Vol.58, n.2: pp. 300-308, March-April 2015
http://dx.doi.org/10.1590/S1516-8913201500356
ISSN 1516-8913 Printed in Brazil

BRAZILIAN ARCHIVES OF
BIOLOGY AND TECHNOLOGY
A N I N T E R N A T I O N A L J O U R N A L

Resistance of Oxidative Stress in Biofilm and Planktonic Cells

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ABSTRACT

This work studied the susceptibility of biofilm produced by E. coli *to oxidative stress, and compared the components of free radicals defences: level of glutathione, catalase and dismutase activities in planktonic and biofilm located cells. Results showed the diversity of responses to oxidative stress in bacterial cells in log or stationary phases in both planktonic and biofilm forms. The bacteria were exposed to free-radical donors (H_2O_2, tBOOH, menadione, SIN-1 or peroxynitrite) in a wide rangeof finalconcentrations, from0.5 to 10mM.Different level of toxicity of individual donors, independence of cell type (planktonic forms or biofilm) and phases of growth were observed. The highest oxidative stress resistance was observed for the cells in logarithmic phase of growth treated with H_2O_2, both in planktonic and biofilm forms, whereas for the cells in stationary phase, the highest resistance was observed for menadione. These results showed higher efficiency of agents based on superoxide anion donors in combating bacteria colonizing abiotic surfaces stainless steel (AISI 316L).*

Key words: reactive oxygen species, biofilm, bacterial colonization, planktonic cells, implant

INTRODUCTION

Progression in implant technology requires new materials with improved properties in respect to broadly understood bio- and thrombocompatibility. However, relatively low attention is paid onto a serious problem resulting from susceptibility of biomaterials surface to colonization by opportunistic microbes. The study on biofilm formation process and the metabolism of biofilm's cells should get more attention, and results can be useful for development of new materials for medical applications and new pharmaceutics usable in biofilm eradication. Numerous antibiotics and other antibacterial drugs use the free radicals mechanism. Thus, it is especially important to understand the effects of oxidative stress on biofilm destruction and correlation of microbial metabolism with components of antioxidative defence. This workstudied the susceptibility of biofilm produced by *E. coli* to oxidative stress, and compared the components of free radicals defences: level of glutathione, catalase and dismutase activities in planktonic and biofilm located cells.

MATERIAL AND METHODS

Material

All the chemicals were of analytical grade and were purchased from SIGMA-ALDRICH. Samples of stainless steel (AISI 316L) were prepared by a lathe processing, followed by mechanical polishing.

Cell culture

Samples for bacterial colonisation, disc shape with diameter of 8 mm and thickness of 2 mm, were placed each into a homemade bioreactor *(*200

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mL)and were immersed in the medium containing NaCl (1%), bactopeptone (1%) and yeast extract (0.5%), pH 7.0. The medium was inoculated using approximately $2x10^3$ of *E.coli* DH5α cells. The cells were culturedat 37°C for 24h in the case of the use of the logarithmic phase of growth or for 7 days to stationary growth phase.

Test of cell survival in the application of free-radical donors

A suspension of bacteria in logarithmic growth phase (optical density of 0.5 measured spectrophotometrically at λ=620), was supplemented by free radical donor (H_2O_2, tBOOH, menadione, SIN-1 or peroxynitrite) to final concentration of 0.5 mm (for a volume of 10 mL). After 30 min incubation at 37°C, the sample was counted living and dead cell.The same procedure was carried out for the final concentrations of 1, 2, 5 and 10 mM.The whole procedure was carried out as a suspension of the cells in stationary phase of growth.In the case of biofilm cells (for both the growth phases cultures), colonized surfaces of steel samples were placed in the same solution as in the case of planktonic cells (0.5, 1, 2, 5 and 10 mM) for 30 min and the cells were stained for counting, distinguishing between the living and dead cells.

Visualization of *E. coli* cells on sample surfaces

Samples removed from the growth medium were washed extensively with deionised water and fluorescently stained with *bis*-benzimide. Each surface was soaked with the dye by applying 20 µL of stock solution (100 µg/mL). The dye was allowed to penetrate the cells and bind to dsDNA. This process was carried out in the darkat 28°C for 10 min. Finally, bacterial cells present on the sample surfaces were detected using fluorescence microscope (Olympus GX71) and photos were taken with a CCD camera (DC 70).Results were recorded for nine samples, processed in three separate experiments, and up to six randomly selected separate areas were inspected for each sample (Jakubowski et al. 2004). In addition, some samples were subjected to visualization by means of a scanning electron microscope (SEM). Briefly, after incubation and washing as above the samples were fixed for an hour at 4°C in 2.5% solution of glutaraldehyde. Next, the samples were washed three times with deionized water and dehydrated with the use of ethyl alcohol in increasing concentration (60–96%), ten minutes in each. Thereafter, the samples were dried at room temperature. The samples were observed with the use of scanning electron microscope HITACHI S-3000N (after sputtering thin gold film in the sputtering apparatus JEOL JEE-4X).

2',7'-dichlorofluorescin oxidation

Cells were spun down from 2.0-mLaliquots of the cultures, washed three times with 2.0mLof 50 mM sodium phosphate buffer (pH 7.4), re-suspended in the 50 mM sodium phosphate buffer (pH 7.4) at a concentration of 2% (v/v) and pre-incubated at 28°C for 15 min. H_2DCFDA (carboxyfluorescindiacetate, Molecular Probes, Eugene, OR) was prepared as a 1 mM stock solutions in ethanol and added to final a concentration of 10 µM (Jakubowski and Bartosz 1997). After incubation (28°C, 20 min), the cell suspensions were spun down again, re-suspended in the 50 mM sodium phosphate buffer (pH 7.4), and broken by shaking the sediment with glass beads (4°C, 10 min). The homogenates were clarified by centrifugation (3500*g*, 3 min) and fluorescence of the supernatant was measured in a Perkin-Elmer LS-5B spectrofluorimeter (excitation and emission wavelengths of 488 and 520 nm, respectively).

Superoxide dismutase (SOD) activity

SOD activity was estimated with xanthine + xanthine oxidase and Nitro Blue Tetrazolium (NBT) (Beauchamp and Fridovich 1971). Cells were spun down from 2.0mLvolume of the cultures, washed three times with 2.0mLof 50 mM sodium carbonate buffer (pH10.2), re-suspended in the 50 mM sodium carbonate buffer (pH10.2) at a concentration of 2% (v/v) and broken by shaking with glass beads (4°C, 10 min). A spectrophotometric cuvette containing 500 µL of the carbonate buffer containing 200 µM xanthine, 50 µM NBT and 200 µM ethylenediaminetetraacetate (EDTA) was supplemented with xanthine oxidase and the 50 mM sodium carbonate buffer (pH10.2) up to the volume of 1000 µL. The amount of xanthine oxidase was adjusted to obtain a rate of absorption increase of 0.0165 per min (λ = 560 nm) at 25°C (after 30 min absorbance value reached 0.5). Next, into the similarly prepared cuvette were added 100 µL homogenate of logarithmic planktonic cells, and was measured the increase in absorbance (t =30min, λ = 560 nm). The difference between the absorbance of the sample without cell homogenate

(Abs_1 = 0.5) and the Abs obtained for planktonic cells logarithmic (Abs_2) was determined as 100% of the control activity of SOD. The level of SOD activity for the other cell types was obtained by measure of difference of the absorbance and recalculated to the percent of control value.

Catalase activity

Cells were spun down from 2.0 mL aliquots of the cultures, washed three times with 2.0mL of 50 mM sodium phosphate buffer (pH 7.4), re-suspended in the 50 mM sodium phosphate buffer (pH 7.4) at a concentration of 2% (v/v) and broken by shaking the sediment with glass beads (4°C, 10 min). Volume of 333 µL of H_2O_2 solution (54 mM) were mixed with 567 µL of buffer (pH 7.0) and quickly mixed with 100 µL of cells homogenate and the rate of decrease of absorbance at 240 nm was measured during 5 min (Jakubowski et al. 2000). For comparison of differences in catalase activity between tested types of bacterial cells, it was assumed that the rate of decrease in absorbance measured during 5 min for the homogenate of logarithmic phase of planktonic cells is regarded as the activity of the control level, and the values obtained in the same conditions for the other types of cells are presented as a percent of control.

Total glutathione (GSH + GSSG)

Cells were spun down from 2.0 mL aliquots of the cultures, washed, re-suspended in 50 mM potassium phosphate buffer (pH 7.4) at a concentration of 10% (v/v) and added with an equal volume of cold 2 M $HClO_4$ containing 4 mM EDTA and mixed thoroughly. After 15min incubation, the suspensions were centrifuged. One hundred microliter volume of the supernatants of KOH-neutralized perchloric-acid extracts were added to cuvettes containing 1000 µL of 100 mM potassium phosphate buffer (pH 7.0) containing 1mM EDTA, 50 µL 0.4% NADPH in 0.5 % $NaHCO_2$, 20 µL 0.15% 5,5'-dithiobis(2-nitrobenzoic acid) and 20 µL of glutathione reductase solution (activity of 6 U/mL). The reaction rate measured as an increase in absorbance at 412 nm is proportional to the glutathione concentration (Akerboom and Sies 1981).

Statistical evaluation

The ANOVA test was used for statistical evaluation of obtained data with significance put on the level of $p<0.05$. The results are presented in figures as MEAN ± SD.

RESULTS AND DISCUSSION

Examples of biofilm visualization are presented in Figures 1A (fluorescence microscopy) and 1B (scanning electron microscopy). Inorder to determine theresistance of planktonic and biofilm cells to ROS individual stressors in a wide rangeof final concentrations, from 0.5 to 10mM, were used. In the case of H_2O_2 a toxic effect was observed for all the used concentrations, but at 10 mM, total mortality was observed for all the samples. At 5mM, total mortality was observed for planktonic form of the cells, while the cells in biofilm exhibited a minimum level of survival (3.1%for the biofilmin the logarithmic growth phaseand 0.8% for the biofilmin the stationary phase).

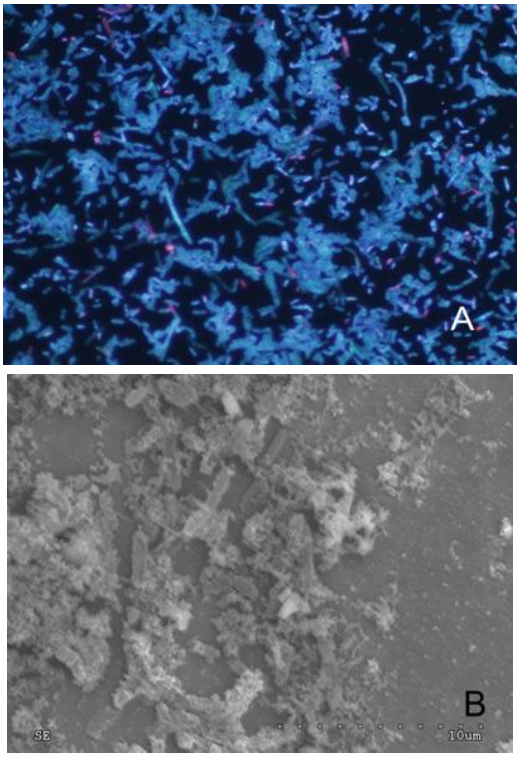
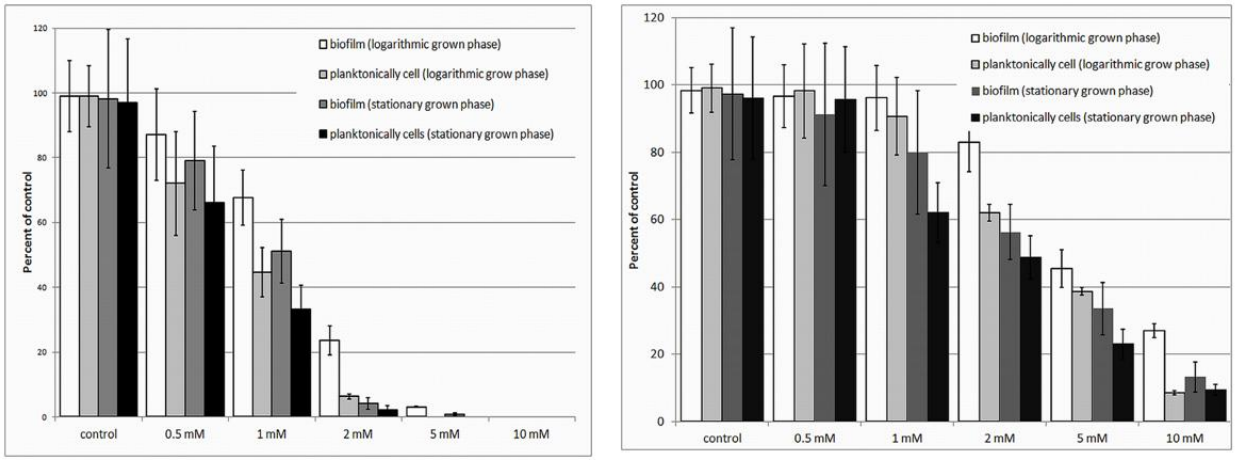


Figure 1 - Observation of biofilm with the use of fluorescence microscope Olympus GX71(A) and scanning electron microscope HITACHI S-3000N (B).

A similar trend was also observed at 0.5, 1 and 2 mM, with a higher resistance to oxidative stress in both planktonic and biofilm cells found in

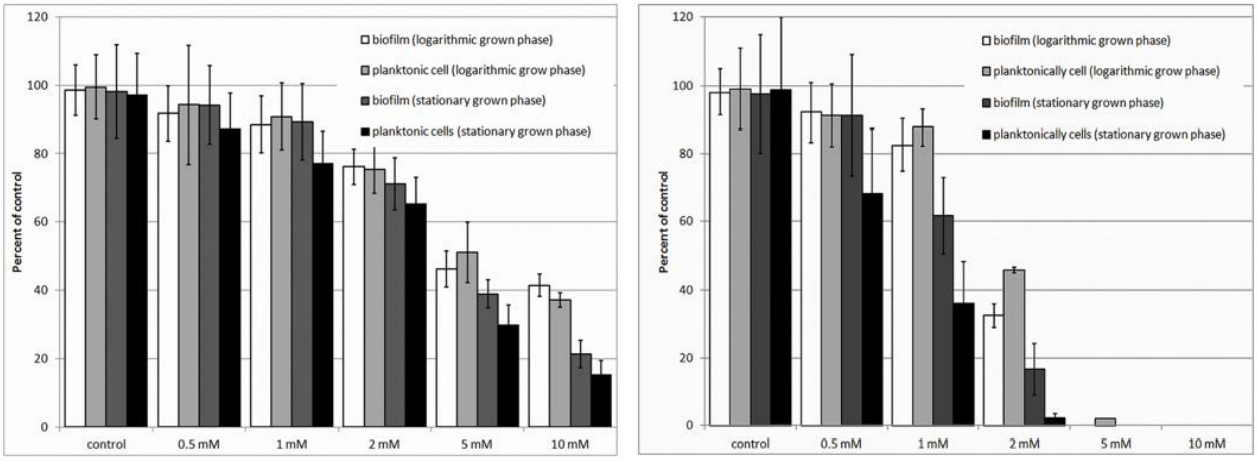
logarithmic phases of grow (Fig. 2A). As shown in Figure 2B, tBOOH also caused death of bacterial cells in the concentration-dependent manner; however, tBOOH was less toxic. The lethal effectof could be observed when tBOOH was used above 1mM, but at the highest concentration (10mM), it did not lead to the total mortality.

A slightly different situation was observed using superoxide anion-generating factor, such as SIN-1. The bacterial mortality was much lower when cells were incubated with SIN-1. As a result of exposure to SIN-1 applied in low concentrations (0.5, 1 and 2 mM), increasing mortality was observed in concentration dependent manner and a higher toxicity was observed for both planktonic and biofilm cells in stationary phase (Fig. 2C). Another compound generating $O_2^{\bullet-}$ - menadione, when used at the two highest concentrations was lethal to all the tested cells. In the case of lower concentrations, more sensitive to menadione were planktonic cells, especially in the stationary phase (Fig. 2D).



A **B**

Figure 2 – (A)The level of cell survival after exposure to H_2O_2. The ANOVA test was used for statistical evaluation of obtained data with significance put on the level of $p<0.05$. The results are presented in figures as MEAN ± SD. (B) The level of cell survival after exposure to tBOOH. The ANOVA test was used for statistical evaluation of obtained data with significance put on the level of $p<0.05$. The results are presented in figures as MEAN ± SD.



C **D**

Figure 2 – (C)The level of cell survival after exposure to SIN-1. The ANOVA test was used for statistical evaluation of obtained data with significance put on the level of $p<0.05$. The results are presented in figures as MEAN ± SD. (D) The level of cell survival after exposure to menadione. The ANOVA test was used for statistical evaluation of obtained data with significance put on the level of $p<0.05$. The results are presented in figures as MEAN ± SD.

Figure 2E showed the results obtained for peroxynitrite – a substance combining features of both menadione and H_2O_2. This compound at 2 mM concentration was 100% lethal to planktonic cells in stationary phase, but planctonic cells in logarithmic growth phase exhibited residual survival (3.6%). Cells in biofilm were more resistant to this compound, especially in stationary phase.

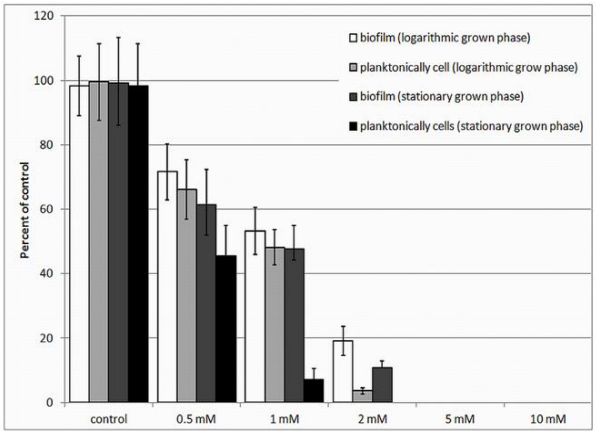


Figure 2 - (E) The level of cell survival after exposure to peroxynitrite. The ANOVA test was used for statistical evaluation of obtained data with significance put on the level of $p<0.05$. The results are presented in figures as MEAN ± SD.

Figure 3 showsthe effects of the exposure of bacteria to radical-generating agents prior to the for formation of biofilms on abiotic surface.

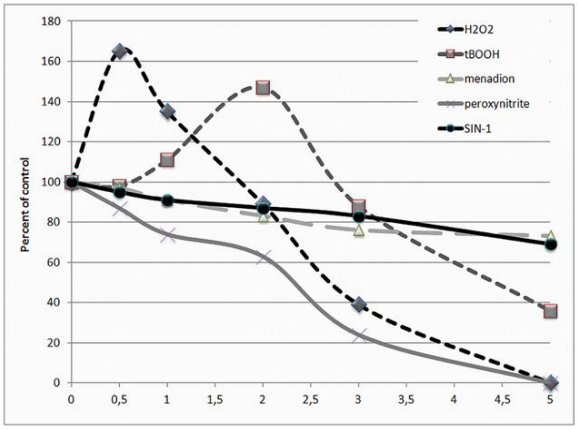


Figure 3 - The formation of the biofilm after exposure to the reactive oxygen species. The ANOVA test was used for statistical evaluation of obtained data with significance put on the level of $p<0.05$.

Results showed differences in the ability to colonize the steel surface between the planktonic cells *E. coli* exposed to the hydroxyl radical, $O_2^{\bullet-}$ or peroxynitride.An exposure of cells to SIN-1 or menadione in the concentrations used (0.5 - 5 mM) caused a slight reduction in the biofilm formation capability. Similar results were observed for the impact of peroxynitrite, which was the most toxic compound among the tested initiators of oxidative stress, and its toxicity was demonstrated at the earlier stage.

Low concentrations of H_2O_2(0.5 and 1 mM) led to an increased formation of bacterial biofilms on abiotic surface being the surface of the medical steel, while the concentration higher than 2 mM caused a rapid reduction in the observed number of adhered cells. Exposure to oxidative stress induced by tBOOH led to the enhancement of biofilm formation at 1 and 2 mM, whereas the 3 mMconcentration caused a decrease in the number of cells observed. At the same time, the agents used at their lowest concentration seemed to have no effect on the biofilm formation rate and the effect was at the limit of observation.

The level of antioxidant ability found in the bacterial cells is presented in Figure 4. The highest level of catalase activity was observed in the cells in biofilm, whereas SOD activity was highest in planktonic cells in logarithmic phase of growth. The key non-enzymatic antioxidant - glutathione, both in reduced and oxidized forms, was found at highest level in the biofilm cells.

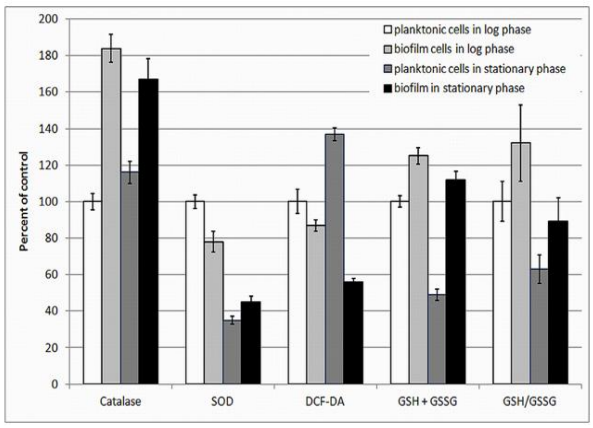


Figure 4 - Elements of protection against oxidative stressThe ANOVA test was used for statistical evaluation of obtained data with significance put on the level of $p<0.05$. The results are presented in figures as MEAN ± SD.

The formation of bacterial biofilm on the surfaces of materials has been considered, with a few exceptions (Jayaraman et al. 1997), as a highly undesirable processes (Geesey 2001; O'Toole 2003; MacKintosh et al. 2006; Subramani et al. 2009; Zeraik and Nitschke 2012). It is particularly undesirable for biomaterial surfaces exposed to a constant contact with the tissues and body fluids of living organism. Abiotic surfaces of implants colonised by the bacteria are, in the majority of cases, the source of opportunistic infections, where the bacterial biofilm develops significantly high resistance pharmaceuticals, making it difficult to eliminate the threat. In the extreme cases, this leads to the need for removing the infected implant, which complicates the patient's process of recovery and results in generating additional costs of the treatment. The formation of hard removable microbial biofilm is not confined to the implant surface (Barbeau et al. 1998; Tenke et al. 2006; Lee et al. 2011), but should be regarded as a serious problem in other cases as the water distribution systems, cooling systems, as it can reduce the performance and functionality of such systems (Williams and Braun-Howland 2003; Chen and Chang 2010; Florjanic and Kristl 2011; Wingender and Flemming 2011; Marangoni et al. 2013). Therefore, it seems to be important to understand the processes that govern the physiology of bacterial biofilm formation and its development.

There are several examples of impediments to combat the bacterial biofilms formed on the surface of biomaterials and there are several explanations for the mechanisms of increased resistance of such a layer of microorganisms to pharmaceutical treatment. The most common explanation of the bacterial biofilm durability is the mechanism of secretion of antibiotic-degrading enzymes, which reduces the possibility of penetration of the antimicrobial substances to the lower layers of cells (Stewart and Costerton 2001; Obst et al. 2006; Chaignon et al. 2007; Hoiby et al. 2010; Banerjee et al. 2011) . Another common argument is based on emerging multi-layer construction of the structure of the biofilm, which also impedes the penetration of antimicrobial substances (Etienne et al. 2005; An et al. 2008). Another reason for the increased resistance of biofilm cells is connected with the observation based on the presence of cells at different stages of development (Chavant et al. 2004; Gad et al. 2004). All those mechanisms are not mutually exclusive, and it seems to be possible that they arise at the same time altogether.

The results of this study suggested the possibility of increased protection of the surface adhered cells – an increased production of elements involved in the protection against reactive forms of oxygen. Significantly lower fluorescence in DCF-DA method showed that the biofilm cells, in comparison to free-living planktonic cells, had indicated a low intensity of oxidative processes (Jakubowski and Bartosz 2000; Jakubowski et al. 2000). This observation could be justified in two ways, either a higher pool of active protection against oxidative stress is present in the cells of the biofilm, or in metabolic processes resulting in reduced levels of generated free radicals. However, one could also combine both the explanations, because there has been a significant decrease in an overall antioxidant protection in planktonic cells during the stationary phase, and a strong reduction in the level of DCF-DA oxidation to its fluorescent form in the stationary phase of biofilm.

Several authors have previously proposed a protective mechanisms for biofilm formed by the microorganisms other than*E.coli*(Albesa et al. 2004; Singer et al. 2009; Arce Miranda et al. 2011; Villegas et al. 2013). The results have shown an interesting relationship - cells of biofilm have a higher resistance to the mediators that produce hydroxyl radical, but in the case of exposure to the superoxide anion, the nature of this relationship is reversed – which means that they are more resistant than the planktonic forms. A possible explanation for this relationship is based on the fact that there are higher levels of catalase in biofilm's cells, which significantly enhance the protection against H_2O_2 and tBOOH. An adhesion of bacteria to the surface and going into a state of a settled lifestyle is associated with the changes in the protein profile (unpublished results). For many microorganisms it was observed that sudden changes in living conditions cause an increase in the level of catalase, and the transition from planktonic form to attached cells form may be accompanied by a similar process (Thieringer et al. 1991; Cianciotto 2001). In addition to the enzymatic degradation of peroxide with catalase, a key role in protecting against H_2O_2 and tBOOH is fulfilled by the glutathione, and the especially important ratio of GSH/GSSG. Our results show a higher level of total glutathione in cells of biofilm when compared to planktonic cells form (in pairs -

biofilm and planktonic cells in the log phase, and biofilm and planktonic cells in the stationary phase). Shown on the figure 4 the reduced level of SOD in cells present on steel surfaces allows substantiating the observation of increased mortality as a result of the exposure of cells to the $O_2^{\bullet-}$ donor (menadione and SIN-1). Moreover, it can also be associated with the observation, reported in the literature, describing the reduced metabolic rate and total amount of protein produced in the settled cells. This slower metabolism and reduction in ROS generation, as well as better protection by the presence of GSH would allow tojustify results of measurements of overall antioxidant protection obtained with oxidation of DCF-DA. A lower level of fluorescence indicates a drop in the pro-oxidant activity within the cells – in both: a better protection effect and a lower internal production of ROS (Jakubowski and Bartosz 2000; de Oliveira and Schoffen 2010).

In this context, it should also be interpreted the results obtained for different types of peroxynitrite treated cells, where the smallest resistance of planktonic cells in log phase weakest correlated with the protection against the effects of exposure to peroxides. The results obtained for different types of the cells treated with peroxynitrite, where the lowest resistance of planktonic cells in logarithmic phase was correlated with the weakest protection against the effects of expose to peroxides, should be interpreted in this context.

The results presented in Figure 3 suggested a significant impact of the factors generating oxidative stress on the process of transition from the form of planktonic cells into the form of settled cells. There was visible a significant increase in the number of cells adhered to the surface of the steel in response to the earlier relatively mild exposure to peroxides (24 h pre-incubation). This added an interesting element to the still unclear mechanism responsible for the promotion of signal causing change of planktonic cells to the sedentary lifestyle. Linking this fact with the previously shown elevated levels of catalase indicated the behaviour associated with the response to stress and transition cells to life giving more chances of survival in the conditions far from optimal. This might be an alternative to the formation of biofilm, but confirmation of this hypothesis would requirefurther study. It should be noted, however, that the effect of superoxide anion used at the same concentration range did not result in intensification of the bacterial biofilm formation by *E. coli* cells.

CONCLUSIONS

The results supplemented the knowledge about the diversity of responses to oxidative stress in the bacterial cells being in log or stationary phases in both planktonic and biofilm forms andplace the hypothesis of higher efficiency of agents based on superoxide anion donors in combating bacteria colonizing abiotic surfaces.

ACKNOWLEDGEMENT

This work was partially supported by the KBN grant no. PBZ KBN 082/T08/13.

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Received: September 09, 2014;
Accepted: January 14, 2015.