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Sae regulator factor impairs the response to photodynamic inactivation mediated by Toluidine blue in *Staphylococcus aureus*

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ABSTRACT

Photodynamic inactivation (PDI) involves the combined use of light and a photosensitizer, which, in the presence of oxygen, originates cytotoxic species capable of inactivating bacteria. Since the emergence of multi-resistant bacterial strains is becoming an increasing public health concern, PDI becomes an attractive choice.

The aim of this work was to study the differential susceptibility to Toluidine blue (TB) mediated PDI (TB-PDI) of *S. aureus* mutants (RN6390 and Newman backgrounds) for different key regulators of virulence factors related to some extent to oxidative stress.

Complete bacteria eradication of planktonic cultures of RN6390 *S. aureus* photosensitized with 13 μM TB was obtained upon illumination with a low light dose of 4.2 J/cm^2 from a non-coherent light source. Similarly, complete cell death was achieved applying 1.3 μM TB and 19 J/cm^2 light dose, showing that higher light doses can lead to equal cell death employing low photosensitizer concentrations. Interestingly, RN6390 in planktonic culture responded significantly better to TB-PDI than the Newman strain.

We showed that deficiencies in *rsbU, mgrA* (transcription factors related to stress response) or *agr* (quorum sensing system involved in copper resistance to oxidative stress) did not modify the response of planktonic *S. aureus* to PDI. On the other hand, the two component system *sae* impaired the response to TB-PDI through a mechanism not related to the Eap adhesin.

More severe conditions were needed to inactivate *S. aureus* biofilms (0.5 mM TB, 157 J/cm^2 laser light). In mutant *sae* biofilms, strain dependant differential susceptibilities are not noticed.

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1. Introduction

Staphylococcus aureus are Gram positive cocci whose natural reservoirs are human skin and mucous membranes [1]. The rupture of any of those barriers can favour the development of *S. aureus*-induced human diseases, including severe chronic or acute infections both in hospitalized patients and community individuals [2]. In particular, *S. aureus* causes several skin infections such as impetigo, cellulitis, foliculitis and infected wounds [3].

The eradication of *S. aureus* is difficult not only due to the emergence of antibiotic resistances (such us methicillin and vancomycin) but also to the ability to form highly organized communities, known as biofilms [4]. For these reasons, alternative therapies are necessary to control the Staphylococcal infections.

Treatment of staphylococcal infections can be approached from different perspectives besides traditional antibiotics, one being the use of photosensitisers (PS) that are activated in the presence of oxygen, when illuminated with the proper wavelength, leading to cell death by inducing unregulated oxidative stress [5]. This treatment is known as antimicrobial photodynamic therapy or photodynamic inactivation (PDI). PDI is particularly good for dental and dermatological applications [6–9], and it has been recently used for treatment of oral infections, periodontal or endodontic infections [10–12], acne vulgaris, non-healing ulcers [13] and burn wound infections [6,14]. In addition, PDI utilization has been suggested for the treatment of colonized catheters and other medical devices [15,16], bacterial induced gastric ulcers [17], craniotomy-derived brain infections or several trauma-associated infections as

Abbreviations: PDI, photodynamic inactivation; PS, photosensitizer; TB, toluidine blue; TB-PDI, toluidine blue mediated photodynamic inactivation.

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Fournier's gangrene, necrotizing cellulitis, etc. [18]. The procedure has been also proposed as a tool for hand hygiene procedures [19]. The appearance of PDI-resistant strains is not likely to happen, since antibiotics multi-resistance is thought to be a completely independent factor regarding PDI susceptibility [18,20,21]. Since the emergence of multi-resistant bacterial strains is becoming an increasing public health concern, PDI becomes an attractive choice.

The cationic PS Toluidine blue (TB) is one of the most widely used PSs [14,16,22,23]. It is highly noteworthy that TB shows certain dark cytotoxicity at high concentrations due to its capacity to cycle between an oxidized cationic form, and a reduced colorless one; reduction that is accompanied by ROS production [24]. This cyclic behavior allows TB to interfere with the respiratory chain of bacteria, inducing oxidative stress [25,26].

While the mechanism underlying the response of *S. aureus* to PDI still remains unclear, a connection between several phenotype features and bacterial susceptibility to photoinactivation has been proposed [27–35], and a correlation between the ability of forming biofilms and the resistance to PDI has been suggested [28,30]. Though, the information about a correlation between PDI susceptibility and virulence factor regulators of *S. aureus* is scarce.

This pathogenic bacteria expresses a plethora of virulence factors accurately regulated by a complex network of different transcriptional factors (e.g.: *mgrA*, σ^B) and several two-component systems (e.g.: *sae*). In addition, the *agr* system is activated in response to cell density or quorum sensing, and is generally related to the positive regulation of extracellular proteins and negative regulation of cell wall-associated proteins [36,37]. It is known that the *agr* locus is involved in several stages of biofilm development, showing a complex, and highly context-dependent dynamic [38]. The synthesis of surface proteins is repressed by *mgrA*, whilst activates the exoproteins production. The σ^B factor of the RN6390 strain is downregulated due to a natural 11-bp deletion in the *rsbU* phosphatase gene [39]. This transcription factor is related to the bacterial ability to cope with stress [40]. In addition, the σ^B transcription factor negatively affects the *agr* system, thus its activity is upregulated in the RN6390 strain [41]. In addition, the *sae* system upregulates a wide variety of virulence factors including proteins that link bacteria to the extracellular matrix of the host, like Eap or FnBPs adhesins [42]. Mutations in *sae* are present in several uncharacterized *S. aureus* strains isolated from patients [43].

Host innate immune systems respond to bacterial infections by generating reactive oxygen and nitrogen species. These oxidative stresses are sensed in parallel signalling pathways through several systems in *S. aureus* [44]. In particular, it was found that a redox-sensing mechanism related to hydrogen peroxide stress is responsible for *mgrA* signalling [44]. Besides both *agr* and *sae* systems have been found to be involved in the response to oxidative stress [45,46].

The aim of this work was to study the differential susceptibility to TB mediated PDI (TB-PDI) of *S. aureus* mutants for different key regulators of virulence factors involved both in planktonic and biofilm lifestyles and which have been related to some extent to oxidative stress.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The following *S. aureus* strains were used in this study: Newman (ATCC 25904), Newman *mgrA*$^-$ (Newman *mgrA*::*ermB*) [47], Newman *sae*$^-$ (Newman Δ*saeRS*) [48], Newman *eap*$^-$ (Newman *eap::ermB*) [49], SH1000 (RN6390 *rsbU* repaired) [50], RN6390 (*rsbU*$^-$) [51] and RN6911 (RN6390 *agr*::*tetM*) [52]. The strains were kindly provided by Dr. Ambrose L. Cheung (Department of Microbiology, Dartmounth Medical School, Hanover, USA) and Dr. Muzaffar Hussain (Institute of Medical Microbiology, University Hospital of Müenster, Müenster, Germany). Bacteria were stored in trypticase soy broth (TSB) (Difco, USA) with 20% glycerol at −20 °C until use. Newman *mgrA*$^-$, Newman *eap*$^-$ and RN6911 bacterial strains were grown on 10 μg/ml erythromycin and 5 μg/ml tetracycline supplemented medium, respectively.

2.2. Photosensitizer

Toluidine blue (TB) (Sigma Chem. Co, USA) was employed as a photosensitizer on concentrations ranging from 0.06 to 13 μM and from 0.3 to 0.5 mM for treatment of planktonic and biofilm cultures, respectively.

2.3. Photoinactivation of S. aureus planktonic cultures

An aliquot from overnight cultures of each strain was grown on TSB at 37 °C under constant shaking until obtaining an optical density at 600 nm (OD) equivalent to *ca.* 10^8 CFU/ml. The bacterial suspensions were centrifuged at 13,000 rpm for 10 min at 4 °C, washed and suspended in sterile PBS to be utilized in PDI assays. One ml of each bacteria inoculum was added into each well of a 12-multiwell plate and cultured in presence of non-toxic (in darkness) concentrations of TB [53]. After 10 min of incubation in the dark at room temperature, the plates containing the bacterial suspensions were illuminated in the presence of the photosensitizer employing a non-coherent light source. Viable cells were quantified by plating an aliquot of serial dilutions on TSA. To assess total bacteria eradication, the whole 1 ml bacteria inoculum was plated. After 24 h incubation of the TSA plates at 37 °C, the number of CFU/ml was determined. To assess total bacteria eradication, the whole 0.5 ml bacteria inoculum was plated. The following treatments were employed as controls: i) bacteria treated with TB but not exposed to light; ii) non TB-treated bacteria exposed to light; and iii) non TB-treated bacteria not exposed to light.

2.4. Non-coherent light source: fluorescent lamps array

Multiwell plates containing bacterial suspensions were placed on a glass slide and exposed from below to the light source at 20 °C with water filters and air-cooling. An array of four Osram L 18W/765 fluorescent lamps, 380 nm–780 nm was employed which provided a homogeneous fluent rate of about 1.76 mW/cm^2 on the surface of the sample measured with a FieldMaster power meter and a LM3 HTP sensor (Coherent Inc., USA). The light dose was modified by switching the exposition time, obtaining fluences between 4 J/cm^2 and 19 J/cm^2 respectively.

2.5. Biofilm formation and photodynamic inactivation

The assessment of biofilm formation was performed as previously described with some modifications [51]. Briefly, *S. aureus* strains were grown overnight and diluted 1:1000 in 0.25% glucose supplemented TSB. An aliquot of this cell suspension was inoculated into sterile 24-well polystyrene microtiter plates (Greiner Bio-One). After 24 h of static incubation at 37 °C, the medium in each well was replaced by 0.25% glucose supplemented sterile PBS. Then, the TB was added onto biofilms and statically incubated for 30 min at 37 °C. After washing with PBS, biofilms were irradiated with a 635 nm laser. Following irradiation, the biofilms were scrapped and the resulting suspensions were homogenized by vortex shaking. Bacterial viability (CFU/ml) was determined by plating on TSA as previously described.

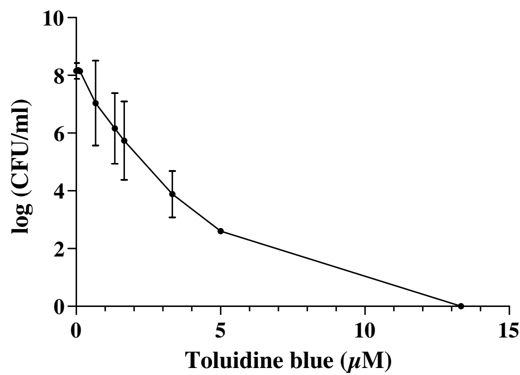

Fig. 1. PDI of *S. aureus* planktonic cultures as a function of TB concentration.
After 10 min of dark incubation with the PS, RN6390 cultures were irradiated with a non-coherent light source (4.2 J/cm^2), employing several TB concentrations up to 13 μM. Viability of post-treated cultures, expressed as CFU/ml, was established by plating on TSA followed by colony counts. Curves are representative of three independent experiments performed in duplicates.

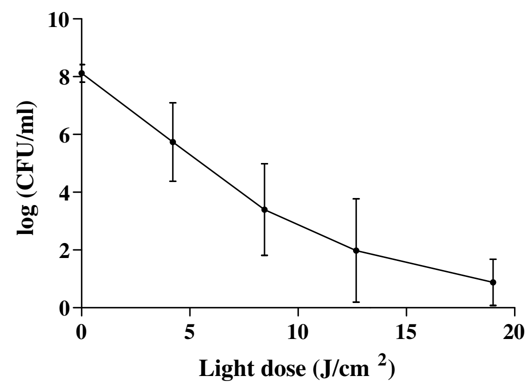

Fig. 2. TB-PDI of *S. aureus* planktonic cultures as a function of light dose.
After 10 min of dark incubation with 1.3 μM TB, RN6390 cultures were irradiated with a fluorescent lamp array, employing several light doses ranging from 4.2 to 19 J/cm^2. Viability of post-treated cultures, expressed as CFU/ml, was established by plating on TSA followed by colony counts. Curves are representative of three independent experiments performed in duplicates.

2.6. 635 nm laser

To photoinactivate *S. aureus* biofilms, a PDT laser (Lumiia; Argentina) was employed coupled to an optic fiber with a flat diffuser. The wavelength of the light emitted by this device is 635 nm ± 0.25%, and was employed on continuous exposition mode and a numerical aperture of 0.22. Biofilms were irradiated from above at 260 mW/cm^2 for 10 min per well, that equals a fluence of 156 J/cm^2.

2.7. Statistical analysis

Differences between two means were evaluated by the unpaired, two-sided Student *t*-test. Statistical tests were carried out using the Graphpad Software (version 5.0; GraphPad Prism). *P* values <0.05 were considered significant. Means ± standard deviations were depicted.

3. Results

Planktonic cultures of the *S. aureus* RN6390 strain were illuminated at a fixed non-coherent light dose (4.2 J/cm^2) in the presence of increasing concentrations of TB (Fig. 1). Upon illumination, CFU counts decreased as a function of the TB concentration. Employing a TB concentration as low as 0.6 μM, we obtained a significant photodynamic effect (6.89 log CFU/ml) respect to the irradiated control without PS (8.22 log CFU/ml, $p < 0.05$). In addition, using the highest TB concentration of 13 μM, complete bacteria eradication was achieved.

We observed an exponential decay in bacterial viability of 1.3 μM TB treated planktonic *S. aureus* RN6390 as a function of the increasing light doses (Fig. 2). At the highest dose employed of 19 J/cm^2, a 6-log reduction in bacterial viability was obtained (0.88 log CFU/ml vs 8.19 log CFU/ml of the non-irradiated control exposed to TB). Conditions of 1.3 μM TB and 19 J/cm^2 light dose were employed in the following experiments.

In order to characterize potential differences in the sensitivity to photodynamic inactivation, the effect of TB-PDI was compared between mutant strains with deficiencies in central regulators. Firstly, we compared RN6390 and Newman *S. aureus* backgrounds (Fig. 3). Both strains were susceptible to PDI treatment. Interestingly, upon TB-PDI treatment RN6390, CFU counts were significantly lower than the ones of the Newman strain ($p = 0.03$).

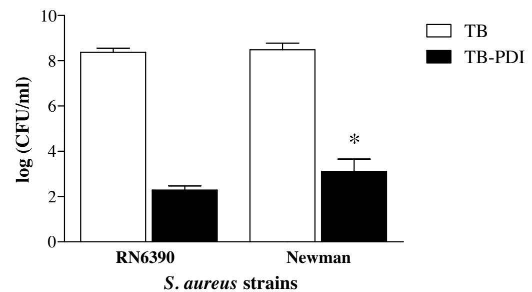

Fig. 3. TB-PDI susceptibility of *S. aureus* RN6390 and Newman strains.
After 10 min of dark incubation with 1.3 μM TB, RN6390 and Newman planktonic cultures were irradiated with a fluorescent lamp array using a 19 J/cm^2 light dose. The control refers to non-irradiated cultures of the respective TB-treated strain. Values are means of 3 independent experiments performed in quadruplicates and bars are SD (Student's *t*-test). * $p = 0.03$ as compared to TB-PDI treated RN6390 strain.

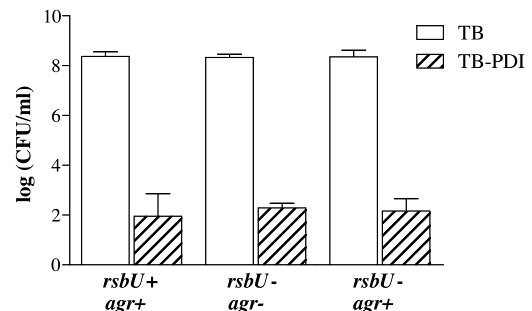

Fig. 4. Effect of key regulatory systems on TB-PDI susceptibility of *S. aureus* RN6390 background.
Planktonic cultures of the wild-type RN6390 strain and two isogenic regulatory mutants, were dark incubated 10 min with 1.3 μM TB and afterwards, were irradiated with a fluorescent lamp array using a 19 J/cm^2 light dose. The control refers to non-irradiated cultures of the respective TB-treated strain. Values are means of 3 independent experiments performed in quadruplicates and bars are SD (Student's *t*-test).

Fig. 4 shows the effect of TB-PDI on the viability of the wild type RN6390 strain and two isogenic mutants. It is clear that the number of colonies of RN6390 (*rsbU*$^-$ *agr*$^+$), SH1000 (*rsbU*$^+$ *agr*$^+$) and RN6911 (*rsbU*$^-$ *agr*$^-$) were significantly reduced by TB-PDI treatment. This reduction of CFU counts varied around 5 and 6 magnitude orders for all the strains analyzed, thus showing that neither

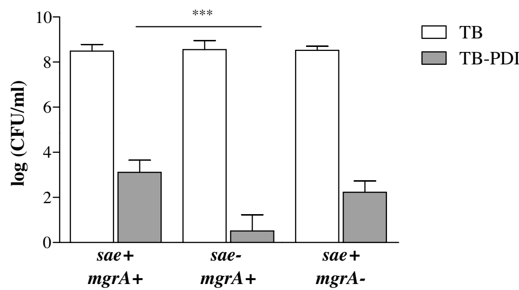

Fig. 5. Effect of key regulatory systems on TB-PDI susceptibility of *S. aureus* Newman background.
Planktonic cultures of the wild-type Newman strain and two isogenic regulatory mutants, were dark incubated 10 min with 1.3 μM TB and afterwards, were irradiated with a fluorescent lamp array using a 19 J/cm^2 light dose. The control refers to non-irradiated cultures of the respective TB-treated strain. Values are means of 3 independent experiments performed in quadruplicates and bars are SD (Student's *t*-test). *** $p = 0.007$ for *mgrA*+ *sae*+ as compared to *mgrA*+ *sae*-.

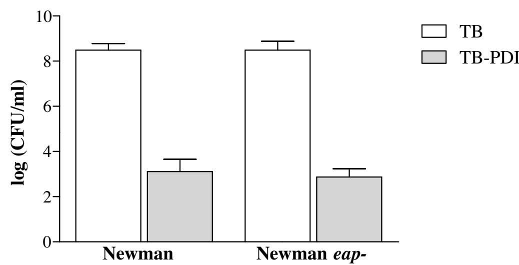

Fig. 6. Influence of mutants for the adhesion Eap on TB-PDI susceptibility of *S. aureus* Newman strain.
After 10 min of dark incubation with 1.3 μM TB, Eap mutants of Newman background planktonic cultures were irradiated with a fluorescent lamp array using a 19 J/cm^2 light dose. The control refers to non-irradiated cultures of the respective TB-treated strain. Values are means of 3 independent experiments performed in quadruplicates and bars are SD (Student's *t*-test).

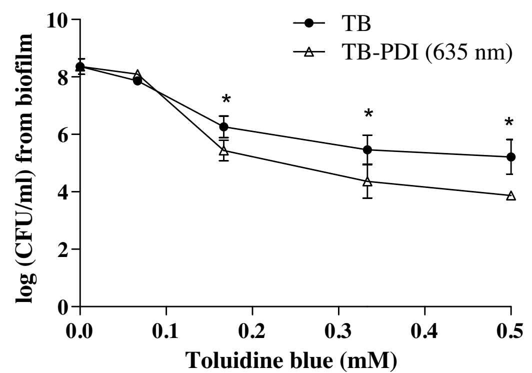

Fig. 7. PDI of RN6390 *S. aureus* biofilms employing TB.
RN6390 biofilms were irradiated with a 635 nm laser at 156 J/cm^2 after 30 min of TB dark incubation. Viability of suspensions derived from post-treated biofilms was established by colony counts. Curves are representative of three independent experiments performed in duplicates *$p < 0.05$ relative to TB-treated non-irradiated biofilms, Student's *t*-test.

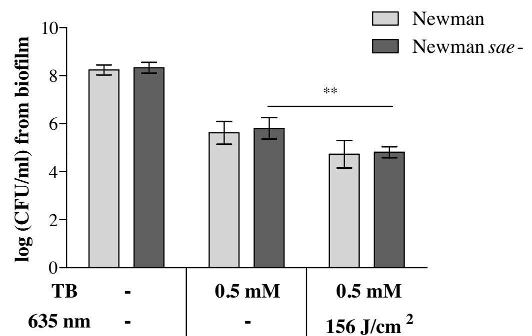

Fig. 8. Effect of *sae* regulatory system on TB-PDI susceptibility of biofilms.
Biofilms developed by the Newman strain and its *sae* isogenic mutant were treated with 0.5 mM TB for 30 min, previous to 635 nm laser irradiation at 156 J/cm^2. Viability of suspensions derived from post-treated biofilms was established by colony counts. Values are means of 4 independent experiments performed in triplicates and bars are SD. ** $p < 0.05$ as compared to the AT treated biofilm non-irradiated (Student's *t*-test).

rsbU nor *agr* modified the response of *S. aureus* to PDI under the assessed conditions.

The viability of bacterial cells of the wild type Newman strain (*sae*$^+$ *mgrA*$^+$) and its isogenic regulatory mutants *sae*$^-$ *mgrA*$^+$ and *sae*$^+$ *mgrA*$^-$ showed to be significantly reduced after TB-PDI treatment as compared with the control (Fig. 5). In addition, no significant differences were observed between the Newman wild-type strain and its *mgrA* isogenic mutant. On the other hand, the TB-PDI treatment induced a CFU impairment in the Newman *sae*$^-$ mutant significantly higher than the one observed in its parental strain ($p = 0.0007$).

To deepen in the role of *sae* regarding PDI protection, the effect of TB-PDI on the *eap* deficient Newman mutant was studied. Eap is an extracellular adhesin that after its secretion interacts with cell surface of the bacteria modulating the charge distribution, and its expression is positively controlled by the *sae* regulatory system. As shown in Fig. 6, *eap* mutant sensitivity to TB-PDI did not differ from the one its parental strain Newman ($p = 0.421$).

It is worth noting that none of the strains employed in the present study showed intrinsic photosensitivity in the absence of PS.

TB-PDI treatment of *S. aureus* biofilms required higher light fluences from a coherent light source as well as higher PS concentrations were needed to achieve photoinactivation. We employed a laser emitting light at 635 nm wavelength, which is within the absorption peak of TB in aqueous solution. Biofilms of all the strains employed did not exhibit sensitivity to light treatment in the absence of PS.

Fig. 7 shows that from 0.16 mM onwards, TB induced per se a significant decrease on cell viability. Moreover, an additional 1-log decrease on biofilm CFU ($p < 0.05$ TB vs TB-PDI) was observed. In addition, the highest decrease of biofilm CFU, was obtained employing the highest TB concentration and light, that is 0.5 mM (3.88 log CFU/ml for TB-PDI (635 nm) vs 5.48 log CFU/ml for TB treatment, $p = 0.03$).

Sensitivity to TB-PDI was analyzed on biofilms developed by Newman, and its *sae*$^-$ mutant, the latter of which probed to be higher responsive to TB-PDI in planktonic state as compared to its parental strain. Fig. 8 shows that all the biofilms were responsive to TB-PDI (0.5 mM TB + 156 J/cm^2) although to the same extent ($p = 0.1958$ for the comparison between Newman and Newman *sae*$^-$ TB-PDI treated) under the conditions employed.

4. Discussion

This work demonstrates that *S. aureus* RN6390 and Newman –two well-characterized reference strains- are sensitive to TB-PDI both as planktonic cultures and biofilms, although more severe conditions were needed to inactivate the last ones, as previously reported [54,55]. Both hindrance of the penetration of light within the biofilms [56] and the inability of the PS to diffuse to the inner regions of the structure [23,28], in addition to the genetic diversity within the biofilm and bacteria communication via quorum sens-

ing [21] contribute to the higher resistance of biofilm structures to PDI treatment. In the present work, to achieve photoinactivation of *S. aureus* biofilms, both higher light doses and concentrations of TB were needed as compared to planktonic cultures. Moreover, TB doses showing dark toxicity were necessary to induce a phototoxic effect on biofilms. In this regard, TB seems to stimulate dark ROS production in *E. coli arc* mutants by deviating electrons from the respiratory chain to O_2, and thus decoupling the electron flux and the energetic metabolism. This mechanism, observed using 0.65 mM TB –similar concentration to the ones here reported as toxic-, could explain the dark toxicity described in our work [25,26].

In order to prevent oxidative damage, bacteria are equipped with enzymes, such as catalase and superoxide dismutase. Despite all this complex defences against ROS-induced damage, many studies showed that Gram-positive bacteria, including *S. aureus*, are very sensitive to PDI. Until now a specific bacteria defence system against singlet oxygen itself is not present in pathogenic bacteria. Therefore it is questionable if bacteria can develop a defence system against singlet oxygen directly [21]. However, sublethal PDI of *S. aureus* was found to affect the expression of several proteins including overexpression of catalase KatA and to enhance the activity of superoxide dismutase [34,57], thus suggesting that PDI is able to induce bacterial response to oxidative stress, although the different defence pathways are unclear [21]. Our results suggest that *S. aureus* Newman was less responsive to PDI as compared to RN6390 strain. This may be ascribed to the downregulation of the σ^B factor in the RN6390 strain [39], which is a stress response factor shown to be involved in H_2O_2 resistance [58]. In concordance, the effective killing of strain RN6390 using photoantimicrobial chemotherapy was attributed to its *rsbU* defect [59]. Moreover, frequent mutations in *rsbU* were identified in clinical isolates of *S. aureus* vulnerable to photoantimicrobial treatments [59].

Through multilocus sequence typing, the *S. aureus* population may be clustered in main six major clonal complexes: CC1 CC5, CC8, CC22, CC30, and CC45 [60]. Rapacka et al. [33] classified the *S. aureus* clonal complexes as suitable or refractory to PDI using Protoporphyrin IX as PS. Both Newman and RN6390 strains belong to the CC8 clonal complex which was described to be susceptible to PDI treatment. Regarding the *spa* type, RN6390 is t211 while Newman is t008, but neither type has been highlighted as particularly refractory or sensitive to Protoporphyrin IX mediated PDI [33].

On the other hand, our results suggest that factors under control of the *sae* system are capable of protecting bacterial planktonic cultures from PDI, whilst the *mgrA* regulator, as well as the *agr* system, would not be related to PDI protection of bacteria adopting a planktonic lifestyle.

One of the *sae* promoters is regulated by hydrogen peroxide, which has been ascribed to play a role in the defence to the phagocytic killing of bacteria [45]. In addition, it was shown that copper resistance to oxidative stress –particularly H_2O_2 scavenging- represses both *sae* and *agr* in *S. aureus* SH1000 strain [61]. Indeed, we have found that *sae* driven protection to PDI is not mediated through the Eap adhesin that links bacteria to the extracellular matrix of the host. Thus, the highest PDI protection observed in the *sae* mutant cannot be attributed to an Eap-mediated charge distribution distortion affecting TB interaction with bacterial surface.

Employing *S. aureus* mutants lacking *agr* system it was shown that *agr* induced certain degree of resistance against PDT [35,62] employing Protoporphyrin diarginate and chlorin e6 as PSs. However, we have shown in the present work that the *agr* locus does not seem to be related to TB-PDI sensitivity. It is therefore important to consider that different PSs elicit photodamage by different mechanisms, and this may be related to the different strain responses to PDI.

It is worth noting that the different susceptibilities to PDI found in the present study are correlated to different phenotypes already present in the *S. aureus* strains analyzed and do not imply complete resistance towards the treatment.

We found that the strain-dependant differences (<1 log unit reduction) in the susceptibilities of *S. aureus* growing in planktonic state are not present when the bacteria are grown in biofilm culture. This is not surprising since only one-fifth of the genome is expressed definitely when bacteria are growing as a biofilm [63]. In addition, higher resistance to PDI growing in biofilm as compared to planktonic form turns undetectable strain dependant differences in susceptibilities.

The key conclusion of our work is that *sae* exerts partial protection from TB mediated PDI on *S. aureus* bacteria growing on planktonic but not biofilm states, though all the strains analyzed were susceptible of being photoinactivated. Further studies with clinical isolates would be necessary to extrapolate our results to a putative differential susceptibilies in a clinical setting.

Acknowledgements

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