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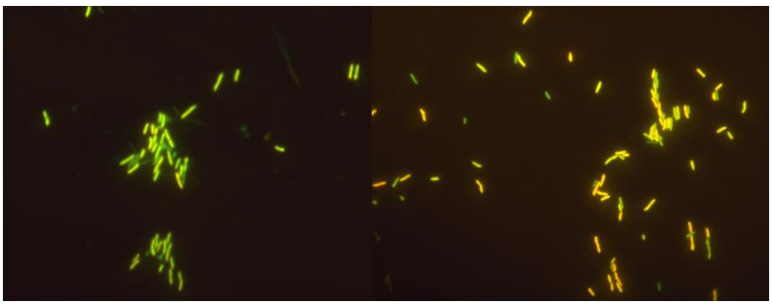

Identification and Characterisation of Efflux Pumps in *Rhodococcus erythropolis*

Ana Carolina Ferreira Vencá

Dissertação para a obtenção do Grau de Mestre em

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Júri

Presidente:	Prof. Arsénio do Carmo Sales Mendes Fialho
Orientador:	Doutora Carla da Conceição Caramujo Rocha de Carvalho
Co-orientador:	Prof. Miguel Viveiros Bettencourt
Vogal:	Doutor Pedro Carlos de Barros Fernandes

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Abstract

This work assesses, for the first time, the presence of efflux pumps as a defense mechanism in *Rhodococcus erythropolis*.

Several known efflux pump inhibitors (EPIs), namely Thioridazine (TZ), Verapamil (VP), Carbonyl cyanide m-chlorophenylhydrazone (CCCP), Omeprazole (OMP) and Sodium orthovanadate (Na_3VO_4), were used to assess efflux activity. This was achieved through the determination of minimum inhibitory concentration (MIC) of EPIs and of the antibiotics vancomycin and ciprofloxacin, in the presence and absence of the EPIs; and through real time fluorometry. The action of efflux pumps in *R. erythropolis* cells was suggested by the decrease of MICs of both antibiotics in presence of the inhibitors, and demonstrated through the decrease of the efflux of ethidium bromide in the presence of EPIs. Since the used EPIs are described as inhibitors of efflux pumps belonging to the ATP Binding Cassette superfamily and to the Major Facilitator superfamily, these results suggest the presence of transporter proteins belonging to these families in strain DCL14.

Previous studies have suggested that the inhibition of proton-motive force-dependent pumps by EPIs may involve not only a direct effect on the pump but also on the transmembrane potential. The interference of the compounds on the membrane potential was assessed by fluorescence microscopy. TZ, OMP and CCCP were shown to promote a higher percentage of depolarized cells. Most of *R. erythropolis* cells were able to repolarize the membrane after 1h exposure to Na_3VO_4 and VP.

The results clearly indicate the presence of efflux pumps as a defense mechanism in *R. erythropolis*.

Keywords: Efflux pumps; *R. erythropolis*; Efflux pump inhibitors; Real time fluorometry; Depolarized cells; Fluorescence microscopy.

Resumo

Este trabalho procura demonstrar, pela primeira vez, a presença de bombas de efluxo como mecanismo de defesa em *Rhodococcus erythropolis*.

Vários inibidores de bombas de efluxo (EPIs) conhecidos, tais como Tioridazina (TZ), Verapamil (VP), Carbonilcianeto m-clorofenil-hidrazona (CCCP), Omeprazol (OMP) e Ortovanadato de sódio (Na_3VO_4), foram usados para avaliar a actividade de efluxo. Tal foi conseguido através da determinação da concentração mínima inibitória (MIC) dos EPIs e dos antibióticos vancomicina e ciprofloxacina, na presença e ausência dos EPIs; e através de fluorometria em tempo real. A acção de bombas de efluxo em *R. erythropolis* foi indiciada pela diminuição das MICs dos antibióticos na preseça dos inibidores, e demonstrada pelo decréscimo do efluxo de brometo de etídio na presença de EPIs. Como os EPIs utilizados são descritos como inibidores de bombas de eflluxo pertencentes às superfamílias ATP *Binding Cassette* e *Major Facilitator*, estes resultados sugerem a presença de proteínas transportadoras pertencentes a estas familias na estirpe DCL14.

Estudos anteriores sugerem que a inibição de bombas dependentes da força motriz protónica por EPIs pode envolver não só um efeito direto na bomba mas também no potencial transmembranar. A interferência dos compostos no potencial de membrana foi avaliada por microscopia de fluorescência. Foi mostrado que TZ, OMP e CCCP promovem uma elevada percentagem de células despolarizadas. A maioria das células conseguiram repolarizar a membrana após 1h de exposição a Na_3VO_4 e VP.

Estes resultados indicam claramente a presença de bombas de efluxo como mecanismos de defesa em *R. erythropolis.*

Palavras-Chave: Bombas de efluxo; *R. erythropolis*; Inibidores de bombas de efluxo; Fluorometria em tempo real; Células despolarizadas; Microscopia de fluorescência.

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ABC – ATP binding cassette;

ATP – Adenosine triphosphate;

CCCP – Carbonyl cyanide 3-chlorophenylhydrazone;

CIP – Ciprofloxacin;

DMSO - Dimethyl sulfoxide;

EPI – Efflux pump inhibitor;

EtBr – Ethidium bromide;

MATE – Multidrug and toxic compound extrusion;

MDR – Multidrug resistance;

MIC – Minimum inhibitory concentration;

MFS – Major facilitator superfamily;

OD_{λ} – Optical density at wavelength λ;

OMP – Omeprazole;

PBS – Phosphate buffer saline;

RFF – Relative final fluorescence;

RND – Resistance-nodulation-division;

rpm – Revolutions per minute;

SMR – Small multidrug resistant;

TB – Tuberculosis;

TZ – Thioridazine;

VAN – Vancomycin;

VP – Verapamil;

XDR – Extensively-drug resistant.

Introduction

1 Introduction

Bacteria are fundamental catalysts in many industrial processes across diverse areas of application. Examples of activities which commonly make use of bacterial processes are environmental, pharmaceutics, biotechnology and food industries. There are several used bacteria to apply in bioremediation that includes Bacilli, Pseudomonads and Methanobacteria (Milton H. Saier 2007). *Lactobacilli* is a bacterium widely used in the food industry (Zacharof, Lovitt and Ratanapongleka 2010) and it widely present, along with *Streptomyces*, in pharmaceutics (Battaracharyya and Sen 2006). In industry, chemical catalytic processes require extreme pH, temperature and pressure conditions. Bacteria are used to achieve the same processes under more moderate conditions regarding these variables. However, the adaptation mechanisms and the versatile metabolism of bacteria can be exploited to further increase their applicability.

The ubiquitous *Rhodococcus* bacteria display a large set of enzymatic capabilities and they can be found in a widespread of environments (Bell, et al. 1998). Strains from deep sea and sea level sediments, alpine soils, and from the Arctic and the Antarctic were isolated (de Carvalho and da Fonseca 2005b). The versatile metabolism conferred by their extended array of enzymes and the resistance displayed by rhodococci makes these bacteria potential candidates to use in environmental and industrial biotechnology (Bell, et al. 1998).

Bacteria have the ability to use several different mechanisms of defense and adaptation against hostile environments. To date, the known adaptation mechanisms of *Rhodococcus* are those associated to the cell wall composition and cell membrane morphology, and metabolic/catabolic pathways (Warhurst and Fewson 1996, Bell, et al. 1998, de Carvalho and da Fonseca 2005b). However, since it has been already shown that rhodococci tolerate elevated concentrations of toxic compounds, it is expected that other defense mechanisms may exist. One of these propose mechanisms is the extrusion of compounds through efflux pumps, since this is known to occur in other Gram-positive bacteria like the mycolata *Mycobacterium smegmatis* (Choudhuri, Sen and Chakrabarti 1999) and *M. tuberculosis* (Amaral, Martins, et al. 2008), and in *Staphylococccus aureus* (Aeschlimann, et al. 1999, Kaatz, et al. 2003). In these studies, efflux pumps have been shown to constitute one of the causes of the Multi-Drug Resistance (MDR) phenomena (Nikaido 2009).

1.1 The genus *Rhodococcus*

The classifying genus name *Rhodococcus* was first proposed by Zopf in 1891 in order to group two red bacteria described by Overbeck in the same year, *Micrococcus erythromyxa* and *M. rhodochrous*.

Winslow and Rogers in 1906 and Molisch in 1907 also suggested this genus name to classify other red cocci, and with *R. rhodochrous* as type strain, the name was maintained in the first four editions of Bergey's *Manual of Determinative Bacteriology*. In the fifth and sixth editions of Bergey's *Manual*, these and other strains were then reincorporated into *Micrococcus* genus. The lack of suitable characters to describe the strains led to their reclassification into the genus *Mycobaterium* and tentatively in *Nocardia*. Besides the changes of the name for this bacterial genus over the years, it was in 1977 that Goodfellow and Alderson revived the name *Rhodococcus*. The genus was recognized in the *Approved List of Bacterial Names* and in the first edition of Bergey's *Manual of Systematic Bacteriology*. The *rhodochrous* species that did not belong to *Nocardia, Corynebacterium* or *Mycobacterium*, had finally been classified (Goodfellow and Alderson 1977, Bell, et al. 1998, Alvarez 2010).

The extensive collected taxonomic data allowed placing the genus *Rhodococcus* in the phylum *Actinobacteria*, sub-order *Corynebacterineae,* family *Nocardiaceae* (Alvarez 2010). In general, the members belonging to this genus are described as Gram-positive, non-motile, mycolic acid-containing and aerobic organisms with an oxidative metabolism, capable of using several organic compounds as sole carbon and energy sources (Bell, et al. 1998, Alvarez 2010). These environmental bacteria can be found in a wide range of environmental niches, from soils to seawater (Bell, et al. 1998, Larkin, Kulakov and Allen 2005).

There are currently 44 species belonging to the genus *Rhodococcus* (Euzéby 8th February 2012). Several of these species are of great interest because of their metabolic diversity (*R. erythropolis* (Larkin, Kulakov and Allen 2005)), industrial applications (*R. erythropolis*, *R. opacus*, and *R. ruber* (Ivshina, et al. 1998)), or potential in bioremediation and fossil fuel biodesulfurization (*R. rhodochrous* (Boyle, et al. 1992), *R. globerulus*, (Asturias and Timmis 1993), *R. corallinus* (Arnold, et al. 1996), *R. ruber* and *R. erythropolis* (Bell, et al. 1998)). Two of these species are of interest because of their pathogenicity, *R. fascians* and *R. equi*.

1.1.1 *Rhodococcus* in Biotechnology

Bacteria belonging to genus *Rhodococcus* have a large set of enzymes that allow them to perform several biocatalytic and degradation reactions with industrial relevance (Bell, et al. 1998, de Carvalho and da Fonseca 2005b, Larkin, Kulakov and Allen 2005). Rhodococci have revealed to be able to degrade a wide range of hydrophobic natural compounds and xenobiotics: short-chain, long-chain, and halogenated hydrocarbons, and aromatic compounds, like polycyclic aromatic hydrocarbons, polychlorinated biphenyls and dibenzothiophenes (DBTs). The demonstrated cellular resistance and the degradation of all these compounds are related to the genome of the cells (Larkin, Kulakov and Allen 2005). The metabolic diversity and efficiency of *Rhodococcus* is acquired by the presence and

mobilization of large linear plasmids and the presence of multiple homologues of enzymes in catabolic pathways (Geize and Dijkhuizen 2004, Larkin, Kulakov and Allen 2005).

1.1.1.1 Bioremediation and Biodegradation

The ability to degrade a large number of organic compounds and their associated tolerance make *Rhodococcus* bacteria a suitable case study to employ in bioremediation and biodegradation of pollutants (Bell, et al. 1998, Larkin, Kulakov and Allen 2005, de Carvalho 2012).

The wastes produced by industries are large in quantity and in variation, and the contamination of soils and water becomes a difficult problem to solve. Rhodococci has been shown to degrade low bioavailable pollutants, ranging from simple hydrocarbons, to chlorinated hydrocarbons, aromatic hydrocarbons, nitroaromatics and chlorinated polycyclic aromatics (Bell, et al. 1998). The reports made about *Rhodococcus* species demonstrates that *R. rhodochrous* can degrade polychlorinated biphenyls (PCBs) (Boyle, et al. 1992) and it is also a sulfur-removing bacterium (Bozdemir, et al. 1996); *R. globerulus* P6 can degrade the chemical pollutants polychlorinated biphenyls (PCBs) (Asturias and Timmis 1993); biological remediation of wastes containing atrazine and s-triazine can be performed by *R. corallinus* (Arnold, et al. 1996); *R. ruber* and *R. erythropolis* were described as capable to remediate environments contaminated with crude-oil (Bell, et al. 1998). The strain *R. erythropolis* DCL14 was described to be able to degrade a wide range of toxic compounds, such *n*-alkanes and aromatic compounds, fuel oil and motor oil (de Carvalho and da Fonseca 2005b, de Carvalho, Parreño-Marchante, et al. 2005, de Carvalho, Fatal, et al. 2007).

1.1.1.2 Biosurfactants Production

As response to the presence of hydrophobic compounds, such as liquid hydrocarbons, bacteria belonging to genus *Rhodococcus* produce biosurfactants. The produced cellular surfactants in rhodococci are predominantly glycolipids (Lang and Philp 1998) and they promote the adherence of *Rhodococcus* cells to hydrophobic phases in two phase systems. This fact decreases the interfacial tension between the phases, which allow an easier entry of hydrophobic compounds into cells. The dispersion of the hydrophobic compounds caused by the surfactants increases the surface area for microbial action (Bell, et al. 1998). The use of biosurfactants has several advantages and complements the treatment of chemical wastes by bioremediation (Kosaric 1992). The strain *R. erythropolis* DCL14 has a glycolipid-base biosurfactant production in the presence of long-chain alkanes (de Carvalho, Wick and Heipieper 2009). Biosurfactants produced by *R. erythropolis*, *R. opacus*, and *R. ruber* can be applied in crude oil, for example for cleaning oil tanks or removal of oil from contaminated sands (Ivshina, et al. 1998).

1.1.2 *Rhodococcus erythropolis*

R. erythropolis have been described as very tolerant bacteria, and so the commercial interest in this microorganism increased. The versatile metabolism of *R. erythropolis* and their properties of the membrane, make this species an appropriated study object to apply in bioremediation and as biosurfactants producer (Bell, et al. 1998, Ivshina, et al. 1998, Kurane, et al. 1992). Besides the environmental applications and the tolerance of the cells to extreme conditions, *R. erythropolis* has also been studied for the production of antibiotics (Kitagawa and Tamura 2008). A new quinolone antibiotic was produced by the strain *R. erythropolis* JCM 6824 (Kitagawa and Tamura 2008b).

The remarkable properties presented by *R. erythropolis* cells have been extensively demonstrated by de Carvalho, particularly the strain *R. erythropolis* DCL14 and their adaptation to extreme conditions (de Carvalho 2012). These bacteria tolerate water-miscible solvents at a relative high concentrations, such as ethanol, butanol and dimethylformamide (DMF) (de Carvalho, da Cruz, et al. 2004), and water-immiscible solvents such as dodecane, bis(2-ethylhexyl) phthalate (BEHP) and toluene (de Carvalho and da Fonseca 2005b, de Carvalho, Fatal, et al. 2007). It has also been demonstrated that *R. erythropolis* DCL14 is a highly adaptive bacterium, usable in bioremediation and biotransformation processes (de Carvalho, Wick and Heipieper 2009). All of these characteristics make *R. erythropolis* a suitable microorganism as a case study for novel biotechnological applications, and possibly as a mycolic acid-containing bacteria model.

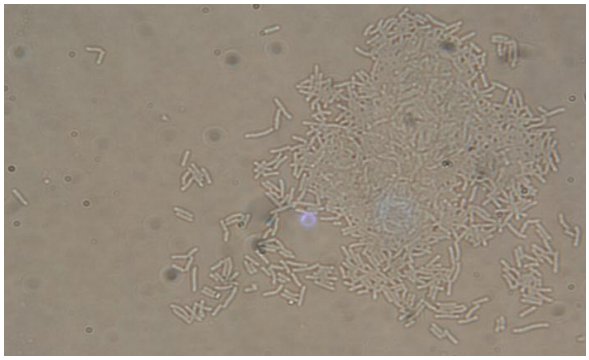


Figure 1 – *Rhodococcus erythropolis* DCL14.

1.1.3 Infections by *Rhodococcus*

R. fascians is a phytopathogenic bacterium that causes fasciation in a wide range of plants, both monocotyledonous and dicotyledonous. The infection of plants by *R. fascians* causes several malformations, ranging from deformation of leaves, to witches' brooms formation, and leafy galls

(Vereecke, et al. 2000, Crespi, et al. 1992). Infections caused by *R. fascians* affect a wide range of crops and plants, such as peas and tobacco, being a significant economic factor (Bell, et al. 1998). Although the infections do not affect the lifespan of the infected plants, the ornamental industry that is based on the plant esthetics is the most affected industry with considerable financial losses due to infections by *R. fascians* (Depuydt, et al. 2008).

R. equi is a worldwide distributed pathogenic bacterium of great interest in veterinary medicine (Prescott 1991). This pathogen causes a disease known as rodococose, a chronic granulomatous pneumonia and lung abscesses that occur in foals under six months old. The manifestation of this infection in adult horses is rare (Prescott 1991, Porto, Fernandes and Barreira 2011). Although foals are the mammals most affected by this bacterium, the tuberculosis- like lesions caused by *R. equi* is also common in cattle and pigs, being the submandibular and other lymph nodes the most affected tissues in these mammals (Prescott 1991, Porto, Fernandes and Barreira 2011, Gyles, et al. 2012). Infections in other mammals are rare, however they may occur, and they are usually the result of immunosppression. It has been reported that *R. equi* can act as an infection agent in patients infected with human immunodeficiency virus (HIV) or organ transplanted patients (Harvey and Sunstrum 1991, Prescott 1991). Usually, *R. equi* is susceptible to several classes of antibiotics, such as erythromycin, rifampin, flouroquinolones, aminoglycosides, glycopeptides, such as vancomycin, and imipenem. For cotrimoxazole, tetracycline, chloramphenicol, clindamycin, and cephalosporins, the susceptibility of the bacterium is more variable, and it can be resistant to penicillin (Weinstock and Brown 2002). Strains of *R. equi* resistant to ciprofloxacin (Niwa and Lasker 2010), to rifampin and macrolide antibiotics (Giguère, et al. 2010) were isolated. The resistance acquired to antibiotics could be an indication of the presence of efflux pumps as a defense mechanism.

1.2 Adaptation Mechanisms Displayed by Bacteria

In general, bacteria present several mechanisms of adaptation, which allow them to survive in hostile environments and in the presence of toxic coumpounds. The most common of these mechanisms are (Figure 2) (Isken and de Bont 1998, de Carvalho, Wick and Heipieper 2009):

i. changes in the cell wall/envelope and membrane composition;
ii. modifications of the physicochemical properties of the cell surface;
iii. conversion of the toxic compound into a non-toxic form;
iv. cell aggregation;
v. production of exopolymeric substances;
vi. activation of efflux pumps.

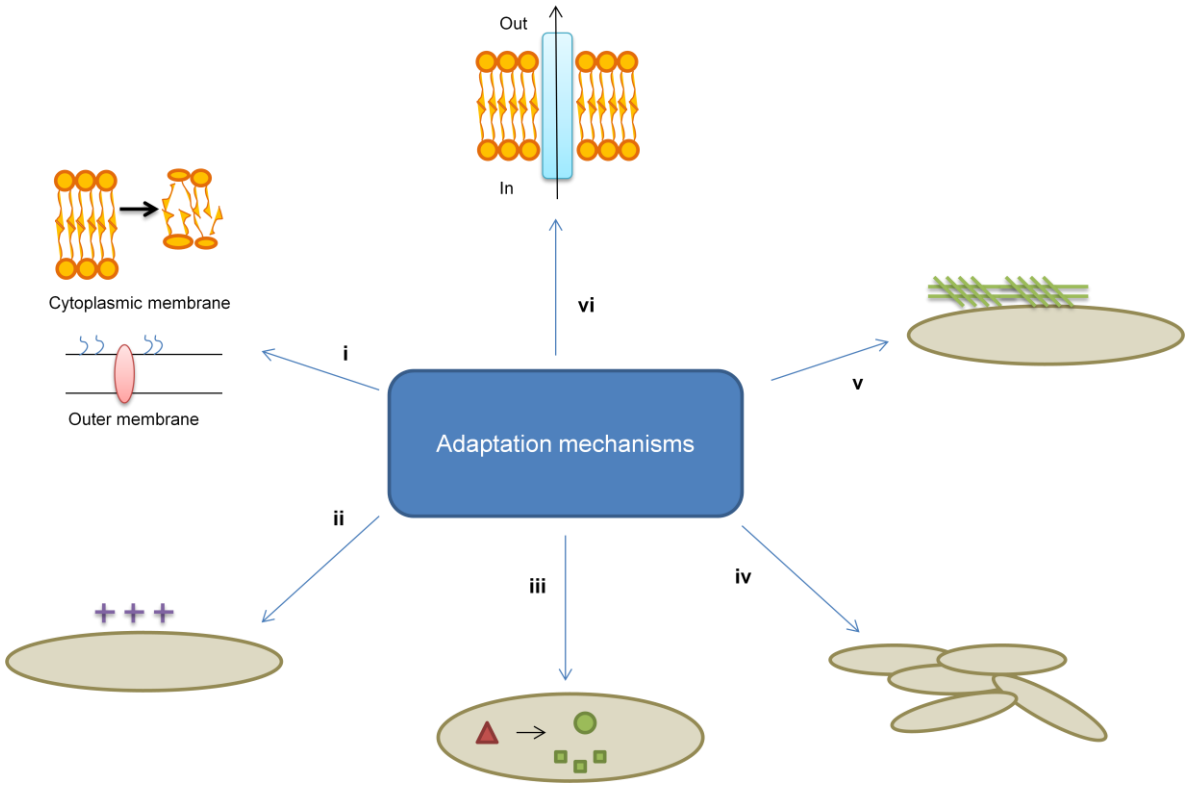


Figure 2 – Adaptation mechanisms displayed by bacteria in hostile environments and in the presence of toxic compounds. i) Modification of the cell envelope and cell membrane; ii) modification of the physicochemical properties of the cell surface; iii) transformation of toxic compound into non toxic; iv) cell aggregation; v) production of exopolymeric substance; vi) activation of efflux pumps. Adapted from (Isken and de Bont 1998).

In cell membranes, changes in the composition of lipids may occur. These changes may include the degree of the saturation of fatty acids, different conformations of the unsaturated fatty acids and alteration of the composition of the lipid head groups (Isken and de Bont 1998, Weber and de Bont 1996). Changes in the outer membrane occur in Gram-negative bacteria, and the most commonly observed modifications were in lipopolisaccharide (LPS) content (Isken and de Bont 1998, Weber and de Bont 1996).

Bacteria have the ability to modify their hydrophobic character, which acts as a way of protection against toxic hydrophilic compounds. Hydrophobic envelopes may promote the aggregation of cells as a mechanism to diminish the exposure and the binding of toxic compounds. This adaptation mechanism was observed for *R. erythropolis* DCL14, and it is characteristic of this strain (de Carvalho, da Cruz, et al. 2004). Another morphological change that may occur is the production of an extracellular hydrophobic carbohydrate capsule, observed, for example, in *Staphylococcus* spp. ZZ1 (Zahir, Seed and Dennis 2006), that repels and prevents toxic compounds, such organic solvents, to reach the cell membrane (Torres, Pandey and Castro 2011).

Resistance to toxic compounds can be promoted by their degradation into a non-toxic or a less-toxic form of the agent. The tolerance of microorganisms to antimicrobial agents and to organic solvents can be mediated by this degradation mechanism (Isken and de Bont 1998, de Carvalho, Fatal, et al. 2007). Another mechanism to decrease the concentration of toxins inside cells is the activation of efflux pump machinery. The active extrusion of toxins from cells is a known defense mechanism for lipophilic cytotoxic agents, especially antibiotics (Isken and de Bont 1998, Nikaido 2001).

1.2.1 The Taxon Mycolata – Cell Envelope as a Defense Mechanism

The taxon mycolata includes several bacteria of clinical and biotechnology relevance, like the genera *Corynebacterium*, *Mycobacterium* and *Nocardia*, besides *Rhodococcus*. The fatty acid that is strongly present, and is characteristic of the cell envelope composition of these bacteria, is the mycolic acid, which differs in its chemical composition and organization in the different genera of mycolata.

Mycolic acids have an important feature in their structure, which is the length of the meromycolate chain compared to the alkyl side branch (Sutcliffe 1998, Alvarez 2010). These molecules are 2-alkyl branched, 3-hydroxy long chain fatty acids that can vary from 22 to 90 carbons of length. The cell envelope of genus *Corynebacterium* has typically the smallest mycolic acids in its composition, containing a size range from 22 to 38 total carbons. In comparison, the mycolic acids that compose the cell envelope of the genus *Mycobacterium* are the most complex, tipically composed with 60 to 90 carbons, containing meromycolate chain functional groups, such as cyclopropane, methoxy- and keto-modifications (Dover, et al. 2004, Alvarez 2010). In genus *Rhodococcus*, these fatty acids have the typical length of 28 to 54 carbons in total. In these bacteria, the alkyl side branch is typically a 10-16 carbons saturated alkyl chain, and the meromycolte chain is a 20-24 carbon chain with up to four double bonds (Figure 3) (Sutcliffe 1998, Gürtler, Mayall and Seviour 2004, Alvarez 2010).

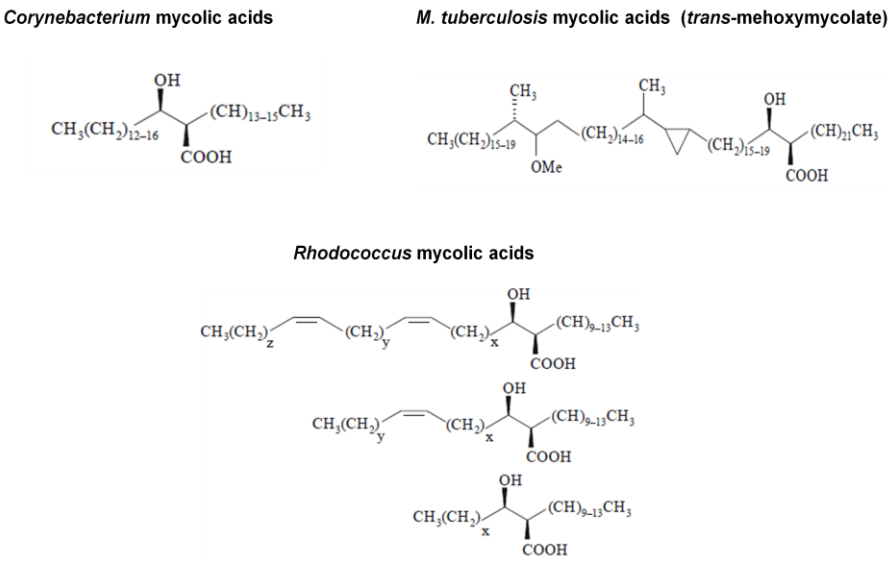


Figure 3 - Mycolic acids chemical structure and composition from the different bacteria genera. Adapted from (Alvarez 2010).

Cell envelope composition is made essentially by mycolic acids that are covalently linked to the cell wall polysaccharide arabinogalactan, which is, in turn, covalently linked to peptidoglycan (Figure 4) (Sutcliffe 1998, Nikaido 2001). The cell envelope architecture of mycolata is further oriented such that mycolic acids form the basis of an outer lipid permeability barrier (Sutcliffe 1998). This turns out to be an advantage of bacteria belonging to this taxon, which does not preclude the modifications of cell membranes under stress conditions.

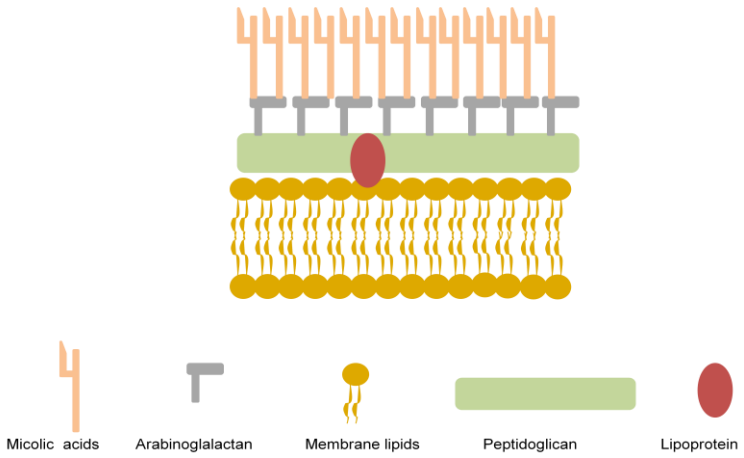


Figure 4 – Cell wall structure of mycolata. Adapted from (Sutcliffe 1998).

The tolerance and adaptation of *Rhodococcus* cells, especially *R. erythropolis,* to several different and aggressive conditions, like the presence of the toxic compounds toluene, carveol and carvone (de Carvalho, Parreño-Marchante, et al. 2005, de Carvalho and da Fonseca 2007) has been related to the high complexity and capacity of modification under stress conditions of the cell membrane this bacterium (de Carvalho, Parreño-Marchante, et al. 2005). Since bacteria have the ability to use several mechanisms of defense against hostile environments, it is important to study the associated mechanisms involved in the resistance of *R. erythropolis.* From the presented adaptive mechanisms, the presence of efflux pumps is the only one that is not described for rhodococci (de Carvalho, Wick and Heipieper 2009, de Carvalho 2012).

1.2.2 Efflux Pumps

Located in the cytoplasmic membrane of bacteria, efflux pumps are transporter proteins that promote the extrusion of drugs out of the cell as drugs enter. Efflux pumps gained great focus essentially in medicine, and emerged as a major challenge in this field for bacterial treatment due to their role in the resistance of bacteria to multiple drugs (Saier, et al. 1998). This defense mechanism has been described for both prokaryotic and eukaryotic cells. The most common structure presented by efflux pumps is a monolithic multidomain transmembrane protein (Figure 5A). However, since Gram-negative bacteria have a more complex membrane, efflux pumps in these bacteria are structurally different. They present a common organization of three components: i) a transporter located in the inner membrane; ii) a periplasmic protein, and iii) a channel on the outer membrane. The efflux is then promoted by the interaction of the transporter of the inner membrane with the channel of the outer membrane, through the preriplasmic protein (Figure 5B) (Marquez 2005, Saier, et al. 1998, Van Bambeke, et al. 2003).

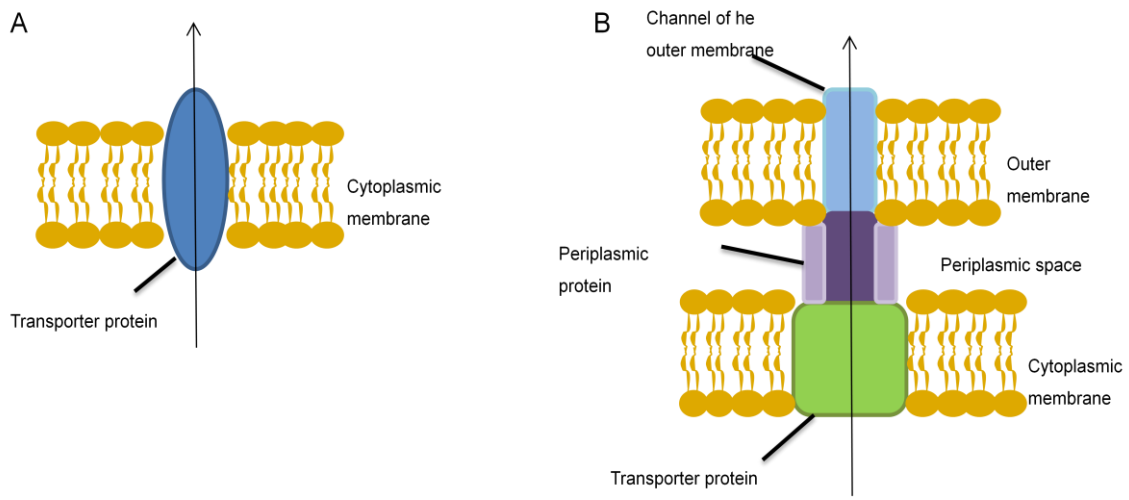


Figure 5 - A) Monocomponent efflux pump; B) Tricomponent efflux pump. Adapted from (Van Bambeke, et al. 2003).

Efflux pumps in bacteria can mediate the efflux of a specific drug or class of drugs, conferring a specific drug resistance to bacteria, or they can mediate the efflux of structurally unrelated drugs conferring a phenotype called Multidrug Resistance (MDR) that is associated to several pathogenic organisms (Mazurkiewicz, Driessen and Konings 2005). The MDR phenomena can be caused by the simultaneous presence of multiple individual resistance mechanisms. The impermeability of the bacterial membrane and active drug efflux can also mediate the MDR. With these defense mechanisms, the drugs cannot reach their cellular targets (Li and Nikaido 2009).

1.2.3 Efflux Pumps in Gram-positive Bacteria

Rhodococcus are Gram-positive bacteria which have not been studied to date regarding the presence of efflux pumps as a defense mechanism system. However, other species of gram-positive bacteria have been studied, and the respective results are useful to establish a characterization of expected efflux pump mechanisms in *Rhodococcus*.

Species of Gram-positive bacteria in which efflux pumps have already been studied, to some extent, are *S. aureus* and some *Mycobacterium* strains.

In *S. aureus,* efflux pumps belonging to four families were described. The efflux pump SAV 1866 belongs to the ATP Binding Cassette (ABC) superfamily (Dawson and Locher 2007) . The pumps QacA and QacB with 14 transmembrane segments (Paulsen, Brown and Littlejohn, et al. 1996) and the pump NorA with 12 transmembrane segments (Paulsen, Brown and Skurray 1996) are described

in the Major Facilitator superfamily (MFS). The first pump belonging to Small Multidrug Resistance (SMR) family was the Smr transporter (Paulsen, Skurray, et al. 1996). With great clinical relevance is the transporter MepA belonging to Multidrug and toxic compound extrusion (MATE) family (Omote, et al. 2006).

The transporter LfrA was described in *M. smegmatis* as an MFS transporter, homologue of the QacA transporter of *S.aureus* (Takiff, et al. 1996) and the Tap pump of *M. fortuitum* (Aínsa, et al. 1998). The Mmr, an SMR pump (Rossi, et al. 1998), DrrAB, an ABC transporter (Choudhuri, et al. 2002), and Tap, an MFS transporter (Aínsa, et al. 1998) were reported in *M. tuberculosis*.

1.2.4 Efflux Pumps Families

Efflux pumps in bacteria differ structurally and in their mode of action, especially in their source of energy to promote the extrusion of drugs. According to their mechanisms of transportation these membrane proteins can be primary active transporters, if the efflux is promoted by the direct use of ATP, or secondary active transporters, if the efflux is promoted by the proton motive force.

According to these differences they may be categorized in different families: the adenosine triphosphate (ATP)-binding cassette (ABC) superfamily; the major facilitator superfamily (MFS); the multidrug and toxic compound extrusion (MATE) family; the small multidrug resistance (SMR) family, which is a subgroup of the drug/metabolite transporter superfamily; and the resistance-nodulation-division (RND) superfamily (Li and Nikaido 2009).With the exception of the efflux pumps belonging to the ABC superfamily, which are primary active transporters, all the other efflux pumps belonging to the remaining families are secondary active transporters (Kuroda and Tsuchiya 2009).

1.2.4.1 Primary Active Transporters

The primary active transporters use the direct energy of ATP to promote the transport of molecules across the membrane against their concentration gradient. In general, the enzymes that perform this type of transportation are transmembrane ATPases (Van Bambeke, Balzi and Tulkens 2000). The ABC superfamily efflux systems belong to this class of transporters.

1.2.4.1.1 ATP-Binding Cassette Superfamily

The transporter proteins belonging to the ATP-Binding Cassette Superfamily have an ATP hydrolysis mechanism involved in the extrusion of drugs from the cell, contrarily to the remaining efflux pumps families (Köhler, Pechère and Plésiat 1999, Nikaido 2009). These efflux pumps are generally composed by a membrane protein with six α- helical transmembrane motifs and an energy coupling protein located in the cytoplasmatic side of the membrane (Saier, et al. 1998). ABC transporters require the involvement of Mg^{2+} in their activity, in prokaryotic cells (Lubelski, Konings and Driessen 2007).

The first reported transporter described for this family in prokaryotes was in *Lactococcus lactis*, and is known as LmrA (Bolhuis, et al. 1994, Putman, Veen and Konings 2000). Other examples of ABC transporters are the efflux pump SAV 1866 from *S. aureus* (Dawson and Locher 2007) and the efflux pump MsbA from *Escherichia coli* (Putman, Veen and Konings 2000).

Primary transporter proteins are implicit in various cellular processes such as the uptake of nutrients and extrusion of cellular metabolites, protection against xenobiotics, osmotic stress and lipid transport. The substrates of these transporters can vary from small ions and small molecules (amino acids, sugars, xenobiotic compounds and vitamins) to large compounds such as polymers (peptides, proteins and polysaccharides). Transporter proteins belonging to ABC superfamily have, therefore, an important biological role not only in drug extrusion but also in the uptake of substrates and necessary nutrients to the cell or extrusion of toxic metabolites (Köhler, Pechère and Plésiat 1999).

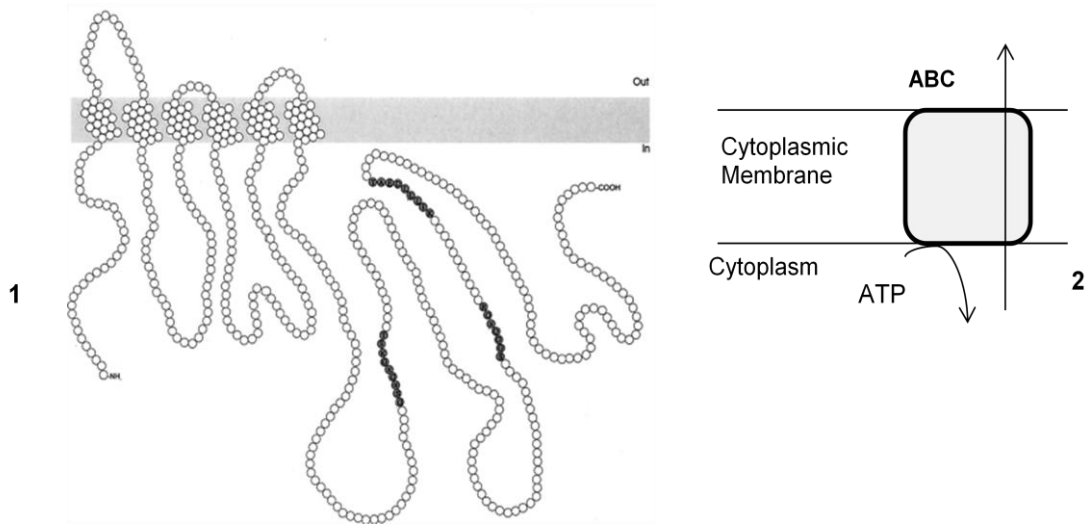


Figure 6 – 1) Model of an efflux pump belonging to the ABC superfamily. Adapted from (Putman, Veen and Konings 2000). 2) Schematic representation of the ABC efflux pump family. Adapted from (Köhler, Pechère and Plésiat 1999).

1.2.4.2 Secondary Active Transporters

Secondary active transporters are membrane proteins that use an electrochemical gradient of cations, the proton motive force, across the membrane coupled to the drug extrusion against its concentration gradient (Van Bambeke, Balzi and Tulkens 2000).

1.2.4.2.1 Major Facilitator Superfamily

The membrane transporters belonging to MFS are found in all kinds of organisms, from bacteria to higher eukaryotes. Their mechanism of action involves the transport of a wide range of substrates, like sugars, Krebs cycle intermediates, organophosphates, oligosaccharides and antibiotics (Pao, Paulsen and Jr. 1998)

Representing one of the largest families of transporters, the MFS contains several important efflux pumps, like QacA and QacB of *S.aureus* and EmrB of *E.coli*. The efflux pumps of this family differ in the number of transmembrane segments (TMS) that constitute them, which may be 14 or 12 transmembrane segments (Nikaido 2009).

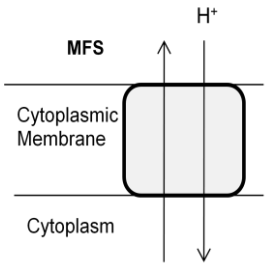


Figure 7 - Schematic representation of the MFS efflux pump. Adapted from (Köhler, Pechère and Plésiat 1999).

MFS with 14 transmembrane segments

The first pumps described inside this class were the QacA and QacB of *S. aureus* (Paulsen, Brown and Littlejohn, et al. 1996). The membrane proteins are constituted by 14 transmembrane segments, containing various amino acids with acidic residues in the transmembrane section. Comparing the sequences of QacA and QacB of *S. aureus* and by directed mutagenesis it was observed that the extrusion of dicationic compounds promoted by QacA, but not by QacB, is dependent on the presence of an aspartate residue in trasmembrane segment 10 (Nikaido 2009). These efflux pumps confer resistance to dyes, quaternary ammonium compounds, diamidines, and biguanidines (Mitchell, Brown and Skurray 1998).

Another characteristic efflux pump belonging to this class is the EmrB of *E. coli.* This extensive studied efflux pump extrudes compounds out of the cell, such as the uncoupler carbonyl cyanide m-chlorophenylhydrazone (CCCP), and the antibiotics nalidixic acid and thiolactomycin (Nikaido 2009).

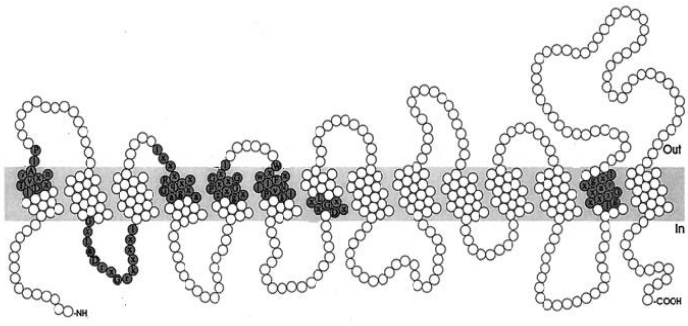


Figure 8 – Model of a 14 TMS efflux pump belonging to the MFS. Adapted from (Putman, Veen and Konings 2000).

MFS with 12 transmembrane segments

The pump NorA of *S. aureus* is the model example of efflux pumps inside this class. Encoded by the bacterial chromosome, this pump confers resistance to fluoroquinolones and cationic inhibitors, including puromycin and tetraphenylphosphonium (Nikaido 2009). The two homologues of this efflux pump are the efflux pumps NorB and NorC also encoded by the chromosome of this bacterium and which also confer similar phenotypes (Nikaido 2009, Paulsen, Brown and Skurray 1996).

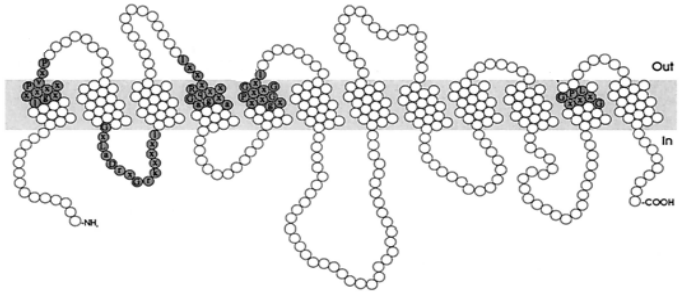


Figure 9 – Model of a 12 TMS transporter belonging to the MFS. Adapted from (Putman, Veen and Konings 2000)

1.2.4.2.2 Small Multidrug Resistance Family

Representing the smallest known efflux pumps, the transporter proteins belonging to this family have typically 110 amino acids residues in length with 4 α-helix transmembrane segments. The first pump belonging to this family was discovered in *S. aureus*, the Smr transporter, being encoded by a plasmid (Paulsen, Skurray, et al. 1996). On the other hand, in Gram-negative bacteria, it was discovered that efflux pumps belonging to this family are encoded by the bacterial chromosome. An example of these transporters is the EmrE efflux pump from *E. coli* (Nikaido 2009, Paulsen, Brown and Skurray 1996).

The model of multidrug efflux mediated by the SMR proteins was proposed by Paulsen et al. They have proposed that the pumps have the first three amphipahtic transmembrane segments with conserved glutamate, serine, tyrosine and tryptophan residues in the helices, which may form part of the passageway of protons and drugs (Paulsen, Skurray, et al. 1996).

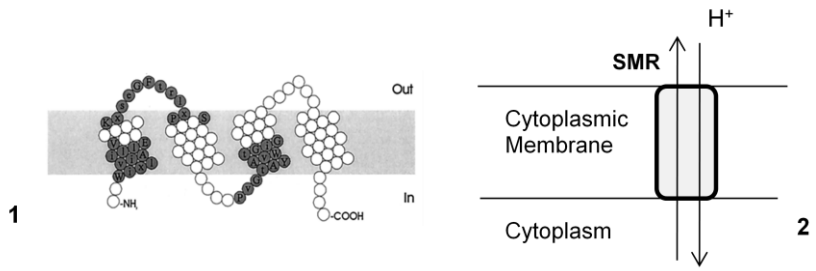


Figure 10 – 1) Model of an efflux pump belonging to the SMR family. Adapted from (Putman, Veen and Konings 2000). 2) Schematic representation of the SMR efflux pump family. Adapted from (Köhler, Pechère and Plésiat 1999).

1.2.4.2.3 Resistance-Nodulation-Division Family

The transporters belonging to this family have a peculiar composition and they confer special resistance to antibiotics in Gram-negative bacteria, although eukaryotes and archea present RND efflux pumps homologues (Nikaido and Zgurskaya 2001).

The transporter proteins of this family are organized in their most common system in Gram-negative bacteria. The protein complexes that span from the cytoplasm to the external membrane are organized in a tripartite system and they are, therefore, composed by a transporter in the internal membrane, an accessory periplasmic protein and a protein in the external membrane, making these efflux pumps examples of a tricomponent transporter protein. The most studied examples of the RND family are the AcrAB-TolC of *E. coli* and MexABOprM of *Pseudomonas aeruginosa* that catalyze the efflux of a large variety of antimicrobial and chemotherapeutical agents (Guglierame, et al. 2006).

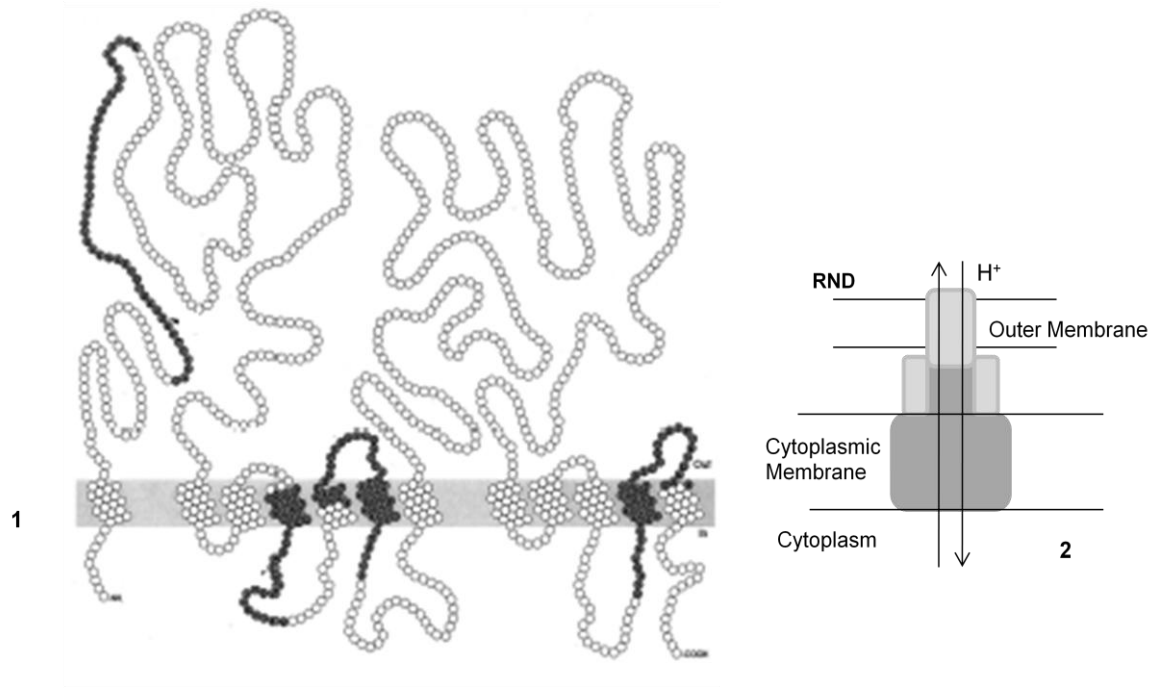


Figure 11 – 1) Model of an efflux pump belonging to the RND family. Adapted from (Putman, Veen and Konings 2000). 2) Schematic representation of the RND efflux pump family. Adapted from (Köhler, Pechère and Plésiat 1999).

1.2.4.2.4 Multidrug and Toxic Compound Extrusion Family

The first described transporters of the MATE family were the NorM of *Vibro parahaemolyticus,* and its homologue in *E. coli* YdhE. Inside this efflux pump family, the transporter protein MepA of *S. aureus* has a great clinical relevance, whose overexpression confers resistance to tigecycline, a developed glycylcycline antibiotic (Omote, et al. 2006).

The MATE transporters have a unique feature for the extrusion of drugs. The efflux pumps belonging to this family confer resistance to multiple toxic cationic agents, like ethidium bromide, using an Na^+ antiport in drug extrusion. The use of sodium motive force as the driving force for the extrusion of drugs is a feature that distinguishes this efflux pump family from other secundary transporter families (Mazurkiewicz, Driessen and Konings 2005). It was proposed that the pumps belonging to this family act as transporters for multiple drugs similar to those extruded by the remaining efflux pumps families, but preferencially for the extrusion of organic cations (Li and Nikaido 2009, Omote, et al. 2006).

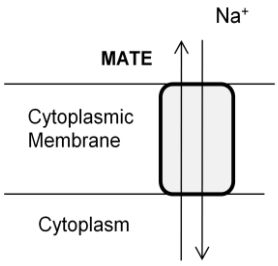


Figure 12 – Schematic representation of the MATE efflux pump family. Adapted from (Köhler, Pechère and Plésiat 1999).

Table 1 - Various families of efflux pumps, their respective efflux pump system, and outflows drugs.

Efflux pumps family	Organism	Efflux pumps	Outflows drugs	References
MFS	*Staphylococcus aureus*	QacA; QacB	monocationic biocides; dyes	(Paulsen, Brown and Littlejohn, et al. 1996)
	Escherichia Coli	EmrB	CCCP; nalidixic acid; thiolactomycin	(Lomovskaya and Lewis 1992)
SMR family	*Staphylococcus aureus*	SMR	cationic compounds; dyes	(Paulsen, Skurray, et al. 1996)
RND superfamily	*Escherichia coli;*	AcrAB-TolC	dyes, detergents, liphofilic antibiotics	(Nikaido and Zgurskaya 2001)
	Pseudomonas aureginosa	MexABOprM		
MATE	*Vibro parahaemolyticus*	NorM	cationic drugs and dyes (ethidium bromide (EtBr), tetraphenylphosphonium, berberine, acriflavine, norfloxacin)	(Omote, et al. 2006)
	Escherichia coli	YdhE		
	Staphylococcus aureus	MepA		
ABC superfamily	*Staphylococcus aureus*	Sav1866	amino acids; sugars; xenobiotic compounds; vitamins; peptides; proteins; polysaccharides	(Köhler, Pechère and Plésiat 1999, Dawson and Locher 2007)
	Lactococcus lactis	LmrA		

1.2.5 Efflux Pumps Inhibitors

Besides the previously presented defense mechanisms displayed by bacteria, the recurrent use of antimicrobial agents in the treatment of diseases has increased the resistance of pathogenic bacteria to multiple drugs, including antibiotics, leading to the MDR phenotype (Berkowitz 1995, Saier, et al. 1998).

The appearance of the MDR phenomena raised the need to develop new antimicrobial agents. The chemical design and re-design of antimicrobial agents has overcome some of the presented adaptation mechanisms, like the enzymatic modification of toxic agents. However, since bacteria have the ability to gain resistance to drugs with their recurrent use, the solution does not consist solely in creating new antimicrobial agents with different structures. Knowing that bacteria have defense mechanisms, the solution is therefore to also create compounds that inhibit these defense mechanisms. An approach to overcome this problem is the inhibition of efflux pump systems, using efflux pump inhibitors (EPIs) (Marquez 2005, Van Bambeke, Pagès and Lee 2010). By blocking the activity of efflux pumps, the concentration of drugs inside the cells will be higher, and the effect of the antimicrobial agents may increase. This new approach against the MDR phenomenon leads to the expectation of decreasing not only the intrinsic resistance but also reversing the acquired resistance of bacteria to drugs and of reducing the frequency of appearance of resistant strains (Marquez 2005). Therefore, to be classified as an EPI, a compound has to satisfy some basic criteria: i) it must promote the activity of multiple substrates of the pump; ii) it should have no activity in strains that do not have the efflux pump; iii) it should increase accumulation and decrease the efflux of the efflux pump substrates; iv) its activity must not affect the integrity of the inner membrane (Lomovskaya and Watkins 2001).

Besides all the recent studies involving EPIs, their mechanism as MDR efflux inhibitors is not entirely understood. Nevertheless, there are several proposals for their mode of action (Zloh and Glenn W. Kaatz 2004, Marquez 2005):

- The inhibitor binds directly to the pump in a competitive or non competitive manner with the substrate, causing the blocking of the efflux pump;
- Inhibitors can cause a depletion of energy, through the inhibition of the biding of ATP or the disturbance of the proton gradient across the membrane;
- Inhibitors have affinity for substrates, and bind them, forming a complex that facilitates the entry of the drug in the cell and prevents its efflux.

There are several EPIs that are commonly used in the study of efflux pumps, *e.g.* verapamil and phenotiazines (Marquez 2005). In general, they have in common the ability to modify the proton-motive force of the membrane that is an essential requirement for the function of efflux pumps

(Mahamoud, et al. 2007). In this present work, five EPIs already tested in previous studies using *Mycobacterium* strains were used: verapamil, thioridazine, omeprazole and sodium orthovanadate and the uncoupler carbonyl cyanide m-chlorophenylhydrazone (CCCP).

1.2.5.1 Verapamil

Verapamil is an efflux pump inhibitor currently used to evaluate the efflux pump activity in Gram-positive bacteria, and it can be also used in Gram-negative bacteria, like *E. coli* (Viveiros, Martins, et al. 2008). This drug is classified as an anti-arrhythmic drug. It is a calcium channel blocker (or a calcium channel antagonist) and is frequently used for the treatment of hypertension, angina pectoris and cardiac arrhythmia (Garvey and Piddock 2008, Marquez 2005, Hoog, et al. 1999). Studies made with verapamil have shown that this drug also inhibits the transportation of potassium ions (K^+) (Hoog, et al. 1999).

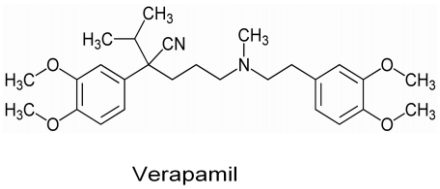


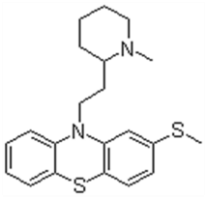
Figure 13 – Chemical structure of verapamil.

Considered as an inhibitor of MDR pumps, verapamil is able to inhibit the **ABC transporters** involved in the extrusion of antibiotics (Mahamoud, et al. 2007). It has been demonstrated that verapamil is effective as an EPI in *M. smegmatis*, in *E. coli* and in *Enterococcus faecalis* (Van Bambeke, Pagès and Lee 2010, Choudhuri, Sen and Chakrabarti 1999, Li and Nikaido 2004).

1.2.5.2 Thioridazine

Belonging to the family of phenotiazines, thioridazine is an antipsychotic drug which is widely used to treat schizophrenia and psychosis. It has been suggested that this class of drugs affect the transporter proteins that depend on the proton-motive force, and like chloropromazine, another phenotyazine, it affects the flux of calcium and potassium across the membrane (Kaatz, et al. 2003)

and inhibits the binding of calcium ions (Ca^{2+}) to enzymes and proteins (Amaral, Martins and Viveiros 2011).



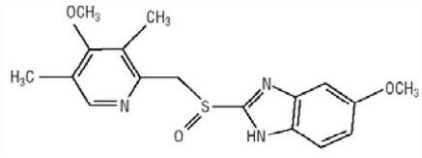
Thioridazine

Figure 14 – Chemical structure of thioridazine.

The effectiveness of phenotiazines, such thioridazine and chlorpromazine, as EPIs has been demonstrated in *M. avium* and *M. semegmatis* (Rodrigues, Wagner, et al. 2008), and in *M. tuberculosis* in order to aid the treatment of patients with tuberculosis (Amaral, Martins, et al. 2008, Amaral, Martins and Viveiros 2011). Phenotiazines are considered as **ABC type** efflux pumps inhibitors (Amaral, Viveiros and Molnar 2004).

1.2.5.3 Omeprazole

Omeprazole is an anti-ulcer drug recurrently used in several gastric pathologies. The mechanism of action of this drug involves the inhibition of the H^+-K^+-ATPase (Wishart 22th August 2011, Aeschlimann, et al. 1999).



Omeprazole

Figure 15 – Chemical structure of omeprazole.

Omeprazole has been described as an effective efflux pump inhibitor of the **MFS** efflux pump NorA of *S. aureus* (Aeschlimann, et al. 1999), and its activity has already been studied using *Mycobacterium* (Suzuki, et al. 2000).

1.2.5.4 Sodium orthovanadate

In biological systems, vanadium compounds have proved to inhibit several enzymes like ATP-phosphohydrolases, ribonuclease, adenylate kinase, phosphofructokinase, squalene synthetase, glyceraldehyde-3-phosphate dehydrogenase, glucose-6-phosphatase and phosphotyrosyl-protein phosphatase (Nechay 1984). These compounds have also the ability to block the flux of ions by inhibition of Na^+, K^+ - ATPase and Ca^{2+} - ATPase, and act as regulators of Na^+ pumps (Nechay 1984, Benabe, et al. 1987). Therefore, vanadium compounds, such sodium orthvanadate, are generally described as inhibitor of ATPases and ATP efflux systems, like the **ABC** efflux pumps (Garvey and Piddock 2008).

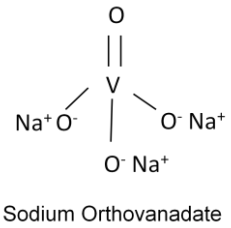
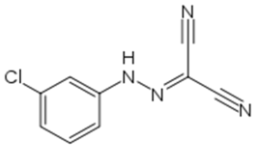


Figure 16 – Chemical structure of sodium orthovanadate.

The activity of vanadate compounds as efflux pump inhibitors was studied and demonstrated in *M. smegmatis* (Choudhuri, Sen and Chakrabarti 1999).

1.2.5.5 Carbonyl cyanide m-chlorophenylhydrazone

The compound CCCP is classified as a protonophore, and as such, the mechanism of action involves the uncoupling of the proton gradient that is established during the normal activity of electron carriers in the electron transport chain, affecting the synthesis of ATP. CCCP considerably affects the energy level of the membrane and cell viability by causing a dissipation of the proton motive force of the membrane, affecting the transporters that depend on this mechanism. Besides its high toxicity for the cell, CCCP is described as a substrate of bacterial efflux pumps (Mahamoud, et al. 2007, Alvarado and Vasseur 1998).



CCCP

Figure 17 – Chemical structure of CCCP.

CCCP has demonstrated inhibition activity in *M. smegmatis* (Choudhuri, Sen and Chakrabarti 1999) and in *M. fortuitum* by inhibition of the **MFS** efflux pump Tap (Ramón-García, et al. 2006).

1.2.6 The Study of Efflux Pumps in Mycolata – Tuberculosis Therapy Using EPIs

The study of efflux pumps has had a major impact on medicine, due to the appearance of the MDR phenomena. Conducted studies made in this area using EPIs were in tuberculosis therapy (Amaral, Martins, et al. 2008, Amaral, Martins and Viveiros 2011, Ordway, et al. 2003).

Nowadays, infectious diseases still remain the leading cause of mortality in the world, for adult people. Infections made by *M. tuberculosis* affect approximately a third of the world's population, although they do not necessarily display symptoms of tuberculosis (TB), which is the most notable pathology associated to this bacterium (Kim, et al. 2005, WHO 23th August 2011).

Global regions where TB is common and therapy is inadequate, have allowed the appearance of strains that resist to first line antibiotic therapy. An efficient therapy for infections by *M. tuberculosis* is using the most effective anti-TB drugs isoniazid (INH) and rifampicin (RIF). However, this therapy is not always enough for the treatment of TB, and a long period of this therapy helps bacteria to develop resistance to these antibiotics. In this situation, *M. tuberculosis* is defined to be multi-drug resistant (MDR-TB) (Kim, et al. 2005, Amaral, Martins and Viveiros 2011). The therapy of TB can lead to a greater resistance by bacteria and MDR-TB can develop into extensively-drug resistant tuberculosis (XDR-TB). The XDR-TB is the *M. tuberculosis* strain resistant to INH and RIF, plus to any fluoriquinolone and to one of the three second line anti-TB drugs (capreomycin, kanamicin and amikacin) (Amaral, Martins, et al. 2008). High frequencies of this strain of *M. tuberculosis* were reported in Eastern Europe, China, South East Asia, Africa and South America. Since infections of XDR-TB have increased over the world and have become an emerging threat, the need for the development of new anti-TB drugs and new therapeutic strategies has also increased (Amaral, Martins and Viveiros 2011, Amaral, Martins, et al. 2008).

A hypothesis proposed and studied by Amaral *et. al* is the use of the EPI thioridazine in the treatment of TB. It has been demonstrated by the research group that this phenothiazine has *in vitro* and *ex vivo* activity against MDR and XDR-TB (Amaral, Martins, et al. 2008). In 2003, it was demonstrated that a concentration of 0.1 mg/L of this drug in the media could kill intracellular MDR-TB (Ordway, et al. 2003) and in 2008 they demonstrated that the same concentration killed intracellular XDR-TB (Amaral, Martins, et al. 2008). Since these studies, the clinical use of TZ with anti-TB drugs has already been implemented as a trial therapy for XDR-TB with some successful treated patients (Amaral, Martins and Viveiros 2011).

1.3 Methodologies to Study Efflux Pumps Systems

The effectiveness of antimicrobial drugs is compromised by the activity of efflux pumps associated to the MDR phenotype. The infections caused by these bacteria have been a major challenged in medicine and caused several therapeutic problems related to the healing process of the infections. In order to avoid these problems, it is necessary to make an early recognition of the MDR bacteria (Lomovskaya, Zgurskaya, et al. 2007). It is therefore necessary to develop methods to understand this type of resistance mechanism, by detection and quantification of drug transport across the bacterial cell wall. During a time that has been called the genomic era, the most common methodology to study the transporter proteins was to sequence the bacteria genome and, by comparison of homologous bacterial genomes and the strategy of knot-out of the genes, to find sequences that would probably encode the efflux pumps (Ryan, et al. 2001, Smith and Romesberg 2007). A method using agar plates to study the behavior and susceptibility of bacteria can be also used, and turns out to be an instrument-free method to study efflux pump activity (Martins, et al. 2006).

Another method that can be used to evaluate the efflux pump defense mechanisms of MDR bacteria is flow cytometry. This method makes use of fluorescent dyes that bind cellular targets to stain the cells. This process facilitates identification and it allows the cells to be distinguished from other particulate matter. This technique is, therefore, most useful for counting and identifying bacteria and to determine the viability of bacteria and their antibiotic susceptibility (Jernae and Steen 1994, Paixão, et al. 2009).

There is a method successfully developed to estimate the activity of efflux pumps in bacteria. This method proposed by Viveiros *et al.* is a semi-automated fluorometric method that monitors the efflux of a common fluorescent substrate such as ethidium bromide (EtBr) (Viveiros, Martins, et al. 2008, Paixão, et al. 2009). This method quantifies the transport of EtBr across the living cell, because it can distinguish conditions of accumulation, a balance between influx and efflux, from conditions that inhibit efflux itself (Figure 8), by measuring the EtBr fluorescence. The kinetics of EtBr transportation will be the base to determine the efflux activity of the cells by this method. EtBr emits weak fluorescence in aqueous solutions, and becomes strongly fluorescent when concentrated in the periplasm of Gram-

negative bacteria and in the cytoplasm of Gram-positive bacteria, due to its binding to cellular components. The use of fluorometry has been revealed to be a powerful method to continuously monitor the transport of the fluorescent substrate across of cell membranes (Paixão, et al. 2009). This method can be used for the screening of EPIs and for the identification of the overexpressed efflux pumps of bacteria, by measuring efflux activity (Paixão, et al. 2009, Viveiros, Martins, et al. 2008). The method estimates, in real-time, the accumulation and efflux of EtBr under varying physiological conditions, such as different temperatures and pH, the presence or absence of an energy source, and the presence or absence of EPIs. It was designed in order to contain several features appropriate to the laboratory, being therefore a simple method assisted by computer, which makes it a user-friendly method, and a precise and reproducible method (Paixão, et al. 2009, Viveiros, Rodrigues, et al. 2010). The described method is sensitive enough to characterize intrinsic efflux pumps systems, a major requirement if there is a need to evaluate the efflux pumps systems mediated by MDR phenotype, and which has already been applied by the research group in the characterization of intrinsic and over-expressed efflux pumps systems of *Escherichia coli*, *Salmonella enteritidis*, *Enterobacter aerogenes*, *Enterococcus faecalis*, *Enterococcus faecium*, *Staphylococcus aureus*, *Mycobacterium smegmatis* and *Mycobacterium avium* (Viveiros, Rodrigues, et al. 2010).

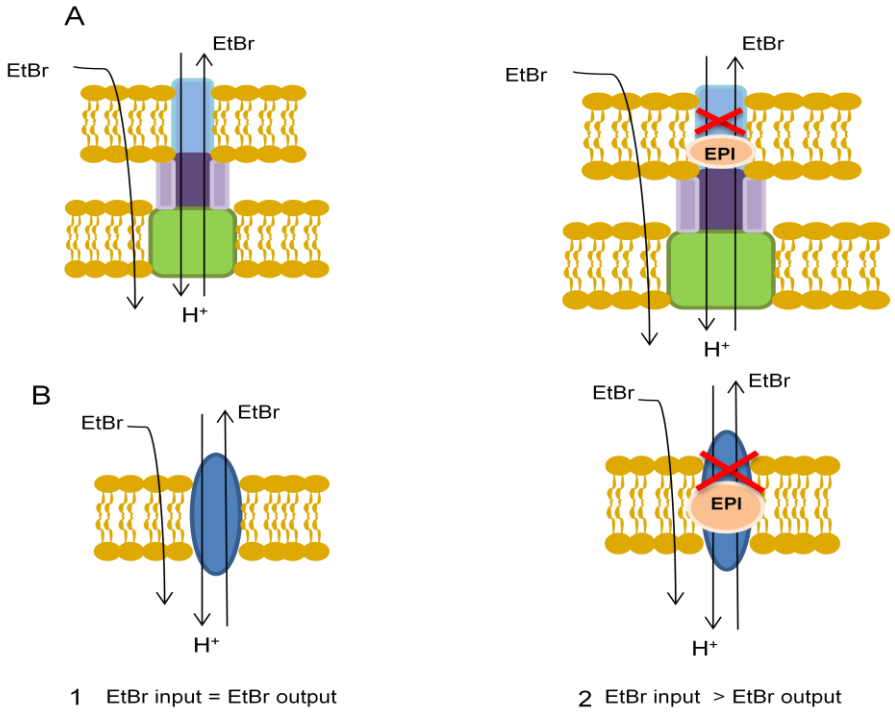


Figure 18 – Model of the transportation of EtBr, or other substrate, across the cell membrane in conditions of accumulation (1) and conditions of efflux inhibition (2), in both Gram-negative (A) and Gram-positive (B) bacteria.

A complementary technique to study the behavior of cells in the presence of EPIs or other hostile conditions is the use of florescence microscopy. This technique, when associated to others, becomes a powerful tool to complement the study of adaptation mechanisms of bacteria by using an appropriated fluorophore, having the advantage of distinguishing the behavior on a single cell event. The evaluation of cell morphology, membrane potential or even cell viability can be made using the indicated available kits. In general, the used fluorophore used changes color according to the cells state, and evaluation is made according to these changes (de Carvalho 2012, de Carvalho, Fatal, et al. 2007, de Carvalho, Parreño-Marchante, et al. 2005). In the present work, the evaluation of membrane potential is made by using 3,3′-diethyloxacarbocyanine iodide ($DiOC_2(3)$) as a fluorophore to evaluate the polarization of cellular membranes. This dye changes color according to the depolarization of the membrane of cells. Green cells correspond to depolarized cells and red/orange cells correspond to polarized cells (Figure 19) (Annex II).

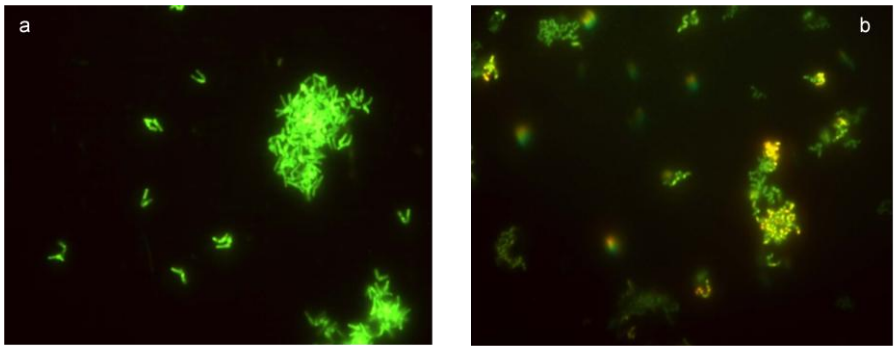


Figure 19 – Example of an assay performed with *R. erythropolis* stained with $DiOC_2(3)$. a) Green cells corresponding to a depolarized state of cells membrane; b) Green cells and red/orange cells corresponding to both depolarized and polarized states, respectively, of cellular membranes.

Table 2 - Advantage and disadvantage of different techniques used in the study of efflux machinery of cells. + advantage; - disadvantage.

Techniques	Time consuming	Simplicity of the method	Reproducibility	Differentiation of influx/efflux conditions	Real time course	Single cell event
Instrument free	+	-	+	-	+	-
Flow cytometry	-	+	+	+	+	+
Real time fluorometry	-	+	+	+	+	-
Fluorescence microscopy	-	+	+	+	+	+

1.4 Objectives

The proposed objective for this master thesis is to demonstrate that the bacterium *Rhodococcus erythropolis* has efflux pumps as an adaptation mechanism, which may be used against toxic compounds.

Three different methods were used in order to assess the action of efflux pumps: through the determination of the minimum inhibitory concentration of antibiotics in the presence and absence of EPIs, which reflects the action of the inhibitors and their ability to decrease the resistance of bacteria to antimicrobial agents; using real time fluorometry, with ethidium bromide as a substrate, in the presence and absence of EPIs, which allows the observation of the accumulation of ethidium bromide or its efflux from the *R. erythropolis* cells; lastly, the use of fluorescence microscopy allows the complementation of this study, demonstrating the effects of the used EPIs on the proton motive force across cellular membranes.

1.5 Thesis Outline

This document is organized as follows:

Chapter 2 presents the materials and methods used in the development of the experimental work associated with this thesis. The strain which will be analyzed and the work conditions and used solutions are presented in this chapter, as well the method which was used to determine the minimum inhibitory concentration of the EPIs and substrates, the real time fluorometry method used to evaluate the inhibitory ability of the EPIs by performing accumulation and efflux assays, and the microscopy fluorescence method used to determine the effect of EPIs in the membrane potential of membrane cell.

Chapter 3 presents the results obtained from the various methods described in the previous chapter and the discussion of these results. In these results, EPIs can be seen to have the ability to: decrease the resistance of *R. erythropolis* to antibiotics, by determination of the MICs; inhibit efflux systems displayed by *R. erythropolis* and their capacity to perform the inhibition in the presence and absence of an energy source, by using the real time fluorometry method; disrupt the proton motive force by evaluation of the membrane potential using fluorescence microscopy.

Chapter 4 present the main conclusions obtained in this work and the future work that can be pursued along this line of research.

Material and Methods

2 Material and Methods

In this section a description is made of the bacteria, solutions, methods and conditions of the laboratorial work done in the development of this thesis.

2.1 Bacteria

The bacterium used in the present work was *Rhodococcus erythropolis* DCL14, obtained from the Division of Industrial Microbiology of the Wageningen Agricultural University, Wageningen, The Netherlands, and maintained at the Institute for Biotechnology and Bioengineering, Lisbon, Portugal (de Carvalho 2012).

2.2 Growth Conditions

R. erythropolis cells were grown in 20 mL of elemental mineral medium pH 7 (Annex I) supplemented with 0.25% (v/v) of ethanol (99.9%, Panreac) (de Carvalho, Parreño-Marchante, et al. 2005), at 28ºC and 200 rpm (Aralab Agitorb 200), in 100 mL Erlenmeyer flasks, until mid-exponential phase, measured at a wavelength of 600 nm until achieving an optical density (OD_{600}) of 0.8 (Hitachi U-2000 Spectrophotometer).

2.3 Compounds

The antibiotics which were used were vancomycin (VAN) (Sigma Aldrich Chemie, Germany; stock solution 10 mg/mL) and ciprofloxacin (CIP) (Sigma Aldrich Chemie, Germany; stock solution 10 mg/mL), prepared in dionized sterile water; and ethidium bromide (EtBr) (Sigma Chemical CO, USA; stock solution 10 mg/mL) as fluorescent substrate used in real-time fluorometry. VAN and CIP stock solutions were maintained at 4ºC. EtBr stock solution was maintained at -20ºC and protected from light.

The efflux pumps inhibitors used in the present work were thioridazine (TZ) (Sigma Aldrich Chemie, Germany; stock solution 10 mg/mL); verapamil (VP) (Sigma Aldrich Chemie, Germany; stock solution 10 mg/mL); omeprazole (OMP) (Sigma Aldrich Chemie, Germany; stock solution 10 mg/mL); sodium

orthovanadate (Na_3VO_4) (Sigma Aldrich Chemie, Germany; stock solution 100 mg/mL); and cyanide m- chlorophenylhydrazone (CCCP) (Sigma Aldrich Chemie, Germany; stock solution 1 mg/mL).
VP, TZ and Na_3VO_4 stock solutions were prepared in dionized sterile water. CCCP stock solution was prepared in deionized sterile water and ethanol (Panreac Quimica SA, Spain) 1:1. OMP stock solution was prepared in dimethyl sulfoxide (DMSO) (Merk KGaA, Germany). The solutions were maintained at 4ºC and CCCP was protected from light.

2.4 Determination of the Minimum Inhibitory Concentrations

The minimum inhibitory concentration (MIC) for each EPI, EtBr and antibiotic was defined as the lowest concentration that inhibited cell growth.

After the growth, the cell suspension was diluted in the same growth broth to equal the McFarland 5 standard. The MICs for the EtBr, antibiotics and EPIs were determined by the broth microdilution method in 96-well microtitre plates according to the CLSI guidelines (CLSI 2011). The broth used to perform the microdilutions was the Mueller-Hinton Broth (Sigma-Aldrich Chemie, Germany). Amounts of 130 µL of Mueller-Hinton Broth were added to the wells of the 96-well microtitre plate. An Additional amount of 50 µL of Mueller Hinton-Broth was added to the wells of the first column, this column being the sterility control. To the wells of the second column additional 110 µl of Mueller-Hinton Broth were added as well as 20 µL of the compounds whose MIC is to be determined, in a total volume of 260 µL. This column corresponds to the highest concentration of the compounds from which serial dilutions of 1:2 were made. An amount of 130 µL was taken from the second column and added to the third column, and so on up to the eleventh column. With the exception of the first column, 20 µL of the previously prepared cells suspension were added, for a final volume of 150 µL. The twelfth column of the plate corresponds to the growth population control. The control of the DMSO was also made to guarantee that this solvent does not affect the growth of cells. The plate was covered with a Breathe-Easy™ sealing membrane (Sigma-Aldrich).

The growth of cells population was monitored at 0, 16 and 18 hours at a wavelength of 600 nm (Molecular Devices Spectra Max 340 PC). The 96-well microtitre plates were kept at 28ºC and 200 rpm during the realization of these assays.

2.4.1 Determination of the Minimum Inhibitory Concentration for the Substrates in the Presence of the EPIs

After the growth of the cells the culture was diluted in the same growth broth to equal the McFarland 5 standard.

Amounts of 110 µL of Mueller-Hinton Broth were added to the wells of the 96-well microtitre plate. An additional amount of 40 µL of Mueller-Hinton Broth was added to the wells of the first column, this column being the sterility control. To the wells of the second column additional 110 µL of Mueller-Hinton Broth were added as well as 20 µL of the substrate, in a total volume of 240 µL. This column corresponds to the highest concentration of the compounds from which serial dilutions of 1:2 were made. An amount of 120 µL was taken from the second column and added to the third column, and so on up to the eleventh column. In order to evaluate the effect of EPIs in presence of substrates, 20 µL of the EPIs were added from second to eleventh column to a final concentration ½ of the determined MIC. With exception of the first column, 20 µL of the previously prepared cells suspension were added to the remaining wells, for a final volume of 160 µL. The twelfth column of the plate corresponds to the growth population control. The plate was covered with a Breathe-Easy™ sealing membrane (Sigma-Aldrich).

The growth of cells population was monitored at 0, 16 and 18 hours at a wavelength of 600 nm (Molecular Devices Spectra Max 340 PC). The 96-well microtitre plates were kept at 28ºC and 200 rpm during the realization of these assays.

2.5 Real Time Fluorometry

In the protocol of this method, cells were grown in elemental mineral medium pH 7 suplemented with 0.25% (v/v) of ethanol (99.9%, Panreac), until mid-log phase (OD_{600} 0.8), and then the cells were centrifuged and washed with a phosphate-buffered solution (PBS). The aliquots were transferred to microtubes, the EtBr was added and then the tubes were ready to be placed in the thermocycler. The obtained data points represent the fluorescence and they were displayed on the monitor as they were obtained, being a real-time data points (Viveiros, Rodrigues, et al. 2010).

This method was carried out using the real-time thermocycler Rotor-Gene™ 3000 (Corbett Research, Sidney, Australia). The excitation and emission wavelengths for EtBr were selected, which are 530 nm band-pass and 585 nm high-pass filters, respectively. The fluorescence of the samples was acquired in cycles of 60 seconds, during a desired interval, at an optimal temperature for this strain (28ºC) (Viveiros, Rodrigues, et al. 2010). The assays were duplicated.

2.5.1 Accumulation Assays

In the accumulation assays, the accumulation of the EtBr within cells was evaluated, which corresponds to the balance between the entry of EtBr into cells and its extrusion. In the presence of an EPI a higher accumulation of EtBr is expected because of the inhibition of the efflux activity (Paixão, et al. 2009). In Figure 20 is represented a graphical example of an accumulation assay of EtBr in the absence and presence of an EPI. The difference of fluorescence (Δ) produced in presence and absence of EPI corresponds to the inhibition of EtBr efflux due the inhibitory activity of the EPI. Therefore, it is possible to evaluate the inhibitory capacity of the EPI being studied (Paixão, et al. 2009).

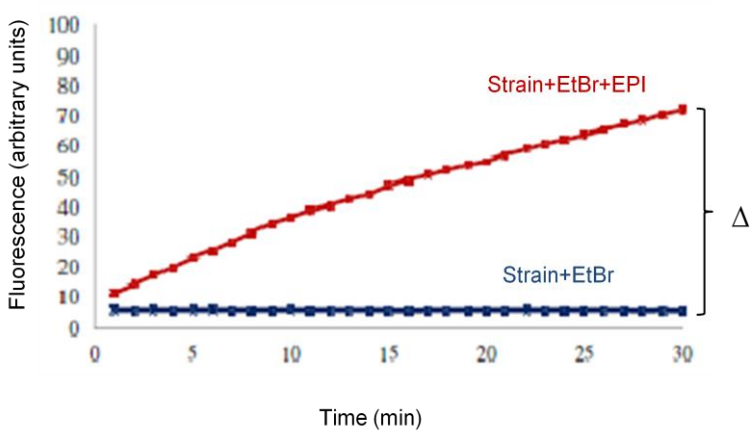


Figure 20 – Example of an EtBr accumulation assay in absence (blue curve) and presence (red curve) of an EPI.

2.5.1.1 Accumulation Assays of EtBr

R. erythropolis cells were grown in 20 mL of mineral medium until they reached the mid-log phase. The cells were centrifuged at 13000 rpm for 3 minutes (Biofuge Pico Heraeus, Kendro Laboratory Products, Osterode, Germany), the pellet was washed twice with 1 mL of PBS and the OD_{600} of the cellular suspension adjusted to *ca* 0.8 (PU8620 UV/VIS/NIR Philips, Cambridge, United Kingdom) (Paixão, et al. 2009).

The accumulation assays were performed in 0.2 mL microtubes, with a final volume of 0.1 mL. A volume of 0.05 mL of washed cell suspension was added to 0.05 mL of EtBr solutions with different concentrations, in the absence and presence of glucose at a final concentration of 0.4%. The solutions of EtBr had final concentrations of 0.25, 0.5, 1, 2, and 3 mg/mL.

After preparation of the solutions, the microtubes were placed in the Rotor-Gene™ 3000. The accumulation assays were performed at a temperature of 28 º C and the fluorescence of the samples was acquired in cycles of 60 seconds, for a duration of 60 minutes.

The slope for each curve was determined and the uptake rate for ethidium bromide was calculated for the conditions of presence and absence of glucose, in order to determine the differences in EtBr accumulation for both conditions.

2.5.1.2 Accumulation Assays of EtBr in presence of EPIs

To perform the accumulation assays of EtBr in the presence of EPI, the cells were treated as described previously in 2.4.1.1. A volume of 0.05 mL of washed cell suspension was added to 0.05 mL PBS solutions in the absence and presence of glucose, the absence and presence of EtBr and the absence and presence of the different studied EPIs in order to obtained the following final concentrations:

i. EtBr 0.5 mg/mL + glucose 0.4%;
ii. EtBr 0.5 mg/mL + glucose 0.4% + EPI ½ of MIC;
iii. EtBr 0.5 mg/mL
iv. EtBr 0.5 mg/mL + EPI ½ of MIC.

After preparation of the solutions, the microtubes were placed in the Rotor-Gene™ 3000. The accumulation assays were performed at a temperature of 28 º C and the fluorescence of the samples was acquired in cycles of 60 seconds, during 60 minutes.

2.5.2 Efflux Assays

To perform the efflux assays, the cells were treated as described previously in section 2.4.1.1. The cell suspension was incubated in test tubes during 60 minutes at 28 º C, 200 rpm, in the presence of EtBr 0.5 mg/mL and in the presence of the selected EPI (VP) at a concentration that was ½ of MIC, in the absence of glucose. This process allows loading cells with EtBr (Viveiros, Martins, et al. 2008).

After the 60 minutes of incubation, the cells were collected, centrifuged at 13000 rpm for 5 minutes, and the pellet washed in cooled PBS, in order to minimize the efflux. A volume of 0.05 mL of washed cell suspension was added in the 0.2 mL microtubes, where 0.05 mL of PBS solutions were previously added, in the absence and presence of glucose and the absence and presence of the different EPIs studied in order to obtain the following final concentrations (Viveiros, Martins, et al. 2008):

i. PBS + glucose 0.4% ;
ii. EPI ½ of MIC;
iii. EPI ½ of MIC solution + glucose 0.4%;
iv. PBS (absence of glucose and EPI).

The microtubes were always kept in ice and the solutions were cooled. After preparation of the suspensions, the microtubes were placed in the Rotor-Gene™ 3000. The efflux assays were performed at a temperature of 28 º C and the fluorescence of the samples was acquired in cycles of 30 seconds, for a duration of 30 minutes.

The obtained fluorescence data points were normalized in accumulation assays, and a relative fluorescence was obtained. This relative fluorescence corresponds to the ratio of fluorescence that remains per unit of time, relatively to the EtBr- loaded cells (Rodrigues, Ramos, et al. 2011), which can be, therefore, determined according to the expression 2.1:

$$\boldsymbol{Relative\ Fluorescence\ (arbitrary\ units)} = \frac{x}{\boldsymbol{Loaded\ Cells\ Fluorescence}} \quad \textbf{(2.1)}$$

Where *x* = Fluorescence (cells + glucose); Fluorescence (cells without glucose); Fluorescence (cells + glucose + EPI).

The fluorescence obtained by cells in the conditions of higher retention of EtBr (loaded cells), was defined as the maximum fluorescence value (relative fluorescence of one, marked as a dash line in Figure 21). The relative fluorescence data allows visualizing the efflux activity and it can distinguish the different effects and capacities of inhibition of the several EPIs in presence and absence of glucose (Figure 21).

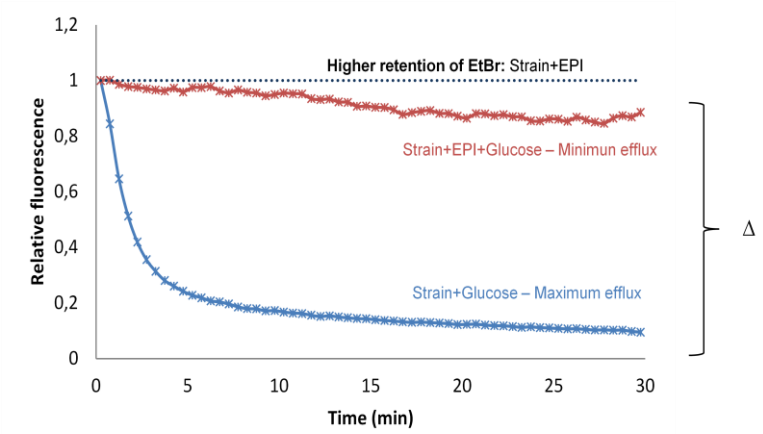


Figure 21 - Example of an EtBr efflux assay in absence (blue curve) and presence (red curve) of an EPI.

The ability of each EPI to inhibit the efflux of Etbr was defined as the relative final fluorescence (RFF) as it is an indicative of the activity of the compounds. The RFF value is obtained according equation 2.2, with the last point of relative fluorescence of the curve in the presence of the inhibitor ($RF_{treated}$) and with the last point of relative fluorescence of the maximum efflux curve ($RF_{untreated}$) (Ramalhete, et al. 2011).

$$RFF\ (\Delta) = RF_{treated} - RF_{untreated} \qquad \textbf{(2.2)}$$

2.6 Fluorescence Microscopy and Image Analysis

Images were observed with an Olympus CX40 microscope, equipped with an Olympus U-RFL-T burner and an U-MWB mirror cube unit (excitation filter: BP450-480; barrier filter: BA515). The 100x-objective lens had a numerical aperture of 1.3. Images were grabbed with an Evolution ™ MP 5.1 CCD color camera using the acquisition software Image-Pro Plus, both from Media Cybernetics (USA). Image analysis was carried out using Visilog 5 from Noesis SA, Les Ulis, France. At least 10 images were taken from each sample (de Carvalho et al. 2003). The evaluation of membrane potential was made using the *Bac*Light™ Bacterial Membrane Potential Kit from Molecular Probes (Invitrogen), which uses $DiOC_2(3)$.

2.6.1 Potential Membrane Assays

The membrane potential assays were done according to the manufacturer's instruction (Annex II). Amounts corresponding to ½ MIC of the EPIs were added to cell suspensions in mid-exponential phase. At the same time, 5µL of $DiOC_2(3)$ were added to the mixture according to the manufacturer. The suspensions were stored protected from light and samples were taken. The observations were made after 15, 30 and 60 minutes.

Results and Discussion

3 Results and Discussion

Rhodococci have several characteristics that indicate the presence of efflux pumps as a defense mechanism, including their tolerance to high concentrations of toxic compounds (de Carvalho, Parreño-Marchante, et al. 2005, de Carvalho, Fatal, et al. 2007), and to antibiotics (Niwa and Lasker 2010, Giguère, et al. 2010). Supporting the hypothesis that efflux pumps are indeed present in *Rhodococcus*, genomic sequences of possible efflux pumps, including a putative arsenic efflux pump from *Rhodococcus* sp B03 (Q0VTU9 UniProt), and a putative amino-acid metabolite efflux pump from *Rhodococcus erythropolis* SK121 (C3JNY3 UniProt), have been reported. The results of the present work will assess this hypothesis.

The resistance of bacteria to antibiotics occurs due to different mechanisms, including the use of efflux pumps (Li and Nikaido 2004). If efflux pumps are inhibited by the action of EPIs, the decrease of the minimum inhibitory concentration (MIC) value of a given antibiotic is excpected, because the cells cannot decrease the concentration of the antibiotic within them. To ensure that an EPI does not act as a bactericide, the determination of the MIC of the compounds was performed. This ensures that if efflux pump fails to operate, this has been achieved by pump inhibition due to EPI activity and not because of cell death.

Using the EPIs to be tested in the present study at half of the MIC as that of the EPIs, the presence of efflux pumps in *R. erythropolis* was assessed, using EtBr as substrate. Works performed at IHMT, Lisbon, by the group of Dr. Miguel Viveiros, proved that the determination by fluorometry, in real time, of the uptake/efflux balance of EtBr is possible, demonstrating that the EPIs verapamil, phenothiazines and CCCP, inhibit the efflux activity in *S. aureus, Mycobacterium* and *E. coli* (Costa, et al. 2011, Rodrigues, Wagner, et al. 2008, Rodrigues, Ramos, et al. 2011, Paixão, et al. 2009). By analogy, in the present study, the evaluation of the existence of efflux pumps in *R. erythropolis* DCL14 was made using real time fluorometry.

The mechanism of action of EPIs can be by depolarization of the cell membrane (Mahamoud, et al. 2007). Therefore, a fluorescence kit was used to evaluate the depolarization of cells by fluorescence microscopy and image analysis.

3.1 Minimum Inhibitory Concentration

As explained in section 1.2.5., the EPIs used in the present work were verapamil, thioridazine, omeprazole, sodium orthovanadate and the uncoupler carbonyl cyanide m-chlorophenylhydrazone.

The antibiotics vancomycin and cirpofloxacin were chosen as model antibiotics because they are used for the treatment of *R. equi* infections (Weinstock and Brown 2002, Niwa and Lasker 2010).

In order to evaluate the susceptibility profile of the strain, the MICs for the EPIs and antibiotics were determined. It is important to perform the inhibition assays always in a concentration below the MIC, to guarantee the viability of cells.

Table 3 – Values of MICs of EPIs and substrates determined for *R. erythropolis*. ½ MIC corresponds to the initial concentration of the EPI used in the following experiments.

EPIs	MIC (µg/mL)	½ MIC (µg/mL)
Thioridazine (TZ)	12	6
Verapamil (VP)	200	100
Omeprazole (OMP)	320	160
Sodium Orthovanadate (Na_3VO_4)	1500	750
CCCP	0.27	0.135
Fluorescene substrate		
Ethidium Bromide (EtBr)	25	
Antibiotics		
Vancomycin	2.5	
Ciprofloxacin	10	

According to the results presented in table 3, the highest MIC was obtained for Na_3VO_4 and the lowest for CCCP. The strain DCL14 was revealed to be, also, more resistant to ciprofloxacin, requiring four times greater concentration of ciprofloxacin than vancomycin for their growth to be inhibited.

Since it is expected that EPIs interfere with the efflux of antibiotics, the MICs of the antibiotics were determined in the presence of ½ of the MIC of EPIs.

Table 4 – Determined MICs of EtBr and antibiotics in the presence of ½ of the MICs of the EPIs and inhibitory effect of each EPI.

EtBr + ½ Mic EPIs	MIC (µg/mL)	Inhibitory Effect
EtBr + TZ	25	0
EtBr + VP	25	0
EtBr + OMP	25	0
EtBr + Na_3VO_4	6.25	4x
EtBr + CCCP	25	0
Vancomycin + ½ Mic EPIs	MIC (µg/mL)	Inhibitory Effect
Vancomycin + TZ	0.31	8x
Vancomycin + VP	0.63	4x
Vancomycin + OMP	0.31	8x
Vancomycin + Na_3VO_4	0.31	8x
Vancomycin + CCCP	0.08	32x
Ciprofloxacin + ½ Mic EPIs	MIC (µg/mL)	Inhibitory Effect
Ciprofloxacin + TZ	2.5	4x
Ciprofloxacin + VP	5	2x
Ciprofloxacin + OMP	2.5	4x
Ciprofloxacin + Na_3VO_4	5	2x
Ciprofloxacin + CCCP	2.5	4x

By analyzing the results in table 4, it can be seen that, in general, the MICs of the antibiotics significantly decrease (2 to 32 fold) in the presence of EPIs, when compared with the determined value for the MIC of that antibiotic in the absence of inhibitors. Since the action of EPIs results in a decrease of 2 to 32x the value of MIC of antibiotics, the results suggest the action of efflux pumps in the resistance of DCL14. Na_3VO_4 was the EPI that promoted the accumulation, and consequent toxic effect, of EtBr, since it was the only EPI that decreased the value of the MIC of the fluorescent substrate. These results suggest that the pumps used for the extrusion of the substrates are different of those used by the cell to extrude antibiotics.

The global analysis of these assays reveals an inhibitory effect in the growth of cells by the action of EPIs, which was evaluated in the presence of antibiotics and inhibitors. This assessment was made after 16 and 18 hours of exposure to both compounds. Although the results suggest the presence of efflux pumps, the evaluated effect of the compounds could have been caused by other mechanisms of action. It becomes, therefore, necessary to perform additional tests, such as real time fluorometry,

which allow the monitoring of the action of EPIs in real time. With this method, the EPIs which are truly effective can be identified.

3.2 Real Time Fluorometry

In the real time fluorometry tests, the accumulation and efflux of substrates was assessed using EtBr as model substrate. In aqueous solutions, out of the cell, EtBr emits weak fluorescence, but when it is bound to cellular components EtBr emits a strong fluorescesnce. The measured fluorescence will correspond, therefore, to the EtBr within cells (Paixão, et al. 2009).

In the EtBr accumulation assays performed with *R. erythropolis* the concentrations of EtBr which were tested were between 4 and 0.25 µg/mL, in the absence and presence of glucose 0.4% (v/v), and in the absence and presence of EPIs at a concentration of ½ of MIC.

These assays were made in order to ensure that the initial fluorescence signal of EtBr in the cell is sufficiently low when the cells are not being inhibited, so that when the EPI is added, and the concentration of EtBr within the cell increases, the fluorescence signal can still be measured.

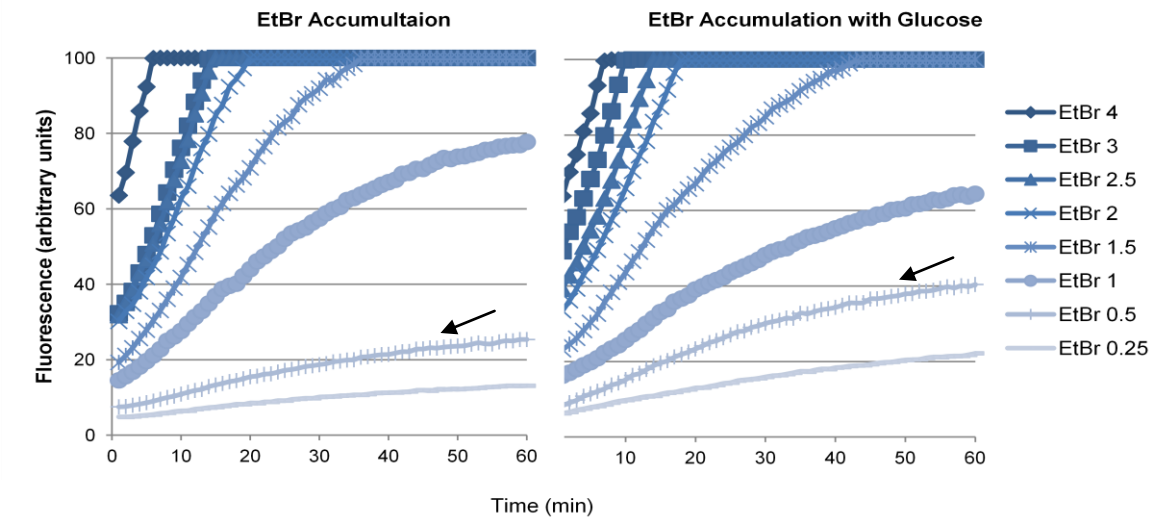


Figure 22 –EtBr accumulation assays, at 28ºC, in the following concentrations: EtBr 4 – 4 µg/mL; EtBr 3 – 3 µg/mL; EtBr 2.5 – 2.5 µg/mL; EtBr 2 – 2 µg/mL; EtBr 1.5 – 1.5 µg/mL; EtBr 1 – 1 µg/mL; EtBr 0.5 – 0.5 µg/mL; EtBr 0.25 – 0.25 µg/mL. The arrow marks the chosen concentration to perform in the remaining assays.

In these accumulation assays it was seen that at an EtBr concentration of 0.5 µg/mL the strain did not evidence a strong accumulation of EtBr by itself, reaching a value of units of fluorescence of 25 in 60 min. This concentration of EtBr was therefore considered to be the ideal concentration to perform the remaining tests (Figure 22).

It was also shown that in the presence of glucose at a concentration of 0.4% (v/v) the uptake rate of EtBr decreased in general for the concentrations 4 to 1 µg/mL. At the lowest studied concentrations for EtBr in presence of glucose, the accumulation of EtBr increased, suggesting that these concentrations of EtBr are not sufficiently toxic for the cells to trigger any defense mechanism that involves the presence of glucose, possibly the efflux pump machinery (Figure 23).

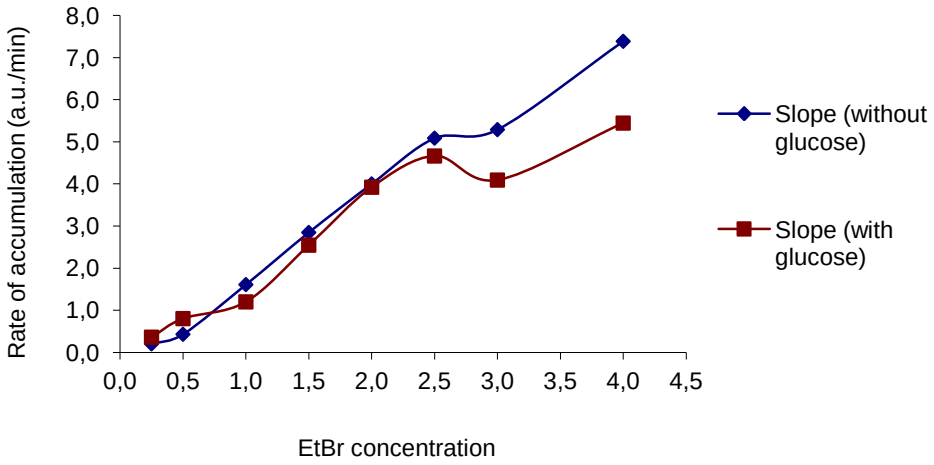


Figure 23 – Slopes of the EtBr accumulation curves.

Once determined the suitable concentration of EtBr to conduct the following tests, the accumulation assays in the presence of the EPIs were carried out. The cells were, therefore, exposed to EtBr at a final concentration of 0.5 µg/mL, and to EPIs, at a final concentration of ½ MIC.

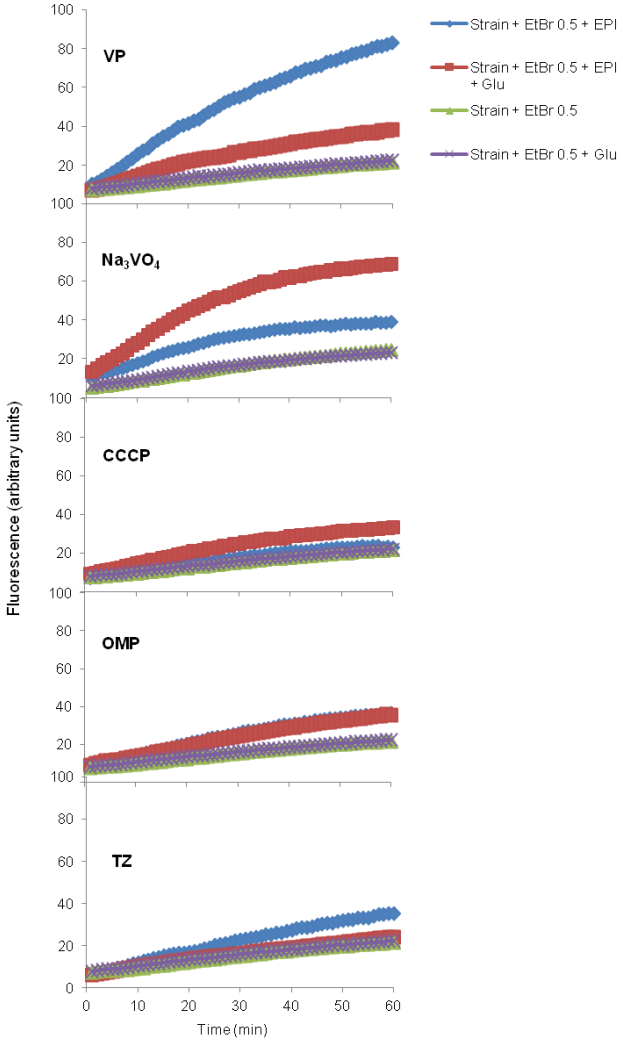


Figure 24 –EtBr accumulation assays in the presence of the various EPIs and in the presence and absence of glucose, at 28ºC.

In presence of VP, the cells presented the highest retention of EtBr, in absence of glucose. The fluorescence significantly decreased when the cells were exposed to glucose. Na_3VO_4 led to the converse result. In presence of glucose the retention of EtBr was highest, rather than in absence of glucose. The remaining EPIs, TZ, CCCP and OMP, did not demonstrate to be the most favorable to accumulate EtBr. In the presence of the inhibitor TZ and OMP, the strain did not accumulate a significant amount of substrate. CCCP was the EPI that retained less substrate, with the lowest fluorescence values obtained. The differences between the conditions of accumulation of EtBr in the presence and absence of EPIs, corresponding to the Δ value, are indicated in table 5. Given the Δ values, it is demonstrated that, with the exception of Na_3VO_4 and CCCP, the florescence signals of the

accumulated EtBr are lower in presence of glucose. VP was chosen to allow a greater accumulation of EtBr in cells required, at the beginning of efflux assays.

Table 5 - Δ values obtained in the accumulation assays for each EPI.

EPIs	Δ value without glucose	Δ value with glucose
VP	61.21	15.84
Na_3VO_4	18.83	37.19
CCCP	1.49	11.07
OMP	14.84	13.19
TZ	13.79	1.76

From the results of the accumulation assays it can be determined that the optimal concentration of EtBr to use in efflux assays is 0.5 µg/mL, and the EPI most favorable to load cells with the substrate is VP.

3.2.1 Efflux Assays

To perform the efflux assays the cells were first loaded with the fluorescent substrate in order to reach the maximum level of accumulation, as described in 2.5.2. After this incubation, the medium was replaced by the following solutions according to previous studies (Paixão, et al. 2009):

i. PBS without glucose (control of the effect of glucose);
ii. PBS containing glucose (condition of maximum efflux);
iii. PBS containing glucose and EPI (condition of minimum efflux);
iv. PBS without glucose containing EPI (control of the effect of EPI).

These assays allowed the monitoring of the efflux of EtBr by cells and the determination of the inhibitory effect of each EPI in real time. In order to optimize the reading of the results, a normalization of the obtained data points was made, as described in 2.4.2.

The effect of the presence of glucose was determined, by exposing the strain without EPIs in the presence (red curve, Figure 25) and absence of glucose (blue curve, Figure 25). The remaining results presented show the strain in the conditions of maximum accumulation, which corresponds to the bacterium in the presence of the inhibitor VP, in the absence of glucose (dash curve, Figure 26

and 27), strain in conditions of minimum efflux (purple curve, Figure 26) and the strain in conditions of maximum efflux (red curve, Figure 26).

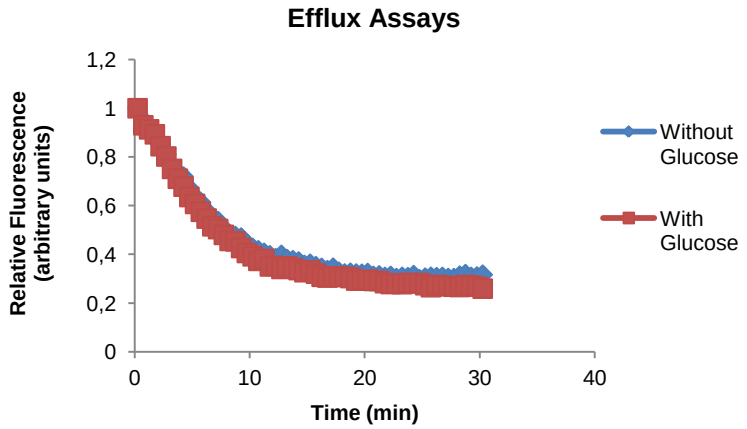


Figure 25 - Efflux assays in the presence and absence of glucose, at 28ºC

The efflux of substrate occurred predominantly in the first 10 min in the absence of EPIs, as shown in Figure 25, and the presence of glucose did not affect the EtBr efflux. The presence of glucose in *S. aureus* has been demonstrated to have an active role in the efflux activity in this Gram-positive bacterium (Costa, et al. 2011), and in the Gram-negative *E. coli* (Viveiros, Martins, et al. 2008). There are differences in the efflux time of the substrate between the Gram-positive and Gram-negative bacteria studied using this method. In *S. aureus* the efflux occurs predominantly in the first 2 to 5 min (Couto, et al. 2008, Costa, et al. 2011). In *M. avium* and *M. smegmatis* the efflux time is extended mainly to the first 10 min (Rodrigues, Wagner, et al. 2008, Rodrigues, Ramos, et al. 2011), which is also demonstrated to occur in *E. coli* (Viveiros, Martins, et al. 2008). The observed differences can be possibly due the complexity of the cell-wall of bacteria.

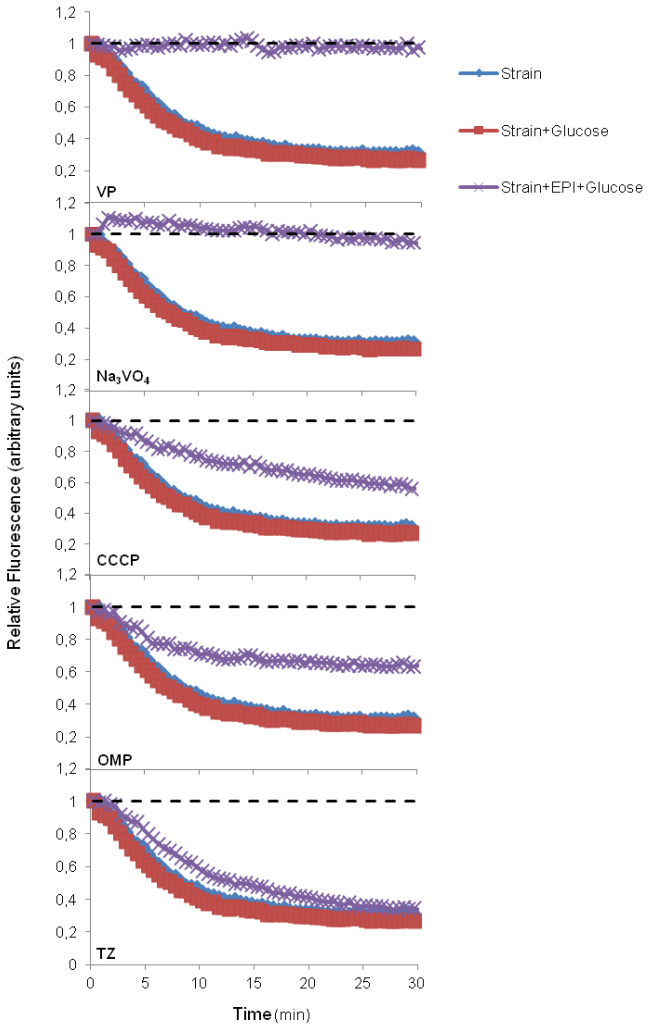


Figure 26 - EtBr efflux assays in the presence of the various EPIs, at 28ºC.

The main conclusion of these assays is that *R. erythropolis* DCL14 showed efflux activity, as can be seen by the maintenance of the signal of EtBr in the presence of EPIs and its decrease in their absence (Figure 26). Na_3VO_4 and VP were demonstrated to have the highest inhibitory effect by preventing the efflux of EtBr from the cells. The EPIs CCCP and OMP had similar effects in inhibition. The relative fluorescence obtained in the presence of these two EPIs in the end of the assay is particularly similar, as well as the relative fluorescence obtained for Na_3VO_4 and VP. This reveals similar inhibitory effects between EPIs. TZ was the EPI with the lowest inhibitory effect. The relative fluorescence obtained in the end of the assay in the presence of TZ was almost the same to the one obtained in the absence of this EPI. This EPI becomes a more effective inhibitor in *Mycobacterium* strains (Rodrigues, Wagner, et al. 2008, Rodrigues, Ramos, et al. 2011). The inhibitors VP and CCCP were already tested in other Gram-positive bacteria using the same method, and were shown to also

have inhibitory effects on efflux activity in *S. aureus* (Costa, et al. 2011), *M. smegmatis* and *M. avium* (Rodrigues, Wagner, et al. 2008, Rodrigues, Ramos, et al. 2011).

In order to study the influence of the glucose in the efflux systems, the efflux assays were made in the presence of EPIs and in the presence (purple curve, Figure 27) and absence (green curve, Figure 27) of glucose. As the previous data, the obtained data for all presented conditions (different EPI and presence and absence of glucose) were normalized as described previously in 2.4.2.

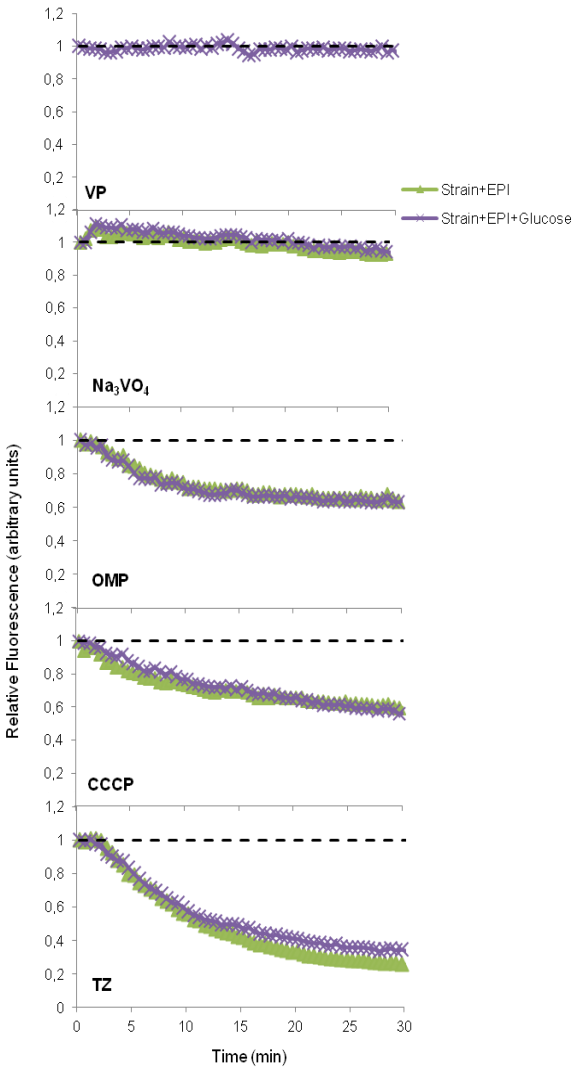


Figure 27 - EtBr efflux assays in the presence of the various EPIs in presence and absence of glucose, at 28ºC.

In Figure 25 it is demonstrated that glucose does not involved in the extrusion of EtBr from cells. In the remaining data (Figure 26 and 27) it is also inferred that the presence of glucose, as a carbon source, does not exert a significant effect in the efflux systems of *R. erythropolis*, regardless of the used EPI.

To quantify the inhibitory effect of each EPI, the Relative Final Fluorescence (RFF) value for each compound was determined (table 6).

Table 6 – RFF values obtained for each EPI in efflux assays.

EPI	RFF
Na3VO4	8.36
VP	7.39
CCCP	4.25
OMP	3.86
TZ	1.47

The results indicate that the compound that promoted the highest inhibitory effect in *R. erythropolis* DCL14 was Na_3VO_4, followed by VP. CCCP and OMP had similar RFF values, and TZ was the inhibitor that led to the lowest inhibitory effect of efflux.

In these assays it was demonstrated that the compounds tested as EPIs promoted the inhibition of the efflux pump systems of this strain, and the inhibitory effect of the tested EPIs, in descending order, is the following: Na_3VO_4 > VP > CCCP > OMP > TZ.

As described in sections 1.2.5.1 to 1.2.5.5, the EPIs Na_3VO_4, VP and TZ are considered to be ABC efflux pump inhibitors, and CCCP and OMP as MFS transporter inhibitors. Therefore, the results indicate that the strain DCL14 has efflux pumps belonging to ABC superfamily and to MFS. The inhibitory effects demonstrated by Na_3VO_4 and VP are similar. From these results, it can be inferred that these EPIs act in a similar mode to inhibit the ABC transporters. The same can be concluded for the EPIs CCCP and OMP.

3.3 Membrane Potential

The depolarization of cell membranes was assessed by fluorescence microcopy and image analysis by using $DiOC_2(3)$ as a fluorophore. The EPIs were added to the cells suspension and the depolarization of cells was observed after 15, 30 and 60 min, which corresponds to the middle of the efflux assays, the end and 30 min after the end of the assays.

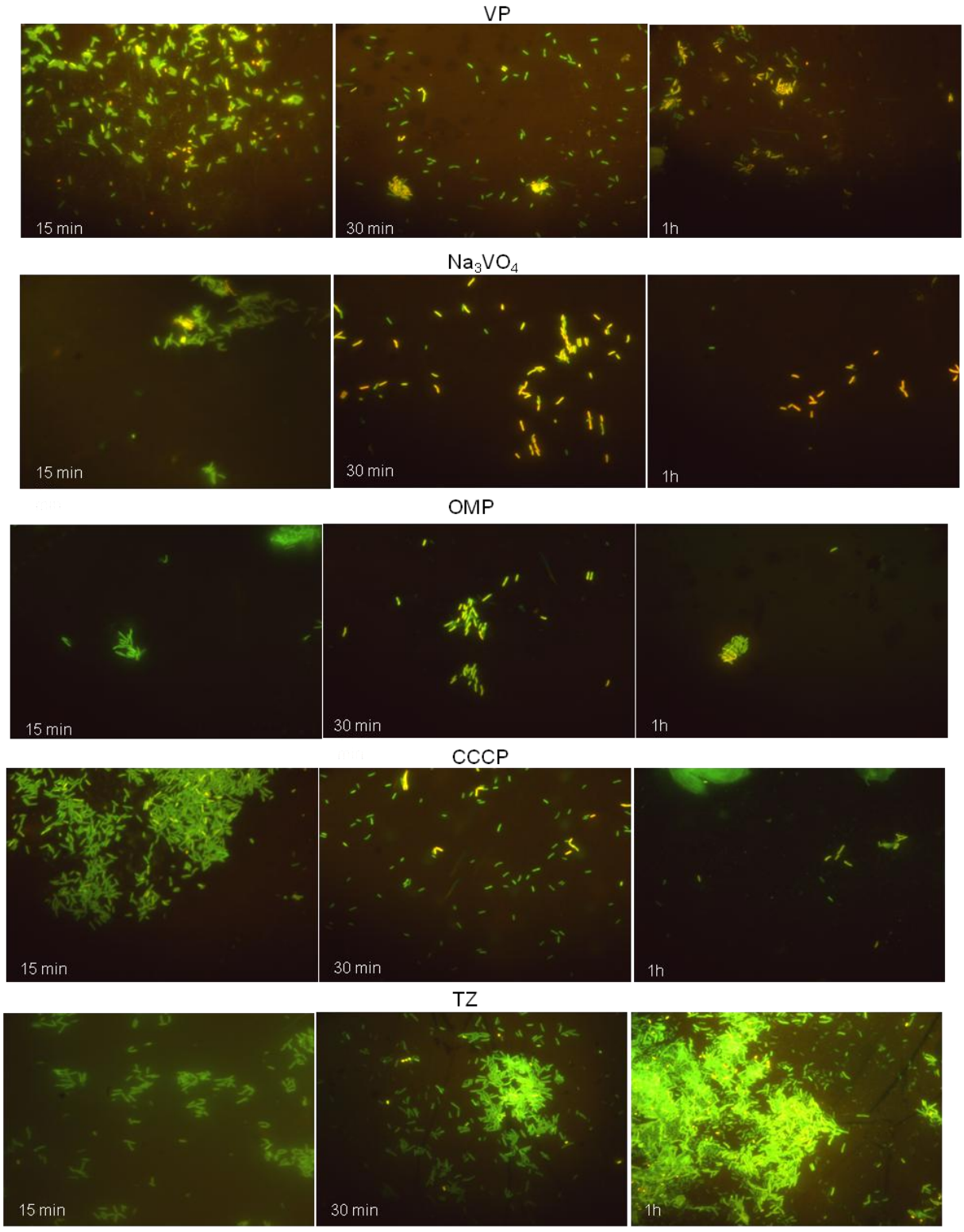


Figure 28 – Fluorescence microscopy images of the membrane potential assays in the presence of the various studied EPIs. Green cells correspond to depolarized cells and orange cells correspond to polarized cells.

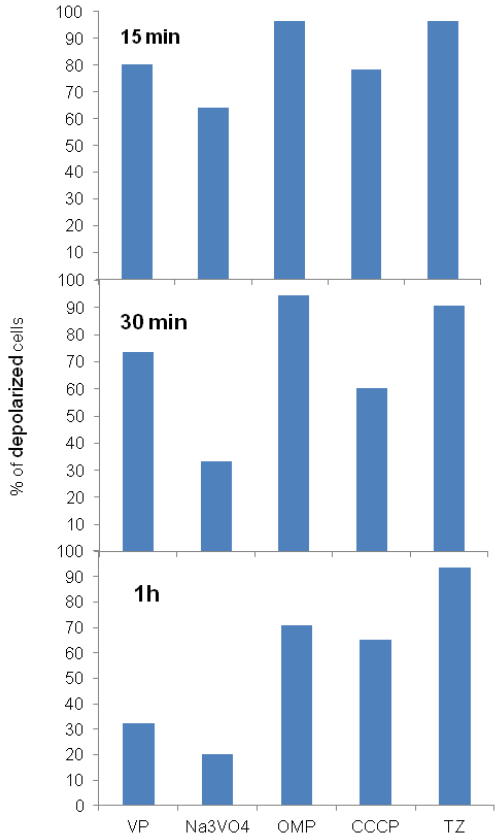


Figure 29 – Membrane potential assays in the presence of the various EPIs.

Fifteen minutes after adding the EPIs, the percentage of depolarized cells was at least 60%, TZ and OMP being the EPIs that promoted the highest percentage of depolarized cells (over 90%), followed by CCCP and VP (over 75%). Na_3VO_4 was the EPI that depolarized the lowest percentage of cells, as demonstrated in Figures 28 and 29.

After 30 min, which corresponds to the end of the efflux assay, the cells presenting the highest percentage of depolarized membranes were those incubated with the EPIs TZ and OMP, in which only 10% of cells recovered. This percentage was maintained for TZ after 1h hour of incubation, revealing that its mode of action leads to the depolarization of the cellular membrane. Although *2*0% of the percentage of cells incubated with OMP recovered after 1h, the elevated percentage of depolarized cells indicates that this EPI also interferes with the gradient of protons across the cellular membrane. The mode of action of CCCP as an uncoupler is already known (Mahamoud, et al. 2007, Alvarado and Vasseur 1998). However, the percentage of depolarized cells incubated with this EPI, after 30 min and 1h of incubation, was relatively low (*ca* 60%) when compared with those achieved with TZ and OMP. This suggests that, at the concentration used in the assays, CCCP did not have the highest activity as uncoupler. Na_3VO_4 and VP were the EPIs that promoted the lowest percentage of depolarized cells. This percentage decreased after 30min and 1h for both EPIs, indicating that their mode of action does

not cause the irreversible depolarization of the membrane of the cells, although they promoted the highest inhibitory effects. Therefore, it can be inferred that these compounds act as EPIs but their mode of action may not be solely by depletion of the energy source promoted by the proton motive force, since *R. erythropolis* cells were able to repolarize their membrane. It may involve direct binding to the pump, or binding to the substrate forming a complex, as explain in section 1.2.5. Further studies are required to elucidate this aspect.

From these results, it can be concluded that TZ and OMP, like CCCP, depolarize the membranes of bacteria by interfering with gradient of protons through the cellular membrane. In the presence of Na_3VO_4 and VP *R. erythropolis* cells were able to repolarize their membrane after 1h exposure to these EPIs.

Conclusions and Future Work

4 Conclusions and Future Work

4.1 Conclusions

R. erythropolis is a highly adaptable bacterium capable of tolerating high amounts of a wide range of toxic compounds and it has, therefore, interesting applications in biotechnology. The tolerance of *R. erythropolis* is associated to its versatile metabolism and the properties of its membrane. The presence of efflux pumps may have an important role in the adaptation mechanisms of this bacterium. To our knowledge, these considerations had never been studied before, although there is supporting evidence to the presence of these transporter proteins, such as the genomic sequences of putative efflux pumps in *Rhodococcus* strains (C3JNY3 and Q0VTU9, UniProt).

In the present work, the presence of efflux pumps in *R. erythropolis* DCL14 was studied by using several EPIs to assess efflux activity. The determination of the minimum inhibitory concentration of antibiotics in the presence of the several EPIs demonstrated a decrease in the MIC values of the tested antibiotics, suggesting the presence of efflux pumps.

The use of real time fluorometry proved to be a good and reproducible method to assess efflux activity in *R. erythropolis*. The obtained results suggest that strain DCL14 has efflux pumps as a defense mechanism, since the tested EPIs acted as inhibitors resulting in an accumulation of EtBr inside the cells. Na_3VO_4 was the EPI that demonstrated the highest inhibitory effect, and, in a descending order, EPIs could be ordered according to their activity as follows: Na_3VO_4 > VP > CCCP > OMP > TZ. The tested EPIs are described as belonging to the ABC superfamily (TZ, VP and Na_3VO_4) and as MFS transporter inhibitors (CCCP and OMP). The results thus suggest that *R. erythropolis* might have efflux pumps belonging to these two families. Nevertheless, further studies are required to confirm the identity of the present efflux pumps. The presence of glucose, used as a carbon source during the assays, did not exercise any effect in the efflux activity, although it had a significant effect in EtBr accumulation, both in the presence and absence of EPIs.

As for the effect of inhibitors in the membrane potential, the results demonstrated that Na_3VO_4 and VP had the lowest effect in the membrane potential, promoting the lowest percentage of depolarized cells, and the ability of cells to repolarize their membrane after 1h exposure. The remaining tested EPIs, TZ, OMP and CCCP, promoted a higher percentage of depolarized cells, indicating that the mechanism of action of these EPIs results in the blockage of the gradient of protons across the membrane, inhibiting, therefore, the efflux.

4.2 Future Work

The data collected in this thesis will be valuable to determine the full mechanisms responsible for the high adaptability of *R. erythropolis* cells. However, several questions have risen during this study which may only be answered in future studies.

In order to evaluate the effect of a carbon source in the energetic of the efflux systems of *R. erythopolis*, further studies are required using compounds such as ethanol or n-alkanes which are preferentially used as carbon sources by this bacterium.

Further studies are necessary in order to determine exactly which efflux pumps are present in rhodococci and to which families they belong to. Genomic analyses are therefore necessary. This involves the sequence of the bacterium genome and, by comparison to homologous bacterial genomes and using the strategy of knot-out of the genes, finding sequences that would probably encode the efflux pumps.

R. erythropolis DCL14 is described as having a high tolerance to toxic compounds, such as toluene and xylene. It will be interesting to assess if adaptation to high concentration of toxic compounds involves the action of efflux pumps.

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Annex I

Elemental Mineral Medium

The elemental mineral medium was the medium used to growth *R. erythropolis* (Wiegant and Bont 1980). This medium is composed by 10 mL of Medium D 100x and 10 mL of phosphate buffer 100x *per* litter, adding deionized water to 1 litter. The media were autoclaved at 121ºC for 20 minutes

Table 7 – Composition of the solutions used for the elemental mineral medium.

Medium D *per* litter at a final concentration 100x in deionized water		
Compound	**Weight (g)**	**Distributer**
EDTA	1	Merk KGaA, Germany
$ZnSO_4.7H_2O$	0.2	Merk KGaA, Germany
$CaCl_2.2H_2O$	0.1	Merk KGaA, Germany
$FeSO_4.7H_2O$	0.5	Merk KGaA, Germany
$CuSO_4.5H_2O$	0.02	Panreac Quimica SA, Spain
$CoCl_2.6H_2O$	0.04	Merk KGaA, Germany
$MnCl_2.4H_2O$	0.1	Sigma Aldrich Chemie, Germany
$(NH_4)_2SO_4$	200	Panreac Quimica SA, Spain
$MgCl_2.4H_2O$	10	Panreac Quimica SA, Spain
Phosphate buffer solution at final concentration 100x in deionized water		
Compound	**Weight (g)**	**Distributer**
K_2HPO_4	155	Sigma Aldrich Chemie, Germany
$NaH_2PO_4.H_2O$	85	Sigma Aldrich Chemie, Germany

Solid Medium

The cells were preserved in petri plates at 4ºC. The medium used in the petri plates were autoclaved at 121ºC for 20 minutes.

Table 8 - Composition of the solutions used for the solid medium.

Medium for plaques *per* litter in deionized water		
Compound	**Weight / Volume**	**Distributor**
Medium D 100x	10 mL	-
Phosphate buffer 100x	10 mL	
Agar	15 g	
Glucose [(a)]	5 g	Sigma Aldrich Chemie, Germany
Yeast extract [(b)]	15 g	Bacto™ Yeast Extract BD, France

PBS and Glucose Solution

The accumulation and efflux assays were performed in phosphate buffer saline (PBS) and in the absence and presence of glucose.

Table 9 – Composition of PBS and glucose.

Solution	**Composition**	**Distributer**
PBS [(a)]	10mM phosphate buffer; 2,7mM KCL; 137mM NaCl; pH 7,4	Sigma Aldrich Chemie, Germany
Glucose [(a)]	20% (w/v) in 100 mL deionized sterile water.	Sigma Aldrich Chemie, Germany

Annex II