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Quorum-sensing-regulated virulence factors in *Pseudomonas aeruginosa* are affected by sub-lethal photodynamic inactivation

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ABSTRACT

Background: Photodynamic inactivation (PDI) is recognized as a new antimicrobial approach. It is likely that in human hosts receiving this therapy, pathogens may encounter sub-lethal doses of PDI (sPDI), which may affect microbial virulence. This study was aimed to evaluate the effect of sPDI using methylene blue (MB) on the expression of genes belonging to two quorum sensing (QS) operons (*rhl* and *las* systems) and two genes necessary for pyocyanin and rhamnolipid production (*phzM* and *rhlA*) under QS control in *Pseudomonas aeruginosa*.

Methods: Ability of pyocyanin and rhamnolipid production of *P. aeruginosa* ATCC 27853 and clinical isolates exposed to sPDI (MB at 0.012 mM and light dose of 23 J/cm^2 was evaluated. The effect of sPDI on expression of *rhlI, rhlR, lasI, lasR, phzM* and *rhlA* were also evaluated by quantitative real time polymerase chain reaction.

Results: sPDI led to the down-regulation of the expression of all four QS genes (*lasI*, *lasR*, *rhlI* and *rhlR*) and rhamnolipid gene (*rhlA*). However, up-regulation of pyocyanin gene (*phzM*) was observed after sPDI. These results were consistent with phenotypic changes.

Conclusion: This study suggests that oxidative stress induced by sPDI can affect QS-regulated virulence factors of *P. aeruginosa* such as pyocyanin and rhamnolipids in different ways.

1. Introduction

Pseudomonas aeruginosa is a common opportunistic pathogen of acute and chronic nosocomial infections that have been responsible for high mortality over the past decade. Nosocomial infections caused by this bacterium are often hard to treat because of both the intrinsic resistance of the species and its extraordinary ability to acquire resistance mechanisms to several groups of antimicrobial agents [1].

During infection, *P. aeruginosa* releases a variety of virulence factors such as pyocyanin, protease, elastase, rhamnolipids and hydrogen cyanide which facilitate microbial dissemination through evading from phagocytosis, modifying the immune response and destroying the host tissue [2,3]. Quorum sensing (QS) operates the release of *P. aeruginosa* virulence factors and the synthesis of biofilm during infection [4]. QS is an intercellular communication system, which can efficiently control the gene expression in a cell density dependent manner [5]. Three distinct, but interacting QS systems (*las*, *rhl* and *pqs*) regulate the expression of virulence factors in *P. aeruginosa*. The three major signaling molecules of *P. aeruginosa* involve *N*-3-oxo-dodecanoyl-L-homoserine lactone (C12-HSL), *N*-butyryl-L-homoserine lactone (C4-HSL) and four-quinolone signal. Once those signals reach the significant levels, they activate their regulatory genes and enhance the transcription of virulence factors [6]. C12-HSL is the basic molecule of *las* system; it triggers the expression of *rhl* and *pqs* circuits in a hierarchical manner [7]. The production of pyocyanin pigment and rhamnolipids is controlled by *rhl* system [8].

Pyocyanin is one of the secondary metabolites of *P. aeruginosa*. Synthesis of this pigment is through complex cascade of reactions involving various genes such as *phzABCDEFG* and *phzHMS* [9]. This blue-green pigment causes oxidative stress to the host, disrupting host catalase, and mitochondrial electron transport [10]. *In vitro*, purified pyocyanin induces apoptosis in neutrophils and inhibits the phagocytosis of apoptotic bodies by macrophages [10,11]. Moreover, it is able to modulate the expression of the chemokines IL-8 by airway epithelial cells and suppress cilia beating [12].

Rhamnolipids are a class of biosurfactants which are produced by *P. aeruginosa* [13]. The genes rhlA, rhlB and rhlC play a direct role in the rhamnolipid biosynthesis pathway [14]. There are some reports of rhamnolipid participation in physiologic processes, including assimilation of insoluble substrates, and changing the hydrophobicity of the

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cell surface [15]; antimicrobial activity [16]; hemolytic activity in human pathogenesis [17]; promotion of swarming motility [18]; maintenance of biofilm architecture [19]; invasion of human airway epithelium; and lysis of polymorphonuclear neutrophils [20,21].

The severity of *P. aeruginosa* infections and limited antimicrobial agents to treat these infections has led to find other possible strategies for prevention and treatment. In recent years, many reports about the efficacy of photodynamic inactivation (PDI) against *P. aeruginosa* have been published [22–24]. PDI is a new antimicrobial approach which has been reported as a fast and effective method to inactivate a broad spectrum of microorganisms, including those resistant to common antimicrobials [25].

PDI causes photo-oxidative stress using a photosensitizer (PS) excited through visible light. Reactive oxygen species (ROS) production happens through transferring electron or energy from the excited PS to oxygen [26]. The generated ROS can trigger microbial cell damage and killing via damage to the cell membrane, inactivation of essential enzymes and proteins, and/or damage to DNA [27].

PDI should be generally used at lethal doses to kill bacteria, however, it is likely that in human hosts receiving this therapy, any microorganism viable at the site of infection would be exposed to sublethal doses of PDI (sPDI). In this situation, sPDI would not result in total cell death and survivors would expose to ROS stress. Exposure to low levels of stress activates protective mechanisms through a complex pathway involving various regulators so that cell death is avoided [28]. For example, *P. aeruginosa* protective mechanisms for defense against ROS include two superoxide dismutases [29], three catalases [30], four alkyl hydroperoxide reductases [31], the mucoid phenotype [32] and pigments [24]. It is also clear that ROS can damage DNA. Such stress also leads to increased mutational events, which can lead to selection for survival of more resistant strains [33].

Consequently, in this study we aimed to assess whether sPDI can affect *P. aeruginosa* ability to produce rhamnolipids and pyocyanin as virulence factors. Thus, the effect of sPDI using methylene blue (MB) on the expression of genes belonging to two QS operons (*rhl* and *las* systems) and two genes necessary for pyocyanin and rhamnolipid production (*phzM* and *rhlA*) under QS control in *P. aeruginosa* was evaluated.

2. Materials and method

2.1. Growth conditions

P. aeruginosa ATCC 27853 and six clinical isolates (P1-P6) from burn wound infections were used throughout the study. Bacteria were routinely grown on nutrient agar (Merck, Germany) under aerobic conditions at 37 °C. To prepare the working inoculum, the overnight cultures of bacteria were suspended in sterile 0.9% saline to reach the turbidity of 0.5 McFarland.

2.2. Photosensitizer and light source

Methylene blue (MB, Sigma-Aldrich) was used as the photosensitizing agent. MB stock solution (3.2 mM) was prepared in 0.9% saline, filter sterilized and stored at 4 °C in the dark no more than two weeks prior to use. Stock solution was further diluted to obtain the desired concentrations.

The light source was a diode laser (AZOR, Russia) with an emission at 650 nm. The total output power provided by the device was 30 mW.

2.3. Evaluation of the effect of sPDI on pyocyanin production

In our preliminary experiments [34], the in vitro lethal and sublethal doses of MB-PDI (MB at final concentrations of 0.006-0.4 mM and light dose of 23 J/cm^2) on bacteria were evaluated. According to the results (not shown), 0.012 mM MB and light dose of 23 J/cm^2, were chosen as "sub-lethal" PDI parameters for other experiments.

For assessing the effect of sPDI on pyocyanin production, bacteria were grown on Luria Bertani (LB) agar overnight. Equal volumes of cell suspensions (1–2 × 10^8 CFU/ml) and MB at a final concentration of 0.012 mM were added to 2 ml sterile microtubes and incubated for 10 min in the dark. Then samples were irradiated with a diode red laser light for 10 min (23 J/cm^2), followed by washing excess dye. 1 ml of LB broth was added to each microtube and 500 µl of that was transferred to a tube containing 10 ml of LB broth supplemented with 1% glycerol and 1% NaCl. Tubes were incubated at 37 °C for 72 h with shaking at 120 rpm. Afterward, 1 ml of each tube was transferred to a sterile microtube and the cells were harvested by centrifugation (9000*g*, 10 min). The supernatant was passed through a syringe filter with 0.2 µm cellulose acetate membrane and pyocyanin in supernatant was extracted with equal amount of chloroform until the medium became colorless. Again, centrifugation was performed in 9000*g* for 10 min, which green color turned to blue-green. The chloroform layer was pooled in a separate tube, following by adding 0.5 ml of HCl (0.02 N) and removing the aqueous layer, so that the top layer turned pink in color. 200 µl of each tube was transferred to the wells of a microtiter plate and read in 545 nm. Pyocyanin production was measured by using an extinction coefficient of 17.064 [35].

2.4. The effect of sPDI on Rhamnolipid production

The blue agar plate method [36] was used to assess the effect of sPDI on rhamnolipid production of bacteria. LB agar plates containing 0.2 g cetyltrimethylammonium bromide (CTAB) and 5 mg MB were punched with a 5 ml sterile pipette to prepare some wells. A volume of 3 µl of bacterial suspensions (exposed to sPDI) was loaded to the wells and plates were incubated at 37 °C for 72 h. Then, they were kept 6 h at the refrigerator. Plates were located on a trans-illuminator and the diameter of rhamnolipid production zone was measured in centimeter.

2.5. Quantitative PCR experiments

Real time-PCR was used to evaluate the transcriptional expression of *lasI, lasR, rhlI, rhlR, phzM, rhlA*. Following by sPDI, the excess MB was removed and cells were washed by 0.9% sterile saline. Total bacterial RNA was extracted from sPDI-treated and untreated suspensions using RNX-Plus solution (Sinaclon, Iran) guidelines. DNase treatment was performed by adding DNase I (Thermo Fisher Scientific, US) to the total extracted RNA. The concentration and purity of RNA samples were assayed on a ND-1000 Nanodrop, and absence of degradation was confirmed on 1% agarose gel. cDNAs were then synthesized through random hexamer primed reactions using a Thermo scientific kit, according to the manufacturer's protocol.

Quantitative PCR experiments were performed on a Rotor-Gene 6000 thermocycler (Corbett, Qiagen Inc., Toronto, ON, Canada), using Green master mix with fluorescent dye (Genaxxon kit, Germany) according to the manufacturer's protocol. The reference gene was *16 s rRNA*. Primers (as shown in Table 1) were used for amplification under the following conditions: 95 °C for 15 min, amplification for 40 cycles with denaturation at 95 °C for 15 s, annealing for 20 s at 61 °C, and extension at 72 °C for 30 min. The specificity of the primers was evaluated using melt curves.

2.6. Statistical analysis

Comparisons between means of treated and untreated groups were analyzed using one-way ANOVA and post hoc (Tukey-dunken) tests. $P < 0.05$ was considered statistically significant.

Real time PCR data was analyzed by ΔΔCt method, and gene expression levels were determined by $2^{-\Delta\Delta Ct}$. Relative changes of *phzM, rhlA, lasI, lasR, rhlI, rhlR* gene expression in samples were evaluated with respect to *16 s rRNA* as internal control by a paired sample t-test.

Table 1
Primer sequence.

Genes		Sequence (5'–3')	Accession number	Amplicon size
lasI	F	CCGTTTCGCCATCAACTCTGG	**NC_002516.2**	153
	R	CGGATCATCATCTTCTCCACGC		
lasR	F	GCAGCACGAGTTCTTCGAGG	**NC_002516.2**	183
	R	GCGTAGTCCTTGAGCATCCAC		
rhlI	F	CCGACGCCTACCTGCTCAAG	**NC_002516.2**	222
	R	TGCGAACGAAATAGCGCTCCA		
rhlR	F	CGCCACACGATTCCCTTCAC	**NC_002516.2**	150
	R	GCTCCAGACCACCATTTCCGA		
phzM	F	AGACTTCTACAGCTACCTGAAGC	**NC_002516.2**	166
	R	GATGGCCTTGGTCAATTCGC		
rhlA	F	GAGCGCTTCGAGGTCAATCAC	**NC_002516.2**	258
	R	CTGATGGTTGCTGGCTTTCAGG		
16S rRNA	F	GGCTCAACCTGGGAACTGCA	**NC_002516.2**	137
	R	CAGTATCAGTCCAGGTGGTCGC		

The correlation between the changes in gene expression before and after sPDI was assessed by SPSS 18 software and R Studio 1.0.136.

3. Results

3.1. Pyocyanin production increased after sPDI

P. aeruginosa ATCC 27853 and only two clinical isolates (P2 and P3) had the ability to produce pyocyanin before exposure to sPDI. Fig. 1 showes pyocyanin production of these bacteria before and after sPDI. MB at 0.012 mM and light dose of 23 J/cm^2 resulted in significant increase in pyocyanin production ability of *P. aeruginosa* ATCC 27853 and two clinical isolates compared to their untreated controls ($P < 0.05$).

3.2. Rhamnolipid production reduced after sPDI

As shown in Fig. 2, rhamnolipid production in *P. aeruginosa* ATCC 27853 and all isolates significantly reduced after exposure to MB (0.012 mM) and light dose of 23 J/cm^2 compared to the untreated controls ($P < 0.05$).

3.3. Expression of QS and QS-regulated virulence factor genes changed after sPDI

Fig. 3 shows fold changes in gene expression of the studied microorganisms after sPDI (0.012 mM MB, 23 J/cm^2) compared to their untreated controls. sPDI led to the down-regulation of the expression of all four QS genes (*lasI*, *lasR*, *rhlI* and *rhlR*) and rhamnolipid gene (*rhlA*). However, up-regulation of pyocyanin gene (*phzM*) was observed after sPDI. These results were compatible with phenotypic changes.

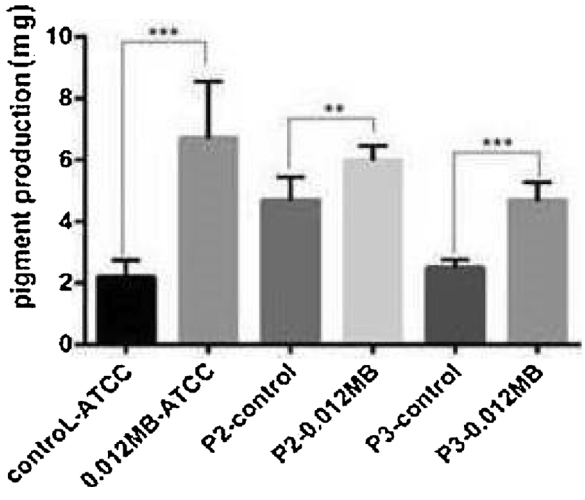

Fig. 1. The effect of sPDI on pyocyanin production of *P. aeruginosa* ATCC 27853 and two clinical isolates (P2 and P3), Control groups: production of pigment before exposure of bacteria to sPDI, 0.012 MB groups: production of pigment after exposure of bacteria to sPDI.

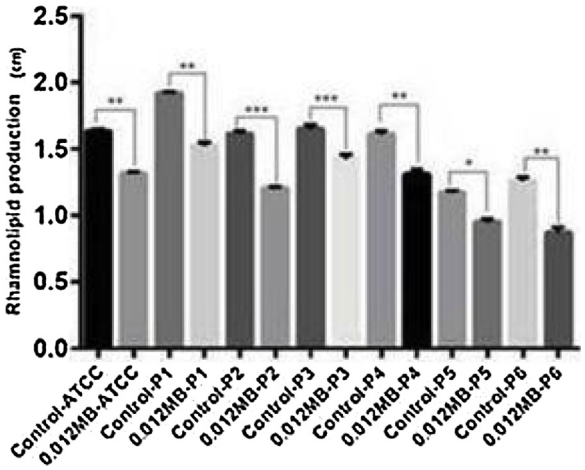

Fig. 2. The effect of sPDI on rhamnolipid production of *P. aeruginosa* ATCC 27853 and six clinical isolates (Control groups: production of rhamnolipid before exposure of bacteria to sPDI, 0.012 MB groups: production of rhamnolipid after exposure of bacteria to sPDI).

4. Discussion

There is a direct link between QS and stress tolerance in *P. aeruginosa* [37]. As rhamnolipid and pyocyanin biosynthesis are also under QS control, we aimed to assess whether oxidative stress induced by sPDI can affect the production of these virulence factors by *P. aeruginosa*.

The four studied QS genes, i.e. *lasI*, *lasR*, *rhlI* and *rhlR*, were down-regulated by a single sPDI treatment with MB at 0.012 mM and light dose of 23 J/cm^2 in *P. aeruginosa* ATCC 27853 and six clinical isolates. A dramatic reduction of gene expression was also observed with the *rhlA* gene. The reduction in rhamnolipid gene expression was confirmed by the reduction in rhamnolipid production zone in CTAB agar plates.

It has been shown that the *rhl* system directly controls the biosynthesis of rhamnolipids through the transcription factor RhlR and its autoinducer C_4-HSL. C_4-HSL forms a complex with RhlR to activate the transcription of *rhlAB* genes to initiate rhamnolipid biosynthesis during stationary phase [38,39].

Our results are in consistent with these findings. According to the results, it seems that a single sPDI treatment led to the down-regulation of rhl*I/R* and *rhlA* genes following down-regulation of *lasI/R* genes, and

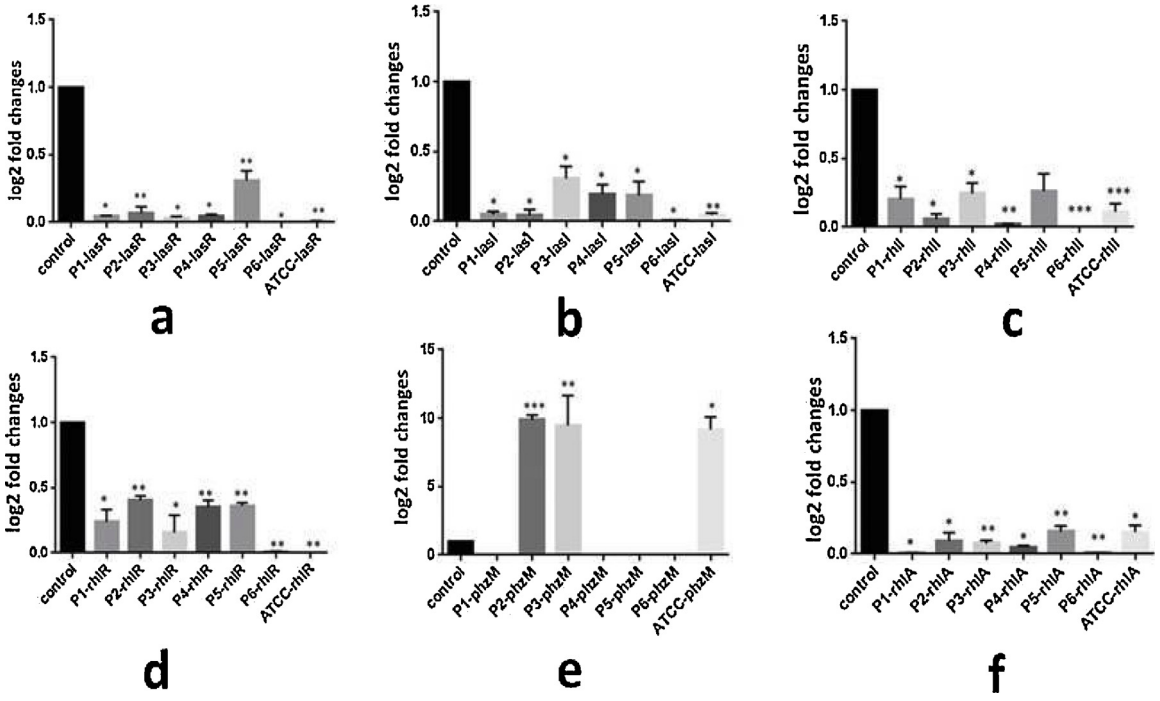

Fig. 3. Gene expression fold changes in *P. aeruginosa* ATCC 27853 and clinical isolates after sPDI (MB at 0.012 mM and light dose of 23 J/cm^2). (a) *lasR* gene expression changes (b) *lasI* gene expression changes (c) *rhlI* gene expression changes (d) *rhlR* gene expression changes (e) *phzM* gene expression changes (f) *rhlA* gene expression changes. Controls in all charts were assumed as gene expression level in bacteria without sPDI treatment.

at the end, it resulted in the reduction of rhamnolipid biosynthesis.

Up-regulation of pyocyanin gene (*phzM*) was observed after sPDI treatment in *P. aeruginosa* ATCC 27853 and two clinical isolates (P2 and P3) in this study. Increased production of pyocyanin was also observed after sPDI treatment.

It has been shown that OxyR is the central transcriptional regulator of the oxidative stress response in many Gram-negative bacteria, which at the time of induction by H_2O_2, undergoes a conformational change allowing oxidized OxyR to obtain DNA-binding capacity and to activate the transcription of antioxidant genes including those encoding catalases, glutathione reductase and alkyl hydroperoxide reductases [40,41,42]. In *P. aeruginosa*, OxyR is recognized to activate the expression of the *katA*, *katB*, *ahpB* and *ahpCF* genes to respond the oxidative damages caused by H_2O_2 [43,44]. After exposure to H_2O_2, the expression of a large number of genes are altered, including an up-regulation of protection mechanisms and a down-regulation of primary metabolism genes [45,30].

It has been shown that OxyR can partially control the production of pyocyanin. Therefore, it seems that pyocyanin plays a protective role against the reactive oxygen and nitrogen species produced by phagocytic cells during infection [46].

On the other hand, LasR induces the expression of RsaL. RsaL inhibits the expression of some QS target genes such as biosynthetic genes of pyocyanin and cyanide [47]. So, according to these reports, it seems that oxidative stress mediated by sPDI has led to the down-regulation of *lasR* gene and up-regulation of *phzM* gene in this study.

A small number of studies in *P. aeruginosa* have proposed the photoinactivation of virulence factors via photodynamic treatment with exogenously administered photosensitizers [48,49]. Tan et al. also investigated the effects of 5-aminolevulinic acid photodynamic therapy (ALA-PDT) on virulence factor secretion (pyocyanin and elastase) by *P. aeruginosa*. ALA-PDT inhibited pyocyanin and elastase secretion and significantly reduced the mRNA expression of QS-related genes (*lasI*, *lasR*, *rhlI*, and *rhlR*) and virulence factor-related genes (*lasB* and *phzH*) in the *P. aeruginosa* biofilm [50].

However, there is a main difference between our study and those studies mentioned above. In those reports, authors used lethal doses of PDI for their experiments and consequently, they observed reduction in virulence factor secretion. As PDI is currently one of the most promising treatments for resistant bacterial infections, we must be cautious by using sub-lethal doses of PDI; because bacteria start to produce several combating strategies to resist in stressful environments as well as changes in their physiology and pathogenesis factors.

In conclusion, this study suggests that oxidative stress induced by sPDI can affect QS-regulated virulence factors such as pyocyanin and rhamnolipids in different ways. Over-expression of pyocyanin can be a possible protective role against sPDI-induced oxidative stress. As the molecular mechanisms of signaling pathways activated by PDI are still unclear, more studies can help finding the keys to clarify bacterial responses to sPDI-mediated oxidative stress.

Conflicts of interest

The authors hereby declare that they have no conflict of interest.

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