Influence of Cell Membrane on Bacterial Persistence

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Abstract This work aimed at studying the appearance of persister bacterial cells in antibiotic exposed populations and the effect of antibiotics on the fatty acid (FA) composition and membrane zeta potential (ZP) of those cells. Two Gram-positive bacteria were studied: *Staphylococcus aureus* and *Rhodococcus erythropolis*. The appearance of persister cells tolerant to vancomycin and teicoplanin, which act by inhibiting cell wall peptidoglycan biosynthesis, was assessed by colony-forming unit counts. For that, bacteria in exponential growth phase were challenged with high antibiotic concentrations: 50 and 100 $\mu g.mL^{-1}$. Biphasic killing kinetics, typical for persister cell enrichment, were observed in both species and for the two tested antibiotics/concentrations. Furthermore, distinct Fourier transform infrared (FTIR) patterns were obtained for regular and persister cells. Population dynamics analysis of the survivor persisters upon antibiotic removal, monitored spectrophotometrically at 600 nm, revealed culture repopulation, confirming persister cells as reversible phenotypic variants of regular cells. Analysis of fatty acid methyl esters (FAMEs) showed that both studied bacteria respond to antibiotics by increasing the percentage of saturated FA while reducing significantly that of branched FA. Besides, the cells decreased the ZP with both time of exposure and antibiotic concentration. Together, these results strongly suggest that bacteria overcome antibiotic action by reorganizing the cell membrane, in order to reduce its permeability to the stress agent. Additionally, enzymatic activities and oxygen consumption rates of persister cells further suggest that they are active rather than 'dormant', refuting previous hypotheses.

Keywords *Staphylococcus* • *Rhodococcus* • vancomycin • teicoplanin • FAMEs • persistence

Introduction

Bacteria have been evolving and optimizing their regulatory networks since they were exposed to selective pressures present in their native ecological niche. The adaptation mechanisms may involve the acquisition of new cellular functionality, due to both gain and loss of function mutations, or via modulation of cellular networks [1]. Importantly, bacteria have evolved in order to respond to antimicrobial agent exposure (*e.g.*, antibiotics; organic pollutants). For that, bacteria developed a variety of molecular adaptations at the level of the membrane cell that allows them to prevent the antimicrobial agent from reaching its target. These molecular adaptations may include a decrease in membrane permeability, due to modifications in the composition of the outer membrane (lipopolysaccharide); a decrease in the number of porins (only valid for Gram-negative bacteria); or the overexpression of efflux pumps (EPs) that recognize the antimicrobial and extrude it [2]. Alterations in the fatty acid composition of membrane phospholipids are an important part of the bacterial adaptation mechanism [3-5]. Since fatty acids are the major constituents of membrane phospholipids, modulation of both number and position of double bonds of acyl chains plays crucial role in preserving a suitable dynamic state of the bilayer during exposure to environmental stress. However, evolutionary advantages of bacteria, rather than be based only in a small, specific set of parameters, are actually composed by a complex arrangement of structural, metabolic, regulatory, microevolutionary, and genome rearrangement features, as well as advantages of growth rate and population size [6]. Following that complexity, it is now known that the more effective strategy adopted by bacterial populations to survive a temporary environmental stress (*e.g.*, antibiotic exposure), and then repopulate it when the conditions improve, is by setting aside a small fraction of the total population, the persister cells, in a non- or slow-growing, but stress-tolerant phenotypic state. These cells are now known as 'persister cells' and were first described by Joseph W. Bigger [7] who studied the action of penicillin against staphylococci. Persister cells are not resistant to antibiotics [8, 9], and therefore do not exhibit an increased MIC. Rather, persister cells are non- or slow-growing reversible phenotypic variants of the wild type, tolerant to bactericidal antibiotics [10-13], which means that they do neither grow nor die in the presence of antibiotics. Furthermore, the fraction of cells that survive antibiotic exposure is highly dependent on the specific antibiotic that is used, suggesting that not only the rate at which cells enter and exit from a 'dormant' state, but also additional physiological changes, are responsible for persister phenotypes [14, 15]. These advances strongly define persistence as an active state, which allows cells to respond to temporary environmental stress, refuting previous theories. Further studies will be of paramount importance to provide detailed information on the role of persisters in all chronic infections. The main objectives of the present studies are thus the following: to study the appearance of persister cells in antibiotic exposed populations; to study the effect of antibiotics on the fatty acid composition of the phospholipids of persister bacterial cells; and to study their effect on the surface properties of the cells.

Materials and methods

Bacterial Strains and Growth Conditions Strain *Staphylococcus aureus* ATCC 25923 was kindly provided by the Unit of Mycobacteriology of the Instituto de Higiene e

Medicina Tropical, Universidade Nova de Lisboa. Strain *Rhodococcus erythropolis* DCL14 was isolated by the Division of Industrial Microbiology of the Wageningen University, The Netherlands [16]. It is deposited and maintained at the Institute for Biotechnology and Bioengineering, Lisbon, Portugal. Both *S. aureus* and *R. erythropolis* cells were suspended in 20 mL of Mueller Hinton Broth (MHB; Fluka) in 100mL Erlenmeyer flasks. *S. aureus* cells were cultured at 37°C and 200 rpm whilst *R. erythropolis* grew at 28ºC and 200 rpm on incubators Agitorb 200 (Aralab, Portugal). The growth of the planktonic cells was monitored by optical density measured using a Merck-Hitashi spectrophotometer at 600 nm.

Reagents and Antibiotics Mueller Hinton powder was purchased from Sigma-Aldrich (Fluka Analytical) and, after prepared, MBH was stored at 4°C for no more than 1 week. The mineral medium solution was prepared as described previously [17]. All media used were sterilized in an autoclave at 121°C for 15 minutes. Vancomycin (Vancomycin hydrochloride from *Streptomyces orientalis* with a potency ≥ 900 $\mu g.mg^{-1}$) and teicoplanin (from *Actinoplanes teichomyceticus* with a purity ≥ 80%) were purchased from Sigma-Aldrich. Both antibiotics were used at concentrations of 50 and 100 $\mu g.mL^{-1}$ in milli-Q water.

Determination of *Minimal Inhibitory Concentration (MIC)* The MICs were determined according to the Clinical and Laboratory Standards Institute (CLSI) guidelines [18]. In summary, antibiotics were serially diluted in two-fold steps (from 100 $\mu g.mL^{-1}$ to 0.037 $\mu g.mL^{-1}$) in 96-well microplates (Sarstedt Inc., Newton, USA) in Mueller-Hinton medium. To 150 µL of medium containing the antibiotic in each well, 50 µL of an exponentially growing cell culture diluted to 0.5 McFarland standard was added. Microplates containing *S. aureus* cells were incubated at 37ºC whilst *R. erythropolis* cells were incubated at 28ºC. The MIC was determined for each antibiotic by measuring the optical density of cell cultures after 16 and 24 h of exposure on a SpectraMax® Plus 384 Microplate Reader spectrophotometer from Molecular Devices (Silicon Valley, CA, USA) at 600 nm. At least two independent tests were performed. The MICs determined for each bacterium and antibiotic were the following: $MIC_{vancomycin\ S.\ aureus}$ = 0.59 $\mu g.mL^{-1}$ and $MIC_{teicoplanin\ S.\ aureus}$ = 0.39 $\mu g.mL^{-1}$; $MIC_{vancomycin\ R.\ erythropolis}$ = 1.17 $\mu g.mL^{-1}$ and $MIC_{teicoplanin\ R.\ erythropolis}$ = 1.56 $\mu g.mL^{-1}$.

Time-Dependent Killing To 1 mL of exponentially growing cells, a given concentration of antibiotic was added in 12 mL pyrex tubes. The tubes containing *S. aureus* were incubated on a shaker at 200 rpm and 37°C for a total of 6 h whilst those containing *R. erythropolis* were incubated at 28ºC and 200 rpm. At certain time intervals (30 min, 1, 2, 3, 4, 5 and 6 h), a 20 µL sample was collected and spread on an agar plate for determination of CFU. The CFU counts were performed after 16-18 h according to CLSI guidelines [18] or after 37-38 h of incubation when growth was slower.

Growth after Antibiotic After challenging each culture with 50 and 100 $\mu g.mL^{-1}$ vancomycin or teicoplanin for 3, 4, 5 or 6 h (as stated before) 1 mL of culture was collected and added to 19 mL of fresh MHB in 100 mL Erlenmeyers. Growth was promoted for each strain at the conditions previously stated and monitored by measuring the optical density of the media at 600 nm until stationary phase was reached.

Cell Viability Analysis Cell viability was assessed, upon exposure to 50 and 100 $\mu g.mL^{-1}$ vancomycin or teicoplanin, by fluorescence microscopy using 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). Cells were stained using a LIVE/DEAD® *Bac*Light™ Bacterial Viabillity Kit (Molecular Probes; Life Technologies; Thermo Fisher Scientific) and images were captured by an Evolution™MP5.1 CCD colour camera using the software Image-Pro Plus (both from Media Cybernetics, Inc., USA). Image analysis was performed as described previously [19]. At least 15 images were taken from each sample.

Enzymatic Activity Analysis Semiquantification of the enzymatic activities of *S. aureus* cells before and after exposure to antibiotics, and subsequent growth in fresh medium after drug removal, was assessed using a API ZYM® kit from BioMérieux (France). According to the manufacturer, a semiquantitative scale was used to classify color intensity: 0 (no activity) to 6 (strong activity).

Oxygen Consumption Analysis To determine the metabolic activity of the cells, oxygen consumption analyses were performed on Oxodish® OD24 microtiter plates, using a SDR Sensor Dish® Reader (all from PreSens, Germany). Each bacterium was exposed to the adequate antibiotic concentration and compared to non-exposed cells. To test the effect of low antibiotic concentrations, *S. aureus* cells were exposed to both vancomycin and teicoplanin at concentrations ranging between 2 and 100 $\mu g.mL^{-1}$, whilst for high antibiotic concentrations 50 and 100 $\mu g.mL^{-1}$ were used. Growth of the cells on fresh medium was assessed after 3, 4, 5 and 6 h of pre-exposure to each antibiotic and concentration. The assays were monitored for up to 48 h. The plates containing *S. aureus* cells were incubated at 37ºC and 200 rpm whilst *R. erythropolis* cells were incubated at 28ºC for 200 rpm. Data acquisition was performed by the software SDR_v37 from PreSens.

Lipids Analysis The FAMEs analysis was performed by gas chromatography (GC). For that, 1 mL of cell suspension was collected from each culture and the cells were recovered by centrifugation at 10,000 g for 5 minutes in 1.5 mL eppendorfs (Eppendorf). Cells were then washed by vortex with 1 mL of milli-Q water and recovered by performing a second centrifugation step at 10,000 g for 5 minutes. The fatty acids contained in the cell pellet were simultaneously extracted and methylated using the Instant FAME™ procedure from MIDI, Inc. (Newark, USA). The FAMEs were analysed on a 6890N gas chromatograph from Agilent Technologies (Palo Alto, CA, USA), with a FID and a 7683 B series injector, and equipped with a 25 m long Agilent J&W Ultra 2 capillary column from Agilent. The gas chromatograph was programmed and controlled by the MIDI Sherlock software package, version 6.2. The FAMEs were identified by the MIDI software, using MIDI calibration standards, a methyl cis-11-octadecenoate standard solution from Sigma-Aldrich and confirmed by using two qualitative standards, one

containing a mixture of bacterial FAMEs and another of polyunsaturated fatty acids (PUFA), both from Supelco. The average error associated with the GC quantification of each FAMEs composition was ± 2.2 %, quoted for a confidence interval of 99.5 %. Errors were calculated based on seven independently prepared standard solutions.

Zeta Potential Analysis The Zeta Potential analysis was performed using a Zetasizer Nano ZS from Malvern Instruments Ltd. (Malvern, UK). For that, 20, 40 or 60 µL of cell suspension was collected from each culture and added to 2 mL of a 1.012 $\mu g.mL^{-1}$ potassium nitrate (KNO_3) solution in 2 mL eppendorfs. Data acquisition was carried out by the software Zetasizer 7.10 from Malvern Instruments Ltd. (Malvern, UK). The assays were done in triplicate and the results represented are average values.

FTIR Analysis To determine the effect of antibiotic exposure to the cells, a Fourier transform infrared (FTIR) spectroscopy analysis was performed on both *S. aureus* and *R. erythropolis* cells before and after a 4 h exposure to antibiotics and subsequent growth in fresh medium after drug removal. Cells were collected by centrifugation at 4,000 g for 10 min and the pellets suspended in demineralized water to obtain an optical density of *ca.* 64. Samples containing 20 µL from each prepared cell suspension were plated on a 96-well zinc selenium plate from Bruker Optics® (Billerica, Massachusetts, USA) and dehydrated in a vacuum desiccator containing silica until full dehydration was achieved, confirmed by visual inspection (*ca.* 45 min). The readings were performed by transmission mode using a HTS-XT module coupled to a Vertex-70 spectrometer from Bruker Optics® (Billerica, Massachusetts, USA), with a resolution of 4 cm^{-1}. A total of 40 scans were performed per sample. The data were analyzed after a spectral pre-processing by a Multiplicative Scatter Correction (MSC), previously implemented [20-22], and applied using Chemometric data preprocessing software from MATLAB® Central (USA) (http://www.mathworks.com/matlabcentral/fileexchange/30765-chemometric-data-preprocessing). The pre-processing was followed by a smoothing using the Savitzky-Golay algorithm ([23, 24]) and a 2nd order derivative via the same algorithm. The final multivariate analysis was performed using Partial Least Squares (PLS) regression or Discriminant Analysis software from MATLAB® Central (USA) (http://www.mathworks.com/matlabcentral/fileexchange/30685-pls-regression-or-discriminant-analysis--with-leave-one-out-cross-validation-and-prediction/content/PL S/pls.m).

Results and Discussion

Bacterial Cultures under Stress Conditions

MICs Determination To select the concentration of each antibiotic that would allow the formation/isolation of high levels of persister cells, and since that process is generally favored upon exposure to high doses (high MIC equivalents) of bactericidal antibiotics [8], the MICs of each one of the two tested antibiotics were determined for both *S. aureus* and *R. erythropolis*. MICs of 0.59 and 0.39 $\mu g.mL^{-1}$ were obtained, respectively, for vancomycin and teicoplanin in the case of *S. aureus* whilst MICs of 1.17 and 1.56 $\mu g.mL^{-1}$ were obtained, respectively for vancomycin and teicoplanin in *R. erythropolis*.

Time-Dependent Killing The typical biphasic killing patterns [25] were observed for both bacteria and for each tested conditions, showing the survival of the subpopulation of persister cells (**Figure 1**). Nevertheless, different survived cells curves, depending on the tested antibiotic/concentration were observed, indicating physiological heterogeneity in terms of robustness or fitness within persister subpopulations. This behavior, observed even for antibiotics with identical mechanisms of action such as vancomycin and teicoplanin, suggests that the fraction of cells that survive antibiotic exposure is highly dependent on the specific antibiotic that is used. Similar results led, recently, Johnson and Levin, and later in the same year Hofsteenge *et al.*, to hypothesize that additional physiological changes/mechanisms, rather than only the entrance in the so called 'dormant state', are responsible for different persister phenotypes or levels of induced persistence [14, 15]. Both the percentage of survived cells and death rates (data not shown) reveal a higher tolerance, regardless of the tested antibiotic/concentration, for *R. erythropolis* when compared to that of *S. aureus*. This enhanced antibiotic tolerance observed might suggest the presence of a higher enrichment in persister cells, which might be, in turn, somehow related to its reduced duplication time (t_d = 0.85 h^{-1}) compared to that of *S. aureus* (t_d = 0.71 h^{-1}). According to Hofsteenge *et al.* [14], the different levels of antibiotic tolerance could result from distinct levels of induced persistence, which might vary from antibiotic to antibiotic. It should be noted that teicoplanin already proved to be partially more active than vancomycin against *S. aureus* and enterococci, suggesting an higher persistence induction capacity [26].

Cell Viability Analysis The percentage of cell viability decreased with the time, especially when the duplication time was reached (1.98 and 3.96 h). Nevertheless, the values obtained for the cell viability assay were higher than those obtained for the percentage of survived cells (CFUs) (**Figure 1**). Consider as an example the values obtained at 5 h: 99.65 %, 87.31 %, 100 % and 99.78 %, respectively for 50 $\mu g.mL^{-1}$ and 100 $\mu g.mL^{-1}$ vancomycin and 50 $\mu g.mL^{-1}$ and 100 $\mu g.mL^{-1}$ teicoplanin, obtained by the cell viability assay (data not shown); and 11.69 %, 2.05 %, 0.26 % and 0 % obtained by CFUs under the same conditions (**Figure 1**). These results can be easily explained by the fact that the bacterial viability kit used in the microscopy assay distinguishes viable (*i.e.*, cells with an intact membrane) from non-viable cells (*i.e.*, cells with a damaged membrane), whilst the CFU counts indicate the number of cells able to divide. Accordingly, the percentage of viable cells obtained by microscopy must always be high than that obtained by CFU counts, since after exposure a given cell might be able to maintain/restore its membrane integrity but not its reproductive capacity.

FTIR Analysis The registered wavelengths revealed the expected pattern for both bacteria, with the spectral bands characteristic of lipids, C–H, C=O and C–O–C bonds, normally appearing at 2960-2852 cm^{-1}, 1745-1715 cm^{-1}, and 1200-900 cm^{-1} (data not shown), respectively [27].

Furthermore, the spectral bands showed an increased intensity in the stationary phase, suggesting the expected equilibrium in the formation and degradation of the respective bonds within the bacterial lipids, opposing to the oscillatory behavior that is expected during the exponential phase of growth. A first examination of the PCA distribution results (data not shown) reveals a clear approximation of the different subpopulations with the culture age (from the exponential to the stationary phase of growth), as expected in the absence of the stress agent, and thus confirming the reversible phenotypic character of persister cells. However, it should be noted that for the highest concentration of antibiotics tested (100 $\mu g.mL^{-1}$) that approximation was not obtained during the time of the experiments, given the more drastic effects on the exposed populations. The initial blank, 'B. 0 h', showed always a distinct profile, resulting in a clear separation from the other data. Interestingly, a change in the composition of the exposed cells with the age was observed for both strains. During the exponential phase, regardless of the antibiotic tested, the separation of the data from cells exposed to a concentration of either 50 or 100 $\mu g.mL^{-1}$ was made along PC2 (x axis), while during the stationary phase that separation was made along PC3 (y axis). These curious results seem to suggest a change at the level of lipid composition and antibiotic response with the age of the culture, as the results from previous sections also indicate. Moreover, cells exposed to a concentration of 50 $\mu g.mL^{-1}$ were represented closer to the non-exposed cells, whilst a concentration of 100 $\mu g.mL^{-1}$ resulted in data further away from non-stressed cells, especially for teicoplanin. These data corroborate the previous observations at the level of *i.* percentage of survived cells (S*. aureus* and *R. erythropolis*)

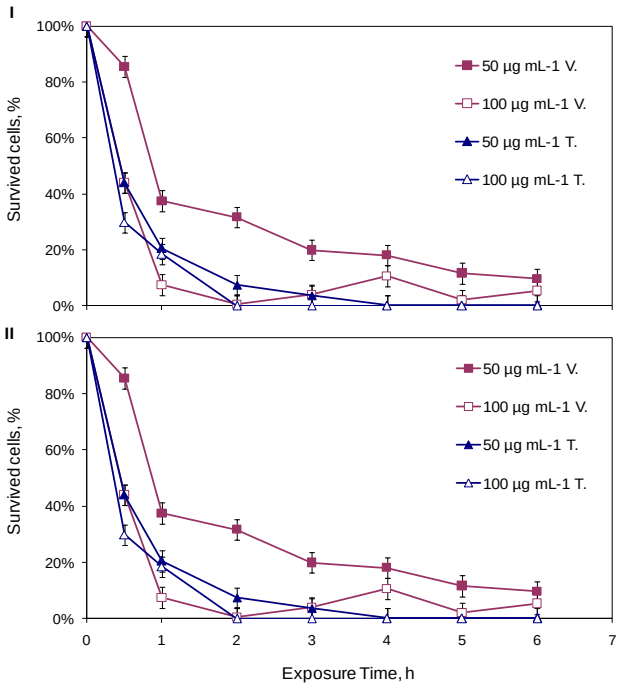

Figure 1 (I) and (II) – Percentages of *S. aureus* **(I)** and *R. erythropolis* **(II)** cells that survived during 6 h of exposure to high concentrations of vancomycin ('V.'; squares) or teicoplanin ('T.'; triangles). Error bars represent the respective standard deviation.

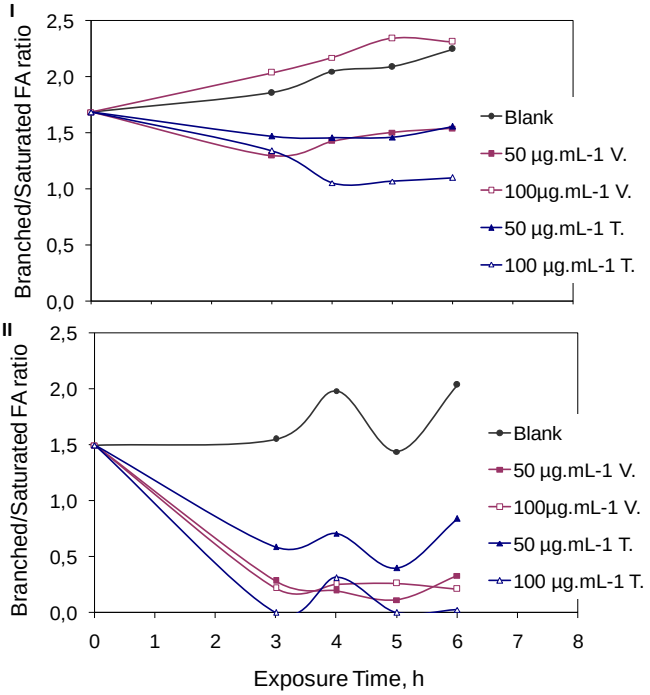

Figure 2 (I) and (II) – Effect of vancomycin ('V.'; squares) and teicoplanin ('T.'; triangles) concentration and time of exposure on the branched/saturated FA ratio of *S. aureus* **(I)** and *R. erythropolis* **(II)** cells. The 'Blank' (circles) curve represents non-exposed culture.

(**Figure 1**), and *ii.* cell viability analysis, upon *S. aureus* exposure (data not shown), suggesting a higher potency for teicoplanin when compared to vancomycin.

Lipid Analysis Non-stressed cells of both strains increased the percentage of branched FA while decreasing the percentage of saturated FA with culture age ('Blank' curve, **Figure 2**). During antibiotic exposure both *S. aureus* and *R. erythropolis* promoted a decrease in the branched/saturated FA ratio (**Figure 2 (I)** and **(II)**, respectively); except for *S. aureus* cells exposed to 100 $\mu g.mL^{-1}$ vancomycin. Moreover, most of the alterations occurred during the first 3 h of antibiotic exposure, indicating relatively fast mechanisms of response. These results strongly suggest that both bacteria respond to vancomycin and teicoplanin induced stress by promoting changes resulting in lower membrane fluidity, thereby confirming what has been generally reported in the presence of toxic organic compounds such as phenol, PAHs, organic solvents and other extreme environmental conditions [3-5]. Importantly, the oscillatory behavior observed reveal an oscillatory action of the antibiotics, which can be explained by the fact the tested antibiotics only act on dividing cells.

Zeta Potential Analysis An overall decrease in the ZP values of the cells was observed with both concentration and time of exposure for both bacterial species studied. A greater response was obtained upon exposure to 100 $\mu g.mL^{-1}$ teicoplanin, with membrane ZP decreasing from -14.8 to -25 mV after a total of 6 h of exposure in the case of *S. aureus* (**Figure 3 (I)**) and from -24.9 to -36.1 mV after the same period of exposure in the case of *R. erythropolis* (**Figure 3 (II)**). Similarly to what was observed at the level of lipid composition (**Figure 2**), the most significant changes occurred during the first 3 h of antibiotic exposure. The

observed increase in the cell wall negative net surface charge as a response mechanism against teicoplanin is expected, since this compound is negatively charged at neutral pH [28]. Therefore, such an increase in the negative character of the cells will result in the repulsion of teicoplanin. On the other hand, the same principle is not valid for the positively charged vancomycin [28], which would that way be attracted to a more negative membrane. However, the behavior observed for vancomycin exposure seems to corroborate the most recent advances on the subject [29, 30]. Moreover, and after equivalent results were obtained both at the levels of percentage of survived cells (**Figure 1**) and membrane FA composition (**Figure 2**) suggested the same, now the increased responses at the level of membrane ZP profile (relatively to those of *S. aureus*) point, as well, towards an enhanced antibiotic tolerance for *R. erythropolis*.

Oxygen Consumption Analysis The oxygen consumption curves at low antibiotic concentrations indicate, as expected and for both antibiotics, that the consume decreases with the increasing antibiotic concentrations (**Figure 4 (I)**; data not shown for teicoplanin). Moreover, three distinct profiles were observed (for 12.5 $\mu g.mL^{-1}$; and for concentrations below and above this value). At conditions lower than 12.5 $\mu g.mL^{-1}$, the cells consumed high amounts of oxygen during the first 10-15 h after which levels successively lowered, until no oxygen was consumed, similarly to what was observed in the absence of antibiotics ('Blank' curve, **Figure 4**). On the contrary, for concentrations higher than 12.5 $\mu g.mL^{-1}$ a distinct behavior was obtained, suggesting an immediate effect on cells' metabolic activity, at least at the level of oxygen consumption. For those concentrations, the cells decreased considerably their levels of oxygen requirements

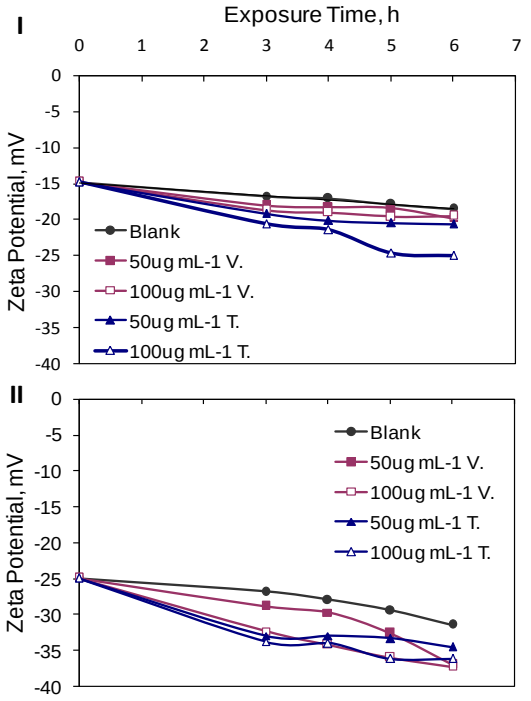

Figure 3 (I) and (II) – Effect of vancomycin ('V.'; squares) and teicoplanin ('T.'; triangles) concentration and time of exposure on the zeta potential of *S. aureus* **(I)** and *R. erythropolis* **(II)** cells. The 'Blank' (circles) curve represents non-exposed culture.

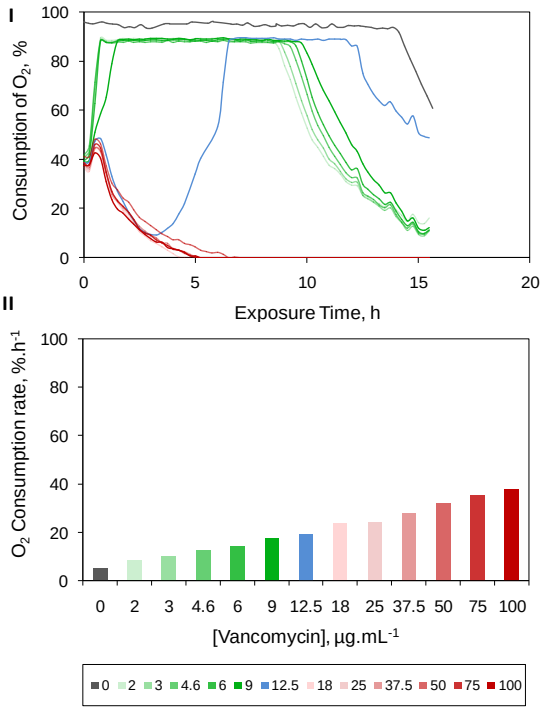

Figure 4 (I) and (II) – Effect of vancomycin ('V.') concentrations on *S. aureus* oxygen consumption **(I)** and oxygen consumption rates **(II)** (at low antibiotic concentration). The 'Blank' curves represent non-exposed cultures. Oxygen consumption rates **(II)** were calculated using the values within the interval of oxygen consumption decay **(I)**.

during the first hour, with the consumption ceasing before 5 h of exposure. Together, the results suggest that antibiotic concentrations as low as 12.5 $\mu g.mL^{-1}$ are enough to trigger changes with repercussion in the metabolic activity of the cells, as observed by oxygen consumption. The oxygen consumption rates increased with increasing antibiotic concentration (**Figure 4 (II)**; data not shown for *R. erythropolis*). When exposed to antibiotics, cells must switch-on a series of oxygen-requiring adaptive mechanisms to counteract the stress caused. Orman and Brynildsen have also shown that even cells in a non-growing state can be metabolically active [31]. The results ultimately indicate that, instead of entering in a 'dormant', inactive state, persister cells are, despite their expected non- or slow-growing phenotype, in a truly active response state. This has also been recently defended by other authors [12, 31].

Oxygen consumption by *S. aureus* cells in the presence of high antibiotic concentrations decreased with the increasing antibiotic concentration (**Figure 5**). After 2-3 h of exposure, the cells decreased the level of oxygen consumption, this time with the consume ceasing around 20 h of exposure. Nevertheless, and although the oxygen consumption decreased once more with the antibiotic concentration, the results reveal an overall increase relatively to that obtained at low antibiotic concentrations (**Figure 4 (I)**), suggesting an active response to such elevated concentrations. However, by looking at the decrease registered in the oxygen consumption rate (**Figure 6 (I)**), it seems more likely that the cells fail to respond to the increase in antibiotics concentration, especially for teicoplanin. These observations might be explained by the fact that, although increasing its metabolic activity, *S. aureus* is still unable to successfully

develop mechanisms that allow it to overcome the antibiotics action, resulting in the death of the large majority of its cells (**Figure 1 (I)**). For *R. erythropolis* the opposite evolution was observed: both the blank and challenged cells maintained the levels of oxygen consumption, for almost 30 h of exposure, at around 80-100% regardless of the tested antibiotic/concentration. Once again, the increased metabolic activity, *i.e.* increased levels of oxygen consumption, experienced by the non- or slow-growing persisters corroborates the recent results of Orman and Brynildsen [31]. This behavior is also in accordance with the results obtained *i.* at the level of percentage of survived cells (**Figure 1 (II)**); *ii.* for membrane FA composition (**Figure 2 (II)**); and *iii.* for cell net surface charge (**Figure 3 (II)**); which together strongly suggest an enhanced antibiotic tolerance for *R. erythropolis*. This tolerance is thus likely achieved thanks to an active metabolic activity (**Figure 5 (II) and Figure 6 (II)**).

Enzymatic Activity Analysis The metabolic activity of the cells was also accessed by comparison of the enzyme activity of 11 enzymes present in the API ZYM® kit (**Figure 7**). A first analysis of the obtained enzyme profiles reveals that, even upon exposure, the survivor, persister cells managed to maintain unchanged the activity of the majority of the prior active enzymes, corroborating the results obtained at the level of oxygen consumption (vide section *Oxygen Consumption Analysis*) and thus refuting the previous theories claiming that this class of cells enters a 'dormant', inactive state. However, a closer analysis shows that, after teicoplanin exposure, the activity of some enzymes such as esterase (C4) (E3), esterase lipase (C8) (E4), leucine, valine and cystine arylamidase (E6 to E8), trypsin (E9) and α-chymotrypsin (E10) were slightly reduced as a direct effect of the presence of the antibiotics. A reduced enzymatic activity was also observed for α-glucosidase (E16) after exposure to

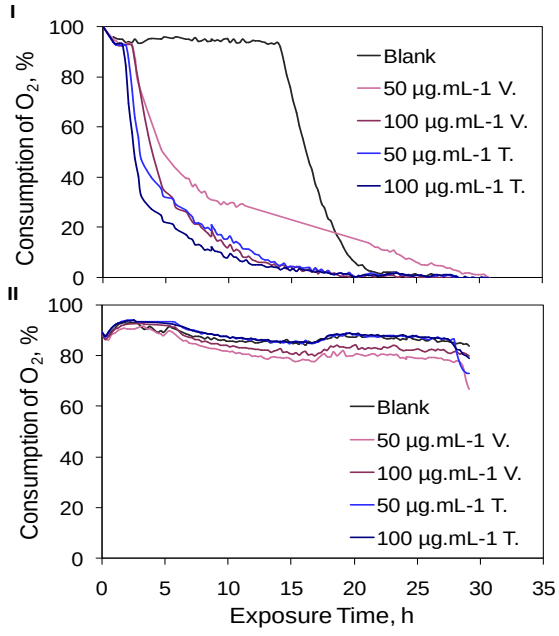

Figure 5 (I) and (II) – Effect of vancomycin ('V.') and teicoplanin ('T.') concentrations on *S. aureus* **(I)** and *R. erythropolis* **(II)** oxygen consumption (at high antibiotic concentration). The 'Blank' curves represent non-exposed cultures.

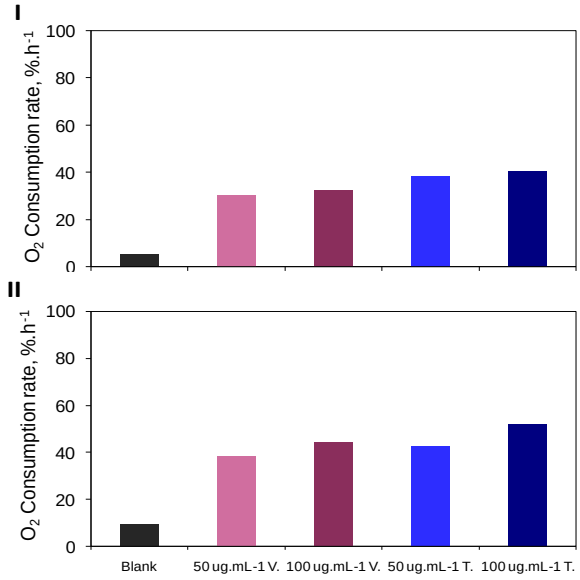

Figure 6 (I) and (II) – *S. aureus* **(I)** and *R. erythropolis* **(II)** oxygen consumption rate during vancomycin ('V.') and teicoplanin ('T.') exposure (at high antibiotic concentration). The 'Blank' bars represent non-exposed cultures.

vancomycin, with no considerable activity being reported in the case of teicoplanin. The specific function of each enzyme will be discussed in section *Enzymatic Activity Analysis* of the next chapter 'Bacterial Re-Growth in Fresh Medium (after Stress Conditions)'.

Bacterial Re-Growth in Fresh Medium (after Stress Conditions)

Repopulation of the Culture after Drug Removal According to Lechner *et al.* [10], persister tolerance is not transferred to the progeny, therefore, a similar behavior to that observed during normal cell growth is expected during the re-growth of the survivor persister subpopulation in fresh, drug-free medium. The curves represented in **Figure 8** show the expected pattern, described by a slow increase in biomass, rather than a fast increase (typical for resistant, mutant cells after acquiring resistance). Therefore, the small subpopulation of cells that managed to survive antibiotic exposure is, most likely, composed of persister cells. The cultures that were in contact with the antibiotic for a longer period of time presented longer *lag* phases (**Figure 1**). Here, the results obtained for *R. erythropolis* re-growth after 100 $\mu g.mL^{-1}$ teicoplanin exposure really stood out from the rest, with cells requiring almost four times the period of time needed for the re-growth of the same bacteria after 50 $\mu g.mL^{-1}$ exposure (**Figure 8 (II)**). Nevertheless, and for both bacteria, the cultures previously exposed to each antibiotic/concentration presented a similar (re-)growth to that of 'Blank', non-exposed cultures, thus confirming the persister nature of the subpopulation of survivor cells (**Figure 8**; data not shown for *S. aureus*).

Lipid Analysis The cells of *S. aureus* grown on fresh media presented FA compositions that depend on the concentration at which the previous population had been exposed to. For the lower, less effective concentrations (from 2 to 37.5

µg.mL^{-1}), the cells FA composition was similar to that of cells prior to exposure: high levels of branched FA and low levels of saturated FA. However, for the higher concentrations tested (75 and 100 µg.mL^{-1} in the case of teicoplanin and, additionally, 50 µg.mL^{-1} in the case of vancomycin), the cells presented different FA composition: the percentage of saturated straight FA increased, whilst the percentage of branched FA decreased (data not shown). A higher degree of saturated FA and a lower percentage of branched FA, should have resulted in less fluid membranes, thus these cells should be able to prevent the entrance of recalcitrant compounds to the cell. What is most significant is that these changes in lipid composition were transmitted to a non-stressed population by the parent cells. Contrarily to resistance mechanisms which are encoded in the DNA sequence, phenotypic modifications have a limited life time but the 'memory' of past conditions and stress-induced molecules may influence the future generations [32].

For the re-growth of both *S. aureus* (**Figure 9**) and *R. erythropolis* (data not shown) cells pre-exposed to high antibiotic concentrations, the changes registered in the percentages of each class of FA showed that the lipid composition of the cells presented high levels of branched FA and low levels of saturated FA, similar to non-exposed cells (data not shown). Furthermore, the harsher the previous conditions of exposure (*i.e.,* higher antibiotic concentration and/or longer period of exposure), the longer time the cells needed to return to its original composition during the re-growth. Nevertheless, after 24 h of re-growth, the cells generally acquired a composition identical to that of the initial non-exposed population.

Zeta Potential Analysis As for the FA composition of the cells, the changes observed at the net surface charge reveal an approximation, along time, to the prior to exposure values ('Blank', **Figure 10**). High antibiotic concentration and/or long periods of exposure resulted in longer times for the cells to

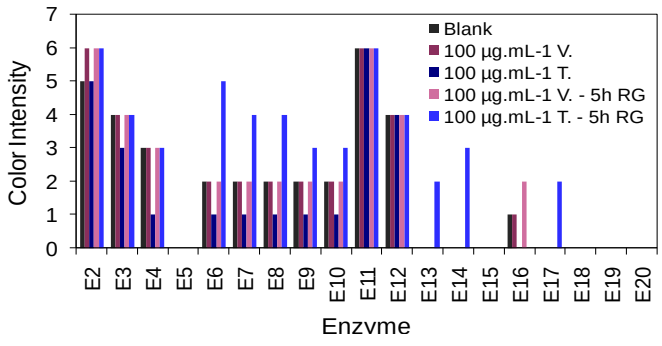

Figure 7 – Enzymatic activity of 14 enzymes in *S. aureus* prior and after 6 h of exposure to high vancomycin ('V.') or teicoplanin ('T.') concentrations. The 'Blank' bars represent non-exposed cultures. Color intensity: 0 (no activity) to 6 (strong activity). The horizontal axis corresponds to the following enzymes: E2, alkaline phosphatase; E3, esterase (C4)**;** E4, esterase lipase (C8)**;** E5, lipase (C14); E6, leucine arylamidase; E7, valine arylamidase; E8, cystine arylamidase; E9, trypsin; E10, α-chymotrypsin; E11, acid phosphatase; E12, Naphtol-AS-BI-phosphohydrolase; E1**3,** α-galactosidase; E14, β-galactosidase: E15, β-glucuronidase; E16, α-glucosidase; E17, β-glucosidase; E1**8,** N-acetyl-β-glucosaminidase; E19, α-mannosidase; E20, α-fucosidase.

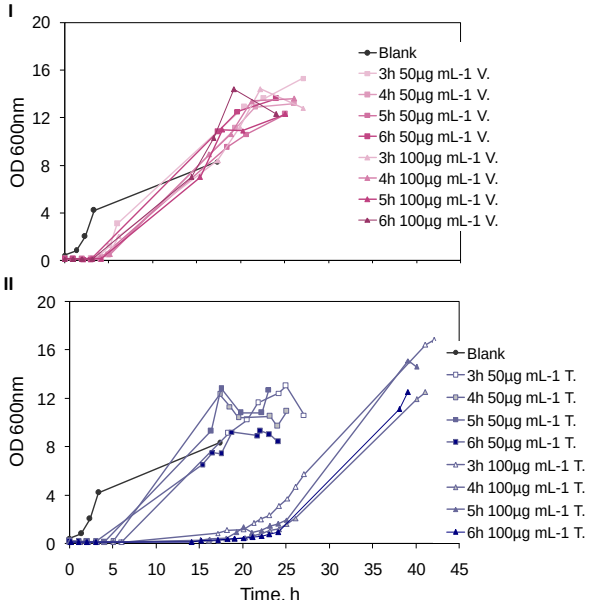

Figure 8 (I) and (II) – Growth in fresh, antibiotic-free medium of *R. erythropolis* cells that had survive exposure for 6 h to 50 (squares) and 100 µg.mL^{-1} (triangles) vancomycin ('V.') **(I)** or teicoplanin (' T.') **(II)**. The 'Blank' curves (circles) represent non-exposed cultures.

return to their initial ZP value during the re-growth. Nevertheless, after 24 h of re-growth cells generally acquired a ZP value (-22.8 and -24.4 mV for cells pre-exposed to 50 and 100 µg.mL^{-1} vancomycin, -21.0 and -20.2 mV for cells pre-exposed to 50 and 100 µg.mL^{-1} teicoplanin in the case of *S. aureus* (**Figure 10**); -25.8 and -25.8 mV for cells pre-exposed to 50 and 100 µg.mL^{-1} vancomycin, -30 and -29.7 mV for cells pre-exposed to 50 and 100 µg.mL^{-1} teicoplanin in the case of *R. erythropolis*; data not shown) similar to that of the initial, non-exposed populations (-20.0 and -29.5 mV, respectively for *S. aureus* and *R. erythropolis*).

Oxygen Consumption Analysis For *S. aureus* cultures pre-exposed to low antibiotic concentrations and grown in fresh, drug-free medium, similarly to what happened in the presence of antibiotics (**Figure 4**), three distinct behaviors can be observed. Here the results for *S. aureus* cells surviving low and high vancomycin concentrations are presented (**Figure 11**). For concentrations lower than 0.0125 µg.mL^{-1}, cells consumed high levels of oxygen (80-100%) for the first 15 h. After that period, cells likely entered the stationary phase, consuming successively less oxygen, until it ceased around 20-25 h of growth. A similar pattern was followed by the non-exposed, 'Blank' cultures, suggesting again that cells can easily tolerate these low antibiotic concentrations. A concentration of 0.0125 µg.mL^{-1} turned out to represent a transition concentration, revealing a behavior somehow between that observed for lower and higher concentrations; in the case of vancomycin exposure, the same was registered for a concentration of 0.018 µg.mL^{-1}. After 20-25 h of growth, cultures pre-exposed to 0.025 and 0.0375 µg.mL^{-1} vancomycin and 0.018 µg.mL^{-1} teicoplanin started to consume high levels of oxygen. This late consume likely points towards a reduced number of initial cells, due to the bactericidal action of the antibiotics tested, and thus a long *lag* phase was required. Furthermore, after pre-exposure to 0.025 µg.mL^{-1} vancomycin the cells were able to

restore their metabolic activity faster than their counterparts pre-exposed to a higher concentration of 0.0375 μg.mL^{-1}, stressing the direct relation between the antibiotic concentration and the bacterial metabolic activity (*i.e.* population re-growth). The results suggest that these *S. aureus* and *R. erythropolis* (data not shown) cells were able to restore the initial, prior to exposure metabolic activity after 15 h of growth in fresh, drug-free medium. Moreover, for the populations pre-exposed to concentrations higher than 0.0375 μg.mL^{-1} vancomycin and higher than 0.018 μg.mL^{-1} teicoplanin, oxygen consumption was never observed during the time of the experiments, supporting the results previously obtained at FA composition, and thus suggesting a higher bactericidal potency for teicoplanin.

For *S. aureus* cultures pre-exposed to high antibiotic concentrations and grown in fresh, drug-free medium, the same three-phase evolution was obtained (**Figure 11 (II)**). High consumption of oxygen was observed during the first 15 h for the cultures pre-exposed to 50 μg.mL^{-1} vancomycin whilst for the last 15 h high consumption was observed for the cells pre-exposed to 100 μg.mL^{-1} vancomycin (**Figure 11 (II)**). In the presence of high antibiotic concentrations, cells will likely increase their metabolic activity in order to develop an effective response. The results obtained during *S. aureus* growth in fresh, drug-free medium follow what was expected: when increasing the antibiotic concentration, cells will increase the oxygen consumed (in this case cells increased the time period during which they consumed high amounts of oxygen rather than the percentage of oxygen consumption), in an attempt to surpass stress action. Furthermore, the longer the time of pre-exposure, the faster cells increased their metabolic activity, suggesting the presence of regulated and sensitive response mechanism(s) against antibiotics action. In the case of previous exposure to teicoplanin, a similar behavior was obtained with the increasing antibiotic concentration and time of exposure (data not shown). However, the two distinct phases – a first one for cells pre-exposed to 50 μg.mL^{-1} teicoplanin and a second one for cells pre-exposed to 100 μg.mL^{-1} teicoplanin – were not observed. Probably due to the more drastic mechanism of action of teicoplanin, which require a more radical response, the cells experienced a faster increase in the metabolic activity. The most effective response mechanisms were obtained for *R. erythropolis* (data now shown). Regardless of the antibiotic/

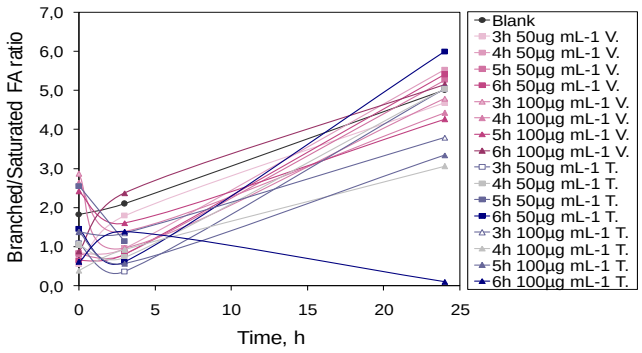

Figure 9 – Branched/saturated FA ratio of *S. aureus* grown in drug-freee medium inoculated with the cells exposed up to 6 h to 50 (squares) and 100 μg mL^{-1} (triangles) vancomycin ('V.') or teicoplanin ('T.'). The 'Blank' curves (circles) represent non-exposed cultures.

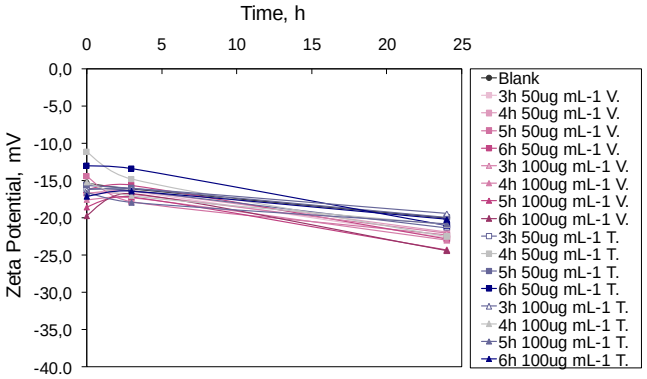

Figure 10 (I) and (II) – ZP values of *S. aureus* grown in drug-freee medium inoculated with the cells exposed up to 6 h to 50 (squares) and 100 μg.mL^{-1} (triangles) vancomycin ('V.') or teicoplanin ('T.'). The 'Blank' curves (circles) represent non-exposed cultures.

concentration tested, very high levels of oxygen consumption were registered (80-100%). Following what was previously observed in the presence of antibiotics (**Figure 5**), *R. erythropolis* cells managed to maintain the metabolic activity (*i.e.* high oxygen consumption) during the re-growth, again corroborating the recent results of Orman and Brynildsen [31]. Non- or slow-growing cells, such as persister cells, can actually be metabolically active.

Enzymatic Activity Analysis For both antibiotics, 5 h of re-growth in fresh medium were sufficient for the survivor cells not only to restore the enzymatic activities previously reduced during the exposure but also to overcome, in some cases the initial, prior to exposure activity (**Figure 7**). This is particularly visible for cells surviving teicoplanin exposure. A detailed analysis shows that this increase was more pronounced for the enzymes leucine, valine and cystine arylamidase; trypsin; α-chymotrypsin; α- and β-galactosidase; and α- and β-glucosidase. Interestingly, all of these enzymes represent hydrolases that lead, as a result of its activity, to the production and subsequently release of energy in the cell [33-35]. Moreover, the enzyme alkaline phosphatase, which is responsible for dephosphorylation reactions (*i.e.*, removal of phosphate groups) has been related (with hyaluronidase and coagulase), to a factor of pathogenicity in *S. aureus* [33]. Likewise, the activity of trypsin enzymes, also energy-producers enzymes, has also been implicated in the enhancement of *S. aureus* virulence as a direct effect of the presence of trypsin-cleavage products [34]. Curiously, the highest increases in activity were registered for the three arylamidase enzymes. Leucine, valine and cystine arylamidase catalyze the release of an N-terminal leucine, valine or cystine, respectively, from a wide range of peptides, amides and arylamides, producing acetyl-coA, a known 'high energy' compound essential for the processing of many energy producing reactions in the cell. Additionally, α- and β-galactosidase proved to be bacterial adaptive enzymes activated only in stress conditions [35]. Glucosidase enzymes are now known to degrade the glucose present in the MH broth starch, eventually leading to the release of energy in the cell. Moreover, β-glucosidase has been reported for some bacteria as one of the most thermostable enzymes identified up to now, often highly resistant to chemical denaturation and to degradation by

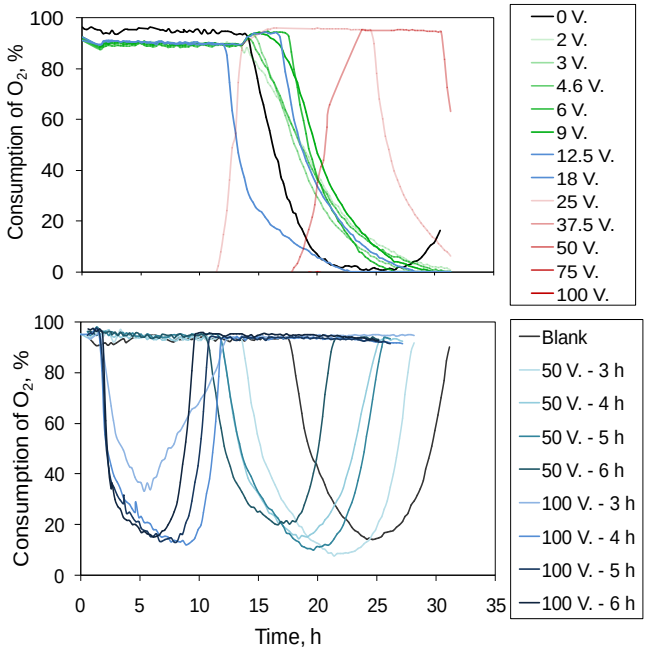

Figure 11 (I) and (II) – Effect of previous antibiotic concentration and time of exposure on *S. aureus* oxygen consumption during re-growth at low (I) and high (II) antibiotic concentration. *S. aureus* oxygen consumption was followed along time of re-growth after pre-exposure. 'Blank' curves represent the non-exposed cultures.

proteases [36]. Ultimately, the observed increase in the activity of these enzymes will increase the levels of energy in the cell. These results therefore clarify the observations made during antibiotic exposure as strictly temporary, proving wrong the theories defending persistence as a 'dormant' phenotype. Together with the evidences collected from the oxygen consumption analysis at high antibiotic concentrations for both *S. aureus* and *R. erythropolis* (**Figure 5** and **Figure 6**), these last data on *S. aureus* increased activities for several 'energizing' enzymes after antibiotics exposure strongly suggest an actual active state, adapted to successfully respond to the presence of stressful conditions (in this case, presence of antibiotics).

Conclusions

S. aureus and *R. erythropolis* cultures were successfully grown in MHB, reaching the corresponding exponential phase in less than 3 h, with duplication times of ca. 0.71 and 0.85 h^{-1}, respectively. To promote persister formation, the cells were exposed to concentrations of both vancomycin and teicoplanin much higher than the MIC. The biphasic killing kinetics observed in both bacteria upon exposure to the two tested antibiotics/concentrations suggest that the majority of the susceptible, regular cells were killed and indicate the survival of a small fraction of tolerant, persister cells in the antibiotic exposed populations. Moreover, and despite the observation of this biphasic profile, typical for persister cells enrichment, the distinct survival rates obtained for different antibiotics/concentrations further indicates physiological heterogeneity in terms of robustness or fitness within persister subpopulations. Importantly, these different persister phenotypes (or levels of induced persistence) support that the small fraction of cells that survive antibiotic exposure is highly dependent on the specific antibiotic that is used, as recently hypothesized by others.

Relatively to the effect of antibiotics on the FA composition of the cells, both strains responded to antibiotic stress by increasing the degree of saturation of the FA of the membrane phospholipids. Contrarily, non-stressed cells increased their levels of branched FA while decreasing the percentage of saturated FA with the age of the culture. The exposed cells thereby responded to the presence of antibiotics at the lipid level as bacterial cells usual respond to a number of toxic organic compounds: by decreasing the fluidity of the cellular membrane. In the case of the net surface charge, values decreased with both time of exposure and antibiotics concentration. The more negative character of the cell surface could contribute to a repulsion mechanism towards teicoplanin but should have the opposite effect towards vancomycin. Nevertheless, an increased positive cell-wall charge as response to glycopeptides has only been observed for immediate and resistant variants of *S. aureus* [30].

Since the most significant alterations in FA composition and ZP values occurred during the first 3 h of antibiotic exposure, the original population should contain persister cells even before stress exposure rather as has been suggested by population dynamics studies. Notably, throughout the entire set of experiments a higher tolerance was obtained for *R. erythropolis* when compared to *S. aureus*. The higher tolerance that this bacterium has evolved towards a more effective adaptability to stress conditions, developing enhanced mechanisms of response (as shown by a large number of published studies). This could result from a higher enrichment in persister cells prior to antibiotic exposure relatively to that of *S. aureus* (as suggested by the higher survival rate during antibiotic exposure). Following these observations, the results obtained during the growth in drug-free medium of the cells which had survived exposure to high concentrations of antibiotics confirm persister cells as reversible phenotypic variants of regular cells which allow bacteria to survive a temporary environmental stress.

Finally, the levels of oxygen consumption observed upon antibiotic exposure, and the enhanced activity of enzymes related to energy production, suggest an active character of persister cells instead of the usually suggested 'dormant' state. Persister cells apparently promote the most effective strategy of stress response, refuting previous hypotheses that their survival is based on 'static' response, deriving only from their nature.

Further work should involve: the study of bacterial response after exposure to other classes of antibiotics; and the analysis of the effect of antibiotic exposure on the action of efflux pumps and the influence of those efflux pumps in persister cells mechanism(s) of formation.

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