Oxidative and nitrosative stress in *Staphylococcus aureus* biofilm

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**Abstract**

Diverse chemical and physical agents can alter cellular functions associated with oxidative metabolism, thus stimulating the production of reactive oxygen species (ROS) and reactive nitrogen intermediates (RNI) in planktonic bacterial physiology. However, more research is necessary to determine the precise role of cellular stress in biofilm. The present study was designed to address the issues of *Staphylococcus aureus* biofilm formation with respect to the generation of oxidative and nitrosative stress. We studied three pathogenic *S. aureus* clinical strains and an ATCC strain exposed to a different range of culture conditions (time, temperature, pH, reduction and atmospheric conditions) using quantitative methods of biofilm detection. We observed that cellular stress could be produced inside biofilms, thereby affecting their growth, resulting in an increase of ROS and RNI production, and a decrease of the extracellular matrix under unfavorable conditions. These results contribute to a better understanding of the processes that enable adherent biofilms to grow on inert surfaces and lead to an improved knowledge of ROS and RNI regulation, which may help to clarify the relevance of biofilm formation in medical devices.

**Introduction**

*Staphylococcus aureus* is one of the pathogens of nosocomial sepsis that is most frequently isolated, especially in patients with indwelling medical devices, and at risk of contracting chronic staphylococcal foreign body-associated infections (Götz et al., 2000; Costerton et al., 2005), mediated by the ability of the microorganism to form biofilms on different surfaces. These biofilm-embedded bacteria are more resistant to stressful conditions and antimicrobial agents than their planktonic counterparts (Schlag et al., 2007; Otto, 2008), with staphylococcal biofilm formation being a multifactorial and dynamic process. The ability of bacteria to form biofilm is strictly related to their capacity to produce an extracellular mucous substance, the main component of which is of a polysaccharide nature and consists of glycosaminoglycans (Götz, 2002). The adherence to biomaterials and the formation of biofilms are affected by a variety of environmental conditions (Pamp et al., 2006), such as nutrient availability (Bowden & Li, 1997; Croes et al., 2009), low oxygen (Cramton et al., 2001), high osmolality (Lim et al., 2004) and subinhibitory antibiotic concentrations (Aiassa et al., 2010; Páez et al., 2010).

Diverse chemical and physical agents can alter the cellular functions associated with oxidative metabolism, thereby stimulating the production of reactive oxygen species (ROS). In *in vivo* and *in vitro* studies have related the toxicity to prokaryotic cells to the generation of ROS, including superoxide (O₂⁻), hydrogen peroxide (H₂O₂), the extremely reactive hydroxyl radical (HO·), peroxyl radical (ROO·) and singlet oxygen (¹O₂) (Aiassa et al., 2010; Páez et al., 2010). However, the production of ROS by *S. aureus* has been investigated in relation to adhesion and biofilm formation, and it could be useful to study the different factors that participate in the physiological characteristics of this bacterium. Another form of stress is termed nitrosative stress, with nitrate (NO₃⁻) and nitrite (NO₂⁻) used as terminal electron acceptors under anaerobic conditions. Schlag et al. (2007) have reported interplay between respiratory nitrate reduction and biofilm formation in *S. aureus* SA113 and *Staphylococcus epidermidis* RP62A and have shown that the presence of nitrite, a product of nitrate respiration, causes a
stress response, which concomitantly involves impairment of PIA-mediated biofilm formation. They have also provided data suggesting that the acidified nitrite derivative nitric oxide (NO), widely used as a defense or signaling molecule in biological systems, is directly or indirectly involved in the inhibition of S. aureus biofilm formation (Schlag et al., 2007).

Although the roles of ROS and reactive nitrogen intermediates (RNI) have been extensively studied in planktonic bacterial physiology, there is still limited information available, and more research is necessary to determine the precise role of cellular stress in biofilm. The present study was designed to address the issues of S. aureus adhesion and inhibition of biofilm with respect to the generation of oxidative and nitrosative stress. For this purpose, an in vitro method of ROS and RNI production was developed, which to our knowledge is the first study that has attempted to correlate biofilm formation with the alteration of ROS and RNI production under stressful conditions.

Materials and methods

Bacterial strains and culture conditions

In our study, three pathogenic S. aureus clinical strains (associated with different indwelling medical devices) and an ATCC 29213 strain (a biofilm control) were used. Stock cultures were maintained in 20% glycerol at −80 °C.

Quantification of biofilm formation by the use of crystal violet (CV)

The biofilm-forming ability of the strains was measured by determination of the adhesion to 96-well plates. The assay for biofilm formation used for this study was adapted from the method of O’Toole & Kolter (1998), which is based on the ability of bacteria to form biofilm on solid surfaces and uses CV to stain biofilms. Briefly, a final cell concentration of approximately 1 × 10⁹ CFU mL⁻¹ was mixed with 200 μL of TSB in each well of flat-bottomed microtiter plates (96-well, Greiner Bio-One, Germany), and then incubated without agitation under different conditions. After incubation, the supernatant was separated for extracellular oxidative and nitrosative stress assay and the plate was rinsed with phosphate-buffered solution (PBS, pH 7.2). After drying, staining for adherent biofilms was performed using CV (1%). Then, the CV was removed and cells were rinsed three times with 300 μL PBS (pH 7.2) before drying for 24 h at room temperature. A quantitative assessment of the biofilm 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. The average OD₅₉₅ nm value was determined by three replicates and was interpreted by the following scale: positive (> 0.24), weak (> 0.12 and < 0.24) or negative (< 0.12) (Deighton et al., 2001). The biofilm biomass unit (BBU) was arbitrarily defined with 0.1 OD₅₉₅ nm = 1 BBU.

Influence of incubation time, temperature, pH, reduction and atmospheric conditions

Biofilm formation was investigated at 12, 24 and 48 h, and the effect of temperature was evaluated at 25, 30 or 37 °C. Static conditions were used at 37 °C for 24 h at different pH values (5–8). The influence of the reduction conditions was assayed in thioglycolate broth and microaerobic conditions with TSB or thioglycolate broth were also studied.

Three wells with 200 μL TSB or thioglycolate were added to serve as negative controls and to obtain a background value, which was then subtracted from the values obtained from the cells in the wells.

Oxidative metabolism of biofilm

The intra- (iROS) and extracellular (eROS) production of ROS was detected by the reduction of nitro-blue tetrazolium (NBT, Sigma) to nitroblue diformazan. The supernatant was separated by measuring the eROS. Then the biofilms in individual wells of sterile 96-well polystyrene microtiter plates were treated with 0.05 mL dimethyl sulfoxide (Merck) to extract the reduced NBT using 0.1 mL NBT (1 mg mL⁻¹) and 0.1 mL TSB (for the final volume) at 37 °C for 30 min, followed by the addition of 0.02 mL hydrochloric acid (0.1 M) to stop the reaction and measure iROS. The reaction is detectable by the byproducts of the assay, which are proportional to the ROS generated in biofilm and were measured by OD at 540 nm (Paraje et al., 2009; Aiassa et al., 2010; Páez et al., 2010).

RNI metabolism

The supernatant under different conditions was separated for extracellular nitrosative stress assay and incubated for NO measurement. The NO was evaluated as nitrite by a microplate assay method using Griess reagent (Paraje et al., 2009). One hundred microliter aliquots of supernatant were mixed with 200 μL Griess reagent [sulfanilamide 1.5% in 1 N HCl and N-1-naphthyl ethylenediamine dihydrochloride 0.13% in sterile distilled water (dH₂O)]. Absorbance was measured at 540 nm in a microplate reader, and the nitrite using NaNO₂ as standard. Each sample was tested in triplicate and results were expressed in μM.
Oxidative and nitrosative stress in biofilm

Biofilm research by optical microscopy and confocal scanning laser microscopy (CSLM)

Biofilms were observed by CSLM as described below (Otto, 2008). Before imaging, the biofilm was formed in microtiter plates (24-well, Greiner Bio-One, Germany), and rinsed with sterile 50 mM potassium phosphate buffer solution (pH 7.2; no autofluorescence detected) for 10 min before being stained with 15 μM propidium iodide (Sigma) for 5 min at room temperature to detect bacterial cells in red. After being washed in PBS, the samples were incubated with 50 μg/mL of fluorescein isothiocyanate-conjugated Con A (FITC–Con A) (Sigma) for 5 min at room temperature to stain the glycocalyx matrix green. The propidium iodide was excited at 520 nm, the emission was monitored at 620 nm, and FITC–Con A at 495 and 525 nm, respectively. Intact biofilms were examined nondestructively using a Fluoview FV1000 Spectral Olympus CSLM (Olympus Latin America, Miami, FL) equipped with UPlanSApo ×100/1.40 oil UIS2 Olympus oil immersion lens. Optical sections of 0.87 μm were collected over the complete thickness of biofilms. Then, for each sample, images from three randomly selected positions were obtained and analyzed using an Olympus Fluoview FV 1000 (Zernotti et al., 2010). For image analysis, three investigators (J.A.M., I.A. and M.G.P.) evaluated the images independently in a blinded retrospective manner.

Statistical analysis

All experiments were performed in triplicate and numerical data are presented as means with error bars representing SDs. The data were statistically analyzed using a one-way ANOVA followed by the Student–Newman–Keuls test for multiple comparisons. Differences between means were assessed with a P-value < 0.05 being considered statistically significant.

Results

Influence of incubation time, temperature and pH on ROS and RNI production by biofilm

A quantitative analysis showed that the S. aureus cells attached to 96-well plates exhibited good biofilm formation (according to the scale described in Materials and methods) after 18 h and remained up to 24 h. However after 48 h, their attachment was significantly reduced (P < 0.005) when tested under the same experimental conditions. The ATCC strain was stable until 48 h (BBU = 2.50), with this strain showing the best biofilm formation between 18 and 24 h. After 48 h, the biofilm of S. aureus was detached from the abiotic surface. No additional biofilm production was detected after 72 h compared with incubation at 48 h. Therefore, 18–24 h was chosen for the other assays (Fig. 1a).

The production of detectable amounts of ROS and RNI (NO) by S. aureus in the biofilm was evaluated by NBT and Griess, respectively. These assays were useful in determining the relation between the intracellular and extracellular metabolite strains, determined by iROS/BBU (Fig. 1b), eROS/BBU (Fig. 1c) and NO/BBU (Fig. 1d), where the BBU were obtained from each strain of S. aureus.

The iROS, eROS and NO in all strains showed an increase at 48 h, except for the ROS in ATCC strain, with the rise in extracellular reactive species (eROS and NO) being more significant than iROS.

To determine the optimal temperature for biofilm formation, the S. aureus attached to polypropylene was studied at different temperatures. We observed that this process was more efficient at 37 °C than at 30 or 25 °C (data not shown).

Adhesion was also studied at different pH ranges (5.6–8.0). At a slightly acidic pH, the biofilm formation was 3.5-fold higher than at the basic pH. However, at the physiological pH range, the biofilm formation was more stable (Fig. 2a).

When different pH values were assayed, the extracellular metabolites (eROS and NO) increased significantly with a rise in pH. However, the increase in iROS was not as important at basic pH (Fig. 2b–d). The level of biofilm formation was inversely related to the extracellular metabolites acquired, and the increase of extracellular reactive species was also more significant than iROS.

Effect of reduced culture medium and aerobic/microaerobic atmosphere on biofilm formation

We compared S. aureus biofilm formation under aerobic and microaerobic growth conditions in TSB and in thioglycolate medium, respectively. When assays were performed with thioglycolate medium in aerobiosis, an increase in biofilm formation was seen with respect to TSB (Fig. 3a). For this condition, the thioglycolate medium produced better biofilm formation, with lower ROS and ON occurring (Fig. 3b–d).

The total production of biofilm with TSB medium was found to be approximately the same for both aerobic and microaerobic conditions at 37 °C. However, incubation under the microaerobic condition in thioglycolate medium resulted in significantly less biofilm formation for all the strains, compared with aerobic incubation (Fig. 4a). In contrast to the aerobic condition, microaerobiosis, the biofilm formation in thioglycolate medium did not strongly stimulate biofilm formation, but produced eROS and NO (Fig. 4c and d) (P vs. TSB < 0.005).

Biofilm fesearch by optical microscopy and CSLM

CSLM staining of the bacterial DNA and the glycopolysaccharide of the matrix was used to quantify structural biofilm
Changes with respect to differences in the culture conditions. Images were obtained using a CSLM microscope and two fluorescence stainings were used (propidium iodide and FITC–Con A). The panel in Fig. 4 shows laser scanning fluorescence images for XY (top) and XZ (bottom), of the glycocalyx matrix (green) and dead cells (red) of *S. aureus* ATCC 29213. Similar images were obtained with clinical strains (data not shown).

Biofilm formation in the thioglycolate medium in aero-biosis was greater than in TSB (5.96 vs. 5.02 μm). In
microaerophilia, in thioglycolate medium less biofilm was formed than for aerobiosis (5.96 vs. 5.25 μm). The presence of microcolonies were observed with more dead cells (40%).

**Discussion**

The strains producing biofilm display greater adhesive abilities in comparison to nonproducing ones (Svensäter et al., 2001; Rollet et al., 2009). Clinical isolates are frequently found to produce catheter-associated infections and also postsurgical infections, with a markedly high proportion of these being biofilm-producing strains. Indeed, the abundance of polysaccharides in virulent clinical isolates emphasizes their importance in colonization (Ammendolia et al., 1999).

Several reports have demonstrated that PIA synthesis, as well as biofilm formation by *S. aureus*, are significantly affected by a number of environmental stresses (Cramton et al., 2001; Pamp et al., 2006; Rode et al., 2007; Agostinho et al., 2009). The present study showed diverse patterns of biofilm formation for four *S. aureus* strains exposed to a different range of culture conditions, including time, temperature, pH, reducing conditions and atmosphere. The MTP method was useful as a quantitative technique to measure the biofilm developed from these studies. Although it is clear that the formation of biofilms had an optimal time (18–24 h), temperature (37°C) and pH (lightly acidic), it is also evident that this bacterium could form biofilms under a wide range of conditions. This property could explain the ability of this pathogen to persist successfully in medical environments, where cells persist on various surfaces such as those of hospital furniture, medical devices or food installations, where small numbers of many different organisms initially attach to microirregularities on surfaces, which in time are able to form micro- and macrocolonies that can enter the blood stream and cause septicemia (Herrera et al., 2007).

Although *S. aureus* is now known to produce biofilm, little is known about the environmental factors that triggers
this formation. We observed that biofilm formation was influenced by different conditions, with there being a close relation with extracellular stress (eROS and NO). The NBT assay was useful in determining the iROS and eROS production in *S. aureus* biofilm and allowed us to observe that the increase in the extracellular stress (eROS and ON) was more significant than that of iROS.

NO is obtained from a product of the anaerobic reduction, with this process resulting in a switch from O$_2$ to NO$^{3-}$, NO$^2$ or nitrous oxide (N$_2$O) as the electron acceptor. Barraud et al. (2006) detected ONOO$^-$ inside microcolonies in *Pseudomonas aeruginosa* biofilms, with ONOO$^-$ being formed from NO oxidation only in the presence of ROS (Barraud et al., 2006). Although it is not clear how ONOO$^-$ is produced inside the microcolonies, O$_2$ gradients can occur, with simultaneous O$_2$ and NO$^2$ respiration having recently been demonstrated for *P. aeruginosa* populations (Chen et al., 2006). Schlag et al. (2007) characterized the response of *S. aureus* to nitrite-induced stress and showed that it involved the impairment of PIA synthesis and biofilm formation. They also provided evidence that nitrite-derived NO played a role in the inhibition of biofilm formation and that biofilm-embedded staphylococci could be efficiently killed by nitrite in an acidic environment. Despite NO exposure being able to reduce staphylococcal viability (Kaplan et al., 1996), *S. aureus* has been described as being relatively resistant to growth inhibition by NO, principally by the observation of efficient NO scavenging mechanisms, where flavohemoprotein Hmp was shown to play a crucial role in countering NO toxicity (Richardson et al., 2006). In this study, we observed that oxidative and nitrosative stress could be produced inside biofilms, thereby affecting their growth under different conditions and resulting in ROS and RNI production, with a decrease of the extracellular matrix.

Our data and those from other authors (Beenken et al., 2004; Resch et al., 2005; Zhu et al., 2007) suggest a strong relation between the incubation atmosphere and biofilm formation. Consistent with previous observations, our data demonstrated *S. aureus* in a biofilm to be growing microaerobically, and after 24 h the residual nitrite concentrations rose in the culture supernatants with respect to the initial levels of nitrate and nitrite. When we compared the effect of microaerobiosis, it was evident that the strains exhibited maximum extracellular stress, with the reduced culture possibly increasing the shelf life of these species and their derivatives in these conditions. As no other report was found in the literature about this effect, the oxidative stress stimuli should now be incorporated into the list of factors involved in the formation of biofilm.

In conclusion, we observed that ROS, RNI and its downstream derivatives could play an important role in biofilm development. This suggests that cellular stress is produced inside biofilms, thereby affecting their growth under different conditions and resulting in ROS and RNI production, with a decrease of the extracellular matrix occurring under unfavorable conditions. These radical oxidizers could then accumulate in an extracellular medium and thus affect the matrix.

These results contribute to a better understanding of the processes that enable adherent biofilms to grow on inert surfaces and lead to an improved knowledge of ROS and RNI regulation, which may help to clarify the relevance of biofilm formation in medical devices.

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**References**


