Characterization of the mechanisms of adaptation of *Mycobacterium* to organic solvents

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Abstract

Cells of *Mycobacterium vaccae* were grown in toluene, ethanol, methyl *tert*-butyl ether (MTBE) and glycerol, exhibiting different degrees of tolerance towards these compounds, and being able to use both ethanol and glycerol as sole carbon and energy sources. Cellular adaptation to ethanol and MTBE led to significant changes in the membrane fatty acid (FA) composition, by modifications in the proportion of straight-chain saturated, monounsaturated, branched-chain saturated and 10-methyl FA. Ethanol-adapted cells had a different morphology than MTBE-adapted cells: regular rod-shaped cells were observed with MTBE but cell shrinkage occurred in the presence of ethanol. Besides, cells in the presence of ethanol formed aggregates. The zeta potential of cells was -49.3 mV before the addition of solvent, becoming less negative after MTBE adaptation (-33.1 mV) and more negative in ethanol-adapted cells (-45.9 mV). After adaptation to these solvents, cells acquired an improved tolerance towards efflux pump inhibitors (EPIs), but became more susceptible to antibiotics.

Keywords: Solvent tolerance; *Mycobacterium vaccae;* fatty acid profile.

1. Introduction

Organic solvents can be extremely toxic to microorganisms, even at concentrations as low as 0.1% (v/v), since they can partition and accumulate in the bacterial cell [1]. Solvent toxicity depends on the solvent hydrophobicity, molecular structure [2] and the characteristics of the cell membranes [3]. Each bacterium is known to possess a strain-specific level of tolerance to organic solvents, either by genetic determination or environmental factors [4]. Bacteria have developed a wide range of mechanisms to cope with toxic organic compounds, being the cytoplasmic cell membrane the most important protective barrier [5]. Some mechanisms include modification of phospholipids headgroups, vesicle formation, adjustments in the fatty acid composition of cells, modifications in the biosynthesis rate of phospholipids and extrusion of solvents through efflux pumps.

These mechanisms have been extensively reviewed for Gram-negative bacteria and reports about tolerance mechanisms in Gram-positive bacteria have received more attention only in the last two decades [6]–[14].

Non-tuberculous mycobacteria (NTM) are a group of saprophytic mycobacteria which colonize a broad range of environments such as soil, water and surgical solutions, being known to cause disease in immuno-compromised patients [15]. Mycobacterial species are well known biocatalysts and biodegraders, having a considerable potential in bioremediation of polluted environments [16]–[19]. *M. vaccae* is known to metabolize acetone, cyclohexane, styrene, benzene, ethylbenzene, propylbenzene, dioxane and 1,2-dichloroethylene and to possess a co-oxidative capacity that results in the formation of intermediate molecules more amenable to mineralization as a result of the degradation of recalcitrant compounds [20]. This is one of the few *Mycobacterium* species known to grow on toluene and to use acetone as carbon source [21].

The aim of this study was to determine which mechanisms *M. vaccae* used to overcome the toxicity of organic solvents and to understand if the previous contact with these chemicals could lead to an improved tolerance towards antibiotics and EPIs, which could ultimately hinder the treatment of mycobacterial infections.

2. Materials and Methods

2.1 Microorganism

Mycobacterium vaccae ATCC 15483 was used in this study. It is deposited at the Institute for Bioengineering and Biosciences (iBB), Lisbon, Portugal, and stored at -80ºC and at 4ºC in agar plates.

2.2 Chemicals

Culture media components were Mueller-Hinton (MH) broth from Sigma-Aldrich and Tween 80 from Merck-Schuchardt. Mineral medium was prepared by adding 10 mL/L of 100x concentrated Phosphate Buffer and 10 mL/L of 100x concentrated Mineral Medium to deionized water. The Mineral Medium solution was prepared by the addition of the following order of salts to 1 L of deionized water: 1 g EDTA; 0.2 g $ZnSO_4.7H_2O$; 0.1 g $CaCl_2.2H_2O$; 0.5 $FeSO_4.\ 7H_2O$; 0.2 g $Na_2MoO_4.2H_2O$; 0.2 g $CuSO_4.5H_2O$; 0.4 g $CoCl.6H_2O$; 0.1 g $MnCl_2.4H_2O$; 200 g $(NH_4)_2SO_4$; 10 g $MgCl_2.6H_2O$ (all from Sigma-Aldrich). 100x concentrated Phosphate Buffer was prepared by adding 55 g/L K_2HPO_4 and 85 g/L $NaH_2PO_4.H_2O$ (Sigma-Aldrich) to deionized water. The media were then autoclaved during 20 minutes at 121ºC, and stored at 4ºC.The agar plates used to preserve the bacterial cells were prepared by adding 10 mL/L of 100x concentrated Mineral Medium, 10 mL/L of 100x concentrated Phosphate Buffer, 15 g/L of agar, 5 g/L of glucose and 3.5 g/L of yeast extract. The solvents used in this work were ethanol (>99.9%) from Panreac, toluene (>99.5%) from Riedel-de Häen, MTBE (>99.5%) and a glycerol solution (86-89%) both from Sigma-Aldrich.

2.3 Growth Conditions

M. vaccae was grown in 100 mL erlenmeyers containing 20 mL of MH broth supplemented with 0.1% Tween 80 at 30ºC and 200 rpm in an Agitorb 200 incubator (Aralab).

Solvent exposure: A certain amount of solvent was added to cell cultures in mid-exponential phase (OD 0.7-1.2)

In MH broth, when cells reached mid-exponential phase (OD 0.7-1.2) solvent was added at different concentrations to each cell culture. Each concentration was tested in duplicate. Growth inhibition was defined as the percentage of growth rate of solvent-exposed cultures compared with control cultures without solvent addition.

Growth inhibition (%): $\frac{\mu 1,solv}{\mu 0,control} \times 100$

Carbon source: Mineral medium (MM) was used to study if the cells were able to use the solvents as sole carbon and energy source. Amounts of 0.25 and/or 1% (v/v) solvent were added to each Erlenmeyer containing 20 mL MM supplemented with 0.1% Tween 80. The flasks were inoculated with mid-exponential cells to an initial OD of 0.086. Cultures were incubated at 30ºC and 200 rpm.

Adaptation: To conduct bacterial adaptation to ethanol and MTBE, 40 mL of MH media supplemented with 0.1% Tween 80 was used. When cells reached the mid-exponential phase, pulses of 1% (v/v) MTBE or 5% (v/v) ethanol were added. When cells seemed to be entering in the exponential phase again, more pulses of solvent were added to the cultures. Growth was monitored and maintained in the same conditions as in the previous experiments.

2.4 Fatty acid composition

Mycobacterial cell suspensions were centrifuged at 10,000 rpm for 5 minutes in a µSpeedFuge SFA13K (Savant Technologies), and the pellet was washed twice with mili-Q water. The cellular fatty acids were simultaneously extracted from the cell pellet and methylated to fatty acid methyl esters (FAMEs). FAMEs were prepared using the instant-FAME method from MIDI, Inc. and analyzed by gas chromatography on a 6890N gas chromatograph from Agilent Technologies, equipped with a 25 m long Agilent J&W Ultra 2 capillary column, a flame ionization detector and an automatic injector 7683B [13].

The saturation degree was calculated dividing the sum of saturated FA by the sum of monounsaturated FA.

2.5 Zeta potential

Cell suspensions (40 µL) were collected before and during solvent exposure, washed 3 times, and suspended in 2 mL of 10 mM KNO_3. The electrophoretic mobility of mycobacterial cells was determined in a Doppler electrophoretic light scattering analyzer (Zetasizer Nano ZS, Malvern Instruments Ltd) at 25ºC, using a clear disposable zeta cell. The zeta potential was determined by using the electrophoretic mobility as an indirect measure of cell surface charge, according to the method of Helmholtz–von Smoluchowski [22]. The zeta potential of the organic solvents was also assessed for the solvents stated in 2.3, with a Glass “Dip” Cell (Malvern). Calculations were automatically performed using the Zetasizer software 7.10, from Malvern Instruments, Ltd.

2.6 Minimum inhibitory concentration determination

The minimum inhibitory concentration (MIC) was determined for antibiotics and EPIs by the broth microdilution method in a 96-well microtitre plate (Sarstedt Inc) according to CLSI guidelines [23] . Briefly, antibiotics and EPIs were serially diluted in two-fold steps,

starting with different initial concentrations: levofloxacin (10 and 7.5 µg/mL), teicoplanin (100 and 75 µg/mL), thioridazine (149.3 and 125 µg/mL) and omeprazole (500 and 400 µg/mL), in 150 µL of MH broth. Afterwards, 50 µL of a cell suspension collected in the exponential phase and diluted in MH broth to a 0.5 McFarland standard was added to each well. The plate was then covered with a Breathe-Easy™ sealing membrane (Sigma-Aldrich), and kept without agitation at 30ºC. OD was measured after approximately 72h, at a wavelength of 600 nm, in a spectrophotometer SpectraMax® 340 PC from Molecular Devices. All experiments were performed in duplicate.

2.7 Fluorescence Microscopy

Cells were observed using a LIVE/DEAD® BacLight™ Bacterial Viability Kit from Molecular Probes which was added to the samples according to the instructions of the manufacturer. The microscope used was an Olympus CX40 equipped with an Olympus U-RFL-T burner and a U-MWB mirror cube unit (excitation filter: BP450-480; barrier filter: BA515). Images were collected with an Evolution™ MP 5.1 CCD color camera using the acquisition software Image-Pro Plus, both from Media Cybernetics. One slide was made per sample and at least five images were taken from each slide.

In order to visualize the EPS (exopolymeric substances) observed in some samples, Nile red was used to stain lipids. A stock solution of 1.3 mg/mL of Nile red (Molecular Probes, Life Technologies) in acetone was used and added to the sample to achieve a 1:100 dilution. This solution was added to selected samples which were kept in dark conditions for 5 min. A U-MWG mirror cube unit (excitation filter: BP510-550; barrier filter: BA590) was used provide the appropriate wavelengths to observe the samples.

3. Results

Cell growth in the presence of organic solvents

Cells in mid-exponential phase (OD of 0.7-1.2) were exposed to different concentrations of toluene, ethanol, glycerol and MTBE. Cells exposed to 1, 2.5 and 5% (v/v) ethanol and 5, 10 and 20% (v/v) glycerol were able to withstand these conditions (Figure 1). The higher the amount of toxic compound, the lower the growth rate of cells. When cells were exposed to 0.1, 0.5 and 1% toluene and 0.5, 1 and 2.5% (v/v) MTBE, concentrations as low as 0.5% toluene caused an immediate

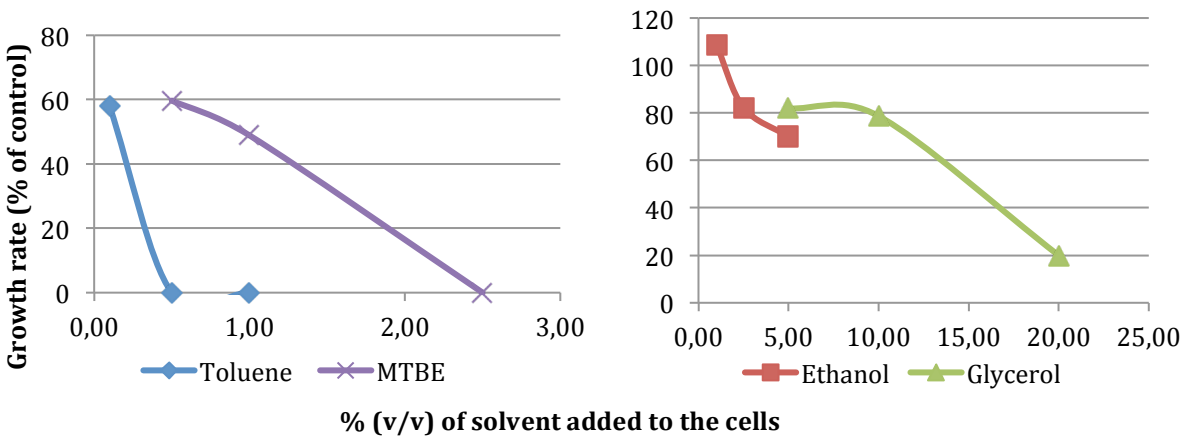

Figure 1 - Growth inhibition caused by the presence of different concentrations of organic solvents (toluene, MTBE, ethanol and glycerol) relative to control.

decrease in the growth rate of cells, thus leading to cell death. Concentrations of 0.5 and 1% were able to sustain celular growth on MTBE, but when 2.5% MTBE was present, mycobacteria were unable to survive.

Alterations in the fatty acid composition of cells

During control conditions, the most common fatty acids present were 18:1 w9c (58-70%), 16:0 (11-13%), 18:0 (7-10%), 12:0 (4%) and 14:0, 14:1 w9c and 18:1 w7c (2-3% of each) and 18:0 10-methyl (1-2%). The most visible alterations under control conditions throughout time were a decrease in the amount of 18:1 w9c (58 to 48%) and 18:0 (10-5%), an increase in the content of 18:0-10 methyl (2 to 9%) and the appearance of 16:1 w6c FA after 7 hours of the beginning of the experiment, increasing from 8 to 11%.

When toluene was present 16:1 w6c was not produced at all. In 1% ethanol this fatty acid (FA) was produced in a content similar to control conditions (approximately 8%), but, in 2.5% ethanol, it was only produced after 11 hours of exposure. In 5% ethanol, the production of this FA was abrogated. Contrarily to the previous cases, 16:1 w6c was always produced in the presence of glycerol, and the cells presented a FA profile very similar to control conditions. In 1% MTBE, this FA appeared only after 11 hours of exposure, and in 2.5% MTBE it was not detected at all. In all cases, there was an overall increase in the saturation degree of cells.

Use of solvents as carbon source

Ethanol was the preferred carbon source under the conditions imposed, since cells were able to grow up to an OD of 3. In the case of glycerol, after 80 hours, a maximum OD of 0.7 was attained. In 0.25% MTBE, this value was lower, being only 0.5. Cells were unable to use toluene as sole carbon and energy sources.

Adaptation to ethanol and MTBE

To conduct bacterial adaptation, 40 mL of MH media were used in order to have enough volume to take samples. Adaptation to ethanol was carried out by adding three pulses of 5% (v/v) ethanol, meaning that cells were able to endure 15% (v/v) ethanol. Cells in the presence of this solvent only grew until a maximum OD of 1.6 and, after the second pulse, formed visible aggregates. After each pulse, there was an immediate decrease in the OD of the culture, followed by a subsequent recovery. In the case of MTBE-adapted cells, an OD of approximately 7 was achieved. A total of 4% (v/v) MTBE was added to the cultures, without the formation of visible aggregates.

Zeta potential

The zeta potential of cells during adaptation was measured at different times of the experiment. At the beginning of the experiment (OD =0.139), the zeta potential of *M. vaccae* cells was approximately -44.975 mV (**Error! Reference source not found.**). When cells were at the exponential phase, the zeta potential became more negative (-49.3 mV). In the presence of ethanol, almost no alteration of

the zeta potential of cells occurred (-45.9 mV), whilst in the presence of MTBE, cells became less negative (-33.1 mV).

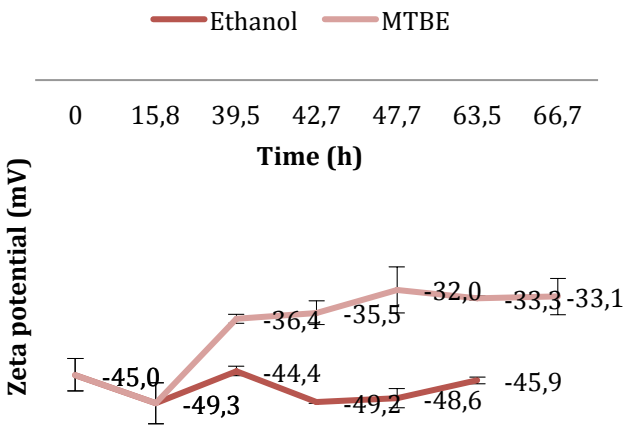

Figure 2 - Zeta potential (mV) of cells exposed to ethanol and MTBE.

Cell morphology

The morphology of *M. vaccae* cells was analyzed using fluorescence microscopy, enabling the visualization of both viable (green) and non-viable (red) cells (Figure 3). The samples were collected after 45 hours of growth, in cells subjected to two pulses of ethanol and three pulses of MTBE. In the case of ethanol-grown cells, two types of cellular features were distinct: the presence of large aggregates and shrinked cells. The amount of red-stained cells was very reduced, being an indicator of a high percentage of viable cells present in the sample. As for MTBE-grown cells, both cellular aggregation and elongated rods were observed. In this case, there was a high amount of red-stained cells, showing that a part of the population lost the viability after an incubation period of 30 hours in MTBE.

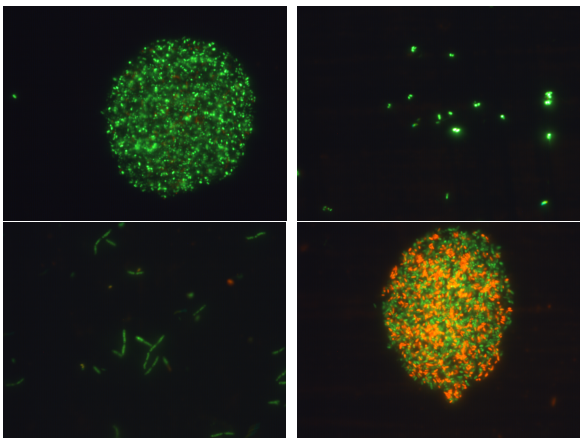

Figure 3 - Morphology of mycobacteria exposed to ethanol (top) and to MTBE (bottom). Magnification: 1500x.

Alterations in the fatty acid composition of cells during adaptation

At the beginning, the most common FA were 18:1 w9c (67%), 16:0 (13%), 18:0 (8%), 12:0 (4%) and 14:0, 14:1 w9c and 18:1 w7c (3% of each). When MTBE was added, *iso* and *anteiso* FA were produced (Figure 4). There was an increase in 13:0 *iso* (1 to 3%), 14:0 *iso* (0.7 to 1.4%), 15:0 *iso* (7 to 13%), 16:0 *iso* (2 to 4%), 17:0 *anteiso* (3 to 4%), 17:0 *iso* (7 to 12%) and 19:0 *iso*. Throughout the adaptation period, the amount of MUFA decreased and BSFA were produced, until they were 42% of the total FA. This led to an increase in the saturation degree of cells. During ethanol adaptation, the most noticeable alterations at the level of the FA were a decrease in the amount of 16:0 (24.5 to 23%), 18:0 (3 to 2%) and an increase in 16:1 w6c (8.7 to 9%), 14:1 w9c (0.5 to 0.9%) and 18:1 w9c (49 to 52%). The saturation degree of cells did not show a linear trend. After the last addition of solvent, the most prominent changes were at the level of 18:1 w9c (58 to 51%), 16:1 w6c (10 to 8%), 16:0 (20 to 21%), 18:0 (2 to 3%) and 18:0 10-methyl (3 to 6%), which led to an increase in the saturation degree of membrane FA.

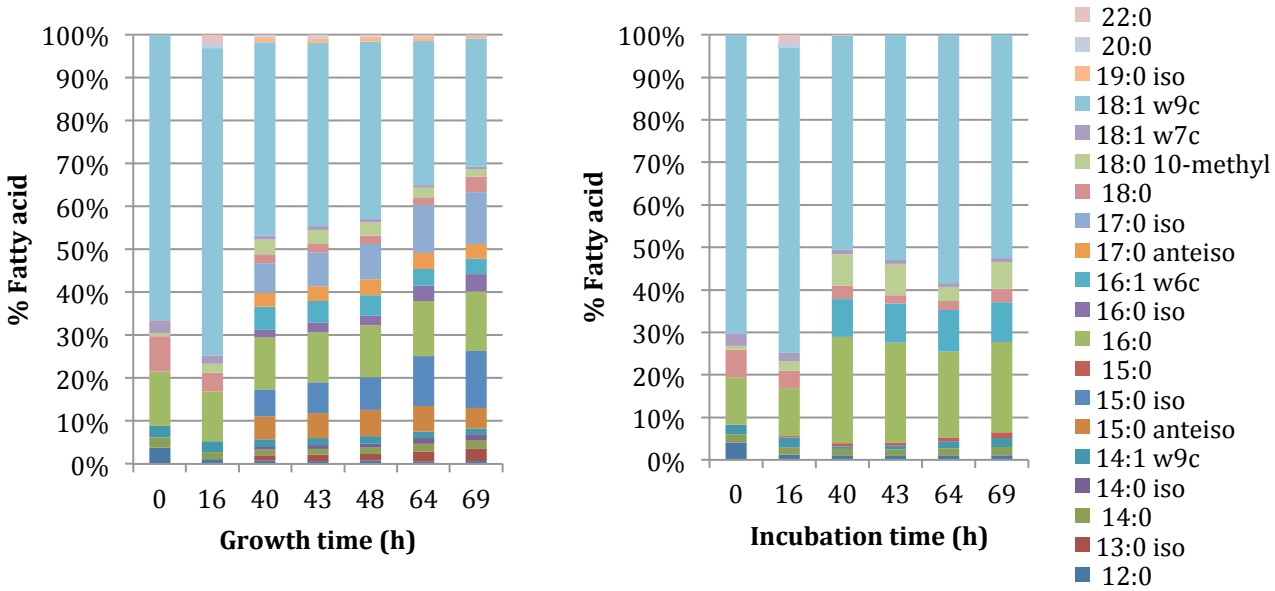

Figure 4 - Alteration at the level of the FA composition of cells in the presence of MTBE (left) and ethanol (right).

MIC determination

Due to the increasing concern towards infections caused by NTM, in 2003 the CLSI has established criteria for antimicrobial susceptibility testing (AST) of NTM. Since this species is a rapidly-growing mycobacteria such as *M. fortuitum*, the same criteria were applied. Non-adapted cells presented higher tolerance levels to teicoplanin than MTBE and ethanol-adapted cells. In the case of levofloxacin, the MIC of non-adapted cells was the same as for MTBE-adapted cells, but this value decreased 15-fold when compared with ethanol-adapted cells (Table 1). This results point to the conclusion that adaptation to these solvents did not lead to increased resistance towards the antibiotics tested, and ethanol-adapted cells were more susceptible to these compounds than MTBE-adapted cells. In the case of EPIs, MTBE and ethanol-adapted cells presented higher MIC values when compared with non-adapted cells (2-fold increase), and both MTBE and ethanol-adapted cells presented equal MIC values towards these compounds.

Table 1 – Minimum inhibitory concentration detected for solvent-adapted and non-adapted cells regarding teicoplanin, levofloxacin, thioridazine and omeprazole.

Antibiotics/EPIs	**Non-adapted cells**	**MTBE-adapted cells**	**Ethanol-adapted cells**
	MIC (µg/mL)		
Teicoplanin	>100	50	25
Levofloxacin	0.6	0.6	0.04
Thioridazine	18.7	74.6	74.6
Omeprazole	250	500	500

4. Discussion

During exposure to different types of organic solvents, there was a dose-dependent inhibition of the cellular growth. The most toxic organic solvents were toluene, followed by MTBE, since concentrations as low as 0.1% and 2.5%, respectively, were able to cause immediate cell death. Toluene and other aromatic hydrocarbons are known to be extremely toxic to living organisms due to its preferential partitioning to cell membranes, leading ultimately to membrane disruption and cell lysis [24]. In fact, concentrations as low as 0.1% (v/v) are usually sufficient to kill most microorganisms [13].

There was an increase in the saturation degree of cells in the presence of toluene, which is in accordance to what was previously described for the closely-related *R. erythropolis* DCL14 [13]. When compared with ethanol and glycerol, concentrations up to 5% and 20%, correspondingly, were sustained. After 25 hours of growth, alterations in the fatty acid composition of cells were observed. Alcohols interact directly with the lipid bilayer of the cells, leading to changes in the degree of freedom or fluidity of the cell membrane. It has been published that bacteria react differently to the presence of short-chain or long-chain alcohols. In the presence of short-chain alcohols such as ethanol, the saturation degree of cells usually decreases, whilst in the presence of long-chain alcohols it increases [14], [25], [26]. This difference may be due to the fact that short-chain alcohols can only penetrate slightly into the hydrophobic center of the phospholipid bilayer, thus causing a swelling effect on the hydrophilic headgroups [26]. Therefore, the insertion of unsaturated fatty acids in order to counteract this effect seems a better adaptive response [27]. A dose-dependent decrease in the membrane saturation degree was observed in *R. erythropolis* DCL14 exposed to ethanol [14]. In this work, the saturation degree of membrane FA increased in the presence of etanol. In general, the main difference regarding the FA composition of cells resides in the production of 16:1 w6c FA. The higher the concentration of solvent used, the sooner the production of this FA was aborted, with exception for glycerol, where it reached similar values when compared with control conditions (approximately 8-9% of the total FA). As an overall response, cells increased the saturation degree in the presence of toluene, MTBE, ethanol and glycerol. This leads to the conclusion that in the presence of the organic solvents tested, cell adjusted their membrane fluidity by favouring more rigid membranes.

It was also shown that *M. vaccae* ATCC 15483 can use ethanol as sole carbon and energy sources, and in a lesser extent, can also use glycerol and MTBE. Glycerol is considered to be the favored carbon source of *M. tuberculosis* and *M. bovis* BCG under laboratorial conditions. The wild type *M. bovis*, however, is unable to use glycerol as sole carbon and energy source due to a nucleotide polymorphism in the pykA gene [28], [29]. Variability can be also found in species that can use MTBE. *M. vaccae* JOB5 (currently *M. austroafricanum* JOB5) does not grow on MTBE when it is supplied as a sole carbon and energy source [30], but *M. austroafricanum* IFP 2012 and *M. austroafricanum* IFP 2015, were able to grow on MTBE as a sole carbon and energy sources and to degrade it with different catabolic efficiencies [31]. *M.*

cosmeticum byf-4 was also reported to use toluene as sole carbon source [8]. When 0.25% toluene was the only carbon source available, even after 60 hours, *R. erythropolis* did not grow, presenting thus a very large *lag* phase. Nonetheless, when concentrations around 0.125% were added, the cells were able to grow [12]. Later, this strain evidenced a remarkable ability to adapt and grow in the present of concentrations up to 50% (v/v) toluene [13]. The concentrations selected for toluene supplementation in the present study might thus have been too high or the experimental time too insuficient, since no growth was observed.

Adaptation of cell populations to stresses is based on the contribution of the most tolerant individuals, due to differences on genetic and physiological diversity. Cell adaptation can be ascribed to: i) enzyme-mediated tolerance (where the toxic compounds are degraded into non-toxic products) [32]; ii) genetic adaptation; iii) physiological state; iv) adaptation of the membrane composition [33]; v) efflux pumps that extrude the toxic compound [34]. During adaptation to ethanol, cells had more negative surfaces and relied in the formation of cell aggregates or the shrinking of the size to overcome the toxicity of the solvent. In MTBE-adapted cells, the opposite was observed, since cells became less negative and rod-shaped cells were observed, together with a lower amount of aggregation. Previous reports stated that the establishment of cell clusters, biofilms or microcolonies provides a better protection against membrane-active compounds. When a population is organized as a microcolony, there is an increase of the total extracellular space inside the colony, together with a reduction of the colony surface area to volume ratio [35]. Also, the cells inside the colony are more protected. A higher viability was achieved in ethanol-adapted cells than MTBE-adapted cells. The difference observed in zeta potential can be related with the prior adjustment of the physicochemical properties of the cell surface in order to overcome the presence of the toxic compounds. Another explanation may reside in the response of the membrane components towards MTBE. Membrane proteins contribute to the total net charge of the cell surface and can alter the zeta potential through variation in their amounts and through the establishment of different intermolecular interactions [36]. MTBE can induce an alteration in the orientation of the positive charges of membrane proteins and other components such as phospholipids, and by exposing these charges, increases the attractive electrostatic interactions between cells and MTBE. The presence of cellular aggregation is a demonstration of the high hydrophobicity caused by the presence of ethanol, thus being in accordance with a more negative zeta potential. MTBE-adapted cells produced branched-saturated FA during adaptation, and increased the saturation degree, whilst in the case of ethanol-adapted cells, the saturation degree did not exhibit such a linear relation and no branched-saturated FA were produced.

MIC determination showed that solvent-adapted cells do not present improved MICs for antibiotics such as levofloxacin and teicoplanin, but in the case of EPIs, higher MICs were obtained, when compared with non-adapted cells. This shows that the use of EPIs in the treatment of mycobacterial infections can be hindered by the previous

contact of environmental mycobacteria with organic solvents, due to adaptation mechanisms.

5. Conclusion

M. vaccae was able to tolerate the organic solvents tested by changing the FA composition of cell membranes, ultimately leading to an increase of the cell rigidity to cope with the toxic effect of the solvents. When cells were adapted to ethanol and MTBE, different tolerance mechanisms were activated, such as changes in the membrane FA, surface net charge and morphology. After adaptation to solvents, cells became more tolerant towards EPIs, but not towards antibiotics, jeopardizing the future use of EPIs in the treatment of mycobacterial infections.

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