



Sub-lethal antimicrobial photodynamic inactivation affects *Pseudomonas aeruginosa* PAO1 quorum sensing and cyclic di-GMP regulatory systems

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ABSTRACT

Background: Antimicrobial photodynamic inactivation (APDI) is a new therapeutic modality which needs more precision during application due to the possibility of exposure of bacteria to sub-lethal doses (sAPDI). In this study, we aimed to evaluate the effect of sAPDI on *Pseudomonas aeruginosa* quorum sensing (QS) and c-di-GMP signaling which are important virulence factor regulatory systems.

Methods: Biofilm formation, pyoverdine, pyocyanin and protease production of *P. aeruginosa* was evaluated before and after a single sAPDI treatment with 0.8 mM methylene blue (MB) plus 1, 2, and 5-min irradiation with red laser light. Fluorescent *lasB*, *rhlA*, *pqsA*, and *cdrA* reporters of *P. aeruginosa* PAO1 and *P. aeruginosa* Δ *mexAB-oprM* were treated individually with sAPDI and the regulatory signals were detected. The gene expressions were also assessed after sAPDI using quantitative real-time PCR analysis.

Results: Morphological observations and molecular assessments indicated that sAPDI with 0.8 mM MB along with 2- and 5-min irradiation led to an increase in the expression of the Las QS system and c-di-GMP signaling, while 1 min irradiation revealed dissimilar results (increase in *lasB* expression and decrease in c-di-GMP levels). Expression of *rhlA* and *pqsA* did not change in response to sAPDI. Further, a severe lethal effect of sAPDI was observed in *P. aeruginosa* Δ *mexAB-oprM* as compared with the wild type strain, whilst there was no difference in QS and c-di-GMP levels as detected by reporters between treated and untreated samples.

Conclusion: The results suggest that sAPDI affects QS and c-di-GMP signaling in *P. aeruginosa* in a time-dependent manner.

1. Introduction

The emergence of bacterial resistance to current antimicrobial therapies has become a major health challenge. As it continues to threaten the treatment of various infections, researchers are looking for alternative approaches to produce potential effective treatments [1]. One promising alternative is antimicrobial photodynamic inactivation (APDI) also referred to as Antimicrobial Photodynamic Therapy (aPDT). APDI is based on the formation of reactive oxygen species (ROS) via the administration of a non-toxic light-sensitive compound, known as photosensitizer (Ps), followed by exposure to light of proper wavelength [2]. ROS are able to kill bacteria via oxidative stress to cellular components. When the balance between APDI-induced ROS and the cellular scavenger systems is interrupted, ROS accumulate inside the cells. If the concentration of ROS reaches the lethal doses, the

cytoplasm and macromolecules degrade and bacterial cell death will occur. If the exposure of ROS to bacteria in the infectious site is not thoroughly uniform and some bacteria experience sub-lethal oxidative stress levels, bacterial regulatory systems may change the expression of protective response components toward overproduction of heat shock proteins (HSPs) [3], anti-oxidant enzymes [4], pigment production [5], efflux pumps [6], and biofilm formation [7].

Pseudomonas aeruginosa is an opportunistic Gram-negative bacterium which can cause various infections, e.g. in cystic fibrosis lungs, burn wounds, and the urinary tract, using several virulence factors like toxins, enzymes, pigments, rhamnolipid, and biofilm formation [8]. Quorum sensing (QS), c-di-GMP, and two component systems are regulatory systems responsible for controlling the production of virulence factors, biofilm formation, and antimicrobial resistance in *P. aeruginosa* [9,10].

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QS is a mechanism that regulates communal behaviors via cell-to-cell signaling and control of density-dependent gene expression through detection of chemical secretory signals called autoinducers (AIs). *P. aeruginosa* employ two classical acyl-homoserine lactone (HSL)-based QS systems, *las* and *rhl*, as well as a *Pseudomonas* quinolone system (PQS) regulatory pathway. The two major signaling molecules are *N*-3-oxo-dodecanoyl-L-homoserine lactone (C12-HSL) and *N*-butyryl-L-homoserine lactone (C4-HSL) for *las* and *rhl*, respectively. AI signal molecules are produced by a synthetase called I protein. The AIs produced by the bacteria bind to the receptor, the R-protein, the AHL-responsive transcriptional activator. Structural genes for PQS production have been identified (*pqsABCDH*) together with a transcriptional regulator (*pqsR*) and the response effector (*pqsE*) [11]. These quorum sensing regulation systems control the expression of the majority of acute and chronic virulence factors in *P. aeruginosa* and are necessary for the survival and the proliferation of this microorganism in the host [12].

Biofilms are structured communities of bacterial cells embedded in a self-produced polymeric matrix which adhere to inert or living surfaces. It has been suggested that the intracellular second messenger molecule 3',5'-cyclic diguanylic acid (c-di-GMP) controls many features of group behavior, including biofilm formation in *P. aeruginosa* [13]. C-di-GMP is synthesized from two GTP molecules by diguanylate cyclases (DGC), and is degraded by phosphodiesterases (PDE) [14]. High intracellular levels of c-di-GMP are associated with biofilm formation, while low c-di-GMP levels lead to a planktonic lifestyle [15].

Previous findings have indicated that *P. aeruginosa* shows decreased potential to produce virulence factors and biofilm formation after sub-lethal photodynamic exposure [16,17]. This highlights the need for further studies on bacterial responses to photodynamic inactivation. In contrast, some studies demonstrated that Bacteria respond to APDI-induced oxidative stress by enhancing gene expression of defense systems [18,4,19], where this response might be a cause for increased tolerance to APDI. On this background, the aim of this study was to investigate the effect of sub-lethal APDI on virulence factor production and gene expression controlled by QS and c-di-GMP regulatory systems of *P. aeruginosa*.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The strains and plasmids used in this study are listed in Table 1. *P. aeruginosa* strains were grown on LB agar and in AB medium supplemented with casamino acid and glucose at 37 °C (ABGluCas). Antibiotics were added where necessary at the following concentrations: 100 µg gentamicin ml⁻¹ (ABGluCasGen) and 200 µg carbenicillin ml⁻¹ (ABGluCasCarb).

2.2. PS and light source

A stock of 51.2 mM MB solution was prepared in miliQ water, filter sterilized, and stored at 4 °C for less than two weeks. The solution was diluted in sterile miliQ water to the final working concentrations (0.006–25.6 µM) prior to each experiment.

A red laser light source with wavelength of 0.66 µm was utilized in this study. The samples were irradiated from above and a distance of 1 cm.

2.3. Viability assays

To evaluate the effect of APDI on *P. aeruginosa* PAO1 and *P. aeruginosa* Δ mexAB-*oprM* viable counts were determined. To this end, overnight cultures of bacteria were diluted in LB medium (with gentamicin for *P. aeruginosa* Δ mexAB-*oprM*) to a final OD₆₀₀ = 0.1. This was followed by adding 500 µl of each MB concentration to equal

volume of bacterial suspension in a sterile 2 ml microtube. Microtubes were incubated in darkness for 30 s, 1, 2, and 5 min at room temperature. Irradiation was performed for same time as dark incubation (1, 2, and 5 min with fluency of 3.8, 7.6, 19, and 38 Jcm⁻², respectively). Then excess MB in each microtube was discarded by centrifuging the samples at 7000 g for 3 min, after which the cells were washed twice and suspended in 1 ml LB medium.

A panel of controls was considered as: Ps treated and dark incubated samples (+Ps, -light), Ps untreated and irradiated samples (-Ps, +light), and Ps untreated and dark incubated samples (-Ps, -light). The experiments were repeated at least three times in triplicate.

A volume of 100 µl of each sample was plated on LB agar and incubated for 24 h at 37 °C. Viable counts were expressed as colony forming unit ml⁻¹ (CFU ml⁻¹) with a detection limit of 30 ≤ CFU ml⁻¹ ≥ 300.

2.4. Crystal violet staining assay

Crystal violet (CV) staining was performed to quantify the amount of biofilm biomass on a well of a 96-sterile microtiter plate. APDI treated and untreated samples were prepared as described above. A volume of 100 µl of each sample was transferred to a well with plates covered and incubated at 37 °C for 24 h. After incubation, the biofilms were stained with CV 0.1% for 10 min, washed twice with 0.9% sterile saline, air dried, and destained with 30% acetic acid. Finally, the plates were read with a plate reader at 590 nm.

2.5. Protease assay

P. aeruginosa PAO1 with starting OD₆₀₀ of 0.01 was incubated at 37 °C with shaking at 200 r.p.m. After 16–18 h incubation, the final OD₆₀₀ of the cultures was measured. SAPDI was performed at desired concentrations where treated and untreated samples were centrifuged at 15,000 g for 15 min. Also, 150 µl of cell-free supernatant was mixed with an Azocasein solution (2% Azocasein wt/v in 2 mM CaCl₂ and 40 mM tris-HCl pH 7.8). After an incubation of 45 min at 37 °C, the reaction was stopped by adding 1.2 ml 10% trichloro-acetic acid which was followed by incubation at room temperature for 15 min. The undigested azocasein was pelleted at 15,000 g for 10 min. Finally, 600 µl of the supernatant was transferred to a plastic cuvette and mixed with 500 µl 1 M NaOH and measured at A440.

The relative protease activity was determined by normalizing A440 of the supernatant to OD₆₀₀ of the culture [[20,21]].

2.6. Pigment assay

Two pigments, pyoverdine and pyocyanin, were chosen to evaluate the effect of sAPDI-induced oxidative stress in pigment production. After sAPDI treatment, cells were washed to remove the excess MB, transferred to tubes containing LB medium and LB supplemented with NaCl and Glycerol for pyoverdine and pyocyanin, respectively. Tubes were further incubated at 37 °C for 24 h. Then, they were centrifuged with the cell-free supernatant measured at 405 nm for pyoverdine and 545 nm for pyocyanin. Pyocyanin production was measured based on an extinction coefficient of 17.064 [22].

2.7. Fluorescence reporter assays

To assess the level of AI and c-di-GMP second messenger in a real-time manner, plasmids containing *gfp* fusions to a total of three QS systems related genes, *lasB*, *rhlA*, and *pqsA*, and one c-di-GMP related gene (*cdrA*) (as presented in Table 1), were transformed into *P. aeruginosa* PAO1 and *P. aeruginosa* PAO1 Δ mexAB-*oprM* using electroporation [23]. Overnight cultures of *P. aeruginosa* PAO1 and *P. aeruginosa* Δ mexAB-*oprM*, containing *las-gfp*, *rhl-gfp*, *pqsA-gfp*, and *cdrA-gfp* reporters and a *gfp*-tagged strain of *P. aeruginosa* PAO1 were treated with

Table 1
Strains, plasmids and primers used in this study.

Strain or plasmid	Relevant features or genotype	Reference or source
Strains		
<i>P. aeruginosa</i>		
PAO1 WT	Sequenced PAO1	Stover (2000) [20]
PAO1-constitutive gfp	gfp tagged on chromosome	Koch (2001) [21]
PAO1- <i>las-gfp</i>	fluorescent reporter of <i>las</i> expression	Hentzer (2002) [22]
PAO1- <i>rhl-gfp</i>	fluorescent reporter of <i>rhl</i> expression	Yang (2009) [23]
PAO1- <i>pqsA-gfp</i>	fluorescent reporter of <i>pqsA</i> expression	Yang (2007) [24]
PAO1- <i>cdrA-gfp</i>	fluorescent reporter of <i>cdrA</i> expression	Rybtko (2012) [25]
PAO1 Δ mexAB- <i>oprM</i>	deletion mutant	
Plasmids		
PcdrA::gfp (mut3)C	pUCP22Not-PcdrA-rbsII-gfp(Mut3)-T0-T1, Ampr Gmr	Rybtko (2012) [25]
pMHLAS	ApR GmR; pMH391 carrying PlacB-gfp (ASV) Plac- <i>lasR</i>	Hentzer (2002) [22]
pMHRA		Yang (2009) [23]
pAC37		Yang (2007) [24]
pCdrA-gfpC	pUCP22Not-PcdrA-RBSII-gfp(Mut3)-T0-T1,AMPr GMR	Rybtko (2012) [25]
Primers		
lasB forward	5'-CGACAACGCGTCGCAGTA-3'	
lasB reverse	5'-AGGTAGAACGCACGGTTGTACA-3'	
rhlA forward	5'-GGCGATCGGCCATCT-3'	
rhlA reverse	5'-AGCGAAGCCATGTGCTGAT-3'	
pqsA forward	5'-CATCTCGCCGAACAGATT-3'	
pqsA reverse	5'-CCAACTGCGTTGTGCTT-3'	
cdrA forward	5'-CAACAGTCAGTTCAACGACCTC-3'	
cdrA reverse	5'-AACGCTGGCTGAAATACTCG-3'	
rpoS forward	5'-CTCCGGGCAACTCCAAAAG-3'	
rpoS reverse	5'-CGATCATCCGCTCCGACCAG-3'	

sAPDI as described above. After removing excess MB, each microtube was filled with 1 ml of ABGluCasGen medium, except for the *cdrA-gfp* reporter strain containing microtube. A volume of 300 μ l of each was transferred to a well of a black transparent bottom 96-microtiter plate. Plates were incubated at 34 °C by a Vector plate reader (Brand). A program was set to read the plate every 15 min for 69 cycles at 490 nm.

To evaluate the expression of *cdrA-gfp*, a microtiter plate was inoculated with 100 μ l of treated and untreated samples and incubated in a Tecan reader at 37 °C where the program was set to a read for every 15 min for 76 cycles at 590 nm.

2.8. Real-time PCR experiments

Real-time qPCR was performed in order to evaluate the QS and c-di-GMP-related gene expression of sAPDI-treated and untreated *P. aeruginosa* PAO1. Total RNA was extracted from samples using the RNeasy mini kit (QIAGEN laboratories, Coutaboeuf, France) in accordance to manufacturer's instructions. A preliminary phase for bacterial envelope lysis was performed using lysozyme for 10 min at room temperature. RNase-free DNase (Promega) was employed for 60 min at 37 °C. Purity and concentration of RNA extracts were analyzed using a nanodrop. CDNA was made from 1 μ g of RNA using high-capacity RNA-to-cDNA master mix (Applied Biosystems). Similarly, the cDNA synthesis was carried out based on the manufacture's protocols.

Oligonucleotide primers used in this study are listed in Table 1. A Power SYBR green master mix (Thermo Fisher Scientific) was used for monitoring cDNA amplification to perform the real time PCR using a Step One Plus thermal cycler (Applied Biosystems). It was carried out by measuring the increase in fluorescence intensity, under the following conditions: 95 °C for 15 min, amplification for 40 cycles with 95 °C for 15 s, and 60 °C for 1 min. The specificity of the primers was evaluated using melt curves. The relative amounts of each target gene were quantified relative to *rpoS* (as an internal standard).

2.9. Statistical analysis

The values were expressed as means \pm standard error of mean (SEM). Analyses of means of groups were conducted through

comparison by one-way ANOVA. $P < 0.05$ was considered statistically significant.

3. Results

Viability count was performed to determine the lethal and sub-lethal doses of APDI. The number of colonies on each plate was counted and a $\geq 3 \log_{10}$ reduction was considered as lethal dose of APDI. The results are presented in Fig. 1. It was found that 1, 2, and 5-min illumination with the concentration of 0.8 mM MB killed 0.5, 1, and 2 \log_{10} of bacteria, respectively, and thus considered as sub-lethal APDI.

Interestingly, the effect of sAPDI against *P. aeruginosa* Δ mexAB-*OprM* was more intense compared to that of the wild type strain. According to the results, 0.95, 1.67, and 3.22 \log_{10} reductions were observed by 0.8 mM MB and 1, 2, and 5-min irradiation with laser, respectively.

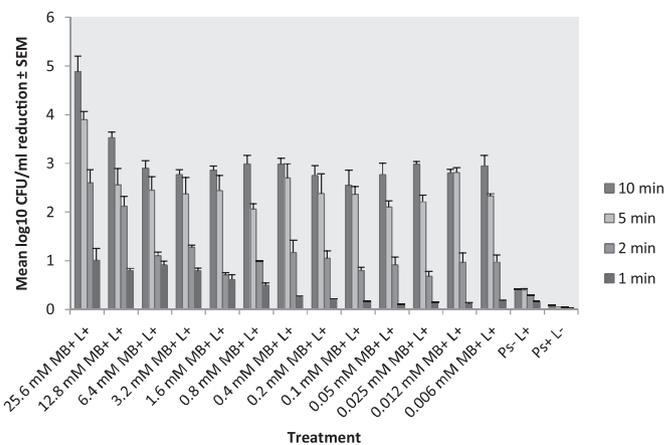


Fig. 1. Effect of APDI treatment on *P. aeruginosa* PAO1 with different MB concentrations and irradiation times.

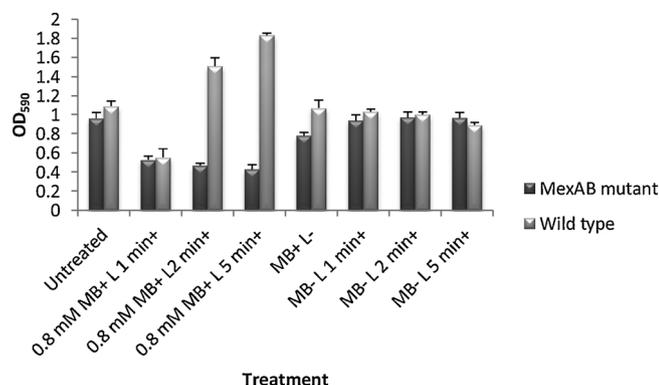


Fig. 2. Effect of sAPDI on biofilm formation of *P. aeruginosa* PAO1 and *P. aeruginosa* Δ*mexAB*:*OprM* (MB = Methylene blue, L = laser irradiation).

3.1. Biofilm formation assay

In order to quantify the extent of biofilm produced by the sAPDI-treated and untreated samples, crystal violet staining was used. After eliminating the excess stain out of the wells, the optical density of each well containing solubilized crystal violet stained cells was read by a microplate reader at 590 nm. As revealed in Fig. 2, sAPDI caused delayed biofilm formation of *P. aeruginosa* PAO1 after a single treatment with 1-min irradiation. The biofilm formation of wild type strain was stimulated with 2- and 5-min irradiation with the same MB concentration. Biofilm formation of *P. aeruginosa* Δ*mexAB*-*OprM* diminished with all MB-irradiation treatments compared to untreated controls.

Control groups did not show any significant decrease compared to the untreated control.

3.2. Effect of sAPDI on virulence factor production of *P. aeruginosa*

We examined the effect of sAPDI directly on the production of the QS controlled virulence factors protease, pyocyanin, and pyoverdine. The protease assay uses a mass correlation between the substrate used and the optical density observed in the post-digestion sample. Pyoverdine and pyocyanin as organic pigments are major secreted QS virulence factors which can be measured based on their spectral properties. The results of the effect of sAPDI on virulence factor production of *P. aeruginosa* PAO1 are reported in Table 2. The results indicated that sAPDI induced protease and pyoverdine production in a time-dependent manner. Increasing in irradiation time led to enhanced protease and pyoverdine production (P < 0.001). However, sAPDI did not affect pyocyanin production significantly (P = 0.082). Also, none of the light controls influenced virulence factor production.

3.3. The effect of sAPDI on *lasB*, *rhlA*, *pqsA*, and *cdrA* expression in *P. aeruginosa* PAO1 and *P. aeruginosa* Δ*mexAB*-*OprM*

Our genetic constructs for detection of QS and c-di-GMP signal molecules relied on *gfp*-fusions to the promoters that are under control of each of these regulatory systems. The reporter genes for OdDHL, HSL, PQS, and c-di-GMP signaling were chosen as *lasB*, *rhlA*, *pqsA*, and

Table 2
The effect of sAPDI on virulence factor production of *P. aeruginosa* PAO1.

Virulence factor Treatment	Total proteases	Pyoverdine	Pyocyanin
Untreated	0.08	0.013	10.4
0.8 mM MB + L 1 min +	0.22	0.18	11.6
0.8 mM MB + L 2 min +	0.23	0.18	11.4
0.8 mM MB + L 5 min +	0.26	0.21	11.9

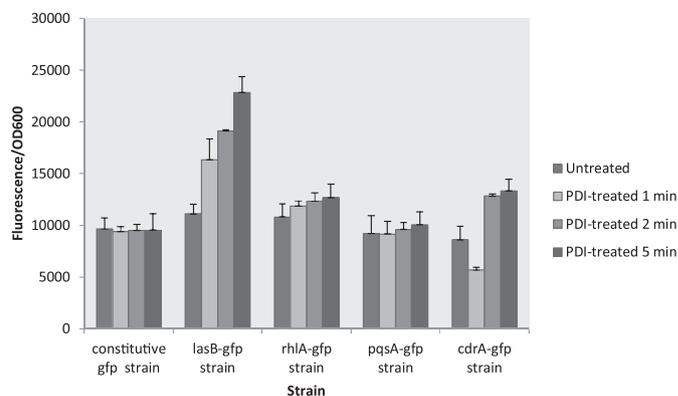


Fig. 3. The effect of sAPDI with 0.8 mM MB and 1-, 2-, and 5-min irradiation times on *P. aeruginosa* PAO1 reporter strains for *lasB*, *rhlA*, *pqsA* and *cdrA* genes.

cdrA, respectively [24,25]. The gene expressions of the four sensors were evaluated for the PAO1 wild type and Δ*mexAB*-*OprM* mutant. After applying sAPDI, incubation was performed with the raw fluorescence intensity and OD600 measured over time. Since the cell density was different given sAPDI treatment between treated and untreated samples, the specific fluorescence intensity was used instead of raw fluorescence intensity to normalize the data.

As shown in Fig. 3, the constitutively expressed *gfp* control, showed no difference in terms of the expression between the untreated and sAPDI-treated samples.

sAPDI was found to induce expression of the *las-gfp* reporter (Fig. 3). The induction of the *las-gfp* reporter appeared to be time-dependent particularly after longer exposure times. With prolongation of illumination time, more expression was detected.

sAPDI-treated and untreated samples did not display significant differences in *rhlA* and *pqsA* expression, suggesting that sAPDI did not induce the Rhl and Pqs QS systems (Fig. 3).

After a single irradiation of 1 min with 0.8 mM MB concentration, a significant decrease in *cdrA* expression levels was obvious between treated and untreated samples (P < 0.001) (Fig. 3). However, applying 2 and 5 min with 0.8 mM MB led to a significant rise in *cdrA* expression in sAPDI-treated samples compared to untreated controls (P < 0.001).

Interestingly, the expression of the four reporter genes in the *P. aeruginosa* Δ*mexAB*-*OprM* mutant showed no significant difference before and after sAPDI treatment (Fig. 4).

There was no difference between the results of light and dark toxicity on treated samples and untreated control across all experiments.

The results of the experiments with fluorescent reporter strains where confirmed with qPCR. qPCR is the technique of combining amplification and detection using SYBR green which correlates the mRNA concentration (extracted from treated and untreated samples) to

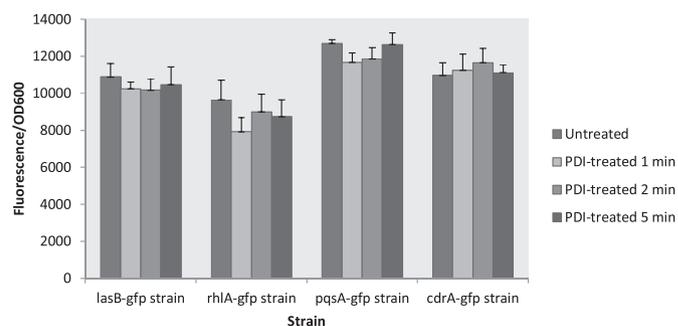


Fig. 4. The effect of sAPDI with 0.8 mM MB and 1-, 2- and 5-min irradiation times on *P. aeruginosa* Δ*mexAB*-*oprM* reporter strains for *lasB*, *rhlA*, *pqsA*, and *cdrA* genes.

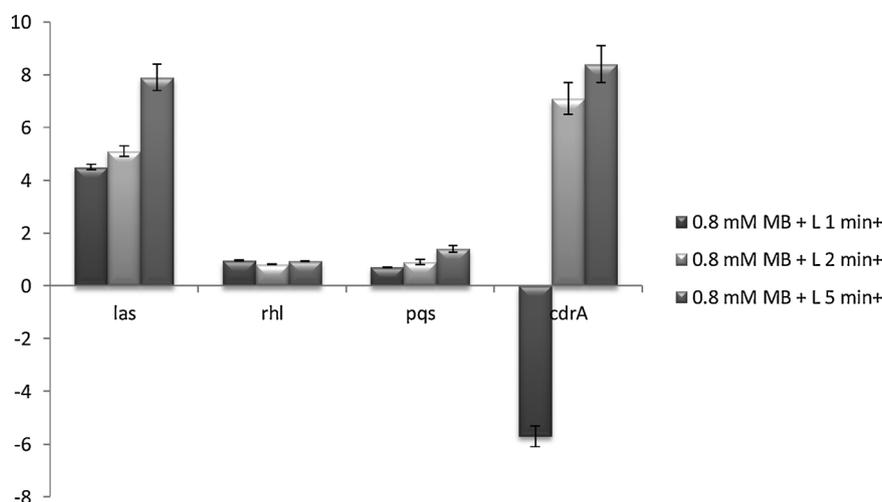


Fig. 5. Gene expression fold changes in *P. aeruginosa* PAO1.

fluorescence intensity.

qPCR analysis clearly showed changes in transcriptional levels of *lasB* and *cdrA* (Fig. 5). An up-regulation of 4.5, 5.1, and 7.9-fold was observed for *lasB* after a single sAPDI with different irradiation times of 1, 2 and 5 min, respectively. Interestingly, the *cdrA* expression levels differed with different fluency of laser light and were in line with phenotypic biofilm formation and reporter results. A 5.7-fold decrease was observed after 1 min irradiation while 7.1- and 8.4- fold increases were detected after 2- and 5-min irradiations.

No significant fold changes were recorded for *rhlA* and *pqsA*. Also, the control groups considered for the four regulatory systems showed no difference with the untreated control group in all cases. The congruence between qPCR and phenotypic results suggests that the transcriptional changes caused by sAPDI directly lead to phenotypic changes.

4. Discussion

Antimicrobial photodynamic inactivation has become a useful practical modality to treat superficial infections caused by antibiotic-resistant bacteria. Although APDI-induced oxidative stress is generally used to kill bacteria in the infection site, sub-lethal oxidative stress may cause pathogens to react to it by expressing resistance mechanisms [26]. In *P. aeruginosa* triggered with ROS, protective mechanisms include production of pigments, efflux pumps, neutralizing enzymes, virulence factors, and biofilm formation, which are mostly under control of regulatory systems like QS and c-di-GMP signaling.

The *cdrA* gene codes for a Psl-binding matrix protein. Earlier work has shown that the *cdrA* transcription levels change positively with the levels of c-di-GMP [[27,25]]. In *P. aeruginosa*, biofilm formation is controlled mostly by c-di-GMP signaling controlled polysaccharide production [28]. Results obtained with the *P. aeruginosa* PAO1 *cdrA* gfp-reporter strain and qPCR suggested a significant decrease in c-di-GMP levels in response to treatment with 800 μ M MB at 3.8 J cm⁻² light fluency, and accordingly a 60% reduction in biofilm formation ability of *P. aeruginosa* PAO1 as assessed by the CV staining method. However, treatment with 800 μ M MB combined with longer light exposures led to increased expression of *cdrA*, suggesting an increase in the c-di-GMP level. Accordingly, these treatments also resulted in increased biofilm formation.

Our finding of a stimulation of biofilm formation by sAPDI is in agreement with prior work. Previous studies have indicated that oxidative stress can stimulate biofilm formation in *E. coli* [29], *Haemophilus influenza* [30], *Campylobacter jejuni* [31], *Staphylococcus aureus* [7], *Streptococcus mutans* [32] and *Azotobacter vinlandii* [33]. Gambino

and Cappitelli speculated that bacteria use ROS as a hint to start adaptation to environmental changes [34]. Schembri et al. reported that exposure of *E. coli* to oxidative stress induced the transcription of *agn43* encoding an adhesion protein. Expression of the Ag43 protein increases biofilm formation and also protects against oxidative stress [29]. In *P. aeruginosa* OxyR, an oxidative stress response protein, helps bacteria to sense environmental ROS. It is also involved in biofilm formation by binding to the promoter region of the bacteriophage Pf4 operon thereby promoting initial biofilm formation [35]. Huang et al. reported that in *Klebsiella pneumonia* RpoS, a general stress response together with SoxR enhances the expression of YjcC, a regulatory protein for oxidative stress response and biofilm formation. This process happens by modulating the levels of c-di-GMP secondary messenger [36].

Another regulatory system which can be affected by ROS is QS. Lumjiaktase et al. hypothesized that QS can control the oxidative stress via regulation of high-density behaviors in *Burkholderia pseudomallei* [37]. The Las system in *P. aeruginosa* has an important role in response to increased population density by associating it to bacterial virulence factor production [38]. Previous studies demonstrated that Las has a protective role by regulating the expression of catalase and superoxide dismutase [39]. In this study, we found that *lasB* expression increased after a single exposure to sAPDI in a time-dependent manner, according results obtained with a *lasB*-reporter strain and qPCR. This indicates that the Las QS system responds to sAPDI induced oxidative stress. In accordance, increase in pyoverdine and protease production was observed in response to sAPDI. Pyoverdine and elastase are two virulence factors controlled by the Las QS system [40,41]. A few have studies examined the effect of APDI against virulence factor production of *P. aeruginosa*. Komerik et al. tested the effect of APDI using TBO and red light on protease activity of *P. aeruginosa*. They found APDI as an effective antimicrobial agent to reduce the virulence of Gram-negative bacteria [42]. Sharma et al. reported similar effects of APDI on phospholipase C and protease activities of *P. aeruginosa* [43].

Pyocyanin production is under the control of *rhl* and *pqs* QS systems in *P. aeruginosa* [44]. According to our results, the pyocyanin production of sAPDI-treated *P. aeruginosa* PAO1 did not change significantly compared to the untreated control. The results of real-time Gfp fluorescence detection of *rhlA-gfp* and *pqsA-gfp* reporter strains were in agreement with the finding that sAPDI did not affect pyocyanin production. These results are consistent with Fila et al. reporting lack of difference in PQS signaling in response to lethal or sub-lethal antimicrobial blue light photodynamic treatment of *P. aeruginosa* [45].

According to Tegos et al., an important responsive agent to APDI is efflux pumps. They demonstrated that amphipathic cations such as MB

are substrates of the MexAB-OprM efflux pump in *P. aeruginosa*. By pumping out MB from the cells, the phototoxicity of APDI drops upon irradiation. The substrates for Mex-Opr pumps are different kinds of biocides, dyes, organic solvents, and signaling molecules of cell-to-cell communication. [6]. A correlation was found between the Las QS system and MexAB-OprM efflux pumps in *P. aeruginosa* as Mex-Opr is able to pump out 3-oxo-AHLs [46]. Therefore, *P. aeruginosa* QS and related virulence factors may be directly controlled by the MexAB-oprM efflux pump. Inhibition of MexAB-oprM activity is associated with low extracellular concentration of C12-HSL, which is at the top of *P. aeruginosa* QS hierarchical order [47]. The inhibition not only causes disorder in the production of virulence factors, but also during APDI treatment results in increased intracellular MB concentrations and severity of damages following irradiation. Accordingly our results indicate that sAPDI had a severe negative effect on viability and biofilm formation of the Δ mexAB-oprM mutant, in contrast to the results with the wild type. Consequently, unlike the wild type, the Δ mexAB-oprM mutant did not display a higher biofilm formation potential and increased *cdrA* and *lasB* gene expression after 2 and 5 min irradiation. In accordance with our results, Tegos et al. also reported an increase in bacterial susceptibility to APDI for an efflux pump knockout mutant in comparison to the wild type strain [6].

Antimicrobial photodynamic inactivation is a promising new alternative approach for eradication of antibiotic-resistant bacteria. Our study shows that sub-lethal photodynamic inactivation might increase bacterial virulence and biofilm formation. This emphasizes the importance of applying APDI in lethal doses throughout the infectious site for the successful treatment of bacterial infections.

5. Conclusion

In this study, the response of *P. aeruginosa* QS and c-di-GMP regulatory systems to sAPDI with 0.8 mM MB and different laser light irradiation times was assessed. We found that QS and c-di-GMP signaling in *P. aeruginosa* change in response to sAPDI in a time-dependent manner. In particular, sAPDI with 0.8 mM MB along with 2- and 5-min light irradiation led to an increase in the expression of the Las QS system and c-di-GMP signaling. These results were corroborated by investigations of the effect of sAPDI on virulence factor production and biofilm formation. In addition, our results confirm that the MexAB-oprM efflux pump is an important resistance factor to APDI.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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