	256	512	**128**
M. avium 386/08	512	512	**128**	512	1024	**256**	**256**	512
M. intracellulare ATCC13950^T	0.25	0.25	0.25	0.25	0.06	0.06	0.06	0.06
M. intracellulare 101/07	8	8	4	8	256	256	256	**64**
M. intracellulare 278/07	8	8	8	8	256	**16**	**16**	256
M. intracellulare 308/07	1	0.5	0.5	1	256	256	256	**16**
M. intracellulare 421/07	1	0.5	0.5	1	256	256	256	256
M. intracellulare 30/08	1	**0.25**	0.5	**0.25**	256	256	256	**64**
M. intracellulare 92/08	2	**0.5**	1	**0.125**	256	256	128	256
M. intracellulare 253/07	1024	1024	**256**	1024	2048	1094	**512**	**256**
M. intracellulare AL2	2048	**256**	2048	1024	2048	**256**	1094	**256**

TZ, thioridazine; CPZ, chlorpromazine; VP, verapamil. EPIs were used at ½ the MIC. A 4-fold reduction was considered to denote significant synergistic effect between the antibiotic and the EPI and is identified in bold. Breakpoints for clarithromycin MIC determination by microdilution in 7H9/pH 6.8: **S** ≤ 16 mg/L; **I** = 32 mg/L; **R** ≥ 64 mg/L (CLSI, 2003).

The MICS determined for each efflux inhibitor tested are shown in Table V.2, and it is important to note that ½ the MIC of each of the agents listed has no effect on the viability of the organisms (data not shown). This is a condition that must be satisfied if the effect of the efflux inhibitor on the MIC of an antibiotic is to be exclusively that against the efflux system itself (Rodrigues L. *et al*, 2008; Viveiros M. *et al*, 2008a). As shown by the results presented in Table V.1, the efflux inhibitors chlorpromazine and thioridazine reduced the MIC of clarithromycin for 12 of the 20 isolates tested, including the *M. avium* reference strain. The effects of these efflux inhibitors on the MIC of erythromycin were also evident for 7 strains. Verapamil promoted significant reduction of the MICs for clarithromycin and erythromycin in 4 and 8 strains, respectively. These results suggest that MAC strains have one or more chlorpromazine and thioridazine sensitive efflux pump(s) that recognise clarithromycin as a substrate. However, in the particular case of the strains with high-level resistance to clarithromycin, the reduction of the MICs promoted by the efflux inhibitors does not reach a level of susceptibility, i.e. an MIC ≤ 16 mg/L.

Screening for mutations in the domain V of the 23S rRNA gene revealed the presence of point mutations in position 2058 in three strains that presented high-level resistance to clarithromycin (*M. avium* 47/07 A→G; *M. intracellulare* 253/07 A→C; *M. intracellulare* AL2 A→C). Substitutions of the adenine at position 2058 by guanine or cytosine has been described in association with high-level resistance to clarithromycin (Griffith D.E. *et al*, 2006; Jamal M.A. *et al*, 2000; Nash K.A. *et al*, 1995). The other isolate showing phenotypic resistance to clarithromycin albeit at a lower level (*M. avium* 386/08), presented a wild-type sequence in this region of the 23S rRNA gene. This phenotype could result from mutation(s) located outside this region, or due to a different resistance mechanism(s), including efflux.

Table VI.2. MICs of EtBr and efflux inhibitors determined for MAC clinical and reference strains.

Strain	MIC (mg/L)			
	TZ	CPZ	VP	EtBr
M. avium ATCC25291^T	20	25	800	6.25
M. avium 104	25	25	500	6.25
M. avium HSB1	25	25	1000	16
M. avium HSB2	25	25	500	8
M. avium HSB3	25	25	1000	16
M. avium HSB4	25	25	1000	16
M. avium HSB5	6.25	25	1000	8
M. avium HSB6	25	25	500	8
M. avium AL1	10	25	500	6.25
M. avium 47/07	12.5	12.5	500	12.5
M. avium 386/08	25	12.5	1000	12.5
M. intracellulare ATCC13950^T	12.5	50	1000	2
M. intracellulare 101/07	12.5	12.5	500	12.5
M. intracellulare 278/07	12.5	12.5	500	6.25
M. intracellulare 308/07	12.5	12.5	500	12.5
M. intracellulare 421/07	12.5	12.5	500	12.5
M. intracellulare 30/08	12.5	12.5	500	12.5
M. intracellulare 92/08	12.5	12.5	500	12.5
M. intracellulare 253/07	6.25	12.5	500	4
M. intracellulare AL2	6.25	12.5	500	4

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

V.3.2 Detection of efflux activity by real-time fluorometry

The efflux activity of MAC reference and clinical strains was assessed by the semi-automated fluorometric method in the presence and absence of the efflux inhibitors, following the procedures explained in the previous chapters. As shown by Figure V.1,

efflux of EtBr was detected in both reference and clinical strains (represented by *M. avium* 386/08 and *M. intracellulare* AL2) at 37ºC in the presence of glucose. Moreover, this efflux activity decreased in the presence of verapamil and thioridazine (Figure V.1). A similar effect was observed with chlorpromazine (data not shown). Whilst both *M. avium* strains showed similar EtBr efflux activity, for *M. intracellulare*, efflux was more evident in the clinical strain, while only a basal efflux activity was registered for *M. intracellulare* ATCC13950^{T}. These results provide further evidence that efflux activity is involved in the resistance to macrolides in both *M. avium* and *M. intracellulare* clinical strains.

V.3.3 Retention of [^{14}C]-erythromycin

Evidence for such activity was also provided by studying the retention of radiolabelled erythromycin in order to compare the intracellular accumulation of this antibiotic in the presence or absence of an efflux inhibitor. As shown in Figure IV.2, the presence of thioridazine and verapamil promotes the intracellular retention of [^{14}C]-erythromycin, in accordance with the results obtained for MIC determination for erythromycin in the presence and absence of these efflux inhibitors (Table V.1). These results confirm the previous assumption that at least one active efflux system is involved in the extrusion of macrolides and contributes to the resistance of MAC to these drugs.

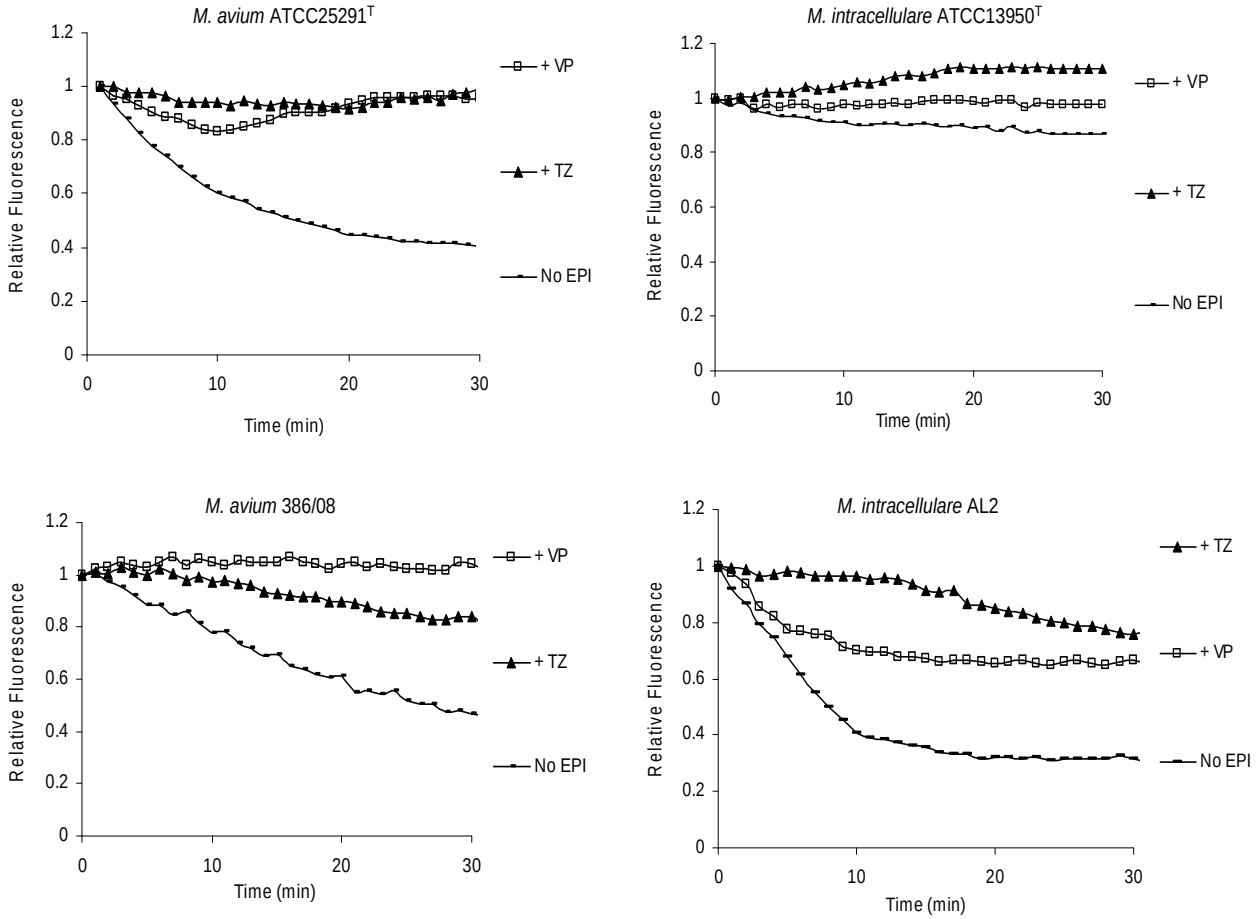

Figure V.1. Efflux of EtBr by *M. avium* ATCC25291^T, *M. intracellulare* ATCC13950^T, and clinical strains *M. avium* 386/08 and *M. intracellulare* AL2. The assays were conducted at 37ºC, with or without an efflux pump inhibitor (EPI). Relative fluorescence was obtained by normalization of data against the conditions of no efflux (presence of efflux inhibitor and no glucose). Efflux of EtBr was inhibited by verapamil (VP) and thioridazine (TZ) used at ½ MIC.

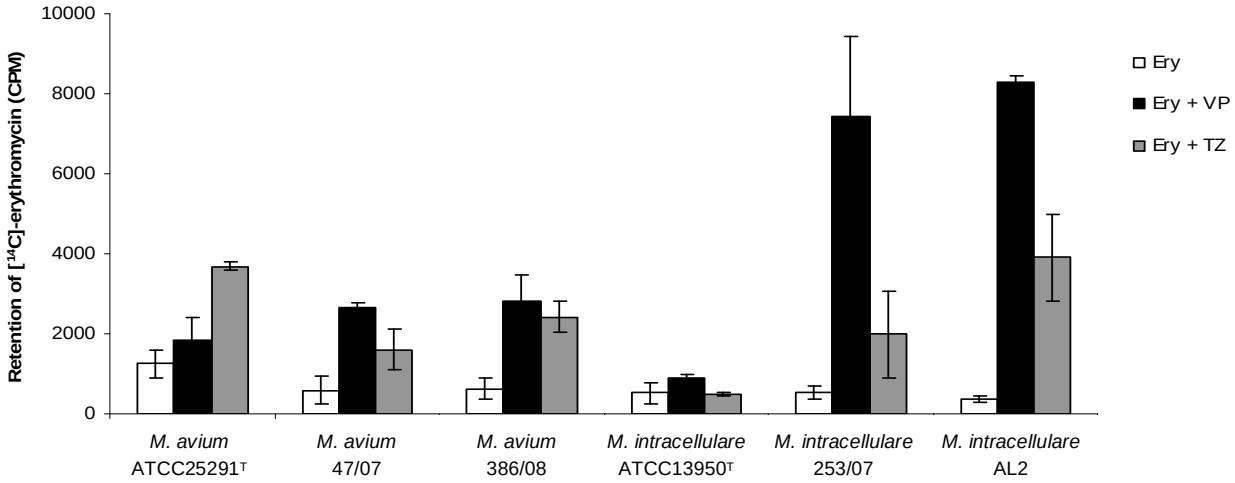

Figure V.2. Retention of [^{14}C]-erythromycin by MAC reference and clinical strains. The intracellular accumulation of [^{14}C]-erythromycin (Ery) was determined in the presence and absence of verapamil (VP) or thioridazine (TZ) at ½ MIC. Values (expressed as counts per minute, CPM) are the average of three replicate tubes. Error bars indicate standard deviations.

V.4 Conclusions

Mycobacteria belonging to MAC are still the most frequent cause of opportunistic bacterial infection in patients with AIDS. Treatment of MAC disease remains difficult and requires months of multiple therapy, although it has been greatly improved with the introduction of the extended-spectrum macrolides clarithromycin and azithromycin, which are far more effective than other antimicrobial agents against MAC infections (Field S.K. *et al*, 2004; Griffith D.E., 2007; Guthertz L.S *et al*., 1989; Primm T.P *et al*., 2004). However, cross-resistance between clarithromycin and azithromycin has been detected and, although monotherapy with clarithromycin (or azithromycin) significantly reduced levels of MAC bacteraemia, it is usually followed by relapses from macrolide-resistant strains. Consequently, other antimycobacterial drugs must be used in combination with clarithromycin to prevent the emergence of macrolide resistance (Heifets LB., 1996).

Efflux of antibiotics has been identified as a relevant contributor to bacterial resistance in clinical practice and is now recognised as an important cause of intrinsic antibiotic

resistance in mycobacteria (Rodrigues L. *et al*, 2008; Rodrigues L. *et al*, 2009; Viveiros M. *et al*, 2003). In this chapter, we investigated the contribution of active efflux to macrolide resistance in reference and clinical MAC strains isolated from AIDS patients. In this work, we have also shown that these efflux inhibitors effectively reduce the MIC of clarithromycin for most strains tested. The same efflux inhibitors were effective in decreasing efflux from MAC cells loaded with EtBr. These efflux assays highlighted the efflux activity in the four high-level resistant MAC strains identified in this study (two *M. avium* and two *M. intracellulare* strains), demonstrating that besides the presence of mutations in the 23S rRNA gene in three of these strains, this high-level resistance also results from efflux activity, as already suggested by the reduction of MICs for clarithromycin and erythromycin in the presence of the efflux inhibitors (Table V.1). Retention of [^{14}C]-erythromycin by the same inhibitors further demonstrated that active efflux contributes to MAC resistance to macrolides. Prolonged exposure to antimycobacterial compounds, such as in MAC therapy, may render the mycobacterial population increasingly resistant by means of increased efflux activity, from which mutants emerge with an increased probability, explaining the relapses from macrolide-resistant strains in patients subjected to monotherapy, especially in AIDS patients where the immune system is weakened; there is thus the need to add other antimycobacterial drugs (Heifets LB., 1996; Tomioka H., 2004). Therefore, agents that inhibit mycobacterial efflux pumps not only render the organism more susceptible to an antibiotic but also reduce the probability of selection of spontaneously arising mutants and may become important antimycobacterial therapy adjuvants and a source of new antimycobacterial compounds.

The results and conclusions obtained in this chapter were summarized in the publications Rodrigues L. *et al*, 2008 and Rodrigues L. *et al*, 2009. We have been able to demonstrate in this chapter that efflux pump activity play an important role in MAC resistance to antibiotics, in particular to macrolides, and this scientific and

methodological approach can now be extended to highly pathogenic mycobacteria such as *M. tuberculosis* complex strains resistant to INH (Chapter VI).

V.5 Materials and Methods

Materials

Middlebrook 7H9 broth and OADC supplement were purchased from Difco (Detroit, MI). EtBr, glucose, PBS, potassium phosphate, lithium chloride, chlorpromazine, thioridazine, verapamil and erythromycin were purchased from Sigma-Aldrich Química SA (Madrid, Spain). Clarithromycin was obtained from Abbott Laboratories (Abbott Park, IL). [N-Methyl-^{14}C]-erythromycin and the liquid scintillation cocktail ULTIMA GOLD F were purchased from PerkinElmer (Waltham,MA). All solutions were prepared on the day of the experiment.

Bacteria

The following MAC strains were used in this study: the reference strains *M. avium* subsp. *avium* ATCC 25291^T and *M. intracellulare* ATCC 13950^T; *M. avium* 104, a common MAC representative whose genome is sequenced; and 17 clinical strains isolated from respiratory specimens from AIDS patients, received in our laboratory from hospitals of the Greater Lisbon area for routine mycobacterial isolation and identification. All specimens were processed by the conventional mycobacteriological NaOH-NALC method (Kent P.T., 1985) and aliquots were collected for acid-fast staining (Ziehl–Neelsen) and inoculation of MGIT tubes of the BACTEC™ MGIT™ 960 system (Becton-Dickinson Diagnostic Instrument Systems, Towson, MD). MAC strains present in full-grown cultures were identified as *M. avium* or *M. intracellulare* by the AccuProbe® system (Gen-Probe Inc., San Diego, CA) according to the manufacturer's

instructions. For the following studies the strains were grown at 37ºC in Middlebrook 7H9 broth or Middlebrook 7H11 solid media, both supplemented with 10% OADC.

Determination of minimum inhibitory concentrations

MICs for the efflux inhibitors thioridazine, chlorpromazine and verapamil and for the antibiotics clarithromycin and erythromycin, alone and in the presence of an efflux inhibitor, were determined by the broth microdilution method according to CLSI guidelines (CLSI, 2003). Briefly, MAC strains were grown in 7H9/OADC medium at 37ºC to an OD_{600} of 0.8. Bacterial cultures were diluted with PBS to McFarland No. 0.5 standard and the inoculum prepared with an aliquot of the suspension was diluted to 1:100. Aliquots of 0.1mL of the inoculum were transferred to wells of a 96-well plate containing 0.1mL of 7H9/OADC medium with two-fold serial dilutions of each agent. The inoculated plates were incubated at 37ºC until growth in the agent-free control well was evident (5–7 days). The MIC was defined as the lowest concentration of compound that inhibited visible growth.

Ethidium bromide efflux assay by a semi-automated fluorometric method

EtBr extrusion from MAC cells was assessed by a semi-automated fluorometric method as described previously (Rodrigues L. *et al*, 2008; Viveiros M. *et al*, 2008a; Viveiros M. *et al*, 2010a). Briefly, mycobacterial cells were grown in 7H9/OADC medium at 37ºC until an OD_{600} of 0.8. The culture was centrifuged at 13000 rpm for 3 minutes, the supernatant was discarded and the pellet was washed once and re-suspended in PBS. After adjusting the OD_{600} to 0.4, mycobacteria were exposed to conditions that promoted maximum accumulation of EtBr: EtBr at ½ the MIC for each strain; no glucose; presence of verapamil at ½ the MIC; and incubation at 25ºC for 60 minutes. EtBr-loaded cells were centrifuged at 13 000 rpm for 3 minutes and re-suspended in EtBr-free PBS. After adjusting the OD_{600} to 0.4, glucose was added at a final

concentration of 0.4%, aliquots of 0.095 mL were transferred to 0.2 mL microtubes and EPIs were added. Fluorescence was measured in a Rotor-Gene™ 3000 real-time thermocycler (Corbett Research, Sydney, Australia) using 530 nm band-pass and a 585 nm high-pass filters as the excitation and detection wavelengths, respectively. Fluorescence data were acquired every 60 seconds for 30 minutes at 37ºC.

Efflux activity was quantified by comparing the fluorescence data obtained for mycobacteria under conditions that allow maximum efflux (incubation at 37ºC, in the presence of glucose and absence of efflux inhibitor) against the data from the control tube that contains the EtBr-loaded cells under conditions that inhibit efflux (presence of an efflux inhibitor and no glucose). Thus, the relative fluorescence corresponds to the ratio of fluorescence that remains per unit of time relative to the EtBr-loaded cells (Rodrigues L. *et al*, 2008; Viveiros M. *et al*, 2008a; Viveiros M. *et al*, 2010a). Each experiment was conducted at least three times and the results obtained did not vary qualitatively.

[^{14}C]-Erythromycin accumulation assay

MAC cultures were grown in 7H9/OADC at 37ºC until an OD_{600} of 0.8. Cells were harvested by centrifugation at 13 000 rpm for 3 minutes, washed once with PBS and the pellet was re-suspended in the same buffer. The OD_{600} was adjusted to 0.4 with PBS containing glucose at a final concentration of 0.4% and 10 µM of [^{14}C]-erythromycin (specific activity 50 mCi/mmol) was added. The bacterial suspension containing radiolabelled erythromycin was split into two replicate sets: one set of triplicate tubes received 0.05 mL of an efflux inhibitor at ½ the MIC, whilst the other set of triplicate tubes received an equal volume of PBS. Following incubation at 37ºC for 16 hours, 1.0 mL aliquots were filtered on Whatman GF/C filters. The filters were washed twice with 3.0 mL of cold 100 mM lithium chloride – 50 mM potassium phosphate buffer (pH 7.0), dried overnight at 37ºC and transferred to scintillation vials

containing the liquid scintillation cocktail ULTIMA GOLD F. The counts per minute (CPM) of each triplicate tube of the replicate sets corresponding to [^{14}C]-erythromycin that remained inside the cells were obtained with the aid of a Beckman LS6500 scintillation counter (Beckman Coulter, Fullerton, CA). The data presented are the average CPM per replicate set of three tubes.

Screening for mutations in the 23S rRNA gene associated with macrolide resistance

Mycobacterial genomic DNA was extracted using a QIAamp DNA Mini Kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions. A 420 bp DNA fragment, spanning positions 1886–2305 of domain V of the 23S rRNA gene, was amplified by polymerase chain reaction (PCR) using primers 23S FI (TTTAAGCCCCAGTAAACGGC) and 23S RIII (GTCCAGGTTGAGGGAACCTT) as described previously (Jamal M.A. *et al*, 2002). The reaction mixture (50 µL) contained 2.5U of Taq polymerase (Fermentas Inc., Ontario, Canada), 1× Taq buffer (Fermentas Inc.), 20 pmol of each primer, 200 mM dNTP and 1.75 mM $MgCl_2$. PCR was conducted in a Mastercycler personal 5332 thermocycler (Eppendorf AG, Hamburg, Germany) and amplification conditions were as follows: DNA denaturation at 94ºC for 4 minutes; 35 cycles of denaturation at 94ºC for 1 minute, annealing at 55ºC for 1 minute and extension at 72ºC for 1 minute; and a final extension at 72ºC for 10 minutes. Amplification products were visualised in 1% agarose gels, purified and sequenced in both strands. Sequences were analysed and aligned using the programs BioEdit version 7.0.9, and ClustalW, respectively, with reference to the 23S rRNA gene of the *M. avium* 104 genome (GenBank accession no. CP000479.1).

Chapter VI

Contribution of efflux activity to isoniazid resistance in *M. tuberculosis* complex

This chapter contains data published in:

o Viveiros M., Portugal I., Bettencourt R., Victor T.C., Jordaan A.M., Leandro C., Ordway D., Amaral L. 2002 Isoniazid-induced transient high-level resistance in *Mycobacterium tuberculosis*. *Antimicrob. Agents Chemother*. **46**(9):2804-2810.

Contribution of efflux activity to isoniazid resistance in *M. tuberculosis* complex

VI.1 Summary

In this work we have induced INH susceptible *M. tuberculosis* H37Rv (ATCC27294) reference strain, *M. tuberculosis* clinical isolates and *M. bovis* BCG to high-level resistance to INH, by gradual exposure to increasing concentrations of this drug. We have used the methodologies developed in the previous chapters to characterize these strains with respect to their efflux pump activity and its contribution to INH resistance. In particular, *M. tuberculosis* strains and *M. bovis* BCG induced to INH resistance were evaluated for their susceptibility to this drug in the presence and absence of the efflux inhibitors thioridazine, chlorpromazine, verapamil and reserpine, using the BACTEC™ MGIT™ 960 system. The efflux activity was assessed by the semi-automated fluorometric method and the expression level of genes coding for efflux pumps was quantified by real-time qRT-PCR. It was demonstrated that the efflux inhibitors decreased INH resistance in the induced strains, namely, verapamil promoted reversal of resistance to susceptibility levels for some of the strains tested. The same efflux inhibitors were able to reduce real-time EtBr efflux. Finally, compared to the non-induced controls, the INH-induced strains showed overexpression of genes coding for efflux pumps. Altogether, these results correlate efflux activity with INH resistance in *M. tuberculosis*. The work presented in this last chapter demonstrates that efflux pumps play an important role in INH resistance and, ultimately, on the emergence of MDRTB, the final goal of this PhD thesis, providing information concerning the role of efflux pumps in the development of INH resistance in *M. tuberculosis* complex. Compounds that inhibit efflux activity may prevent the development of this resistance and provide the basis for new anti-mycobacterial compounds.

VI.2 Introduction

As already discussed in Chapter I Introduction, TB is still a major public health problem accounting for eight million new cases and two million deaths each year (WHO, 2009). The emergence of MDRTB and XDRTB has increased the concern of public health authorities around the world. MDRTB is defined by the WHO as TB caused by *M. tuberculosis* resistant to at least INH and RIF, whereas XDRTB is resistant not only to INH and RIF, but also to any fluoroquinolone and to at least one of the three injectable drugs amikacin, kanamycin and capreomycin (WHO, 2008). INH is one of the most effective first-line drugs against *M. tuberculosis* (Bernstein J. *et al*, 1952), having MICs that range from 0.02 to 0.06 mg/L (Youatt J. 1969). The mechanism of action of INH has been studied since the 1950's and several studies demonstrate that this drug inhibits the synthesis of the mycolic acids by targeting the enzyme InhA (Vilchèze C. *et al*, 2007; Slayden R.A. et al, 2000). Resistance to anti-TB drugs is often due to the occurrence of spontaneous mutations in target genes or regulatory domains, followed by selection of the resulting resistant mutants by subsequent treatment with anti-TB drugs for which the mutants are resistant (Ramaswamy S. *et al*, 1998 Louw G.E. *et al*, 2009). In the particular case of INH, resistance has been associated with mutations located in: (i) *katG*, the calase-peroxidase responsible for the convertion of INH into its active form; (ii) *inhA*, coding for the target enzyme InhA; (iii) *kasA*, encoding KasA, a β-ketoacyl ACP synthase; (iv) *oxyR-ahpC*, an intergenic region responsible for oxidative stress response; and (v) *ndh* that codes for NADH dehydrogenase (Vilchèze C. *et al*, 2007; Louw G.E. *et al*, 2009). However, the described mutations do not explain all the cases of INH resistance, in fact, approximately 20-30% of clinical resistant *M. tuberculosis* isolates do not have mutations in any of the known genes associated with INH resistance (Louw G.E. *et al*, 2009; Ramaswamy S. *et al*, 1998; Ramaswamy S. *et al*, 2003). This suggests that other mechanism(s) are involved in the development of drug resistance, one of which could be the presence of active efflux pump systems that

extrude the anti-TB agent to the exterior of the cell, preventing access to its target (Nikaido H., 2001).

In previous studies, we have induced INH susceptible clinical *M. tuberculosis* isolates, as well as the H37Rv (ATCC27294) reference strain, to high-level resistance to INH by gradual exposure to increasing concentrations of this drug (Viveiros M. *et al*, 2002). The induced strains were resistant to an INH concentration of 20 mg/L and the transfer of these strains to drug-free medium, followed by repeated passages in that medium, yielded organisms whose susceptibility to INH was identical to that of the original strains. Moreover, this induced high-level resistance to INH could be reduced with a subinhibitory concentration of reserpine, a known efflux inhibitor.

In this chapter, we have continued the work started in 2002, now equipped with the rational and technical approaches developed and tested in the previous chapters of this thesis, and characterized these strains with respect to their efflux pump activity using the following approach: (i) *M. tuberculosis* strains (reference strain H37Rv and 2 clinical isolates) and *M. bovis* BCG induced to high-level resistance to INH were evaluated for their susceptibility to INH in the presence and absence of the efflux inhibitors thioridazine, chlorpromazine, verapamil and reserpine; (ii) efflux pump activity in the induced strains was detected using the semi-automated fluorometric method; and (iii) the expression level of genes coding for efflux pumps was quantified by real-time qRT-PCR.

VI.3 Results

VI.3.1 Inducement of resistance to INH in *M. tuberculosis* complex

The process of inducement of INH resistance in *M. tuberculosis* complex was previously described and is represented in Figure VI.1 using *M. tuberculosis* H37Rv as

an example (Viveiros M. *et al*, 2002). The antibiotic susceptibility assay, performed with the BACTEC™ 460TB and BACTEC™ MGIT™ 960 systems, for *M. tuberculosis* H37Rv (ATTC27294), two *M. tuberculosis* clinical strains and *M. bovis* BCG strain Pasteur indicated that each of these strains was susceptible to 0.1 mg/L of INH as well as to RIF, PZA, STR and EMB. These cultures remained inside the BACTEC instrument for an extended period of time that exceeded the completion of the antibiotic susceptibility assay until they yielded evidence of growth. By the end of 28 days after the beginning of the INH susceptibility assay, the growth in all of the cultures with initially susceptible isolates reached a level comparable to that for the individual controls grown in drug-free medium (Figure VI.1).

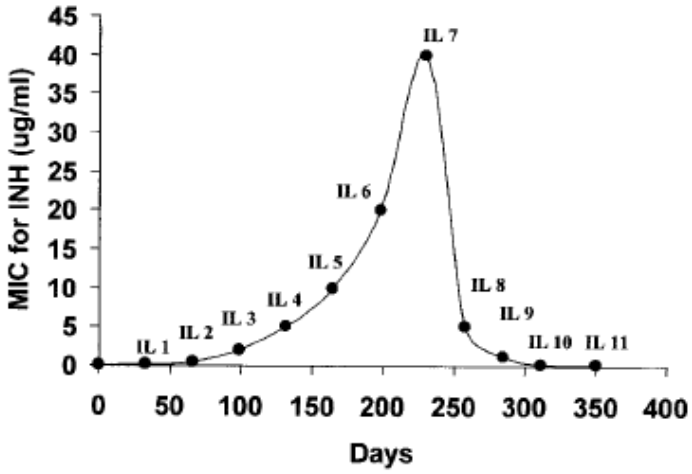

Figure VI.1 Induction and reversal of resistance to INH of *M. tuberculosis* H37Rv reference strain (Viveiros M. *et al*, 2002). The strain (original MIC of 0.05 mg/L) was incubated in medium containing 0.1 mg/L of INH for a period of time beyond that required for antibiotic susceptibility testing. Growth was observed after 28 days and the strain presented a MIC of 0.2 mg/L, being thus designated as having induced INH resistance (induction level 1, IL1). The mycobacteria were then exposed to INH at concentrations that corresponded to the MIC for the previous passage and after 21 days the resulting cells presented an MIC of 0.5 mg/L (IL2). This process was repeated until IL7, at which time the strain was able to grow in the presence of 20 mg/L of INH (MIC 40 mg/L). After this point, the strain was repeatedly transferred to drug-free medium and the MICs gradually decreased (IL8, IL9, and IL10), reaching the MIC for the initial strain (0.05 mg/L) at IL11. The other strains tested were subjected to the same induction procedure, with little difference in the amount of time required for the manifestation of induced resistance at each induction level.

The determination of the MICs for INH showed that these strains presented a higher MIC (0.2 mg/L) than the parental INH susceptible strains (0.05 mg/L) and were defined as having induced INH resistance (induction level 1, IL1). Aliquots of these cultures at IL1 were transferred to fresh BACTEC vials containing a concentration of INH that corresponded to the new MIC (0.2 mg/L). These cultures reached full growth by the end of 10 to 21 days and were subjected to MIC determination, after which time they served as inoculum for the next passage in medium containing a concentration of INH that corresponded to the new INH MIC for the strains in the previous passage. This process was repeated for five additional serial passages, with each succeeding passage containing a concentration of INH that corresponded to the previous MIC for the strain with INH induced resistance at IL2, IL3, IL4, IL5, IL6, and IL7. These passages were also conducted with solid medium containing equivalent concentrations of INH and the plates were evaluated for the numbers of colony forming units (CFUs). This procedure yielded strains that were capable of growing in liquid or solid medium containing 20 mg/L of INH.

Direct examination of the contents of each vial by the use of identification probes indicated that all of the organisms were *M. tuberculosis* complex. However, unlike the parental strains, which were all positive by staining with the Ziehl-Neelsen stain, the cells from any given INH containing vial presented variations in acid-fast staining, ranging from negative to positive staining status. This variation in acid-fast staining could be due to some inhibition of cell-wall mycolic acid synthesis (Vilchèze C. *et al*, 2007). The possibility that the potency of INH in the vials had been affected by prolonged incubation was ruled out since the medium, when filtered free of cells, was as effective as freshly made INH containing medium against *M. tuberculosis* isolates with proven INH susceptibility (data not shown).

After reaching IL7 induction point at which the INH induced strains were capable of growing at 20 mg/L of INH (MIC 40 mg/L), the strains were repeatedly transferred to drug-free medium. This promoted a gradual decrease of the MIC for INH (IL8, IL9, and IL10), reaching the MIC for the original parent strains (0.05 mg/L) at IL11.

VI.3.2 Detection of efflux activity in INH induced *M. tuberculosis* complex strains

The efflux activity of *M. bovis* BCG INH induced strain (*M. bovis* BCG_{INH}) was assessed by the semi-automated fluorometric method and compared with the parental non-induced strain. This was only determined for *M. bovis* BCG due to the biosafety requirements concerning *M. tuberculosis* (biosafety level III). By this manner, all the real-time fluorometric assays for the detection of efflux activity were performed using *M. bovis* BCG (biosafety level II) as a model for *M. tuberculosis*. The accumulation of increasing concentrations of EtBr by *M. bovis* BCG and *M. bovis* BCG_{INH} is presented in Figure VI.2. As already demonstrated in previous studies, this assay allows the selection of the highest concentration of EtBr that does not cause accumulation of EtBr and defines the baseline transport equilibrium between influx and efflux of EtBr, as explained in Chapter III of this thesis (Paixão L. *et al*, 2009; Rodrigues L. *et al*, 2008; Viveiros M. *et al*, 2008a). In this case, accumulation of EtBr under conditions that are considered to maximize efflux, *i.e.*, presence of glucose and 37ºC, begins to take place at a concentration of 0.125 mg/L of EtBr for the *M. bovis* BCG non-induced strain, whereas for *M. bovis* BCG_{INH} accumulation of EtBr starts only at 0.25 mg/L. Moreover, the INH induced strain shows decreased accumulation even at higher EtBr concentrations when compared to the non-induced strain (Figure VI.2. - Compare the accumulation curves for the same EtBr concentration, between the two strains). This increased ability of *M. bovis* BCG_{INH} to handle higher concentrations of EtBr further supports the hypothesis that the INH induction process renders the bacteria resistant to this drug by the overexpression of efflux system(s) and our assumption that EtBr and

INH are substrates of the same panel of efflux pumps. These results are in accordance with the MICs of EtBr determined for these strains (Table VI.1). Moreover, the accumulation of EtBr, used at the above concentrations, increased in the presence of the efflux inhibitors verapamil, thioridazine and chlorpromazine, but only marginally with reserpine. It is important to note that, as previously explained, each efflux inhibitor was used at ½ of the MICs determined for test their inhibitory potential. From the analysis of Figure VI.4, it is possible to conclude that the increase in accumulation of EtBr seen in Figure VI.3 is due to the inhibition of efflux pumps. Efflux of EtBr is observed at 37ºC in the presence of glucose and is inhibited in the presence of the same efflux inhibitors. These results suggest the presence of an intrinsic efflux system in *M. bovis* BCG that extrudes EtBr and is inhibited by thioridazine, chlorpromazine and verapamil. In *M. bovis* BCG_{INH} this efflux system(s) present an increased activity that enables the bacteria to extrude higher concentrations of EtBr than the parental strain.

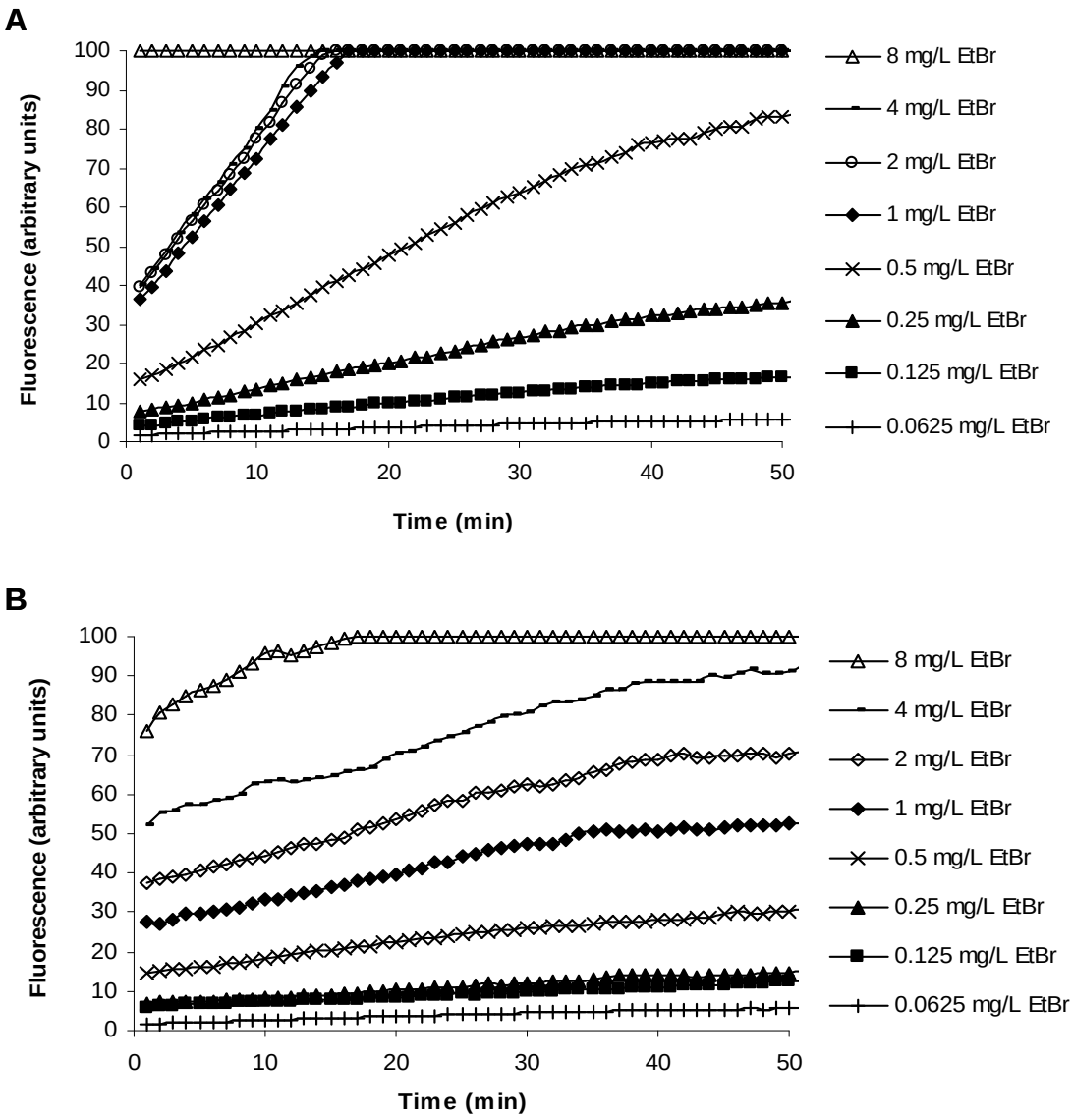

Figure VI.2. Accumulation of increasing concentrations of EtBr (0.0625 – 8 mg/L) by (A) *M. bovis* BCG and (B) *M. bovis* BCG_{INH}.

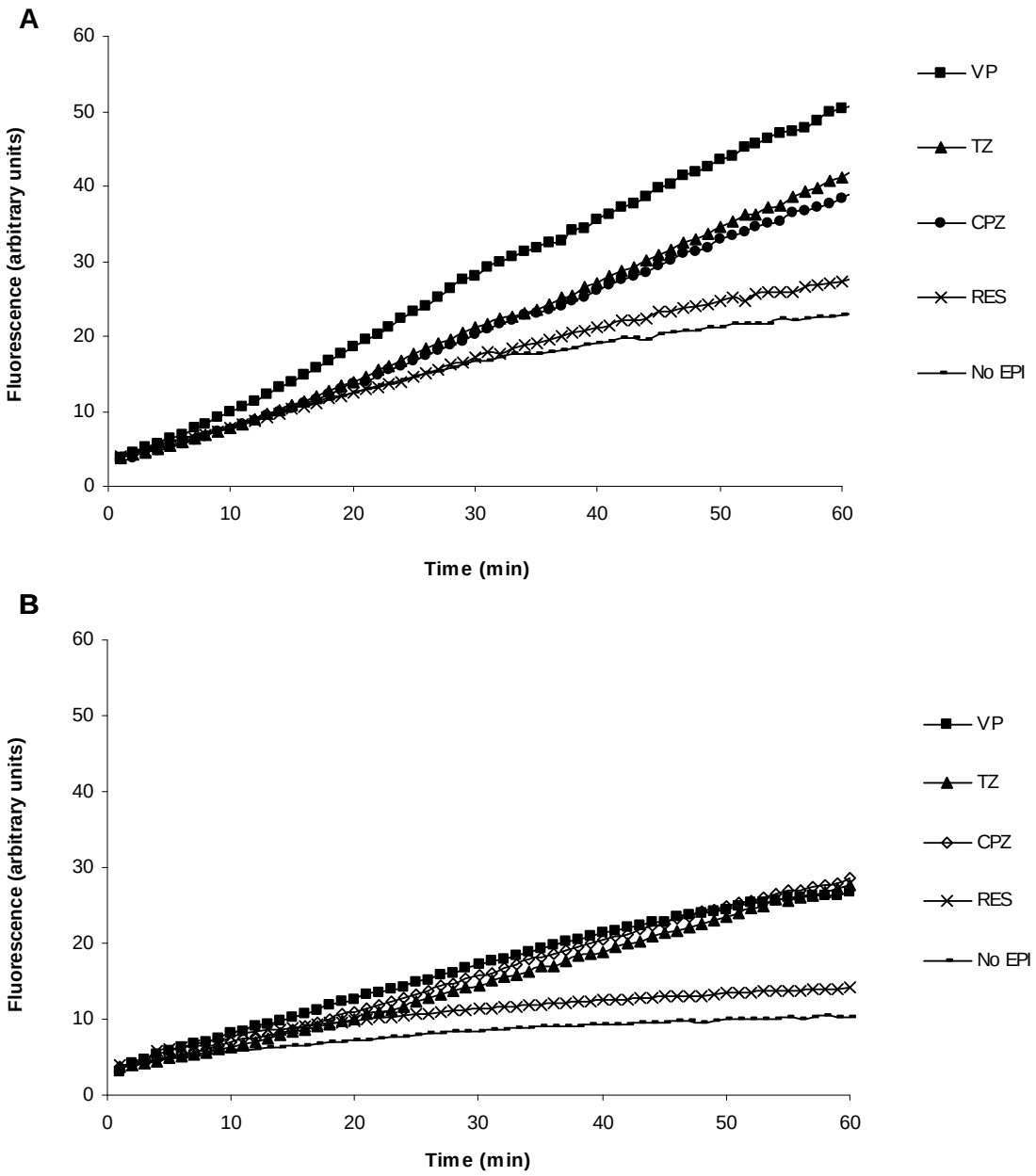

Figure VI.3. Effect of verapamil (VP), thioridazine (TZ), chlorpromazine (CPZ) and reserpine (RES) in the accumulation of EtBr by (A) *M. bovis* BCG (0.125 mg/L EtBr) and (B) *M. bovis* BCG_{INH} (0.25 mg/L EtBr).

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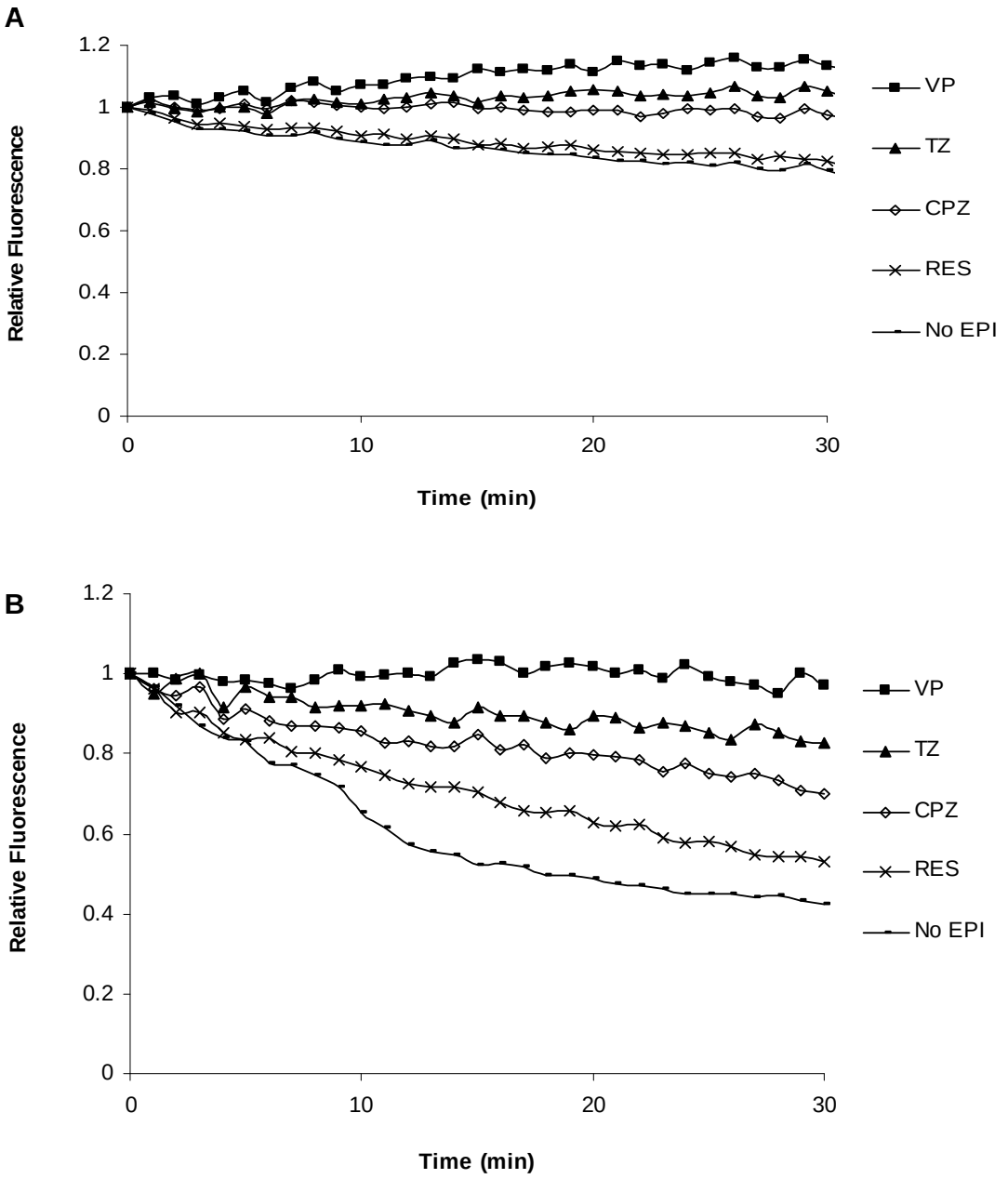

Figure VI.4. Efflux of EtBr by by (A) *M. bovis* BCG and (B) *M. bovis* BCG_{INH}. *M. bovis* BCG was loaded with 0.125 mg/L of EtBr, whereas the INH induced strain was loaded with 1 mg/L of EtBr, both in the presence of verapamil at ½ MIC. Efflux of EtBr took place at 37ºC in the presence of glucose and was inhibited by the efflux inhibitors used at ½ MIC.

EPI, efflux pump inhibitor; CPZ, chlorpromazine; RES, reserpine; TZ, thioridazine; VP, verapamil.

VI.3.3 Evaluation of gene expression in INH induced *M. tuberculosis* complex strains

The quantification of the expression of selected genes coding for efflux pumps *M. tuberculosis* complex by qRT-PCR analysis was performed for the *M. bovis* BCG and *M. tuberculosis* strains induced to INH. As shown in Figure VI.4, exposure to INH promoted the over-expression of *mmr*, *mmpL7*, *tap*, *efpA* and *p55* in *M. tuberculosis* $H37Rv_{INH}$, $737/99_{INH}$ and $711/99_{INH}$, although at a lesser extent in the latter. In the particular case of *M. bovis* BCG_{INH}, only *p55*, *mmpL7* and *mmr* showed overexpression. It is important to stress the absolute level of overexpression (20-40 times) reached at certain measurements once compared to their non-induced counterparts in the absence of INH. It clearly demonstrates that overexpression of efflux pumps is an intrinsic mechanism of resistance that *M. tuberculosis* uses to reduce the level of INH that reaches the activation process inside the mycobacterial cell and further on inhibits the mycolic acid synthesis.

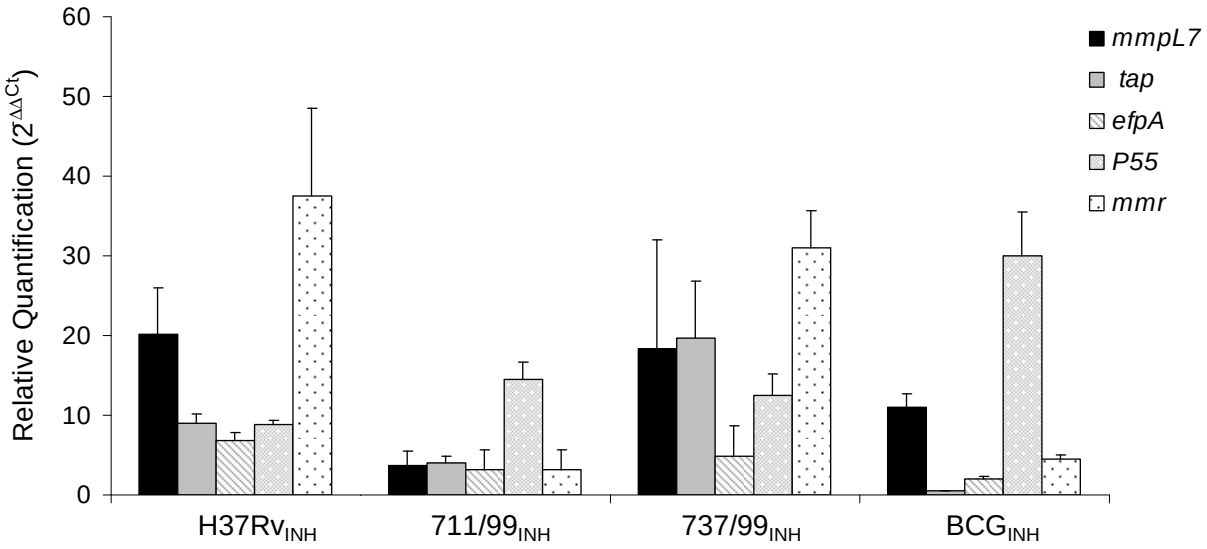

Figure VI.5. Relative expression of genes coding for efflux pumps in *M. tuberculosis* H37Rv and clinical strains and *M. bovis* BCG induced to INH compared with their non-induced counter parts in the absence of INH. A ratio of 1 corresponds to no alterations in expression compared with untreated control cells.

The efflux pumps MmpL7, Tap and EfpA have been associated with INH efflux in other studies (Doran J.L. *et al*, 1997; Pasca M.R. *et al*, 2005; Siddiqi N. *et al*, 2004). In the case of P55 although no study until now has demonstrated its association with INH extrusion, this pump is responsible for efflux-mediated resistance to other compounds such as aminoglycosides, TET and RIF (Ramon-Garcia S. *et al*, 2009; Silva P.E. *et al*, 2001). Concerning Mmr, a recent study based in microarray analysis has reported the overexpression of *mmr* along with other genes coding for efflux pumps after exposure to a combination of INH and EMB. Moreover, this pump has been associated with resistance to TPP, EtBr, ERY and acriflavine (De Rossi E. *et al*, 1998b). In summary, these results further demonstrate that the INH induced strains present an increased efflux activity that is associated with INH resistance.

VI.3.4 Effect of efflux inhibitors in INH resistance of INH induced *M. tuberculosis* complex strains

Since the induction of resistance to INH may be due to the activation or induction of an efflux mechanism(s), the MICs of INH for the induced strains at 20.0 mg/L of INH were also determined in the presence of the efflux inhibitors thioridazine, chlorpromazine, verapamil and reserpine. These inhibitors were used at ½ the MIC (Table VI.1) in order to ensure bacterial viability and their effect of the MIC of INH for these strains is summarized by Table VI.2. The efflux inhibitor verapamil promoted a significantly reduction (at least a 4-fold reduction) of the MIC of INH in all of the strains tested. Moreover, it caused the reversal of resistance to INH to a level similar to the fully INH susceptible wild-type counterparts of the induced $H37Rv_{INH}$ and BCG_{INH}. Concerning the other inhibitors tested, chlorpromazine only had a significant effect in the case of BCG_{INH}, whereas reserpine and thioridazine caused only a marginal reduction (one dilution factor) of the MIC for all of the strains tested. The lower inhibitory activity detected in this study for the efflux inhibitor reserpine, compared with the studies of

2002 (Viveiros M. *et al*, 2002), might be explained by the use in this study of a different and much more accurate growth system, the BACTEC™ MGIT™ 960 system equipped with the Epicenter V5.53A software and the TB eXIST module.

Table VI.1 MICs for thioridazine, chlorpromazine, verapamil, reserpine and EtBr against *M. tuberculosis* H37Rv and clinical strains induced to resistance to INH.

Strains	MICs (mg/L)				
	TZ	**CPZ**	**VP**	**RES**	**EtBr**
M. tuberculosis H37Rv – 711/99 – 737/99	10	20	320	320	0.5
M. tuberculosis $H37Rv_{INH}$	10	20	320	320	4
M. tuberculosis $711/99_{INH}$	10	20	320	320	4
M. tuberculosis $737/99_{INH}$	10	20	320	320	4
M. bovis BCG	10	20	320	320	0.5
M. bovis BCG_{INH}	20	20	320	320	4

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

Table VI.2 MICs for INH in the presence and absence of thioridazine, chlorpromazine, verapamil and reserpine against *M. tuberculosis* H37Rv and clinical strains and *M. bovis* BCG induced to resistance to INH.

Strains	MICs (mg/L)				
	INH	**INH + TZ**	**INH + CPZ**	**INH + VP**	**INH + RES**
M. tuberculosis H37Rv – 711/99 – 737/99	0.05	0.05	0.05	0.05	0.05
M. tuberculosis $H37Rv_{INH}$	40	20	20	**0.0625**	20
M. tuberculosis $711/99_{INH}$	40	20	20	**1.25**	20
M. tuberculosis $737/99_{INH}$	40	20	20	**1.25**	20
M. bovis BCG	0.05	0.05	0.05	0.05	0.05
M. bovis BCG_{INH}	40	20	**10**	**0.0625**	20

CPZ, chlorpromazine; INH, isoniazid; RES, reserpine; TZ, thioridazine; VP, verapamil. Data in bold represents at least a 4-fold reduction, considered to denote significant synergistic effect between the antibiotic and the EPI.

VI.4 Conclusions

The process of *in vitro* inducement of INH resistance by exposure to INH may reproduce what occurs during anti-TB therapy using INH and may account for the remainder of the INH resistance that is not caused by mutations. In fact, only about 70 to 80% of INH resistant *M. tuberculosis* isolates present mutations in any of the genes associated with INH resistance, which suggests that other mechanisms, such as efflux activity, can be responsible for the emergence of resistance to this drug.

The results presented in this chapter show that when INH susceptible *M. tuberculosis* complex strains are gradually exposed to increasing concentration of this drug for a prolonged period of time, they can become resistant to 20 mg/L of INH (MIC 40 mg/L). This induced resistance to INH occurs *via* a mechanism that does not involve mutations in any of the genes associated with INH resistance, but by an efflux system that is sensitive to efflux inhibitors. This induced resistance can also be reversed by consecutive passage of the induced strains in drug-free medium.

The use of the semi-automated fluorometric method applied to *M. bovis* BCG (as a model for *M. tuberculosis*) allowed the detection of an increased efflux of EtBr in the INH induced strains, which was inhibited in the presence of thioridazine, chlorpromazine and verapamil. The same inhibitors also reduced the MIC of INH in the induced strains and, in particular, verapamil caused reversal of resistance in some of the strains tested. These results were further supported by the quantification of the expression level of genes coding for efflux pumps that showed overexpression of *mmr*, *p55*, *mmpL7*, *tap* and *efpA* for the tested strains relatively to the non-induced parental strains.

In conclusion, efflux is an important factor in the development of INH resistance in *M. tuberculosis* complex that creates conditions for the survival of distinct elements of the *M. tuberculosis* population infecting the patient, those that are able to overexpress efflux pumps and resist due to this mechanism. This response stresses the need for

compliance of the multidrug regimen against TB, especially at the first 2 months. If this subpopulation survives the initial treatment there will be an increased possibility of emergence of genetic mutants to INH that can progress to acquire mutations to other anti-TB drug, namely RIF, and thus establish MDRTB.

With the increase of MDRTB strains and the emergence of XDRTB, there are fewer alternatives left to treat the most serious cases of TB. Thus, the identification and development of efflux inhibitors that can restore the antimicrobial activity of the antibiotic subject to efflux, is an approach that can be useful in order to prevent the emergence of this resistance and for the development of new effective anti-TB drugs.

The results and conclusions obtained in this chapter are presently being object of compilation for publication.

VII.5 Materials and Methods

Materials

Middlebrook 7H9 broth and OADC supplement were purchased from Difco (Detroit, MI., U.S.A.). INH, EtBr, glucose, PBS, Tris base, ethylenediaminetetraacetic acid (EDTA), chlorpromazine, thioridazine, verapamil and reserpine were purchased from Sigma-Aldrich Química SA (Madrid, Spain). All solutions were prepared on the day of the experiment. Materials and equipment used to test the antibiotic susceptibilities of the *M. bovis* BCG and *M. tuberculosis* strains tested with the BACTEC™ 460TB and BACTEC™ MGIT™ 960 systems were purchased from Becton Dickinson Diagnostic Instrument Systems (Sparks, Md., U.S.A.) and were prepared according to the recommendations of the manufacturer.

Bacteria

M. tuberculosis H37Rv (ATCC27294) and two *M. tuberculosis* clinical strains, isolated from untreated patients and shown to be susceptible to INH, RIF, STR and EMB, were previously induced to high-level resistance to INH by consecutive passages of the mycobacteria into media containing increasing concentrations of the antibiotic (Viveiros M. *et al*, 2002). *M. bovis* BCG strain Pasteur was also induced to high-level resistance to INH in the present study.

Determination of minimum inhibitory concentrations in the BACTEC™ MGIT™ 960 system

The MIC for the efflux inhibitors thioridazine, chlorpromazine, verapamil and reserpine and for INH in the presence and absence of an efflux inhibitor was determined by the BACTEC™ MGIT™ 960 system equipped with the Epicenter V5.53A software and the TB eXIST module (Springer B. *et al*, 2009). Briefly, the mycobacteria were grown in MGIT tubes containing Middlebrook 7H9 broth until the growth index (GI) reached 100-200 and used as the inocula. MGIT tubes were prepared with serial dilutions of each compound. All of the drug-containing vials were inoculated with 0.5 mL of the bacterial suspensions prepared as described above. Two drug-free controls were included with each test; one inoculated with 0.5 mL of the suspension and the other inoculated with 0.5 mL of a 1:100 dilution of the suspension. The vials were incubated at 37℃ and read in a BACTEC™ 960 reader every day until the GI in the control diluted 1:100 reached 100-200. MIC was defined as the lowest dilution that was negative in drug-containing tubes when the control diluted 1:100 turned positive.

Inducement of resistance to isoniazid

The induction of *M. tuberculosis* H37Rv (ATCC27294) reference strain and four clinical strains to a high-level of INH resistance (20 mg/L) was performed in a BACTEC™

460TB (Becton Dickinson), as previously described (Viveiros M. *et al*, 2001). Briefly, the susceptibilities of the strains to INH were determined by the BACTEC™ 460TB system and the vials retained in the incubator for an additional period of time. By the end of 28-34 days, all of the vials yielded evidence of growth and a new MIC determination was performed. Aliquots of 0.1 mL from cultures growing at an INH concentration just below the MIC were inoculated into fresh vials containing the lowest INH concentration that had completely inhibited the growth of the previous population. This process was repeated for five additional consecutive passages, with each passage containing a stepwise increase in the concentration of INH corresponding to the lowest concentration that had inhibited growth in the previous passage. This process yielded *M. tuberculosis* isolates that were capable of growing in medium containing 20 mg/L of INH.

In this study we have also induced *M. bovis* BCG strain Pasteur to INH resistance (20 mg/L) using the BACTEC™ MGIT 960. Briefly, the susceptibility of the strain to INH was determined by the BACTEC™ MGIT™ 960 system and the MGIT tube (containing 0.1 mg/L of INH) was retained in the BACTEC instrument for an additional period of 34 days, after which a GI of 75 was reached indicating evidence of growth. The MIC for INH was determined and aliquots of 0.1 mL from cultures growing at an INH concentration just below the MIC were inoculated into new MGIT tubes containing the lowest INH concentration that had completely inhibited the growth. This procedure was repeated for seven consecutive passages, each containing a stepwise increase in the INH concentration corresponding to the lowest concentration that had inhibited growth in the previous passage. At the end of this process, the *M. bovis* BCG strain Pasteur with an original MIC for INH of 0.05 mg/L was capable of growing in medium containing 20 mg/L of INH (MIC 40 mg/mL).

Semi-automated fluorometric method

The semi-automated fluorometric method was used for the detection and quantification of efflux pump activity in the studied mycobacterial strains.

(i) Accumulation assay. Mycobacterial cultures at mid-log phase ($O.D._{600}$ of 0.8) were centrifuged at 13000 rpm for 3 minutes, the supernatant discarded and the pellet washed and resuspended in PBS. After adjusting the $O.D._{600}$ to 0.4, glucose at 0.4% was added and, in order to determine the lowest concentration of EtBr that caused accumulation, aliquots of 0.005 mL were transferred from stock solutions of EtBr to yield final concentrations of EtBr that ranged from 0.0625 to 10 mg/L. Aliquots of 0.1 mL were distributed to replicate sets of 0.2 mL PCR microtubes, which were placed into a 36-well rotor in the Rotor-Gene™ 3000, and the fluorescence measured 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 60-90 minutes. After the selection of the highest EtBr concentration that did not cause accumulation, the effect of the efflux inhibitors thioridazine, chlorpromazine, verapamil and reserpine on the accumulation of EtBr was evaluated at 37ºC, in the presence or absence of glucose.

(ii) Efflux assay. The mycobacteria were loaded with EtBr using the conditions that promoted accumulation: accumulation of EtBr in the presence of the most effective efflux inhibitor; absence of glucose; and incubation at 25ºC for 60 minutes (Rodrigues L., 2008; Viveiros M., 2008). In the *M. tuberculosis* strains tested in this work the most effective EPI was verapamil (Figure VI.3). Subsequently, the EtBr-loaded cells were centrifuged at 13000 rpm for 3 minutes and resuspended in EtBr-free PBS with or without 0.4% glucose. After adjusting the $O.D._{600}$ to 0.4, aliquots of 0.095 mL were transferred to 0.2 mL microtubes and the efflux inhibitors added to the respective tubes. The fluorescence emitted was measured on a real-time basis, as described for the accumulation assay. The efflux activity was quantified by comparing the data

obtained under optimum efflux conditions (incubation at 37ºC, presence of glucose and absence of efflux inhibitor) with the data relative to the EtBr-loaded cells in conditions that minimize efflux (presence of efflux inhibitor and no glucose). Thus, the relative fluorescence corresponds to the ratio of fluorescence that remains per unit of time, relative to the EtBr-loaded cells.

Quantification of gene expression by real-time qRT-PCR

The quantification of the expression level of genes coding for efflux pumps by real-time qRT-PCR was carried out by comparing the mRNA levels extracted from the strains induced to resistance to INH with the mRNA extracted from the susceptible non-induced counterparts (normalised against a reference gene). Total RNA was isolated in an RNase-free environment with the aid of the RNeasy Mini Kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions. Mycobacteria were grown in the BACTEC™ MGIT™ 960 system until the GI reached 100-200. Aliquots of 0.5 mL were taken from the cultures and added to 1 mL of RNA Protect Bacteria Reagent. After 5 minutes of incubation at room temperature, the bacteria were harvested at 9000 rpm for 10 minutes, the bacterial pellet ressuspended in 0.1 mL of TE buffer (10 mM Tris-HCl, 1 mM EDTA pH 8.0) and incubated for 10 minutes at 95ºC. The tubes were cooled at room temperature and lysozyme at a final concentration of 3 mg/mL was added. After an incubation period of 10 minutes at room temperature, buffer RLT + 1% β-mercaptoethanol (0.350 mL) was added and the tubes sonicated for 15 minutes at room temperature. After sonication, the samples remained for 15 minutes at room temperature, followed by centrifugation at 13000 rpm for 2 minutes. The supernatant was transferred to a new microcentrifuge tube and 0.250 mL of ethanol was added. The sample was applied to an RNeasy mini column (placed in a 2 mL collection tube) and the RNA extracted with the RNeasy mini kit (QIAGEN), according to the manufacturers instructions. Digestion of contaminant DNA with DNAse I was carried

out for 2 hours at room temperature. Purified RNA was stored in RNase-free water and maintained at -20ºC until quantification was performed.

The primers (table VI.3) were designed using Primer Express 1.5 Software (Applied Biosystems, CA, USA) based on the sequence entries in the GenBank for *M. tuberculosis* H37Rv complete genome (accession number NC_000962).

Table VI.3: Primers used in this study

Gene	Primer sequence (5'-3')	Length of amplicon	Ref.
16S rDNA	Fw_CAAGGCTAAAACTCAAAGGA Rv_GGACTTAACCCAACATCTCA	197	This study
***rv2942* (*mmpL7*)**	Fw_TACCCAAGCTGGAAACAA Rv_CCGTCAGAATAGAGGAACAG	214	This study
***rv1258c* (*tap*)**	Fw_AGTTATAGATCGGCTGGATG Rv_GTGCTGTTCCCGAAATAC	268	This study
***rv1410c* (*p55*)**	Fw_AGTGGGAAATAAGCCAGTAA Rv_TGGTTGATGTCGAGCTGT	198	This study
***rv2846c* (*efpA*)**	Fw_ATGGTAATGCCTGACATCC Rv_CTACGGGAAACCAACAAAG	131	This study
***rv3065* (*mmr*)**	Fw_AACCAGCCTGCTCAAAAG Rv_CAACCACCTTCATCACAGA	221	This study

The assay was performed in a Rotor-Gene™ 3000 thermocycler (Corbett Research) strictly adhering to manufacturer recommendations of the QuantiTect SYBR Green RT-PCR Kit (QIAGEN, Hilden, Germany). Briefly, each 0.2 ml microfuge tube contained 10 μL of the 2x QuantiTect SYBR Green RT-PCR master mix, 0.2 μL of 10x QuantiTect RT mix, 5 pmol of each primer, approximately 20 ng of total RNA and RNase-free water to complete a final volume of 20 μL. Thermal cycling conditions were as follows: reverse transcription for 30 minutes at 50ºC; Initial activation step of 15 minute at 95ºC;

40 cycles of denaturation at 94ºC for 30 seconds, annealing at 52ºC for 30 seconds and extension at 72ºC for 30 seconds; a final extension step at 72ºC for 5 minutes; and an additional step at 52ºC for 15 seconds followed by melt analysis (50-99ºC). The relative quantities of the mRNA of each gene of interest were determined by the use of the comparative threshold cycle (C_T) method (Livak K.J. *et al*, 2001).

Chapter VII

Final Conclusions and Future Perspectives

Final Conclusions and Future Perspectives

VII.1 Final Conclusions

This thesis focused on the study of the contribution of efflux systems to the development of bacterial drug resistance, with the final objective consisting in the evaluation of the role of efflux activity in the resistance of *M. tuberculosis* to INH. In order to detect efflux activity and to evaluate its contribution to the development of drug resistance, several techniques had to be developed and optimized using experimental models like *E. coli*, *M. smegmatis* and *M. avium* complex prior to their application to *M. tuberculosis*. This approach had to do with the limitations associated with *M. tuberculosis* manipulation in the laboratory: this organism is not only a biosafety level III pathogen, but also presents a very slow growth rate (replication cycle of 24 hours). Using experimental models it is possible to develop and optimize the basic experimental conditions in a shorter period of time, thus minimizing the time consuming aspect of working with *M. tuberculosis*. At the same time, the use of *E. coli*, *M. smegmatis* and *M. avium* complex as models allowed to gain more insight of their efflux-mediated resistance mechanisms that was expected to be fairly similar to what occurs in *M. tuberculosis*.

The development of a qRT-PCR protocol in *E. coli* provided the basis for the later application of this methodology to *M. tuberculosis*, but also allowed further insight into the mechanisms that are responsible for the interplay between efflux activity and permeability of the cell envelope that occur after exposure of *E. coli* to TET. By this manner, it was possible to create a model showing the sequence of events regarding the regulation process that increase the expression of efflux pumps and decrease the porin content of the cell wall. The model shows that there is an early stress response to exposure to non-lethal concentrations of TET that promotes the activation of global

regulators like MarA, SoxS and Rob and of periplasmic proteases that regulate the two major outer membrane proteins OmpC and OmpF. This is followed by a long-term adaptative response caused by the increase of MarA that is responsible for (i) the overexpression of AcrAB-TolC and other efflux systems and (ii) the overexpression of MicF and OmpX, downregulators of OmpC and OmpF. This process results in a reduced permeability due to a decreased porin content and in an increased efflux activity, thus establishing the basis for the development of an MDR phenotype (Viveiros M. *et al,* 2007).

The other method that was developed through the course of this thesis was the semi-automated fluorometric method, which allowed the correlation of the results of gene expression quantification with the real-time detection of efflux activity. This method allows not only the detection of efflux of EtBr, but also the screening of compounds for activity against efflux pumps (Viveiros M. *et al*, 2008a, Viveiros M. *et al*, 2008b, Viveiros M. *et al*, 2010a). Moreover, this method was also used to develop a mathematical model that quantifies efflux activity, using EtBr as a substrate. The use of *E. coli* as a model allowed the standardization of the experimental conditions, but also showed the method's ability to detect differences in the EtBr efflux activity between strains that differ in their efflux pump expression (Paixão L. *et al,* 2009). Moreover, the use of the semi-automated method applied to *M. smegmatis* strains that differ in their porin content or efflux activity, demonstrated the main pathways for entry and extrusion of EtBr from these mycobacteria (Rodrigues L. *et al*, 2010). In particular, the role of MspA was put in evidence as being extremely important for EtBr entry into the cell, whereas the efflux pump LfrA was shown to be highly associated with EtBr extrusion, as already reported by other studies (Li X.Z. *et al*, 2004).

Encouraged by the previous results, the work evolved towards more pathogenic mycobacterial species, in this case *M. avium* as a pathogenic model for *M. tuberculosis*. The study of the role of efflux pumps in antimicrobial resistance in *M. avium* complex provided evidence that efflux plays a role in drug resistance in this

clinical relevant mycobacteria (Rodrigues L. *et al*, 2008; Rodrigues L. *et al*, 2009). In particular, this efflux-mediated resistance was demonstrated in *M. avium* complex clinical isolates resistant to macrolides, such as clarithromycin and erythromycin (Rodrigues L. *et al*, 2009). In this study, the semi-automated method coupled with the determination of MICs in the presence and absence of efflux inhibitors allowed the detection of an increased efflux activity in *M. avium* complex clinical isolates and that was inhibited in the presence of efflux inhibitors. Moreover, the efflux assays highlighted the efflux activity in four high-level resistant *M. avium* complex strains (two *M. avium* and two *M. intracellulare*), demonstrating that besides the presence of mutations in the 23S rRNA gene in three of these strains, this high-level resistance also results from efflux activity, as already suggested by the reduction of MICs for clarithromycin and erythromycin in the presence of the efflux inhibitors. Furthermore, the tested inhibitors also caused an increased retention of radiolabelled erythromycin inside the mycobacterial cell, further supporting the presence of active efflux pump system(s) (Rodrigues L. *et al*, 2008; Rodrigues L. *et al*, 2009). Based on their activity in the reduction of resistance of *M. avium* complex to macrolides, these efflux inhibitors were selected for further studies in *M. tuberculosis*.

The final goal of this thesis was the application of the developed methodologies to the study of the contribution of efflux pumps to INH resistance in *M. tuberculosis* complex. *M. bovis* BCG strain Pasteur and *M. tuberculosis* reference and clinical strains were induced to resistance to INH by a step-wise increase of antibiotic concentration over a prolonged period of time until they became resistant to 20 mg/L of INH (Viveiros M. *et al*, 2002). The evaluation of efflux pump activity in the *M. bovis* BCG INH induced strain by the semi-automated fluorometric method demonstrated an increased efflux of EtBr that was inhibited in the presence of thioridazine, chlorpromazine and verapamil. These inhibitors also promoted the reduction of the MIC of INH in the induced strains. In particular, verapamil was responsible for the reversal of resistance in some of the

strains tested. Moreover, qRT-PCR analysis showed that the INH resistance inducement process caused the overexpression of genes coding for the efflux pumps *mmr*, *p55*, *mmpL7*, *tap* and *efpA* in the induced strains relatively to their non-induced counterparts. Altogether, these results suggest that efflux pumps are involved in the development of INH resistance and that efflux inhibitors can be used to prevent this resistance and, ultimately, the emergence of MDRTB (Rodrigues L. *et al*, unpublished data).

In conclusion, the main objective of this thesis was the demonstration that efflux mechanisms are involved in the development of multidrug resistance in *M. tuberculosis* and how phenotypic resistance, mediated by efflux pumps, correlates with genetic resistance. This objective was achieved and the tools developed and optimized in this thesis connected with molecular modelling can lead to the development of new anti-TB drugs or enhancers of the activity of the already existing anti-TB drugs, as well as new methods for the *in vitro* testing of putative efflux inhibitors. These non-antibiotic inhibitors would restore the activity of the antibiotic subject to efflux, by acting directly upon the protein structure of the pump or by a decrease in the level of activity and expression of the efflux pump, and therefore provide an easy way to enhance the activity of already existent anti-TB drugs.

As a corollary of this work, we have also been able to demonstrate the fact that increased activity of efflux pumps can occur in response to prolonged exposure to subinhibitory concentrations of anti-TB compounds, a common situation that results from inadequate TB therapy, which allows the survival of a population increasingly resistant to the anti-TB compound and might be the basis for the maintenance of an increased mycobacterial population in the patient undergoing therapy.

VII.2 Future perspectives

The results gathered in this thesis demonstrate that induced resistance of *M. tuberculosis* to INH is not due to the selection of a mutation that bestows INH resistance, but rather to the inducement of efflux activity by some efflux pump which extrudes the antibiotic prior to its reaching its intended target.

The primary and most obvious perspective to expand this work will be the genetic characterization and the description of the mode of action of the *M. tuberculosis* efflux pumps we have been able to demonstrate are connected with INH resistance.

Secondly, because efflux inhibitors have the ability to reverse the induced INH resistance, further proven by others (Rossi E. *et al*, 2006; Gumbo *et al*, 2007), the potential use of efflux inhibitors as helper-compounds in the anti-TB standard therapy to improve the compliance and the outcome, as well as to decrease the probability of emergence of acquired resistance, is the obvious perspective to be explored in the future. The *in vitro* situations simulated in this thesis are considered to mimic what takes place in the patient who receives an anti-TB drug for a long period of time and each time at a dose level that allows the survival of the bacteria. The demonstration of extrusion of a large variety of agents by phenotypically MDR bacteria has fostered a search for agents that can inhibit efflux pumps and, hence, render ineffective antibiotics effective again.

Thirdly, and because efflux pumps of prokaryotic and eukaryotic cells share a high degree of functional homology (Amaral L. *et al*, 2007), efflux inhibitors will also affect the efflux pumps present in eukaryotic cells and in recent studies it has been demonstrated that many of these compounds, like verapamil or the phenothiazines, inhibit the transport of Ca^{2+} and K^{+} by eukaryotic efflux pumps and, when used at therapeutically dosages, cause non-killing macrophages to kill phagocytosed bacteria by this double effect on the bacteria cell and on the host cell (Martins M. *et al*, 2009, Amaral L. *et al,* 2010, Viveiros M. *et al,* 2010b). Therefore, since pulmonary TB is

mainly an intracellular infection of the alveolar macrophage, a cell that has little killing activity of its own, *M. tuberculosis* can reside in a viable state within its phagosome prison for many years. If a compound is to be effective for the therapy of a TB patient, the activity of the compound against the *M. tuberculosis* strain isolated from the patient would be best evaluated if the macrophage that is to phagocytose the organism is obtained from that same patient. Employing this approach should result in the best predictive situation for the selection of effective anti-TB agents. This approach requires the preparation of human non-killing macrophages obtained from the MDRTB/XDRTB infected patient's peripheral blood prior to the patient receiving any therapy, allowing the rapid screening of new anti-TB drugs, the evaluation of effectiveness of combined therapies and the patient's directed selection of the most effective therapy against TB and/or MDRTB/XDRTB (Viveiros M. *et al,* 2010b).

Finally, it's our future perspective to contribute to the enormous task of controlling and preventing the spread of drug resistant TB.

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