Identification and Characterisation of Efflux Pumps in *Rhodococcus erythropolis*

Ana C. F. Vencá[a, b], Miguel Viveiros[c], Carla C. C. R. de Carvalho[a,b]

[a] *IBB-Institute for Biotechnology and Bioengineering, Centre for Biological and Chemical Engineering, Instituto Superior Técnico, Av. Rovisco Pais, 1049-001 Lisboa, Portugal*
[b] *Department of Bioengineering, Instituto Superior Técnico, Universidade Ténica de Lisboa, Portugal*
[c] *Unit of Mycobacteriology, Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa, Rua da Junqueira 100, 1349-008 Lisboa, Portugal*

Abstract

This work assesses, for the first time, the presence of efflux pumps as a defense mechanism in *Rhodococcus erythropolis* cells. Several known efflux pump inhibitors (EPIs), namely Thioridazine (TZ), Verapamil (VP), Carbonyl cyanide 3-chlorophenylhydrazone (CCCP), Omeprazole (OMP) and Sodium orthovanadate (Na_3VO_4), were used to assess efflux activity. This was achieved by 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 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 promoted 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* cells.

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

1. Introduction

Bacteria are fundamental catalysts in many industrial processes across diverse areas of application. The resistance displayed by rhodococci and the versatile metabolism conferred by their extended array of enzymes makes these bacteria potential candidates to use in environmental and industrial biotechnology (1). In particular, *R. erythropolis* have been described as very tolerant bacteria, and so the commercial interest in this microorganism increased (2; 3).

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 (4; 1; 2). However, there are several characteristics of these bacteria that indicate the presence of efflux pumps as a defense mechanism, including their tolerance to high concentrations of toxic compounds (5; 6), and to antibiotics (7; 8). 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 R. erythropolis SK121 (C3JNY3 UniProt), have been reported.

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 (9). These transporters differ structurally and in their mode of action. 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 (10).

There is a recent method successfully developed to estimate the efflux pumps activity in bacteria in real time. This is a semi-automated fluorometric method that monitors the efflux of a common fluorescent substrate such as ethidium bromide (EtBr), which emits weak fluorescence in aqueous solutions (outside cells) and a strong fluorescence inside cells, when concentrated in the periplasm of Gram-negative bacteria and in the cytoplasm of Gram-positive bacteria. 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, by measuring the EtBr fluorescence (11; 12; 13). To assess efflux activity it is commonly use efflux pump inhibitors (EPIs) (14), and this method can be use for the screening of EPIs and for the

identification of the overexpressed efflux pumps of bacteria, by measuring the efflux activity (12; 11). 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). The EPIs Na_3VO_4 (15; 16), VP (17) and TZ (18; 19) are considered to be ABC efflux pump inhibitors, and CCCP (20; 16) and OMP (21; 22) are MFS transporter inhibitors. In general, these compounds 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 (23). A complement 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, it turns to be a powerful tool to complement the study of adaptation mechanisms of bacteria by using an appropriated fluorophore, having the advantage of distinguishes the behavior on a single cell event.

In this present work, a fluorescence kit was used to evaluate the depolarization of cells by fluorescence microscopy and image analysis. In general, the fluorophore used changes color according to the cells state and evaluation is made according to these changes. The evaluation of membrane potential is made by using 3,3′-diethyloxacarbocyanine iodide ($DiOC_2(3)$) as a florophore. This dye changes color according to the depolarization of the membrane of cells. Green cells correspond to depolarized cells and red cells correspond to polarized cells (24; 6; 5).

The present study assesses the presence of efflux pumps in *Rhodococcus erythropolis* as an adaptation mechanism. Three different methods were used in order to evaluate the action of efflux pumps: through the determination of the minimum inhibitory concentration of antibiotics in presence and absence of the 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.

2. Materials and Methods

2.1. Bacterial strain and growth conditions

Rhodococcus erythropolis DCL14 was 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 (24).

R. erythropolis cells were grown in 20 mL of elemental mineral medium pH 7 (25) supplemented with 0.25% (v/v) of ethanol (99.9%, Panreac) (5), at 28ºC and 200 rpm, 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.

2.2. Compounds

The antibiotics 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 the ethidium bromide (EtBr) (Sigma Chemical CO, USA; stock solution 10 mg/mL) as fluorescence 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 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.

2.3. Determination of minimum inhibitory concentrations

The determination of MICs of TZ, VP, OMP, CCCP and Na_3VO_4, as well of the antibiotics studied alone and in the presence of an EPI, was conducted by the broth microdilution method according to the CLSI guidelines (26). After the growth, the cell suspension was diluted in the same growth broth to equal the McFarland No.5 standard turbidity standard. Amounts of 0.1 mL of the final cell suspension were transferred to each well of the 96-well plate that contained 0.1 mL of each agent at concentrations prepared from 2-fold serial dilutions in Mueller-Hinton broth. The growth of cells population was monitored at 0, 16 and 18 hours at a wavelength of 600 nm. The minimum inhibitory concentration (MIC) for each EPI, EtBr and antibiotics used was defined as the lowest concentration that inhibited cell growth.

2.4. Semi-automated fluorometric method

The assessment of efflux activity in *R. erythropolis* cells was performed by using the semi-automated fluorometric method, by evaluating the extrusion and the accumulation of the EtBr substrate in real-time (11; 12; 13).

2.4.1. Accumulation assays

R. erythropolis cells were grown in 20 mL of mineral medium until an OD_{600} of 0.8. The cells were centrifuge at 13000 rpm for 3 minutes, the pellet was washed twice with 1 mL of PBS and the OD_{600} of the cellular suspension adjusted to *ca* 0.8. 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 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, 2.5, 3 and 4 mg/mL (12). 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.

The effects of the EPIs on the accumulation of EtBr was determined by adding a volume of 0.05 mL of washed cell suspension to 0.05 mL PBS solutions in absence and presence of glucose, absence and presence of EtBr and absence and presence of the different EPIs studied 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 (12).

Fluorescence of the assays was measured in Rotor-Gene™ 3000 (Corbett Research, Sidney, Australia), and it was selected the excitation and emission wavelengths for EtBr which are 530 nm band-pass and 585 nm high-pass filters, respectively. Fluorescence data was acquired every 60 seconds for 60 minutes at 28°C (11).

2.4.2. Efflux assays

To perform these assays, cells were exposed to conditions that promote maximum accumulation of EtBr: EtBr at concentration of 0.5 µg/mL; no glucose; presence of the efflux inhibitor that caused maximum accumulation, in this case verapamil; and incubation at 28°C at 200 rpm for 1h. This process allows loading cells with EtBr (11; 12; 13).

After the incubation, cells were collected by centrifugation 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 were previously added 0.05 mL of PBS solutions, in absence and presence of glucose and absence and presence of the different EPIs studied in order to obtained the following final concentrations: 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) (11). The microtubes were always kept in ice and the solutions were cooled. After preparation of the suspensions, the microtubes were placed in 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, during 30 minutes.

The 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 (27). The fluorescence of cells in the conditions of higher retention of EtBr (loaded cells), was defined as the maximum fluorescence value (relative fluorescence of one). The relative fluorescence data allows visualization of the efflux activity and it can distinguish the different effects and capacities of inhibition of the several EPIs in presence and absence of glucose. 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 with the last point of relative fluorescence of the curve in the presence of inhibitor ($RF_{treated}$) and with the last point of relative fluorescence of the maximum efflux curve ($RF_{untreated}$), by the expression (28):

$$RFF = RF_{treated} - RF_{untreated}$$

2.5. 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™ MP5.1 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 (29). The evaluation of membrane potential was made using the *Bac*Light™ Bacterial Membrane Potential Kit from Molecular Probes (Invitrogen), which uses $DiOC_2(3)$. The membrane potential assays were done according to the manufacturer's instruction. Amounts corresponding to ½ MIC of the EPIs were added to cell suspensions in mid-exponential phase. At the same time, 5µL of $DiOC_2(3)$ was 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.

Table 1 - 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)	Fluorescene substrate	MIC (µg/mL)
TZ	12	EtBr	25
VP	200	**Antibiotics**	
OMP	320	Vancomycin	2.5
Na_3VO_4	1500	Ciprofloxicin	10
CCCP	0.27		

Table 2 - Determined MIC of EtBr and antibiotics in the presence of ½ of MIC of EPIs and inhibitory effect of each EPI.

EtBr + EPIs ½ MIC	**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 + EPIs ½ MIC	**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 + EPIs ½ MIC	**MIC (µg/mL)**	**Inhibitory Effect**
Ciprofloxcin + TZ	2.5	4x
Ciprofloxcin + VP	5	2x
Ciprofloxcin + OMP	2.5	4x
Ciprofloxcin + Na_3VO_4	5	2x
Ciprofloxcin + CCCP	2.5	4x

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 (5; 6), and to antibiotics (7; 8). Supporting the hypothesis that efflux pumps are indeed present in *Rhodococcus*, genomic sequences of possible efflux pumps have been reported **(**Q0VTU9 UniProt and C3JNY3 UniProt).

3.1. Minimum inhibitory concentration

The resistance of bacteria to antibiotics occurs due to different mechanisms, including the use of efflux pumps (30). If efflux pumps are inhibited by the action of EPI, it is expected the decrease of the minimum inhibitory concentration (MIC) value of a given antibiotic, because the cells cannot decrease the concentration of the antibiotic within them. To ensure that EPI does not act as a bactericide, the determination of the MIC of the compounds was performed. This ensures that if efflux pumps fails to operate, this has been achieved by pump inhibition due to EPI activity and not because of cell death. 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 MIC, to guarantee the viability of cells (table 1). The antibiotics vancomycin and cirpofloxacin were chosen as model antibiotics because they are used for the treatment of *R. equi* infections (31; 7). Since it is expected that EPIs interfere with the efflux of antibiotics, the MICs of the antibiotics in the presence of ½ of MIC of EPIs were determined (table 2). By analyzing the obtained results, it can be seen that, in general, the MICs of antibiotics have a significant decrease in the presence of EPIs, when compared with the determined value for the MIC of 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. The EPI Na_3VO_4 promoted the accumulation, and consequent toxic effect of EtBr, being the only EPI that decreased the value of MIC of the fluorescent substrate. These results suggest that the pumps used for the extrusion of the substrates are different from those used by the cells to extrude antibiotics.

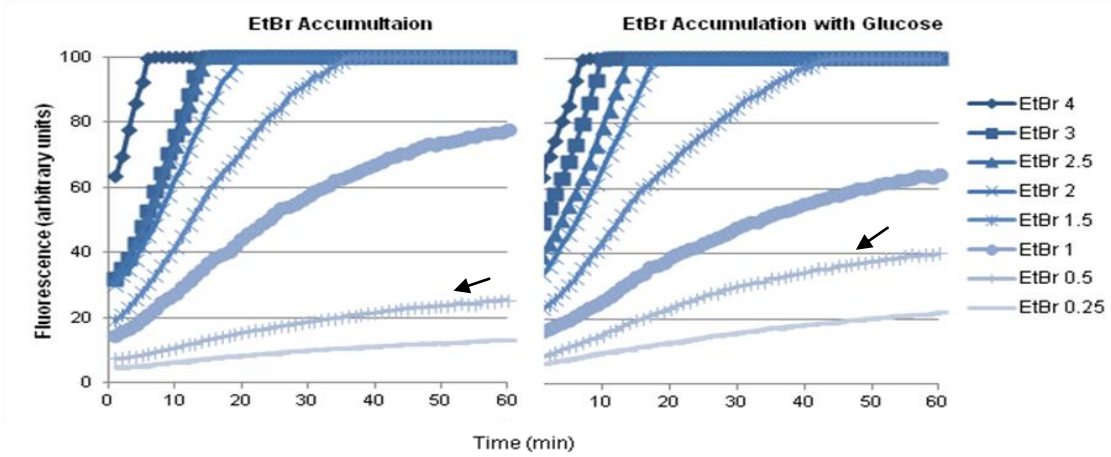

Figure 1 - EtBr accumulation assays, at 28ºC, in the following concentrations: EtBr 4 – 4 µg/mL; EtBr 3 – 3 µg/mL; EtBr 2.5 – 2.5 µg/mL; EtBr 2 – 2 µg/mL; EtBr 1.5 – 1.5 µg/mL; EtBr 1 – 1 µg/mL; EtBr 0.5 – 0.5 µg/mL; EtBr 0.25 – 0.25 µg/mL. The arrow marks the chosen concentration to perform in the remaining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

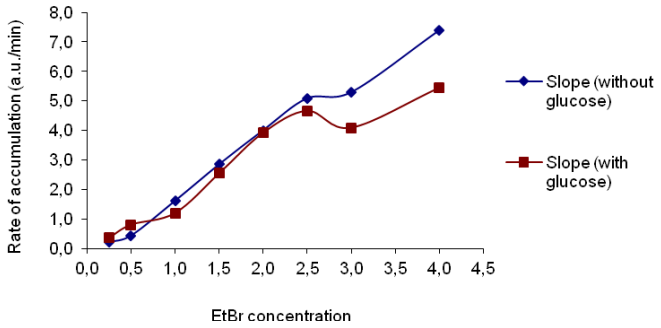

Figure 2 – Slopes of the EtBr accumulation curves.

3.2. Real-time Fluorometry

In the real time fluorometry tests, accumulation and efflux of substrates was assessed using EtBr as model substrate. This method allows the monitoring of the action of EPIs in real time (12).

3.2.1. Accumulation Assays

In the EtBr accumulation assays performed with *R. erythropolis* the concentrations of EtBr 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. In this assay, it could be seen that at a concentration of EtBr of 0.5 µg/mL the strain did not evidence a strong accumulation of EtBr by itself, reaching a value of fluorescence of 25 units in 60 min. This concentration of EtBr was therefore considered to be the ideal concentration to perform the remaining tests (Figure 1). 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 (Figure 2). At the lowest studied concentrations of EtBr in presence of glucose, the accumulation of EtBr increased (Figure 2), suggesting that these concentrations of EtBr are not sufficiently toxic for cells to trigger any defense mechanism that involves the presence of glucose, possibly the efflux pump machinery.

Once determined the suitable concentration of EtBr to conduct the accumulation assays in the presence of the EPIs, they 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 3). In presence of VP and absence of glucose, the cells presented the highest retention of EtBr. The fluorescence significantly decreased when the cells were exposed to glucose. Na3VO4 led to the converse result. In the presence of glucose the retention of EtBr was higher, than in its absence. The remaining EPIs, TZ, CCCP and OMP, did not demonstrate to be the most favorable to promote EtBr accumulation. In presence of the

inhibitor TZ and OMP, the strain did not accumulate a significant amount of the 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 presence and absence of EPIs, corresponding to Δ value, are indicated in Table 3. Given the Δ values, it is demonstrated that, with exception of Na3VO4 and CCCP, the fluorescence signal of the EtBr accumulated are lower in presence of glucose. VP was chosen in the following assays since it allows a greater accumulation of EtBr in the cells which is required at the beginning of efflux assays.

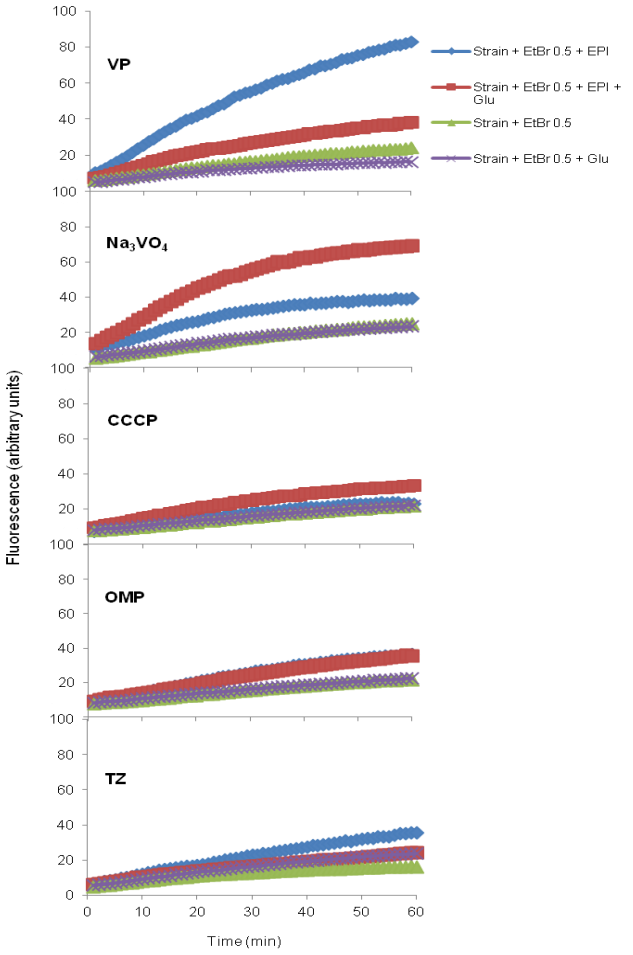

Figure 3 - EtBr accumulation assays in the presence of the various EPIs and in the presence and absence of glucose, at 28ºC.

Table 3 - 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

3.2.2. Efflux Assays

To perform the efflux assays the cells were first loaded with the fluorescence substrate in order to reach the maximum level of accumulation, in this case, with verapamil in absence of glucose. After this incubation, the medium was replaced by the following solutions according to previous studies (12): i) PBS without 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.

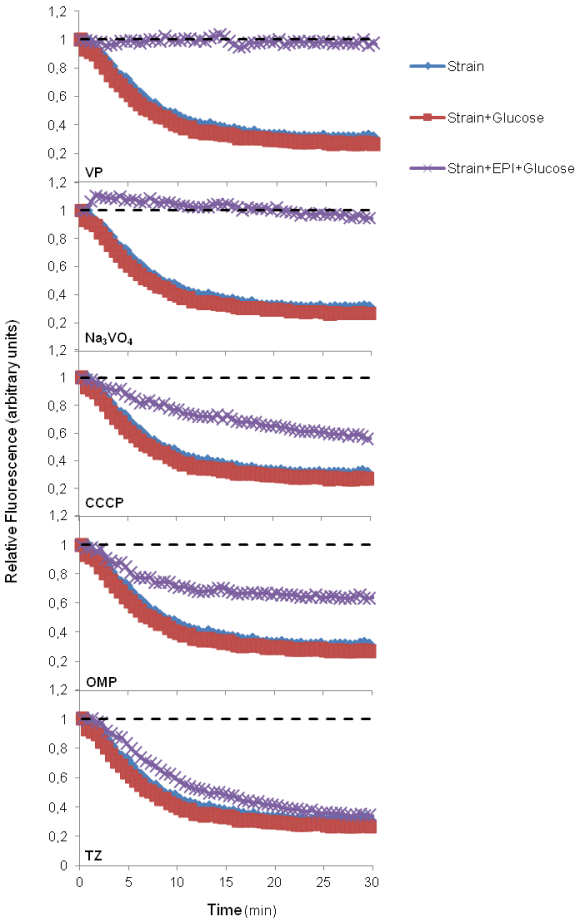

Figure 4 - EtBr efflux assays in the presence of the various EPIs, at 28ºC.

The efflux of the substrate occurred predominantly in the first 10 min in the absence of the EPI, and the presence of glucose did not affect the EtBr efflux. 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 4). Na_3VO_4 and VP demonstrated to have the highest inhibitory effect by preventing the efflux of EtBr from the cells. The EPIs CCCP and OMP had similar effect in inhibition. The relative fluorescence obtained in the presence of these two EPIs at the end of the assay is particularly similar, like 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 at the end of the

assay in the presence of TZ was almost the same to the one obtained in the absence of this EPI (difference of 1.47 units).

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 and absence of glucose (Figure 5).

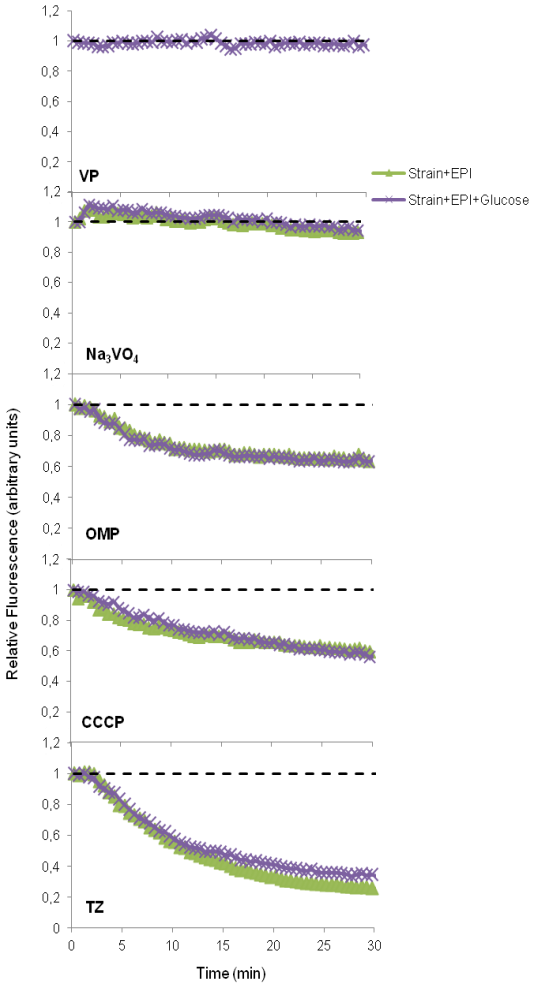

Figure 5 - EtBr efflux assays in the presence of the various EPIs in presence and absence of glucose, at 28ºC.

It is demonstrated in this assay that glucose does not exercise an effect in the efflux of EtBr. In these assay 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 EPI used.

To quantify the inhibitory effect of each EPI, the Relative Final Fluorescence (RFF) value for each compound was determined (table 4).

Table 4 – 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

These assays showed 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 previously mentioned, 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. By the 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 mechanism of action of EPIs can be by depolarization of cell membrane (23). Therefore, a fluorescence kit was used to evaluate the degree of depolarization of cells (figure 5) by fluorescence microscopy and image analysis.

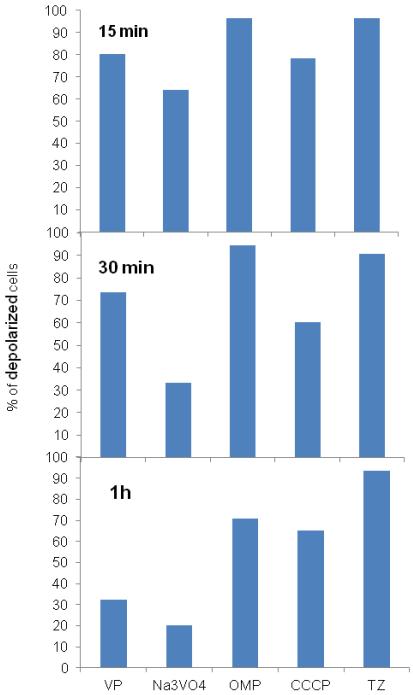

Figure 6 – Membrane potential assays in the presence of the various EPIs.

Fifteen minutes after adding the EPIs, the percentage of depolarized cells was at least 60%, being TZ and OMP 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 Figure 6. After 30 min, which corresponds to the end of the efflux assay, the cells presenting a higher 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 uncoupler is already known (23; 32). 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. It may be by direct binding to the pump, or to by binding to substrate forming a complex, as explain in section 1.2.5. Further studies are required to elucidate this aspect. With these results, it can be concluded that TZ and OMP, like CCCP, depolarize the membranes of bacteria by interfering with gradient of protons through cells 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.

4. Conclusion

R. erythropolis is a highly adaptable bacterium capable of tolerating high amounts of a wide range of toxic compounds and it has, therefore, interesting appications in biotechnology. The tolerance of *R. erythropolis* is associated to its versatile metabolism and its membrane properties. 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.

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 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 suggested 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 presence an 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.

5. References

1. *The genus Rhodococcus.* **Bell, K.S., et al.** 1998, Journal of Applied Microbiology, 85, pp. 195–210.

2. *The Remarkable Rhodococcus erythropolis.* **de Carvalho, Carla C.C.R. and da Fonseca, M. Manuela R.** 2005b, Applied Microbiology and Biotechnology, 67: 715–726.

3. *Adaptation of Rhodococcus erythropolis Cells for Growth and Bioremediation Under Extreme Conditions.* **de Carvalho, Carla C.C.R.** 2012, Research in Microbiology, 1-12.

4. *Biotransformations Catalyzed by the Genus Rhodococcus.* **Warhurst, A.M. and Fewson, C.A.** 1996, Critical Reviews in Biotechnology, 14: 29–73.

5. *Adaptation of Rhodococcus erythropolis DCL14 to Growth on N-alkanes, Alcohols and Terpenes.* **de Carvalho, Carla C.C.R., et al.** 2005, Applied Microbiology and Biotechnology, 67: 383–388.

6. *Adaptation of Rhodococcus erythropolis Cells to High Concentrations of Toluene.* **de Carvalho, Carla C.C.R., et al.** 2007, Applied Microbiology and Biotechnology, 76: 1423–1430.

7. *Mutant Selection Window and Characterization of Allelic Diversity for Ciprofloxacin-Resistant Mutants of Rhodococcus equi.* **Niwa, Hidekazu and Lasker, Brent A.** 8, 2010, Animicrobial Agents and Chemoterapy, 54: 3520–3523.

8. *Determination of the Prevalence of Antimicrobial Resistance to Macrolide Antimicrobials or Rifampin in Rhodococcus equi Isolates and Treatment Outcome in Foals Infected with Antimicrobial-resistant Isolates of R. equi.* **Giguère, Steeve, et al.** 1, 2010, Journal of the American Veterinary Medical Association, 237: 74-81.

9. *Evolutionary Origins of Multidrug and Drug-specific Efflux Pumps in Bacteria.* **Saier, Milton H., et al.** 1998, The FASEB Journal, 12: 265-274.

10. *Efflux-Mediated Drug Resistance in Bacteria - An Update.* **Li, Xian-Zhi and Nikaido, Hiroshi.** 12, 2009, Drugs, 69: 1555-1623.

11. *Demonstration of Intrinsic Efflux Activity of Escherichia coli K-12 AG100 by an Automated Ethidium Bromide Method.* **Viveiros, Miguel, et al.** 2008, International Journal of Antimicrobial Agents, 31: 458-462.

12. *Fluorometric Determination of Ethidium Bromide Efflux Kinetics in Escherichia coli.* **Paixão, Laura, et al.** 18, 2009, Journal of Biological Engeneering, 3: 1-13.

13. **Viveiros, Miguel, et al.** Evaluation of Efflux Activity of Bacteria by a Semi-automated Fluorometric System. [book auth.] Stephen H. Gillespie. [ed.] Stephen H. Gillespie and Timothy D. McHugh. *Antibiotic Resistance Protocols: Second Edition.* s.l. : Humana Press, 2010, 642, 12: 159-172.

14. *Bacterial Efflux Systems and Efflux Pumps Inhibitors.* **Marquez, Béatrice.** 2005, Biochimie, 87: 1137–1147.

15. *The Efflux Pump Inhibitor Reserpine Selects Multidrug-Resistant Streptococcus pneumoniae Strains That Overexpress the ABC Transporters PatA and PatB.* **Garvey, Mark I and Piddock, Laura J. V.** 5, 2008, Antimicrobial Agents and Chemoterapy, 52: 1677–1685.

16. *Isoniazid Accumulation in Mycobacterium smegmatiss Modulated by Proton Motive Force-Driven and ATP-Dependent Extrusion Systems.* **Choudhuri, Baisakhee Saha, Sen, Susmita and Chakrabarti, Parul.** 3, 1999, Biochemical and Biophysical Research Communications, 256: 682–684.

17. *Inhibitors of Bacterial Efflux Pumps as Adjuvants in Antibacterial Therapy and Diagnostic Tools for Detection of Resistance by Efflux.* **Van Bambeke, Françoise, Pagès, Jean-Marie and Lee, Ving J.** 2010, Frontiers in Anti-Infective Drug Discovery, 1: 1-38.

18. *Thioridazine and Chlorpromazine Inhibition of Ethidium Bromide Efflux in Mycobacterium avium and Mycobacterium smegmatis.* **Rodrigues, Liliana, et al.** 2008, Journal of Antimicrobial Chemotherapy: 1-7.

19. *Antimicrobial Activity of Phenothiazines.* **Amaral, L., Viveiros, M. and Molnar, J.** 2004, in vivo, 18: 725-732.

20. *Characterization of Tetracycline Resistance Mediated by the Efflux Pump Tap from Mycobacterium fortuitum.* **Ramón-García, Santiago, et al.** 2006, Journal of Antimicrobial Chemotherapy, 57: 252–259.

21. *Effects of NorA Inhibitors on In Vitro Antibacterial Activities and Postantibiotic Effects of Levofloxacin, Ciprofloxacin, and Norfloxacin in Genetically Related Strains of Staphylococcus aureus.* **Aeschlimann, Jeffrey R., et al.** 2, 1999, Antimicrobial Agents and Chemotherapy, 43: 335–340.

22. *Efect of Proton Pump Inhibitor Alone or in Combination with Clarithromycin on Mycobacterial Growth in Human Alveolar Macrophages.* **Suzuki, Katsuhiro, et al.** 2000, FEMS Microbiology Letters, 182: 69-72.

23. *Antibiotic Efflux Pumps in Gram-negative Bacteria: the Inhibitor Response Strategy.* **Mahamoud, Abdallah, et al.** 2007, Journal of Antimicrobial Chemotherapy, 59: 1223–1229.

24. *Adaptation of Rhodococcus erythropolis Cells for Growth and Bioremediation Under Extreme Conditions.* **de Carvalho, Carla C.C.R.** 2012, Research in Microbiology, 1-12.

25. *A New Route for Ethylene Glycol Metabolism in Mycobacterium E44.* **Wiegant, Wim M. e Bont, Jan A. M. de.** 1980, Journal of General Microbiology, 120: 325-331.

26. *Susceptibility Testing of Mycobacteria, Nocardiae, and Other Aerobic Actinomycetes; Approved Standard - Second Edition.* **CLSI.** 5, Wayne, PA : s.n., 2011, Clinical and Laboratory Standards Institute, 31: M24-A2.

27. *Ethidium Bromide Transport Across Mycobacterium smegmatis Cell-wall: Correlation with Antibiotic Resistance.* **Rodrigues, Liliana, et al.** 35, 2011, BMC Microbiology, 11: 2-10.

28. *Inhibition of Efflux Pumps in Meticillin-resistant Staphylococcus aureus and Enterococcus faecalis Resistant Strains by Triterpenoids from Momordica balsamina.* **Ramalhete, Cátia, et al.** 2011, International Journal of Antimicrobial Agents, 37: 70–74.

29. *A Simple Method to Observe Organic Solvent Drops with a Standard Optical Microscope.* **de Carvalho, Carla C.C.R. e da Fonseca, M. Manuela R.** 4, s.l. : Microscopy Research and Technique, 2003, 60: 465-466.

30. *Efflux-Mediated Drug Resistance in Bacteria.* **Li, Xian-Zhi and Nikaido, Hiroshi.** 2, 2004, Drugs, 64: 159-204.

31. *Rhodococcus equi: An Emerging Pathogen.* **Weinstock, David M. and Brown, Arthur E.** 2002, Emerging Infections, 34: 1379-1385.

32. *Direct Inhibitory Effect of CCCP on the Cl--H+ Symporter of the Guinea Pig Ileal Brush-border Membrane.* **Alvarado, Francisco and Vasseur, Monique.** 1998, American Journal of Physiology - Cell Physiology, 274: 481-491.