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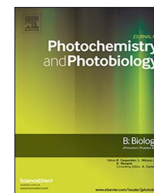

Evaluation of membrane fluidity of multidrug-resistant isolates of *Escherichia coli* and *Staphylococcus aureus* in presence and absence of antibiotics

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A B S T R A C T

In the face of the serious problem of antimicrobial resistance and the global dissemination of multidrug-resistant (MDR) bacteria, it is relevant to deeply study such bacteria both genetically and phenotypically. It is well known that bacteria have the ability to modify the biophysical properties of their cytoplasmic membranes, namely fluidity, in order to survive and thrive in hostile environments. The aim of this study was to assess and compare the membrane fluidity among multidrug-resistant (MDR) isolates of *Escherichia coli* and *Staphylococcus aureus* in absence and in presence of antibiotics (ceftazidime or ciprofloxacin). The membrane fluidity was monitored at 24-h intervals up to three days and at the sixth day, by measuring the anisotropy of 1,6-diphenyl-1,3,5-hexatriene (DPH) and the generalized polarization (GP) of Laurdan (6-dodecanoyl-2-dimethylaminonaphthalene). The anisotropy values as well as the Laurdan excitation GP (GP_{exc}) values obtained from all three *E. coli* strains (two MDR isolates and one susceptible reference strain) were quite similar and indicative of a very alike membrane phospholipid composition, regardless harboring or not resistance to multiple antimicrobials. Nonetheless, in the case of *S. aureus*, the anisotropy values were more increased in methicillin-resistant *S. aureus* (MRSA) isolates in comparison to the reference strain, meaning they have a less fluid membrane. Equally, GP_{exc} values were statistically different among the three *S. aureus* strains and showed that the two MRSA isolates had more rigid membranes than the susceptible strain. The exposition of MDR isolates of *S. aureus* to subinhibitory concentrations of ciprofloxacin did not affect neither the anisotropy values nor the GP_{exc} values, therefore, not altering membrane fluidity. Membrane integrity, assessed by the Live/Dead staining, of all strains and conditions studied was maintained over the six days. Thus, these preliminary studies on membrane properties of MDR isolates demonstrate that i) MRSA seem to have a more rigid membrane that susceptible *S. aureus* and ii) the presence of subinhibitory concentrations of antibiotics does not significantly alter the membrane fluidity of *S. aureus*, regardless being MDR or susceptible, but slightly affect the membrane fluidity of *E. coli*.

1. Introduction

The development of resistance is a natural process, a consequence of the vast genetic plasticity of bacterial pathogens, which allows mutational adaptations, acquisition of genetic material or alteration of gene expression [1]. The fact that bacteria have been continuously challenged by antimicrobial agents, has accelerated, however, the phenomenon of antimicrobial resistance. Resistance to one antimicrobial class can usually be achieved through a combination of different resistance mechanisms. In recent years, the prevalence of bacteria resistant to multiple antibiotics has been steadily increasing worldwide [2,3].

Most antibiotics have intracellular bacterial targets, meaning that they need to cross the cell envelope, a complex multilayered structure that has fundamental differences allowing the distinction of two groups of bacteria, the Gram-positives and the Gram-negatives [4]. Gram-negative bacteria possess an outer membrane, whose permeability largely affects the uptake of antimicrobial agents. β-Lactams, tetracyclines and some fluoroquinolones are hydrophilic molecules that are particularly affected by changes in permeability of the outer membrane since they often use porins to cross this barrier [1]. Besides permeability, cytoplasmic membrane fluidity also influences the ability of most compounds (nutrients and antibiotics) and ions to cross the bacterial cytoplasmic membrane (present in both Gram-negative and Gram-positive

Abbreviations: CAZ, ceftazidime; CPX, ciprofloxacin; DPH, 1,6-diphenyl-1,3,5-hexatriene; GP_{exc}, excitation generalized polarization; Lo, liquid ordered; Ld, liquid disordered; MDR, multidrug-resistant; MIC, minimum inhibitory concentration; MRSA, methicillin-resistant *S. aureus*; NB, nutrient broth

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bacteria) by diffusion and active transport [5–7]. Bacteria have the ability to adjust membrane lipid composition and, thus, to control membrane homeostasis when they are subjected to stressful environmental changes [7,8]. Alteration in lipid composition can be reflected in changes in the overall membrane fluidity [9].

Membrane fluidity can be measured using fluorescent probes to estimate membrane anisotropy and polarization values. The fluorescent probe 1,6-diphenyl-1,3,5-hexatriene (DPH) has been largely used in membrane anisotropy and membrane polarization assays, since it inserts itself into the bilayer core and aligns with the lipophilic tails of phospholipids in the cytoplasmic membrane [5,10]. At low concentrations, DPH preserves the morphology of membrane structures allowing *in vivo* measurements of stress responses and interpretation of cell membrane physiology [6]. Similarly, Laurdan (6-dodecanoyl-2-dimethylaminonaphthalene) is another fluorescent molecule that detects changes in membrane phase properties through its sensitivity to the polarity of its immediate environment. Polarity changes are detected by shifts in the Laurdan emission spectrum, and the Generalized Polarization (GP) function can be defined as a way of measuring wavelength displacements [11]. Changes in membrane fluidity had been traditionally described as the result of gel to liquid-crystalline phase transitions [10]. However, recently, the classical terms of gel and liquid crystalline phases used to describe the membrane have been replaced by liquid ordered (Lo) and disordered (Ld) phases, composed of saturated and unsaturated lipids, respectively [11,12].

Thermal and osmotic changes have been disclosed as stressors that can affect biophysical properties as well as the composition of the membrane, and consequently transport mechanisms (permeability) and cell shape and integrity, with subsequent adverse effects on proliferation and survival [7,10]. However, there is a paucity of information concerning the influence of subinhibitory concentrations of antibiotics on cytoplasmic membrane biophysical properties, namely membrane fluidity, especially of multidrug-resistant bacteria. Equally, to the best of our knowledge, membrane biophysical properties have not been compared between multidrug-resistant (MDR) isolates and susceptible strains.

Thus, the aim of this study was to evaluate biophysical properties of MDR *Escherichia coli* and *Staphylococcus aureus* throughout time (up to six days of incubation) in the absence and presence of antibiotics. Two antibiotics were used: ciprofloxacin, a fluoroquinolone, and ceftazidime, a third-generation cephalosporin. Cytoplasmic membrane fluidity was assessed using fluorescent probes, DPH and Laurdan, to estimate membrane anisotropy and GP values, respectively. Additionally, membrane integrity was assessed by fluorescence microscopy after Live/Dead staining.

2. Materials and Methods

2.1. Antibiotics and Fluorescent Probes

Both antibiotics used, ciprofloxacin (CPX) and ceftazidime (CAZ), as well as the fluorescent probes, DPH and Laurdan, were all purchased from Sigma-Aldrich (St. Louis, Missouri, USA).

2.2. Bacterial Strains and Growth Conditions

The bacterial strains used is this study were: *E. coli* ATCC 25922, *S. aureus* ATCC 25923, two MDR clinical isolates of *E. coli*, EC2 and EC3, and two methicillin-resistant *S. aureus* (MRSA) clinical isolates, Sa1 and Sa3.

Bacterial cells were grown in Nutrient Broth (NB – Liofilchem s.r.l., Roseto degli Abruzzi, Italy). For each experiment, 50 mL of NB were inoculated with exponentially growing cells in order to obtain a cell suspension with an optical density at 600 nm (OD_{600}) of 0.1. Strains were also used to inoculate NB containing sub-minimum inhibitory concentrations (sub-MICs) of antibiotics, specifically 1/2× MIC of CPX or CAZ, in the case of *E. coli* strains, or 1/2× MIC of CPX, in the case of *S. aureus* strains. The corresponding MIC values are shown in the Supplementary material (Table S1). All inocula were incubated at 37 °C with shaking (100 rpm) in a B.Braun Certomat WR shaking water bath (BBI Biotech, Berlin, Germany) for up to 144 h (six days).

2.3. Sample Preparation for Membrane Fluidity Studies

At 24-h intervals (24 h, 48 h, 72 h) and after 144 h, samples were taken to measure OD_{600} (these values were used to construct growth curves), and subsequently diluted in NB to obtain an OD_{600} of 0.4 [5,13]. Then, aliquots of 1.5 mL were centrifuged at 10000 rpm for 8 min, washed twice in 15 mM Tris-HCl buffer (pH 7.4), and resuspended in 10 μM of DPH (1.02 mM stock solution in chloroform) or in 0.5 μM of Laurdan (0.2 mM stock solution in dimethylformamide). The concentrations of the organic solvents in the suspensions were low enough to avoid altering the biological samples. Each suspension was incubated in the dark, at 37 °C with shaking (500 rpm) for 1.5 h. A 1-mL aliquot of unlabeled and labeled samples was transferred to a 1-cm quartz cuvette in order to measure fluorescence anisotropy of DPH as well as to obtain Laurdan emission spectra. A Varian Cary Eclipse fluorescence spectrofluorometer (Agilent Technologies, Santa Clara, California, USA) equipped with a temperature controller and a magnetic cuvette stirrer was used. The temperature was set at 37.0 ± 0.1 °C.

2.4. Anisotropy Measurements

For determining fluorescence anisotropy of DPH-labeled bacteria, excitation and emission wavelengths were set at 358 and 428 nm, respectively. Slit widths were 10 nm for both excitation and emission. Data were recorded using the Cary Eclipse Software. Blank samples (with bacteria but no fluorescent probe added) were recorded to account for light scattering. For each condition, at least three independent measurements were performed, each in four replicate readings.

2.5. Laurdan GP Measurements

Emission spectra of Laurdan-labeled bacteria were obtained at an excitation wavelength of 350 nm using emission wavelengths from 360 to 600 nm. Blank samples were also used and their emission spectra were subtracted to the respective sample spectrum. The excitation GP (GP_{exc}) was calculated using the emission spectrum at an excitation wavelength of 350 nm: $GP_{exc} = \frac{I_{440} - I_{490}}{I_{440} + I_{490}}$. In the equation, I_{440} and I_{490} indicate fluorescence intensities at 440 and 490 nm, respectively [11].

2.6. Assessment of Membrane Integrity

Samples were taken at the same time points mentioned above and diluted to an $OD_{600} = 0.4$. One mL aliquots of these cell suspensions were washed twice in PBS (pH 7.4), and resuspended in 500 μL of the appropriate mixture 1:1 of SYTO 9 and propidium iodide stains, followed by 30 min of incubation at room temperature in the dark, as indicated by the manufacturer's instructions (Live/Dead BactLight bacterial viability kit -Molecular probes, Thermo Fisher Scientific). 5 μL of the stained suspensions were immediately examined using a fluorescence microscope (Zeiss Axio Imager Z1 Apotome).

2.7. E. coli Liposome Preparation and Laurdan GP Measurements

Escherichia coli liposomes were prepared using *E. coli* total lipid extract (Avanti Polar Lipids, Alabaster, Alabama, USA) as previously described [14]. The only modification regarded the buffer used to redisperse the lipidic film, in this study 15 mM Tris-HCl (pH 7.4) was used instead of HEPES buffer. The suspensions of liposomes obtained (large

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