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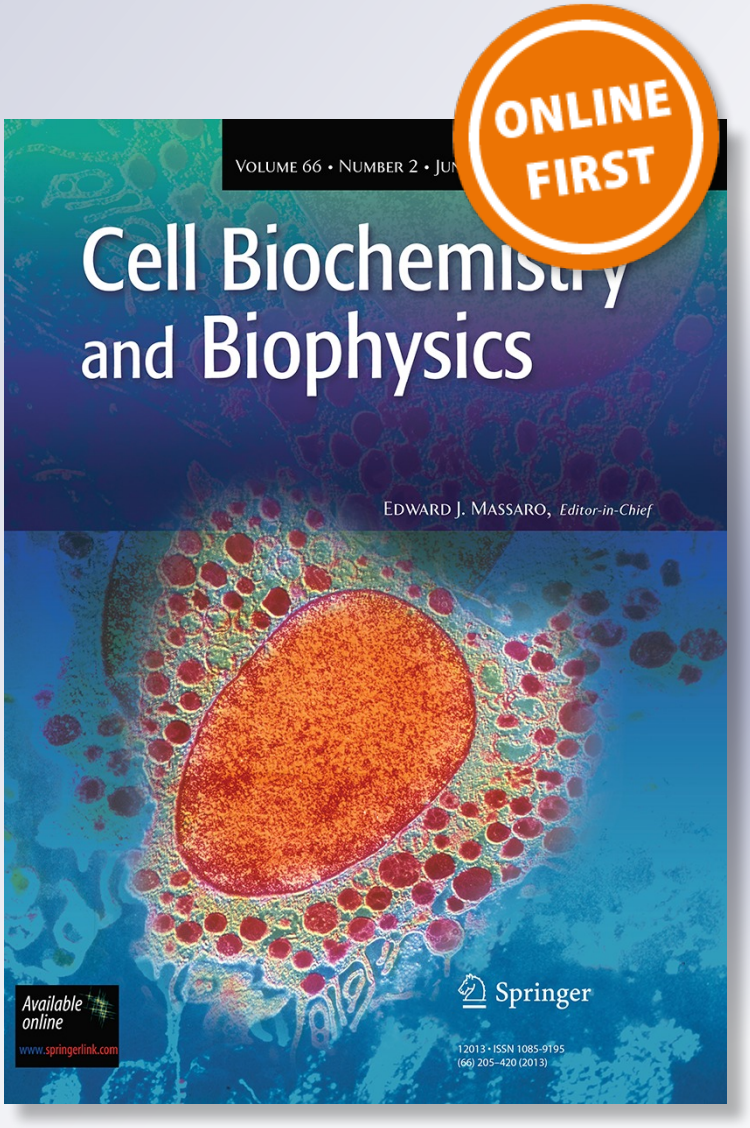

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ORIGINAL PAPER

Macromolecular Oxidation in Planktonic Population and Biofilms of *Proteus mirabilis* Exposed to Ciprofloxacin

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Abstract Diverse chemical and physical agents can alter cellular functions associated with the oxidative metabolism, thus stimulating the production of reactive oxygen species (ROS). Proteins and lipids may be important targets of oxidation, and this may alter their functions in planktonic bacterial physiology. However, more research is necessary to determine the precise role of cellular stress and macromolecular oxidation in biofilms. The present study was designed to evaluate whether ciprofloxacin (CIP) could oxidize the lipids to malondialdehyde (MDA) and the proteins to carbonyl residues and to advanced oxidation protein products (AOPP) in planktonic populations and biofilms of *Proteus mirabilis*. Incubation with CIP generated an increase of lipid and protein oxidation in planktonic cells, with a greater effect found in sensitive strains than resistant ones. Biofilms showed higher basal levels of oxidized macromolecules than planktonic bacteria, but there was no significant enhancement of MDA, carbonyl, or AOPP with antibiotic. The results described in this article show the high basal levels of MDA, carbonyls, and AOPP, with aging and loss of proliferation of biofilms cells. The low response to the oxidative stress generated by CIP in biofilms helps to clarify the resistance to antibiotics of *P. mirabilis* when adhered to surfaces.

Keywords *Proteus mirabilis* · Ciprofloxacin · Planktonic population · Biofilms · Protein oxidation · Lipid oxidation

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Introduction

The aim of this work was to investigate changes in biofilms of *Proteus mirabilis* at level of generations of malondialdehyde (MDA), carbonyls residues, and advanced oxidation protein products (AOPP) due to the action of ciprofloxacin (CIP). In addition, this study postulates that a better understanding of reactive oxygen species (ROS) regulation and the consequent macromolecular oxidation in biofilms could help to clarify the resistance of adherent bacteria to antibiotics. The injury by oxidation of macromolecules may be one of the mechanisms of action of CIP. This might include modifications of lipid and protein membrane, which could lead to structural changes by increasing the noxious action described for CIP.

Oxidative stress results from an imbalance between the production of reactive species and antioxidant defenses; and can be defined as a disturbance in the pro-oxidant–antioxidant balance in favor of the former, thus leading to possible damage [1]. Diverse chemical and physical agents can alter the cellular functions associated with the oxidative metabolism, thereby stimulating the production of ROS. In vivo and in vitro studies have related toxicity in prokaryotic cells to the generation of ROS, including superoxide, hydrogen peroxide, the extremely reactive hydroxyl radical, peroxyl radical, and singlet oxygen [2, 3]. These species are able to oxidize proteins and lipids, resulting in the formation of carbonyl groups in some amino acid residues such as AOPP and MDA as a result of lipid peroxidation.

The most frequently used biomarker of protein damage is the carbonyl assay. Although carbonyls are relatively difficult to induce compared to other oxidative modifications, once present they are irreversible alterations [4] which have been successfully employed to measure oxidative stress in tissue, cell, and protein samples [5].

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As proteins are critical targets for oxidants, also a spectrophotometric assay has been previously developed to detect AOPP, which correspond to several chromophores that include dityrosine and carbonyls, among others [6].

Free radicals are able to attack the polyunsaturated fatty acids in membranes directly, and thereby initiate lipid peroxidation. The primary effect of this is a decrease in the membrane fluidity, which consequently alters the membrane properties and can significantly disrupt membrane-bound proteins. This can also act as an amplifier, with more radicals being formed and polyunsaturated fatty acids degraded into a variety of products. Among the many different aldehydes resulting from lipid peroxidation, the most intensively studied has been MDA, and it is now accepted that this is produced endogenously through the process of lipid peroxidation, which then acts as a mutagen in bacteria cells [7, 8].

Bacterial biofilms infections are particularly problematic because sessile bacteria can often withstand the host immune responses and are generally much more tolerant to antibiotics, biocides, and hydrodynamic shear forces than their planktonic counterparts [9]. It was observed that *P. mirabilis* has the greatest ability to attach to catheters out of all the Gram-negative organisms [10] and thereby establish and maintain infections of the urinary tract and lead to colonization of catheters related to this *Proteus* species can adapt to the catheterized urinary tract and produce an arsenal of strictly regulated virulence factors [11].

Although macromolecular oxidation has been studied in planktonic bacterial physiology using several antimicrobials, there is still limited information available, and more research is necessary to determine the precise role of cellular stress and the subsequent oxidation of macromolecules in biofilms. The present study was designed to investigate macromolecule oxidative damage, with the aim of obtaining a better understanding of the effects of the oxidative stress generated by CIP in planktonic bacteria and in biofilms of *P. mirabilis.* This study postulates that the reduction in the level of ROS and macromolecular oxidation with CIP in biofilms contributes to the antibiotic resistance of adherent bacteria.

Materials and Methods

Bacterial Strains, Culture Conditions, and Minimum Inhibitory Concentration (MIC) Assay

Proteus mirabilis 1 and 2 isolates from human infection were obtained from Hospital Tránsito Cáceres de Allende, Córdoba, Argentina. Ciprofloxacin resistant variant strains (CRV) 1X, 1Y, 2X, and 2Y were prepared in our laboratory as described by Aiassa et al. [2]. All strains were cultivated at 37 °C in trypticase soy agar (TSA) and maintained in this medium.

The MIC was determined according to the norms of the Clinical and Laboratory Standards Institute (CLSI) [12], using the standard tube dilution method in Mueller–Hinton broth.

Quantification of Biofilms Formation by the Use of Crystal Violet (CV)

The biofilms-forming ability of the strains was measured by determination of the adhesion to 96-well plates. The assay for biofilms formation used for this study was adapted from the method of O'Toole and Kolter [13], which is based on the ability of bacteria to form biofilms on solid surfaces and uses CV to stain the biofilms. Briefly, a final cell concentration of approximately 1×10^9 CFU/ml was mixed with 200 µl of trypticase soy broth (TSB) in each well of flat-bottomed microtiter plates (96-well, Greiner Bio-One, Germany), and this solution was incubated without agitation at 37 °C. The supernatant was then discarded and the plate was rinsed with phosphate-buffered solution (PBS, pH 7.2). After drying, staining for adherent biofilms was performed using CV (1 %), which was then was removed and cells were rinsed three times with 300 µl PBS before drying for 24 h at room temperature. A quantitative assessment of the biofilms formation was obtained by extracting the CV with 200 µl per well of the bleaching solution: ethanol/glacial acetone (70:30). The intensity of the coloration was determined at 595 nm using a microplate reader (Model 680 BioRad, Hercules, CA). All strains were tested in three independent experiments on different days and the average OD_{595nm} value was determined from three replicates and interpreted using the following scale: positive (>0.24), weak (>0.12 and <0.24), or negative (<0.12) [14].

Biofilms Preparation for MDA, Carbonyl Residue, and AOPP Determination

Overnight planktonic cultures of *P. mirabilis* were diluted to an OD_{600} of 0.2 in PBS. Then, 50 µl of culture dilution were added to Millipore membrane filters (diameter 25 mm, pore size 0.2 µm) resting on TSA, and incubated for 24 h at 37 °C [15]. The biofilms were then transferred to antibiotic containing agar (4 µg/ml of CIP), and the agar plates were incubated at 37 °C for 2 h. The membrane supported biofilms were placed in 2.0 ml of PBS, and the mixture was vortexed at high speed for 1 min with a FBR Vortex mixer (by Decalab) before performing assays of MDA, carbonyls, and AOPP determination.

Lipid Peroxidation

Proteus mirabilis planktonic and biofilms were incubated with PBS (control) or 4 μg/ml of CIP at 37 °C. These incubations were stopped after 2 h by employing 1 ml of trichloroacetic acid (TCA) 35 % (p/v) in the absence of light. After 20 min, 1 ml of 0.5 % (p/v) thiobarbituric acid (TBA) was added and the samples were heated to 80 °C for 30 min. An ice bath was then used to cool the samples, which were then centrifuged at 1,500×*g* and the absorbance of the supernatant was determined at 535 nm. A calibration curve of MDA solutions was applied to estimate the lipid oxidation, with MDA levels being expressed per mg of protein (nmol MDA/mg protein) [16].

Carbonyl Group Determination

Proteus mirabilis planktonic and biofilms were incubated with PBS (control) or 4 μg/ml of CIP for 2 h at 37 °C. Then, 1 ml of the samples was incubated with 1 ml of 0.1 % 2,4-dinitro-phenylhydrazine solution in HCl 2 M for 60 min. The proteins were precipitated from the solution by the use of 0.25 ml trichloroacetic acid 0.5 %, and the protein pellet was washed three times with ethanol and ethyl acetate (1:1) before being suspended in 1 ml of 6 M guanidine at 37 °C for 30 min. The carbonyl content was determined by spectrophotometry from the absorbance at 364 nm (molar absorption coefficient, 22 mM^{-1} cm^{-1}) [5].

Advanced Oxidation Protein Product (AOPP) Determination

Planktonic and biofilms of *P. mirabilis* were incubated with PBS (control) or 4 μg/ml of CIP for 2 h at 37 °C. 1 ml of the samples was removed, and 50 μl of IK (1.16 M) and 100 μl of acetic acid were added. The final product of the reaction was read at 340 nm, with chloramine-T (50 μM) being used as the standard. The concentrations of AOPP were expressed as chloramine-T milliequivalents per mg of proteins (meq chloramine T/mg protein) [17].

Protein Determination

The quantity of protein in the bacterial suspensions was determined by the Folin–Ciocalteu assay [18].

Statistical Analysis

All experiments were performed in triplicate, and numerical data are presented as means with error bars representing standard deviations (SDs). Differences between means were assessed with a P value < 0.05 being considered statistically significant.

Results

Determination of the Susceptibility to CIP and Biofilms Quantification

Proteus mirabilis 1 and 2 exhibited sensitivity to CIP with MICs of 0.125 and 2 μg/ml, respectively, whereas CRV 1X, 1Y, 2X, and 2Y were obtained with MICs of 16, 4, 8, and 4 μg/ml, respectively.

A quantitative analysis showed that the *P. mirabilis* cells attached to 96-well plates exhibited good biofilms formation according to the scale described in the "Materials and Methods" section, since the average OD_{595nm} value determined from three replicates was >0.24 (Fig. 1). Moreover, no significant differences were observed in the ability to form biofilms among the six strains under study.

Lipid Peroxidation

The oxidation of the lipids was evaluated by means of MDA formation, in the presence and absence of CIP in the planktonic bacterium and biofilms of *P. mirabilis*. Incubation with CIP revealed different effects on planktonic cells and biofilms lipid oxidation.

The sensitive planktonic parental strains 1 and 2 experienced a rise in lipid oxidation with increases of 37.5 and 17.9 %, respectively over the control values after 2 h of incubation with 4 μg/ml of CIP, whereas CRV did not demonstrate increased MDA levels with antibiotic. Next, it was investigated whether lipid peroxidation occurred in biofilms, with MDA being determined for the purpose of comparing this behavior with planktonic cells. In biofilms, there was no rise in MDA with respect to the control level after 2 h of incubation with CIP in sensitive parental sensible strains 1 and 2. However, the lipid oxidation in basal conditions was significantly higher than that

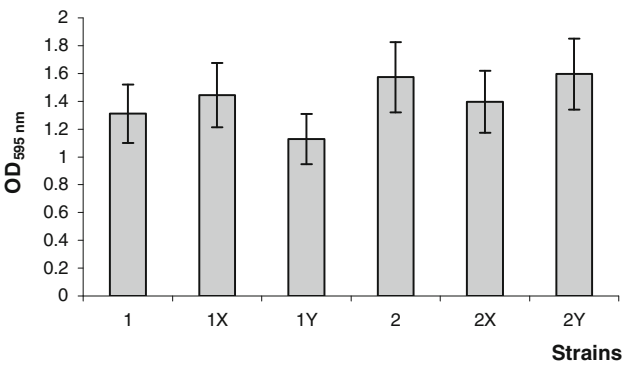

Fig. 1 Quantification of biofilm formation by the use of crystal violet (CV) in the six strains of *P. mirabilis* under study

observed in the planktonic state or after incubation with CIP (Fig. 2).

Carbonyl Assays for Determination of Oxidative Modified Proteins

The carbonyl residues were increased after 2 h of incubation with CIP respect to the control in five of the six planktonic strains of *P. mirabilis*, with the sensitive strains (1 and 2) showing the greatest increases of 70.2 and 40.5 %, respectively (Table 1).

We also investigated the protein oxidation to carbonyl residues in biofilms in order to compare this behavior to the planktonic bacterium. As in the case of lipid oxidation the carbonyl concentration did not change significantly in biofilms after incubation with CIP. However the carbonyls in biofilms with or without CIP were significantly greater than those in planktonic bacterium.

Comparison of AOPP Levels in Planktonic and Biofilmic *P. mirabilis*

In planktonic *P. mirabilis*, the AOPP were increased by CIP action at 2 h of incubation in sensible and CRVs strains. However, as shown Fig. 3 this rise was not evident in any strains while they were forming biofilms. When AOPP levels were compared between biofilms and planktonic cells, it was observed that biofilms had far higher values of AOPP than their planktonic counterparts, since the highest value reached by the planktonic cells was only 104.3 meq chloramine T/mg protein (strain 1) in presence of CIP whereas the maximum value attained by biofilms was 740 meq chloramine T/mg protein (strain 2X).

Table 1 Comparison of carbonyl residues in planktonic and biofilms of *P. mirabilis* in the absence and presence of CIP 4 μg/ml 2 h

Strain	Conditions	mM carbonyls/mg protein	
		Planktonic	Biofilms
1	Control	4.59 ± 0.45	6.03 ± 0.75*
	4 μg/ml CIP	7.81 ± 0.56$^{\#}$	6.43 ± 0.92
1X	Control	3.79 ± 0.19	5.91 ± 0.74*
	4 μg/ml CIP	5.73 ± 0.34$^{\#}$	6.64 ± 0.14*
1Y	Control	3.99 ± 0.31	6.32 ± 1.22*
	4 μg/ml CIP	5.50 ± 0.55$^{\#}$	6.37 ± 1.25*
2	Control	4.88 ± 0.39	9.67 ± 1.83*
	4 μg/ml CIP	7.20 ± 0.36$^{\#}$	6.25 ± 0.41
2X	Control	3.58 ± 0.39	10.59 ± 2.05*
	4 μg/ml CIP	4.80 ± 0.29$^{\#}$	7.77 ± 0.34*
2Y	Control	2.05 ± 0.06	11.52 ± 1.88*
	4 μg/ml CIP	2.39 ± 0.09	9.36 ± 2.45*

Data represent the mean ± SD of three independent trials. $^{\#}$ $P < 0.05$, respect to control without CIP. * $P < 0.05$, biofilms respect to planktonic bacterium

Discussion

The ROS effects on eukaryotic cell macromolecules in relation to the phototoxicity of quinolones has been previously investigated [19] with another study relating the levels of ROS produced by antibiotics in bacteria with the oxidation of lipids and proteins [20]. On the basis of our previous work [2] which indicated that CIP provoked little increase of ROS in *P. mirabilis* biofilms, it was expected that the different stimuli of ROS generated by CIP in planktonics and biofilms cells may generate different degrees of lipid and protein oxidation. Effectively, the present study showed dissimilar behaviors among planktonic populations and biofilms of

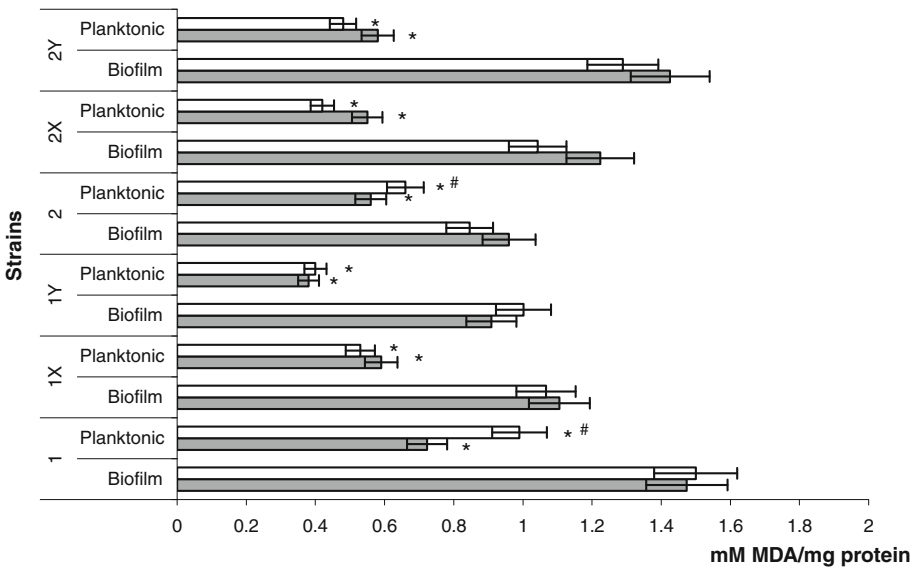

Fig. 2 MDA quantification in sensitive and CRV *P. mirabilis* in absence (*filled bar*) and presence of CIP (*open bar*). Data represent the mean ± SD of three independent experiments. $^{\#}P < 0.05$ respect to control without CIP. $^{*}P < 0.05$ planktonic respect to biofilm

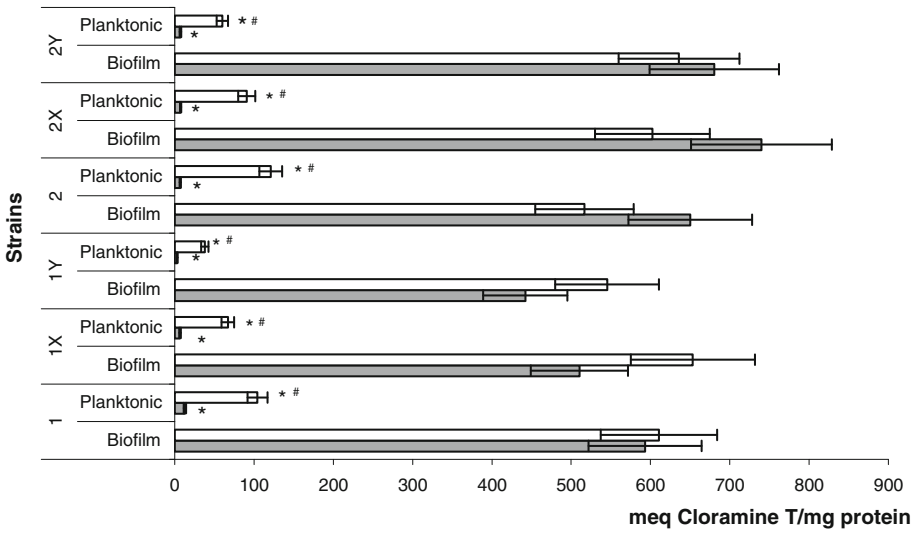

Fig. 3 Quantification of AOPP in sensitive and CRV *P. mirabilis* in the absence (*filled bar*) and presence of CIP (*open bar*). Data represent the mean ± SD of three independent experiments. $^{\#}P < 0.05$ respect to control without CIP. $^{*}P < 0.05$ planktonic respect to biofilm

P. mirabilis, since the results revealed that the incubation with the antibiotic did not increase the levels of oxidized lipids or proteins in biofilms. Nevertheless, the biofilms had higher concentrations of oxidized macromolecules than their planktonic counterparts, in both the absence and presence of CIP.

These results can be explained by taking into account the differences in the physiologies and metabolism between planktonic cells and bacteria that integrate a biofilms. Whereas biofilms are not growing as they are in a state similar to that of a stationary one, planktonic bacteria are actively growing, and consequently during the self-replication of the bacteria, the oxidized components of the cytoplasm are dispersed randomly and non-conservatively [21]. During replication of planktonic cells, the cytoplasm components are divided randomly into the two daughter cells, resulting in equal amounts of old, damaged and potentially non-functional protein, which causes a dilution of altered macromolecules and thereby prevents the aging of stem cells. Nevertheless, in populations of microorganisms that are not constantly growing, as is the case of the cells in biofilms, these are constantly exposed to ROS leading to an accumulation of oxidative damage [22]. This theory reinforces our observations referring to the higher basal levels of MDA, carbonyls, and AOPP in biofilms, derived from the loss of proliferation and aging of the bacteria.

Previously, it has been reported that cells, in response to the increased oxidation of macromolecules and to lessen the danger of ROS, develop defense mechanisms such as the synthesis of antioxidant enzymes to reduce ROS levels and increase the enzymes that repair DNA and proteins [23]. This is consistent with the increase in antioxidant defense mechanisms found in biofilms described in one of our previous works [2]. Consequently, the important antioxidant machinery of biofilms prevents the increase in ROS against an exogenous oxidizing agent such as the CIP, and avoids the subsequent oxidation of more lipids and proteins in presence of this antibiotic. Thus, the low response of *P. mirabilis* in biofilms to oxidative stress generated by CIP contributes to clarify the resistance to antibiotics described as a characteristic of these structures.

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