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Resistance to ciprofloxacin by enhancement of antioxidant defenses in biofilm and planktonic *Proteus mirabilis*

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ABSTRACT

Antibiotic resistance and antioxidant defense were induced by ciprofloxacin in planktonic *Proteus mirabilis* and compared with the natural antibiotic resistance of biofilm. Resistant variants (1X and 1Y) were obtained from cultures of the sensitive wild type "*wt*" strain 1 in the presence of the antibiotic. Planktonic strain 1 exhibited oxidative stress with increases in the reactive oxygen species (ROS) and consumption of NO in the presence of ciprofloxacin, whereas 1X and 1Y suffered non-significant rises in ROS generation, but produced and consumed more NO than sensitive strain 1. The two resistant variants were more resistant to telluride than *wt* and showed increased levels of intracellular superoxide dismutase (SOD) and glutathione (GSH). However, ciprofloxacin did not stimulate oxidative stress in biofilm. The production of ROS and NO with or without ciprofloxacin was less significant in biofilms than in an equivalent number of planktonic bacteria; sensitive and resistant strains did not present differences. On the other hand, SOD and GSH were more elevated in the biofilm than in planktonic bacteria. In summary, these results indicate that ciprofloxacin can induce resistance by the enhancement of antioxidant defense in planktonic bacteria, similar to the natural resistance occurring in biofilm. This feature may be added to the factors that regulate the susceptibility to this antibiotic.

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Introduction

Bacterial pathogens have notably increased their virulent and antibiotic resistance, thus implying a particular challenge to chemotherapy. In addition, the colonization of indwelling devices by antibiotic resistant bacteria such as *Proteus mirabilis,* an important pathogen infecting the urinary tract [1,2], demonstrates the need for further investigation of the factors involved in biofilm associated diseases.

In general, the formation of bacterial biofilms is an effective barrier against the influx of antibiotics and induces multiple antibiotic resistance. The biofilm of urease producing bacteria, in particular of *P. mirabilis*, causes blockage and encrustation in catheters [3]. However, although antimicrobials are ineffective against stabilized biofilms, the development of biofilms can be slowed down by antibiotics due to their ability to affect planktonic bacteria prior to biofilm formation [4].

Research previously carried out in our laboratory with planktonic bacteria has indicated that other microbial species suffered oxidative stress in the presence of antibiotics [5,6]; whereas biofilms required a higher antibiotic concentration in order to produce even a slight increase of ROS generation [7]. However, the effect of antibiotics on reactive oxygen species generation requires further investigation because the effects of antibiotics have been traditionally studied for specific target sites, such as gyrase for ciprofloxacin. Furthermore, the general role of oxidative stress in the damage caused by antibiotics needs more study. Related to this, other investigators recently demonstrated the participation of ROS in the action of antibiotics [8]. Moreover, ciprofloxacin actions involved oxidative DNA damage [9], showing that antibiotics contribute to the oxidation of bacterial macromolecules.

Although, previous work with respect to stress agents in *P. mirabilis* has been related to telluride, there are no investigations on antibiotics that can cause oxidative stress in this bacterium. However, this relation should be studied due to the fact that resistance to telluride can be induced in response to oxidative stress, and therefore this induction may be associated with protection against other forms of oxidative stress [10].

In the present study, we investigated the resistance to ciprofloxacin in planktonic *P. mirabilis* and biofilm, with the purpose of ana-

Abbreviations: wt, wild type; ROS, reactive oxygen species; SOD, superoxide dismutase; GSH, glutathione; TSA, trypticase soy agar; MIC, minimum inhibitory concentration; CLSI, Clinical Laboratory Standards Institute; MHA, Mueller Hinton Agar; cfu/ml, colony forming units per milliliter; MHB, Mueller Hinton Broth; PBS, phosphate buffer; NBT, NitroBlue Tetrazolium; lucigenin, bis-*N*-methylacridinium nitrate; TSB, trypticase soy broth; DMSO, dimethylsulfoxide; RLU/s, relative light units per second; DTNB, 5,5′-dithiobis-2-nitrobenzoic acid; O_2^-, anion superoxide.

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lyzing the effects of the stimulation of antioxidant defenses, such as SOD and GSH, by the changes occurring in ROS and NO. The results obtained reinforced the hypothesis that resistance to oxidative stress can protect *P. mirabilis* against ciprofloxacin action.

Materials and methods

Bacterial strains and growth conditions. P. mirabilis 1 isolate from human infection was obtained from Hospital Tránsito Cáceres de Allende, Córdoba, Argentina. Ciprofloxacin resistant strains 1X and 1Y were prepared in our laboratory as described below. All strains were cultivated at 37 °C in trypticase soy agar (TSA) and maintained in this medium.

Antimicrobial and telluride susceptibility determination. The minimum inhibitory concentration (MIC) was determined using the standard tube dilution method as outlined by the Clinical Laboratory Standards Institute (formerly National Committee for Clinical Laboratory Standards) (CLSI 2006). Strains from cultures of 24 h in Mueller Hinton Agar (MHA) were diluted to 10^6 colony forming units per milliliter (cfu/ml), incubated for 10 min at 37 °C, and then ciprofloxacin (Parafarm) or telluride was added at different concentrations (0.125–512 μg/ml). Bacteria development was observed at 24 h of incubation, according to CLSI indications. The MIC was defined as the lowest concentration of drug that inhibited visible growth after 24 h of incubation at 37 °C.

Development of ciprofloxacin resistant variants. Resistant variants 1X and 1Y were obtained by overnight culture in Mueller Hinton Broth (MHB), from sensitive strain 1 transferred onto MHB containing 0.0625 μg/ml of ciprofloxacin in five successive cultures, and 1 μg/ml in the following culture. Then, a subsequent isolation of resistant strains in plate with MHA was performed with 2, 4, 8, 16 or 32 μg/ml of antibiotic.

Biofilm preparation. Overnight planktonic cultures of *P. mirabilis* were diluted to an OD_{600} 0.2 in phosphate buffer (PBS), pH 7.2. This culture dilution was then used to form biofilms by two methods, as follows: (a) 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 [11]. The biofilms were then transferred to antibiotic containing agar (4 μg/ml of ciprofloxacin) and the agar plates were incubated at 37 °C for 2 h. The membrane supported biofilm was 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 SOD, GSH and NO; (b) polystyrene microtiter plates were inoculated with culture dilution (overnight culture further diluted 1/100) and incubated for 24 h at 37 °C without shaking, and then NitroBlue Tetrazolium (NBT) assays were performed. Biofilms formation was detected and quantified by Crystal Violet staining.

Determination of oxidative stress by chemiluminescence. The chemiluminescence assay was quantified by the light emitted when ROS gave electrons to bis-*N*-methylacridinium nitrate (lucigenin, Sigma). Samples of overnight cultures of planktonic *P. mirabilis* in trypticase soy broth (TSB) were centrifuged at 10,000*g* for 20 min to separate bacteria. These bacteria containing pellets were then washed with PBS, centrifuged three times and suspended in PBS. Aliquots of 0.3 ml of the bacterial suspension were added to 0.3 ml of 0.125, 4.0 or 16.0 μg/ml concentrations of ciprofloxacin. Then, 0.3 ml of 0.145 mM lucigenin was added followed immediately by 0.1 ml of dimethylsulfoxide (DMSO, Anedra), to yield a final count of 10^6 cfu/ml. The light emitted by superoxide anion (O_2^-) was instantly measured by a Bio-Orbit model 1253 luminometer and expressed as relative light units per second (RLU/s). Controls were performed with bacteria in the absence of the antibiotic.

Nitric oxide assay. Planktonic bacteria or biofilm cells (475 μl with 10^6 cfu/ml) were incubated with 4 μg/ml of ciprofloxacin for 2 h at 37 °C. NO, converted to nitrite (NO_2^-) in aqueous solutions, was assayed by reduction of NO_2^- using granulated cadmium. This was followed by spectrophotometric analysis of total NO_2^- at 540 nm using Griess's reagent with sodium nitrite as the standard [12]. The results were expressed in μM NO_2^-/mg of protein, with the quantities of protein being determined by a Folin-Ciocalteau assay.

Superoxide dismutase determination. P. mirabilis planktonic or biofilm was incubated with PBS (control) or 4 μg/ml of ciprofloxacin for 2 h at 37 °C. After centrifugation for 15 min at 15,000*g*, supernatants (extracellular SOD) were separated from cells, which were then diluted with 0.1 ml of DMSO and 0.1 ml of PBS (intracellular SOD). Assays with 150 μl of each fraction were treated with 450 μl of 13 mM methionine (Sigma), 150 μl of 75 μM NBT (Sigma), 450 μl of 100 nM EDTA, and finally 450 μl of 400 μM of riboflavin (Sigma) in the presence of light (445 nm). After 6 min, absorbance was determined at 560 nm. A unit of SOD was defined as the quantity of enzyme required to produce a 50% inhibition of NBT reduction under the conditions specified above. Activity was expressed as units of SOD per mg of protein (U SOD/mg of protein).

Assay of total glutathione. P. mirabilis planktonic or biofilm was incubated with PBS (control) or 4 μg/ml ciprofloxacin for 2 h at 37 °C. Then, the bacterial GSH level was determined by spectrophotometry on the basis of the absorbance of the reaction product of reduced GSH and 5,5′-dithiobis-2-nitrobenzoic acid (DTNB, Sigma). Bacterial suspensions (OD_{600} 1, 0.10 ml) were incubated with 20 μl of glutathione reductase (6 U/ml, Sigma), 50 μl of NADPH (4 mg/ml, Sigma), and 20 μl of DTNB (1.5 mg/ml, Sigma). The absorbance was then determined at 412 nm. Glutathione levels were expressed in mM GSH/mg of protein.

NitroBlue Tetrazolium assay. (a) A bacterial suspension (400 μl of $OD_{600} \sim 1$) was incubated with 100 μl of ciprofloxacin (final concentration 0.125 μg/ml) in the presence of 500 μl of NBT (1 mg/ml) for 30 min at 37 °C. This reaction was then stopped with 100 μl of 0.1 N HCl. Centrifugation at 10,000*g* for 30 min allowed the separation of the bacteria from the supernatant, which was then treated with 0.4 ml DMSO and 0.8 ml of PBS, pH 7. Reduced NBT was measured as formazan blue at 575 nm.

(b) The biofilm formed in polystyrene microtiter plates was washed four times with PBS, before 100 μl of ciprofloxacin 0.125 μg/ml were added in the presence of 100 μl of NBT (1 mg/ml) and the solution incubated for 30 min at 37 °C. The reaction was then stopped with 20 μl of 0.1 N HCl, and treated with 50 μl of DMSO. Reduced NBT was measured as formazan blue at 575 nm. Results were expressed at an $OD_{575\ nm}/10^6$ cells.

Statistical determinations. A statistical analysis was performed using ANOVA, with $p < 0.05$ taken as statistically significant. The experiments were repeated at least three times, with means and standard deviations being calculated.

Results

The induction of resistance by repeated cultures with ciprofloxacin increased the levels of the resistant variants 1X and 1Y reaching a MIC values of 16 and 4 μg/ml, respectively, with the sensitive strain 1 *wt* having a MIC of 0.125 μg/ml. In addition, 1Y and 1X became more resistant to telluride (MIC values of 128 and >512 μg/ml telluride, respectively) than strain 1 (MIC 64 μg/ml telluride). Resistance to ciprofloxacin was accompanied by resistance to this oxidant agent.

Resistant variants suffered lesser stimuli of ROS with ciprofloxacin than sensitive *wt*. The planktonic culture incubated with ciprofloxacin raised the generation of ROS in strain 1 at lower concentrations of antibiotic than 1X or 1Y. The levels of RLU/s were highest in the sensitive strain 1. Table 1 shows that 0.125 μg/ml of

Table 1
Reactive oxygen species (ROS) assayed by chemiluminescence in planktonic bacteria at 2.5 min of incubation with 4 μg/ml ciprofloxacin or with the MIC of each strain.

	RLU/s with ciprofloxacin			MIC (μg/ml)
	With 0 μg/ml	With 4 μg/ml	With each MIC	
1	0.011 ± 0.002	0.051 ± 0.021	0.084 ± 0.041	0.125
1X	0.008 ± 0.002	0.017 ± 0.010	0.016 ± 0.016	16
1Y	0.006 ± 0.003	0.007 ± 0.006	0.007 ± 0.006	4

Table 2
Production of ROS (RLU/s), NO (μM NO_2^-/mg protein) and stimuli of ROS respect to NO in planktonic *P. mirabilis* in the absence of antibiotic or with 4 μg/ml of ciprofloxacin.

	0 μg/ml ciprofloxacin			4 μg/ml ciprofloxacin		
	ROS	NO	ROS/NO	ROS	NO	ROS/NO
1	0.011	1.82	0.0060	0.051	1.38	0.0370
1X	0.008	14.67	0.0050	0.018	4.68	0.0038
1Y	0.006	53.16	0.0001	0.007	26.15	0.0003

ciprofloxacin increased 8-fold the RLU/s in strain 1, while 1X and 1Y exhibited lower values of ROS even for concentrations of 16 and 4 μg/ml, respectively. Furthermore, 1X and 1Y showed low productions of ROS in the absence of ciprofloxacin. The maximum generation of ROS in strain 1 was obtained at 0.125 μg/ml and above this concentration (4 μg/ml) ROS decreased due to the bactericide effect of a supraMIC level of antibiotic.

Inversely, resistant variants 1X and 1Y presented higher levels of NO than sensitive wt in the absence of antibiotic. NO produced by planktonic *P. mirabilis* 1X and 1Y without ciprofloxacin treatment was 8- to 29-fold greater than the NO generated by strain 1 (Fig. 1). The resistant variants in planktonic cultures with ciprofloxacin produced more NO ($p < 0.05$) than sensitive strain 1 (Table 2), but the levels of NO were lower in the presence of antibiotic since there was consumption of NO resulting from the reaction with the ROS stimulated by ciprofloxacin. The relationship ROS/NO was higher in strain 1 than in 1X and 1Y, with a more elevated generation of ROS found with respect to NO in the presence of ciprofloxacin. The resistances of 1X and 1Y showed less ROS and a higher NO than that of sensitive strain 1.

The results indicate that the formation of biofilm led to low stimulation of ROS generation in the presence of ciprofloxacin, with no differences being found among the biofilms of resistant strains (1X and 1Y) and strain 1. Table 3 compares the maximum stimulation of ROS generation obtained by NBT assay with 0.125 μg/ml of ciprofloxacin in planktonic bacteria and biofilm. The sensitive strain *wt* only suffered stimulation of ROS generation in the planktonic cells, since biofilms of this strain did not increment ROS generation in presence of antibiotic. In addition, NO was lower in biofilm than in planktonic bacteria, and no significant differences were found among strains without ciprofloxacin (Fig. 2). The resistance to ciprofloxacin in the biofilms was associated to lower stimuli of ROS and NO.

The antibiotic generated antioxidant defenses in *P. mirabilis* with the stimulus of a defensive response against oxidative stress being obtained during induction by repeated cultures at subMIC ciprofloxacin. Both the resistant variants obtained (1X and 1Y) showed increased levels of GSH (Fig. 3), with the formation the biofilms increasing GSH in all the strains respect to the planktonic

Table 3
Production of ROS by 10^6 planktonic bacteria and 10^6 bacteria in biofilm incubated with 0.125 of ciprofloxacin during the NBT assay.

	MIC (μg/ml)	Planktonic ($OD_{575\ nm}$/10^6 cells)	Biofilm ($OD_{575\ nm}$/10^6 cells)
1	0.125	0.276 ± 0.005	0.050 ± 0.003
1X	16	0.104 ± 0.006	0.060 ± 0.005
1Y	4	0.100 ± 0.003	0.050 ± 0.003

cultures, thus suggesting a possible reason for the resistance to the oxidative stress caused by ciprofloxacin in free bacteria.

Finally, 1X presented a high superoxide dismutase (SOD) activity (Fig. 4A), and the planktonic cultures of the strains showed more extracellular than intracellular SOD/mg of protein. The formation of biofilms greatly increased the extracellular SOD in 1X and 1Y (Fig. 4B), thereby reinforcing the relation between antioxidant capacity and resistance to the antibiotic.

Discussion

Ciprofloxacin was chosen to investigate its effect on the oxidative stress of *P. mirabilis*, taking into consideration that fluoroquinolones are routinely applied in infections caused by this species, due to the fact that these antibiotics display high and persistent levels of urinary bactericidal activity against Gram negative bacteria [13]. The research carried out in the present study showed that the induction of resistance by incubation with ciprofloxacin generates an enhancement in MIC and antioxidant defenses. In addition, the present results reinforce the role of oxidative stress in the mechanism of injuries caused by ciprofloxacin.

The resistance to antibiotic induced in planktonic *P. mirabilis* and the resistance acquired by biofilm formation were both associated to low stimulation of ROS generation with a resultant decrease in the relationship ROS/NO. The notoriously high production of NO by planktonic resistant variants 1X and 1Y reinforces the concept that NO can be a factor that contributes to the resistance to ciprofloxacin. These results indicate that 1X and 1Y, obtained by repeated cultures

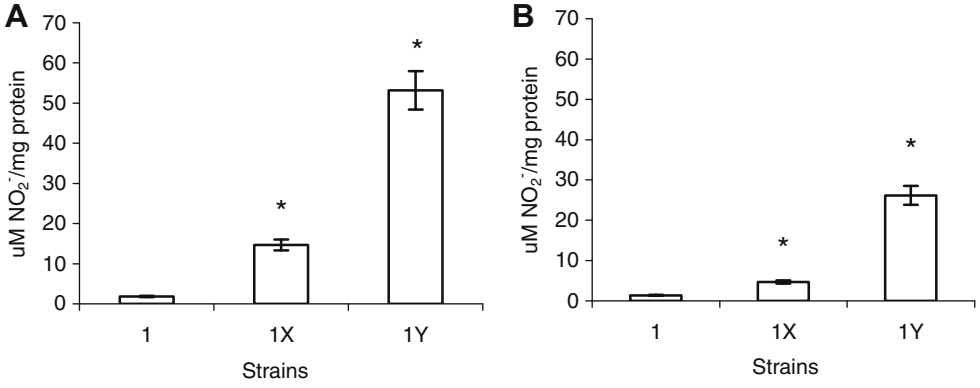

Fig. 1. Production of NO (μM NO_2^-/mg protein) by planktonic bacteria: (A) without ciprofloxacin, (B) with 4 μg/ml of ciprofloxacin (*$p < 0.05$).

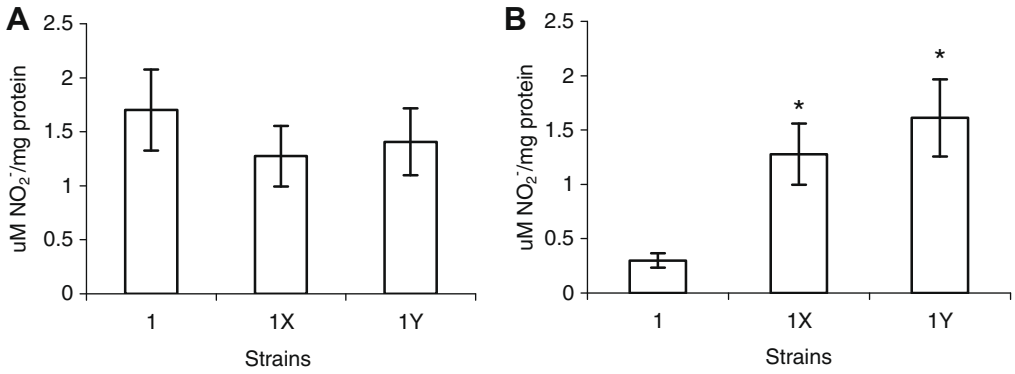

Fig. 2. NO generated by biofilm: (A) without ciprofloxacin, (B) with ciprofloxacin for 2 h expressed as μM NO_2^-/mg protein (*$p < 0.05$).

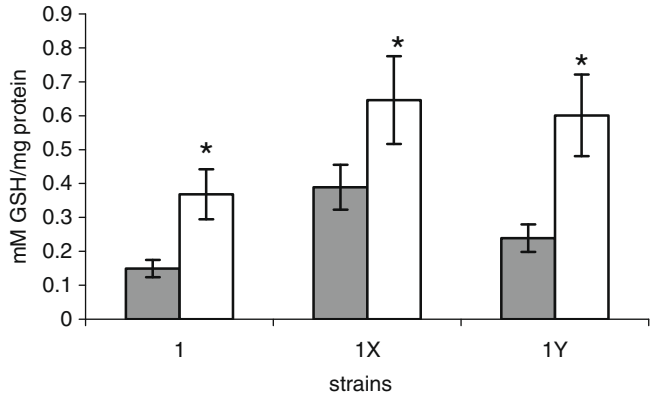

Fig. 3. GSH in planktonic *P. mirabilis* (■) and biofilm (□) without antibiotic expressed as mM GSH/mg of protein (*$p < 0.05$).

with ciprofloxacin, were able to control the oxidative balance by means of increasing the NO respect to the sensitive strain 1, as NO is an important regulator of diverse biological functions**.** Moreover, it has recently become accepted that NO is associated with an "express" cytoprotective system in bacteria, which immediately protects microbial cells from oxidative stress, thus playing a role in the defense of microorganisms. Other antioxidant systems can also protect bacteria from the increased levels of ROS during prolonged oxidative stress, but in general these systems require newly synthesized proteins, in a time and energy dependent form, whereas the reduction of ROS by means of NO was previously found to be associated to fast protective defense [14].

Although, NO is a bactericidal at high concentrations, the physiological production of NO by bacteria is beneficial when NO increases in response to oxidant agents, with an equilibrium forming between the synthesis and consumption of NO and ROS [15]. The decrease of NO observed in planktonic cells with ciprofloxacin can be explained by bearing in mind that ROS reacts with NO, particularly the anion superoxide (O_2^-). This consumption of NO was higher and the levels of ROS lower in the resistant variants than in the sensitive strain 1. It should be pointed out that in 1X and 1Y biofilm there was no evidence of NO consumption in the presence of ciprofloxacin, because ROS did not increase as in planktonic bacteria with antibiotic, and consequently NO was not consumed.

Another objective of the work was to discover if the resistance to antibiotic in biofilm could be related to high antioxidant defenses. The enhancement of the antioxidant metabolism and their transcribing genes within biofilms has been previously described [16], although the physiological relationship between antioxidant enzymes and defenses against antibiotic damage still needs to be explained. The improvement of antioxidant defenses in biofilms is a curious characteristic, because the metabolic activity of bacteria within biofilms, has been reported to be much lower than in planktonic bacteria. This selective enhancement of the antioxidant pathway suggests that bacteria needed to increase their defenses against oxidative stress in biofilms. The results obtained in the present work support the hypothesis that resistance to oxidative stress protects *P. mirabilis* against ciprofloxacin action in planktonic bacteria and biofilm, because cultures with low concentrations of ciprofloxacin were able to generate an enhancement of GSH and SOD which contributed to resist ciprofloxacin in *P. mirabilis*; a feature that may be added to the list of factors that regulate the susceptibility to this antibiotic.

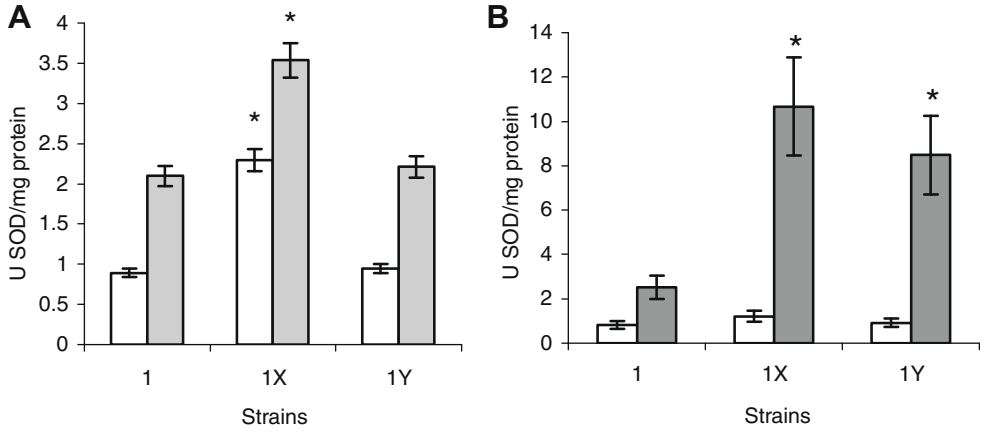

Fig. 4. Intracellular SOD (□) and extracellular SOD (■) without ciprofloxacin: (A) in planktonic of *P. mirabilis*, (B) in biofilm of *P. mirabilis* (*$p < 0.05$).

The enhancement of GSH in 1X and 1Y is consistent with the concept that detoxification from oxidant agents in prokaryotic cells takes place by defense systems similar to those found in eukaryotic systems. In fact, glutathione is the most abundant intracellular thiol in all living cells and is known to be involved in many biological processes, including protein and DNA synthesis, cell transport, enzyme activity modulation, and cellular metabolism, as well as in defense against reactive oxygen species [17]. Concerning this, a relevant finding in *P. mirabilis* describing the modulation of glutathione S-transferase B1-1 expression by different stress factors [18] must be cited. The regulation of GSH also plays a key role in the detoxification of bacteria, since it provides protection from damaging compounds [19].

The results of the GSH determinations add weight to the proposition that antioxidant systems play a role in the defensive reaction of *P. mirabilis* to the damage caused by ciprofloxacin. Moreover, the formation of biofilms increased the bacterial GSH in parallel with the antibiotic resistance, with the contribution of oxidative stress in the mechanism of action of ciprofloxacin seeming to be significant. In addition, the antioxidant action of GSH might be included in the factors that participate in the resistance to oxidative stress provoked by this antibiotic, taking into consideration that there was a correlation between MIC and resistance to stress, with the more resistant strain 1X showing the highest GSH and the lowest value being in sensitive strain 1.

The function to detoxify ROS has been previously found to be associated with genetic regulons, such as soxR/S and marA, and it was shown that exposure to oxidative stress induced bacterial genes including stress related sigma factors [20]. Our results are coherent with the observation that SoxS protein, characterized as a superoxide response regulon, can increase the resistance to antibiotics, with both the mar operon of multiantibiotic resistance and the sox operon being able to give rise to multiple antibiotic resistances [21]. Additionally, the results obtained in the present work show that 1X achieved a high resistance to the oxidant action of telluride, similar to the resistance to oxidative stress caused by ciprofloxacin. It must be emphasized, however, that resistance to telluride was induced by successive subcultures in ciprofloxacin; an aspect that shows the relation between resistance to ciprofloxacin and to oxidative agents. Strain 1Y was less resistant to ciprofloxacin than 1X and did not exhibit resistance to telluride, a result that demonstrates the relation between susceptibility to this oxidant and to ciprofloxacin. Furthermore, resistance to telluride in *Proteus* isolates can differ from patient to patient, in the same way that occurs with susceptibility to antibiotics [10].

Although the production of SOD was described to be associated with the antioxidant defenses of *P. mirabilis* [22], this enzyme has not been previously studied as a defensive response opposed to ciprofloxacin action. In the present work, a relation between antioxidant defenses and resistance to ciprofloxacin is proposed, with the more ciprofloxacin resistant strain exhibiting a greater antioxidant defense, since 1X acquired higher levels of SOD and GSH than 1Y. Another significant result was the increase in SOD, especially the extracellular form, when the bacteria acquired resistance to ciprofloxacin by the formation of biofilm. This observation suggests that SOD plays an important role in the defense against extracellular anion superoxide, as diverse bacteria display an anaerobic metabolism in biofilms, with less generation of ROS than planktonic bacteria, even in non-fermentative species [23]. Consequently, bacteria in biofilms must protect themselves from extracellular ROS rather than intracellular ROS, as suggested by the results obtained in the assays of intra- and extracellular SOD.

In conclusion, the present findings reinforce the importance of a good antioxidant defense in the resistance to ciprofloxacin of *P. mirabilis*, thereby strengthening the likelihood of a role of oxidative stress in the action of this antibiotic. Thus, future investigation could analyze the possible synergy between antibiotics and drugs able to harness the stress caused by antimicrobials.

Acknowledgments

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