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Role of Mn-Cofactored Superoxide Dismutase in the aptitude of *Pseudomonas aeruginosa* to form biofilm, under UV-C Radiations

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Abstract – The biofilm formation and development might vary in presence of UVc radiations, since this biofilm could be considered as a form of adaptation and resistance of microbial organisms to stress exposure. The study of bacterial biofilm was investigated to determine the potential UVc effects on bacterial biofilm formation and development. Moreover, the protective role of superoxide dismutase (SOD) against UVc radiations has been studied in wild-type and mutant strain of *Pseudomonas aeruginosa* lacking cytosolic Mn-SOD (*sodM*), Fe-SOD (*sodB*), or both SODs (*sodMB*), forming biofilm. *Pseudomonas aeruginosa* PAO1 and its isogenic mutants *sodM, sodB* and *sodMB* were exposed to incremental UVc doses. To measure biofilm formation, samples of suspension were taken at indicated times during irradiation and used for both qualitative and quantitative biofilm tests.
Our results showed that inactivation of *sodM* gene increase the sensitivity of *P. aeruginosa* toward stress induced by UVc radiations. Furthermore, our results showed an enhancement of biofilm production, after exposure to UVc radiations, in wild-type and *sod B* mutant which maintained significantly higher levels of biofilm production than *sodM* mutant strain. The overall results showed that Mn-SOD isoform has a protective role against a stress induced by UVc radiations in *P. aeruginosa* biofilm.

Keywords: Biofilm, *P. aeruginosa*, sod mutants, UV.

1. Introduction

Pseudomonas aeruginosa is a bacterium that can be isolated from many different habitats, including water, soil, and plants. *P. aeruginosa* is also an opportunistic human pathogen that causes serious nosocomial infections (Campos-Garci'A et al. 1998). Biofilm formation by *P. aeruginosa* is of particular interest because of its clinical relevance (Donlan and Costerton 2002). In effect, *Pseudomonas aeruginosa* infections generally endure despite the use of long-term antibiotic therapy. This is due to the formation of an antibiotic-resistant biofilm, consisting of bacterial communities embedded in an exopolysaccharide (EPS) matrix (Drenkard and Ausubel 2002), which facilitate the adherence of microorganism (Jain et al. 2008). Biofilm-forming organisms rely on extracellular polymeric substance, also known as matrix, which is essential for colonization of surfaces and volumes (Sutherland, 2001a, b; Flemming et al. 2007).
Though germicidal UV radiation is widely applied for disinfection of water and food, it may also be used to prevent bacterial growth and colonization on surfaces within engineered systems. Emerging UV source technologies, such as ultraviolet-C (UVC) LEDs, present new opportunities for deterring biofilms within certain devices, including medical equipment, food and equipment (Torkzadeh et al. 2020). Moreover, UVc radiation increases the reactive oxygen species (ROS) levels in eukaryotic and prokaryotic organisms. Antioxidant enzymes are involved to deal with oxidative stress. Major component of the antioxidant enzyme system include superoxide dismutase (SOD) (Meng et al.2009). It was previously demonstrated that *P. aeruginosa* possesses two isoforms Fe-SOD and Mn-SOD. Fe-SOD activity is present under all growth conditions, while Mn-SOD activity is elevated only when the organism is starved for iron and/or when it is producing alginate (Hassett et al.1995).
In this context, we studied the biofilm producing potential of *P.aeruginosa* strain PAO1 and its isogenic mutants (*sodM*, *sodB*, and *sodMB*). Congo red agar (CRA) and quantitative microtiter plate (MTP) methods were used for detection of biofilm production, to better understand the roles of Fe-SOD and Mn-SOD, in the aptitude of *P. aeruginosa* wild-type strain to produce biofilm, under stressful conditions.

2. Materials and methods

2.1. Bacterial Strains and Plasmids

Strains used in this study were *P. aeruginosa* PAO1 (wild type strain) and its isogenic mutants *sodM* (*sodM*: Xaac Gmr), *sodB* (*sodB*: XTc Tcr), and *sodMB* (*sodM*: Xaac.Gmr *sodB*: XTc Tcr). Bacteria were kindly provided by Dr. Kazuhiro Iiyama, Institute of Biological Control, Kyushu University, Japan (2007).

2.2. Bacterial irradiation

P. aeruginosa PAO1 and its isogenic strains (*sodM*, *sodB* and sod *MB*) were grown in 10 ml of TSB at 37°C for 24 h without shaking. After incubation, different cultures were measured and adjusted, by spectrophotometer (Spectro UVS-2700 Dual BEAM LABOMED, INC) to an optical density (OD_{600}) of 0.6 equivalent to 1×10^7 Colony Forming Unit per milliliter (CFU/ml). Then, the bacterial cells were harvested by centrifugation at 4500 *g* for 15 min. The bacterial cells were washed with sterile physiological water (0.9% NaCl) and re-centrifuged. The pellet was resuspended in sterile saline solution and transferred to a glass Petri dish. The doses of irradiation were given cumulatively using the same bacterial suspension. To measure biofilm formation, samples of suspension were taken at indicated times during irradiation and used for both qualitative and quantitative tests.

The laboratory UV device was built with the cooperation of the company Guy Daric S.A (Aubervilliers, France). The UVc prototype used contains a sliding rack, with an irradiation board that held six Petri dishes (90 mm diameter). UV-C exposure was carried out using a germicidal low-pressure mercury vapor discharge lamp (length = 900 mm, diameter = 13 mm, power of UV emission at 253.7 nm = 55 W). The incident intensity UV-C rays levels were measured with a selective detector for UV joined to a digital radiometer (Vilbert-Lourmat, Norme la Vallée, France). The UVc doses were calculated as the product of radiation intensity (I: mW/cm^2) and the exposure time (t: s) following the formula recognized by Hassen et al (2000):

$$\text{UVc dose } (mW.s.\ cm^{-2}) = \text{Radiation intensity } (mW.cm^{-2}).\ \text{Exposure time (s)}.$$

UVc exposure times used in this study are ranging from 5 to 30 minutes.

2.3. Biofilm formation

Effect of UVc exposure on the ability of *P. aeruginosa PAO1* and its isogenic mutants *sodM*, *sodB* and *sodMB*, to produce biofilm was evaluated in vitro by two different methods: phenotypic expression of colonies on CRA and quantification of biofilm production by the colorimetric assay. Bacterial suspensions of untreated and UVc treated *P. aeruginosa* strains were used to inoculate the CRA and the polystyrene microtiter plates.

2.3.1. Morphotypes on Congo Red Agar (MCRA)

Colony morphology was studied by the cultivation of *P. aeruginosa* strains (PAO1, *sodM*, *sodB*, and *sodMB*), on CRA as previously described by Freeman et al. (1989). The constituents of media were: brain heart infusion broth 37 g/l, sucrose 0.8 g/l, agar–agar 10 g/l and Congo red stain 0.8 g/l. All the chemicals were supplied from Himedia, Mumbai, India. Congo red stain was prepared as a concentrated aqueous solution, autoclaved separately and added to the media when the agar had cooled to 55 °C. Plates of the medium were inoculated and incubated aerobically for 24 h at 37 °C. After inoculation with both untreated and UVc-treated cultures, the plates were incubated for 24 h at 37°C in aerobic conditions. The slime producer isolates originated black colonies, while the non-producers develop red/pink colonies (Salman et al. 2012). These black colonies were considered as indicative of slime production, while strains producing very red, red and burgundy colored colonies were classified as non-slime producing bacteria.

2.3.2. Microtiter plate (MTP) method

The MTP method was done according to the protocol of Moretro et al. (2003). Briefly, an overnight culture grown in TSB at 37 °C was diluted 1:100 in TSB. 200 µl of this was transferred to 96 well plates. Plates were incubated for 24 h at 37 °C. After incubation, the broth was removed from each of the wells. The wells were then washed 3 times with Phosphate buffer saline (PBS) to remove non-adherent cells. Plates were air dried in inverted position. Wells were stained with 100 µl crystal violet. Plates were then washed with PBS to remove unbound strain and read on ELISA plate reader multiscan (labs systems, flow labs, Finland) on absorbance of 595 nm. Results were scored as follows: If $A_{595}<0.2$, bacteria is considered as negative for biofilm production; if A_{595} is ranging from 0.2 to 0.9, bacteria is considered as weak biofilm producer and if $A_{595}> 0.9$, bacteria is considered as strong biofilm producers. Each strain was tested in triplicate and total test time was 48 h.

2.4. Statistical analysis

Statistical analysis was performed using SPSS version software (10.5 version, Texas, USA). Chi square test was used for comparison of proportion. Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of CRA method were calculated.

3. Results and Discussion

3.1. Biofilm production in *P. aeruginosa* PAO1 and its isogenic mutants *sodM, sodB* and *sodMB*

The results of adhesion on glass tubes, the photos of different morphotypes observed on CRA and the Optical density (O.D) values after culturing on 96-well ELISA plates and also of exopolysaccharides (EPS), for WT, *sodM*, *sodB* and *sodMB* are shown in figure 1.

The PAO1 strain shows colonies with an orange pigment in the center, surrounded by an autolysis area (Figure 1-B) with absence of adhesion to glass (Figure 1-A). The *sodB* displays a rough morphotype with a circle of autolysis (Figure 1-B) and no adhesion to glass is noted (Figure 1-A). A lower production of exopolysaccharides (Figure 1-C) and a biofilm production much higher compared to the WT strain are noticed.

For the *sodM* mutant, the colonies are mucoid (Figure 1-B), associated with a maximum production of exopolysaccharides (212 µg / ml) and an O.D value, relative to biofilm production, equal to that noted for *sod B* mutant. On glass tubes, the film is clearly visible (Figure 1-A). For the *sodMB* double mutant, the colonies are bright. This morphotype, being the most frequent, is associated with a very important adhesion on glass (Figure 1-A) but also with a significant increase ($p < 0.05$) of the O.D, related to the production of biofilm on microtiter plate (from 0.3 to 1.6), as seen in figure 1-C.

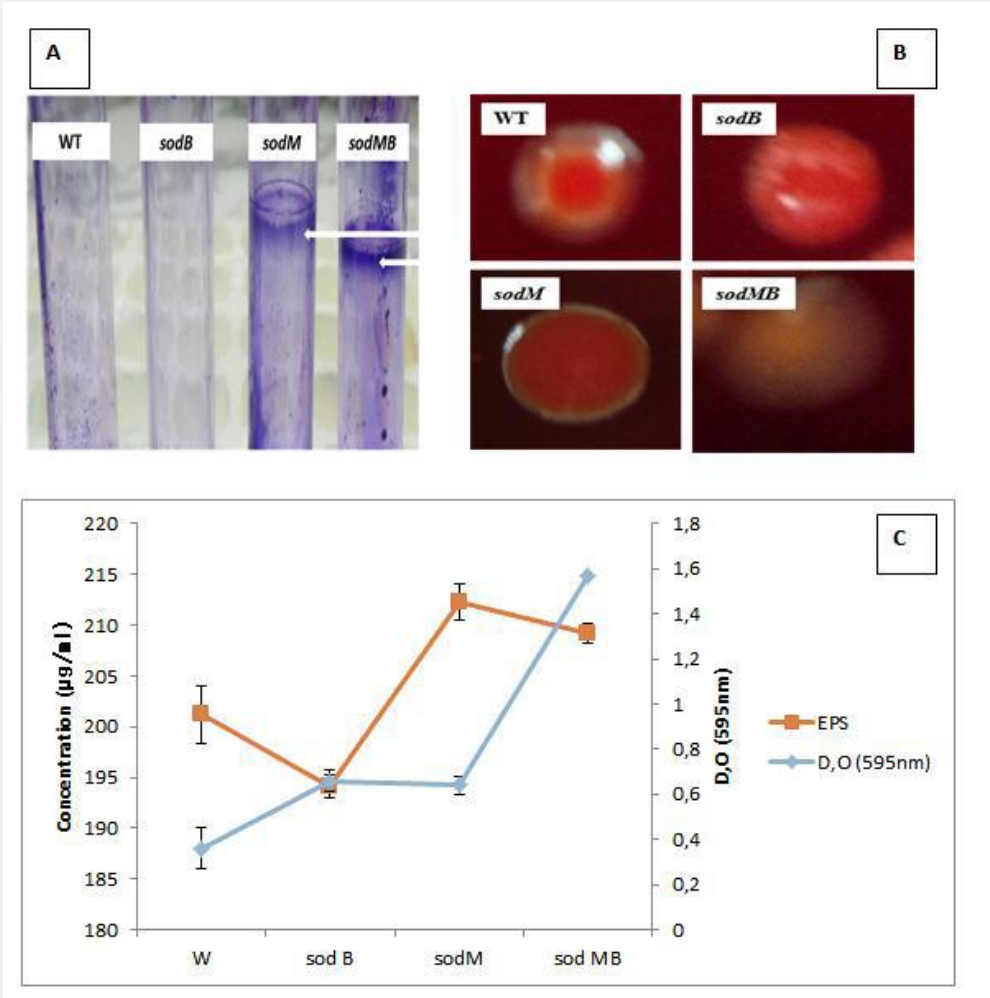

Figure 1: Adhesion on glass tubes (A), morphotype on Congo Red medium (B) and correlation between production of biofilm (O.D at 595nm) on Polyvenylglycol (PVC) microplates and the content of exopolysaccharides (EPS) (C) in WT, *sodB*, *sodM* and *sodMB*.

3.2. Effects of UVc radiations on Biofilm formation

3.2.1. Effects of UVc radiation on biofilm production in the wild strain PAO1

The results of quantitative methods show that for the wild type strain (WT), biofilm production on microtiter plates is significantly ($p < 0.05$) stimulated after 5 minutes of treatment. At the same time, an overproduction of exopolysaccharides is maintained for up to 15 minutes of exposure to UVc radiations (Figure 2-C). The morphotype on Congo Red agar medium shows an area of autolysis all around a pigment orange concentrated in the center (Figure 2-B-T5). After 15 minutes of treatment, the formation of biofilm on microtiter plate is significantly ($p < 0.05$) inhibited while the adhesion to glass tubes is maximum (Figure 2-A-T15). After 30 minutes of exposure to UVc radiations, the morphotype is damaged and the autolysis circle is more external (Figure 2-B-T30). In parallel, EPS production decreases significantly ($p < 0.05$) and adhesion to glass is low. We then find that there is a correlation between the results of quantitative and qualitative methods. In effect, the wild type (WT) strain developed a different morphotype from that observed before irradiation. Adhesion to glass is remarkable, after 15 and 30 minutes of UVc treatment. However, biofilm production on MTP is disturbed, from 15 minutes exposure. This response is similar to the results of Myriam et al. (2011) who demonstrated that for the majority of the 5 tested *P.aeruginosa* strains, isolated from wastewater, biofilm production presents a progressive increase, as seen for WT strain, in function of an increasing of exposure UVc dose until a threshold UV dose . The values of threshold UV doses were different in relation with the response of each bacteria strain to UVc dose (dose/response).

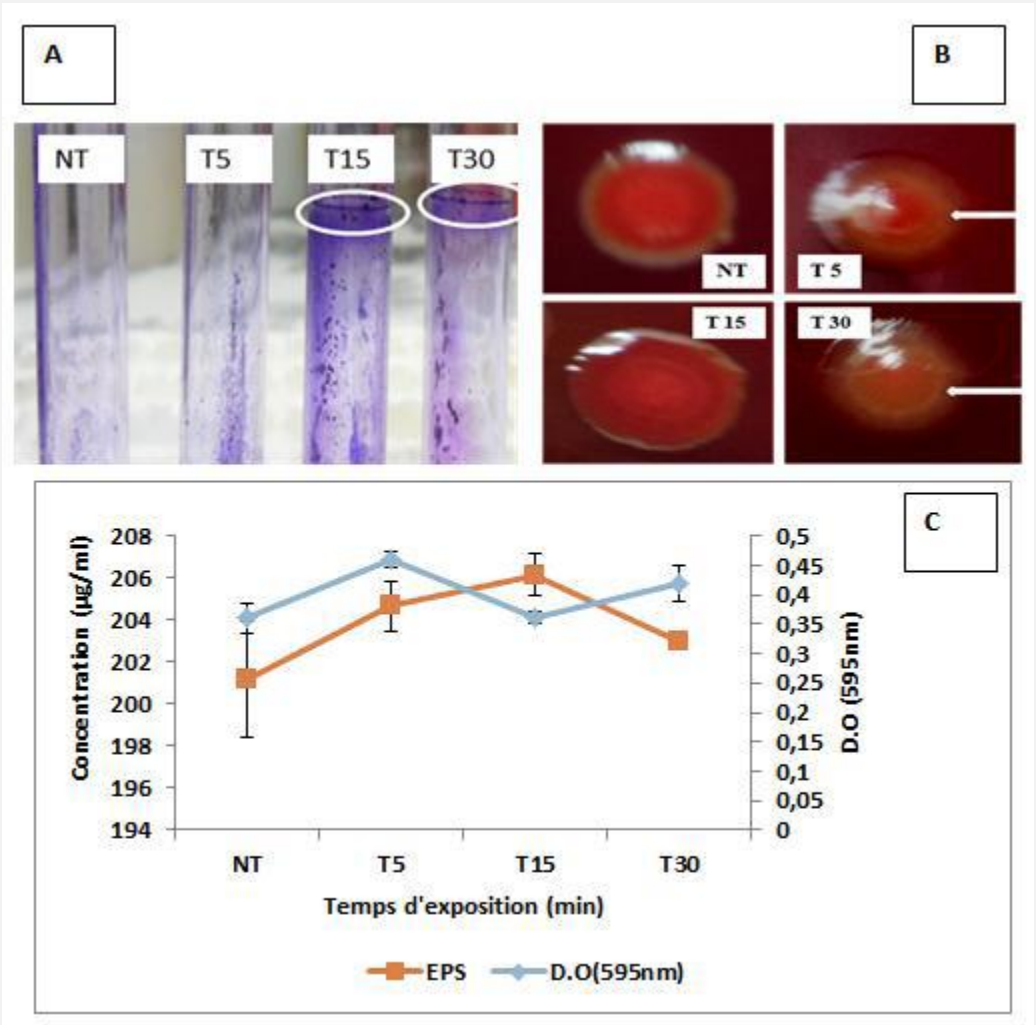

Figure 2: Effects of UVc radiation on adhesion on glass (A), morphotype on CRA (B), biofilm production (O.D at 595nm) in Polyvenylglycol (PVC) microplates and on the content of exopolysaccharides (EPS) (C) in WT. NT: Not treated, T5: treatment for 5 minutes, T15: treatment lasting 15 minutes, T30: treatment lasting 30 minutes.

3.2.2. Effects of UVc Radiation on Biofilm Production in the *sodM* Mutant

In the *sodM* mutant, the production of biofilm on a microtiter plate as well as the content of EPS, are sensitive to short exposure to UVc radiation (5 and 15 minutes). These two parameters decrease after 5 and 15 minutes of treatment (Figure 3-C). On glass tubes, adhesion is important after 5 and 15 minutes of treatment (Figure

3-A). Concerning the morphotype on RC, non-mucoid colonies are observed after 5 and 15 minutes of treatment. However, after 30 minutes of exposure the colonies are necrotic on RC (Figure 3-B-T30) and biofilm production is significantly ($p < 0.05$) stimulated alongside a slight increase in EPS.

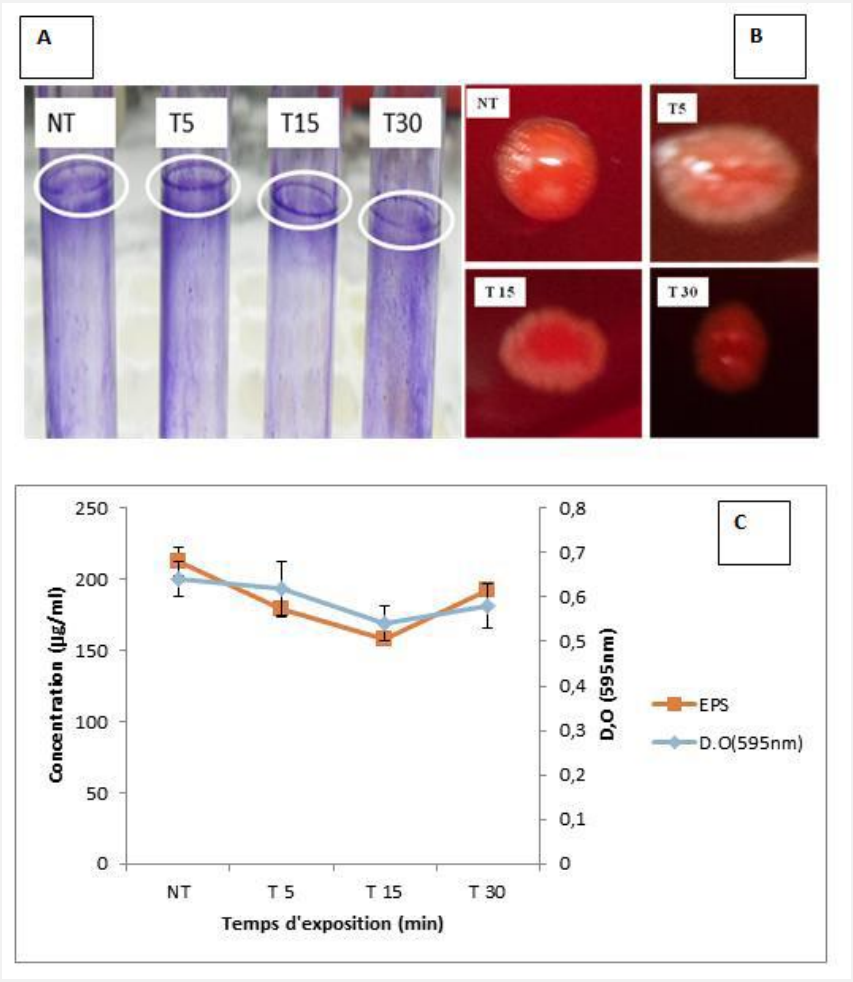

Figure 3: Effects of UVc radiation on adhesion on glass (A), the morphotype on red medium Congo (B), biofilm production (D.O at 595nm) in Polyvenylglycol (PVC) microplates and on the content of exopolysaccharides (EPS) (C) in *sodM*. NT: Not treated, T5: treatment for 5 minutes, T15: treatment lasting 15 minutes, T30: treatment lasting 30 minutes.

It can be seen that biofilm production in *sodM* mutant is severely affected by exposure to UVc radiation, at 5 and 15 minutes of exposure. This shows that Fe-sod isoform alone cannot protect the *sodM* mutant from oxidative stress induced by UVc radiation, hence the phenomenal drop in biofilm production after 5 and 15 minutes of exposure. In parallel, it has been shown in later studies (Kloula et al. 2016) that CAT activity has increased significantly after 5 and 15 minutes, replacing the loss of Mn-SOD activity, in *sodM* mutant. However, in the present study and at these exposure times (5 and 15 minutes) biofilm production remains affected. We can then conclude that, the increase in Catalase levels, may protect Fe-SOD from damage mediated by lipid peroxidation, at longer exposure time to UVc, thing not seen at shorter exposure times. Hence the slight increase in biofilm production observed after 30 minutes of exposure and the phenomenal drop in biofilm production, at 5 and 15 minutes exposure to UVc .

3.2.3. Effects of UVc radiations on biofilm production in the *sodB* mutant

In *sodB* mutant, adhesion to glass and biofilm production on microtiter plate are optimal, after 5 minutes of treatment (Figure 4-A and C), like in WT strain. This stimulation is concomitant with a crackdown on the production of EPS (Figure 4-C). On RC medium, colonies are few shiny and the autolysis circle is absent (Figure 4-B). The ability to produce a biofilm after 5 minutes of exposure to UVc radiations is stimulated and would be due to a control of the oxidative stress induced by UVc radiations. Indeed, it was later demonstrated that the 87% loss of SOD activity in the *sodB* mutant is compensated by an increase in CAT activity with a peak observed at 5 minutes of exposure (Kloula et al.2016). In effect, the control of oxidative

stress induced by UVc radiation, by high CAT activity and also a progressive enhancement of EPS production which prevents UVc radiations to reach biofilm and could explain the maintenance of the ability to produce a biofilm at 5 minutes of exposure. However, after 15 minutes of treatment, the expression of biofilm production is swapped. Indeed, the production of EPS is maximum (206 µg / ml) while the O.D at 595nm decreased (0.85) (Figure 4-C). The comparative analysis of these parameters shows that biofilm and EPS production are inversely proportional, even after 30 minutes of exposure.

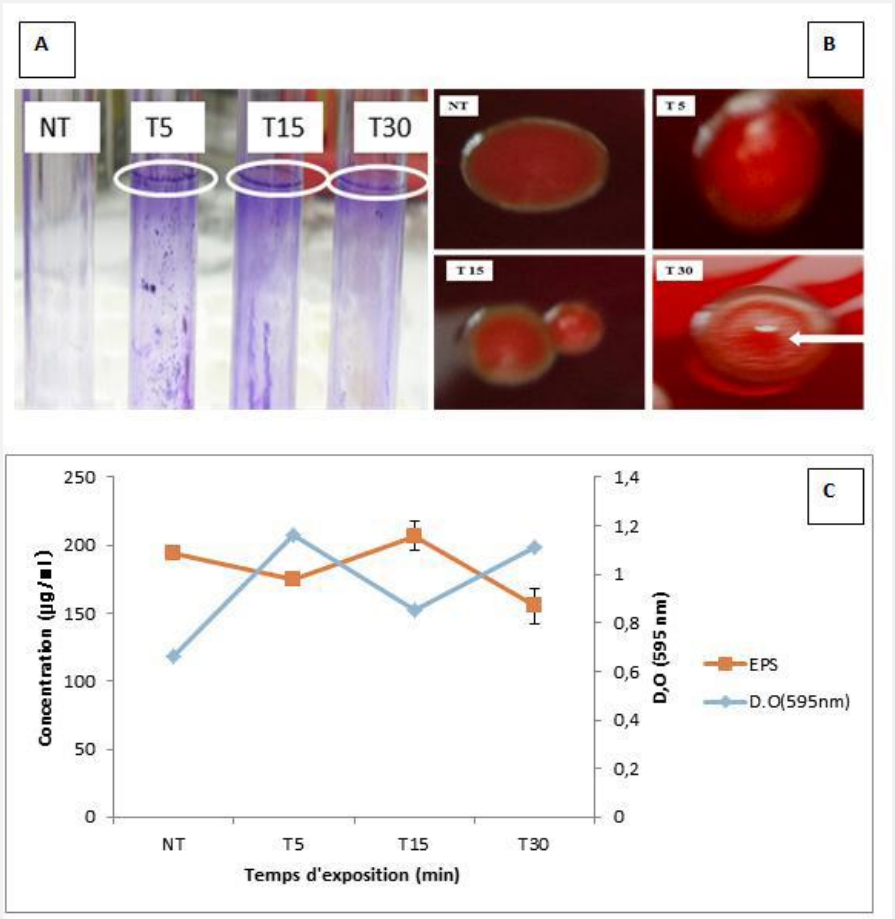

Figure 4 : Effects of UVc radiation on adhesion on glass (A), morphotype on Red medium Congo (B), biofilm production (D.O at 595nm) in Polyvenylglycol (PVC) microplates and on the content of exopolysaccharides (EPS) (C) in *sodB*. NT: Not treated, T5: treatment for 5 minutes, T15: treatment lasting 15 minutes, T30: treatment lasting 30 minutes.

In this work, we have demonstrated the protective role of SOD against stress induced by UVc radiations in *P. aeruginosa* aptitude to form biofilm. For WT and *sod B*, the two curves of biofilm production on Microtiter plates ($O.D_{595}$) show the same profile and can be superimposed for different exposure times. We can conclude that *sodB* mutant keeps the same power of biofilm production as the wild strain, despite the lack of the Fe-sod isoform and the loss of 87% of total SOD activity (Hassett et al.1995). This says that Mn-sod isoform and catalase activities which are involved in the protection against oxidative stress, generated by the exposure to UVc, which explains the maintenance of the power to produce a biofilm, under stress induced by UVc exposure.

3.2.4. Effects of UVc radiations on biofilm production in the *sodMB* mutant

Comparative analysis of adhesion to glass (Figure 5-A) and biofilm production on microtiter plate, in the double mutant *sodMB*, shows the importance of this production before treatment (1.57). However, after UVc treatment the biofilm production is gradually inhibited (from 1.57 to 0.37) (Figure 5-C). This response is expected due to the lack of the Mn-sod isoform, confirmed to be at the origin of the protective role under stress conditions in the simple *sodB* mutant.

The production of EPS drops significantly ($p < 0.05$) after 30 minutes of treatment (Figure 5-C). It is then assumed that the biofilm production in the *sodMB* double mutant is sensitive to prolonged exposure UVc radiation. Indeed, the morphotype changes radically after treatment; the colonies with an orange pigment in the center, surrounded by a circle of autolysis become very mucoids after 15 minutes (Figure 5-B).

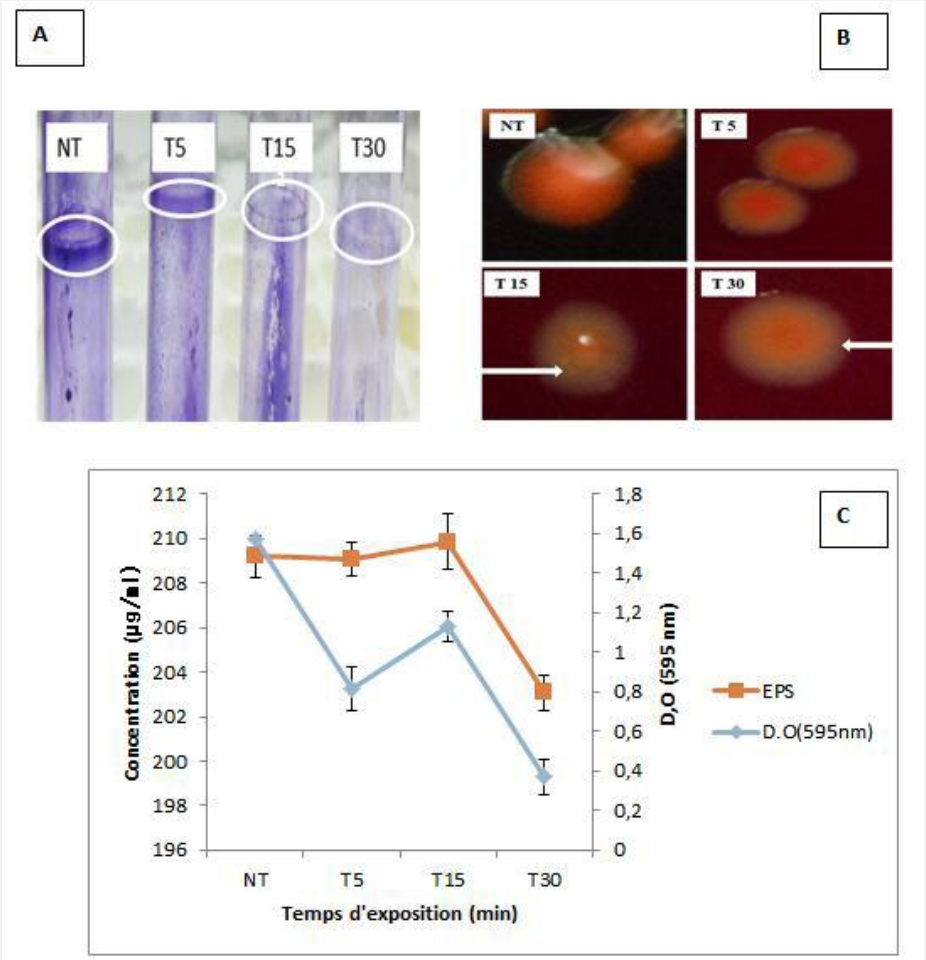

Figure 5: Effects of UVc radiation on adhesion on glass (A), morphotype on Red medium Congo (B), biofilm production (D.O at 595nm) in Polyvenylglycol (PVC) microplates and the content of exopolysaccharides (EPS) (C) in *sodMB*. NT: Not treated, T5: treatment for 5 minutes, T15: treatment lasting 15 minutes, T30: treatment lasting 30 minutes.

The peak production of EPS, in response to prolonged exposure to UVc radiation (15 minutes), in the wild-type strain (WT), the *sodB* and the *sodMB* double mutant, underlines the role of EPS under stress conditions while EPS production is minimal at this exposure time, in *sodM* mutant. The study of Chen et al, (2004) conducted on the same bacterial species, showed that the production of EPS was strongly increased following oxidative or osmotic stress. It seems that the survival rate is much higher for EPS producing cells than for non producing strains. Other roles have also been associated with EPS; the mucositis of *P.aeruginosa* is due to the production of exopolysaccharide. Indeed, in the present study, the mucoid morphotype observed on RC medium, in the mutant *sodM*, is associated with a maximum production of EPS (212 µg/ml). Similarly, for the *sodMB* double mutant, a mucoid phenotype was observed after 15 minutes of treatment and will be associated with significant EPS production (209 µg/ml). Bacteria enclosed in a layer of expolysaccharides are protected by 13% from UVc radiation (Elasri and Miller, 1999). It is then assumed that following the prolonged exposure to UVc radiation, the production of EPS is stimulated, to ensure a better protection against UVc rays. Indeed, in the wild type (WT), the production of EPS is stimulated after 5 and 15 minutes of treatment and seems to be at the origin of the resistance to UVc. Similarly for the *sodB* and *sodMB* mutants, the production of EPS after 15 minutes of treatment further protects these mutants from the effect of UVc. The study of the adhesion of *P.aeruginosa* and its isogenic mutants *sodM*, *sodB* and *sodMB*, on glass also gave an idea of the architecture of the biofilm after exposure to UVc radiation and to link it to the quantitative and quantitative production of the biofilm as well as to the production of EPS.

4. Conclusion

The goal of this study was to evaluate the sensitivity of Fe-SOD and Mn-SOD to UVc radiations, in *P. aeruginosa* biofilm. In this work, we have demonstrated the protective role of SOD against stress by UVc radiations in *P. aeruginosa* aptitude to form biofilm. In effect, it was confirmed that Mn-sod isoform and catalase activities are involved in the protection against oxidative stress, generated by exposure to UVc. Moreover, EPS overproduction limits the penetration of UVc radiation and may protect bacteria cells within

biofilm from oxidative stress induced by UVc radiations. This may explain the maintenance of the power to produce biofilm, under stress generated by UVc radiations.

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