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The mode of action of the PSIR-3 photosensitizer in the photodynamic inactivation of *Klebsiella pneumoniae* is by the production of type II ROS which activate RpoE-regulated extracytoplasmic factors

Vanessa Bustamante [a], Iván A. González [b], Paulina Dreyse [c], Christian Erick Palavecino [a,*]

[a] Laboratorio de Microbiología Celular, Instituto de Investigación e Innovación en Salud, Facultad de Ciencias de la Salud, Universidad Central de Chile, Lord Cochrane 418, Post Cod: 8330546, Santiago, Chile
[b] Instituto de Investigación e Innovación en Salud, Facultad de Ciencias de la Salud, Universidad Central de Chile, Chile
[c] Departamento de Química, Universidad Técnica Federico Santa María, Av. España 1680, Casilla 2390123, Valparaíso, Chile

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ABSTRACT

Background: Due to increased bacterial multi-drug resistance (MDR), there is an antibiotic depletion to treat infectious diseases. Consequently, other promising options have emerged, such as the antimicrobial photodynamic inactivation therapy (aPDI) based on photosensitizer (PS) compounds to produce light-activated local oxidative stress (photooxidative stress). However, there are scarce studies regarding the mode of action of PS compounds to induce photooxidative stress on pathogenic γ-proteobacteria such as MDR-*Klebsiella pneumoniae*.
Methodology: The mode of action exerted by the cationic Ir(III)-based PS (PSIR-3) to inhibit the growth of *K. pneumoniae* was analyzed. RT-qPCR determined the transcriptional response induced by PSIR-3 on bacteria treated with aPDI. The expression levels of genes associated with a bacterial oxidative response, such as *oxy*R and *sod*A, and the extracytoplasmic, regulators *rpo*E and *hfq* were determined. Also, were determined the transcriptional response of the extracytoplasmic factors *mrk*D, *acr*B, *mag*A, and *rmp*A.
Results: At 17 μW/cm^2 photon flux and 4 μg/mL of the PSIR-3 compound, the *K. pneumoniae* growth was inhibited in 3 $\log_{10}$. Compared with untreated bacteria, the transcriptional response induced by PSIR-3 occurs via the extracytoplasmic sigma factor *rpo*E and *hfq*. In contrast, no participation in the *oxy*R pathway or induction of the *sod*A gene was observed. This response was accompanied by the upregulation of the extracytoplasmic virulence factors *mrk*D, *mag*A, and *rmp*A.
Conclusions: PDI aPDI produced by PSIR-3 kills *K. pneumoniae* and may induce damage to the bacterial envelope. The bacterium tries to avoid this injury by activation of extracytoplasmic factors mediated through the *rpo*E regulon.

1. Introduction

The global crisis due to the emergence of bacterial multi-drug resistance (MDR) is one of the most pressing threats to human health [1]. To focus efforts, the WHO published a priority list of microorganisms that require new antimicrobial therapies [2,3]. In first place is the extended-spectrum β-lactamase (ESBL) producers of carbapenemase bacteria, such as *Klebsiella pneumoniae* (KPC$^+$) [2,4]. *K. pneumoniae* is associated with infection of the urinary tract (UTI), pneumonia [5,6], and healthcare-associated infections (HAIs) [7]. The antibiotic crisis is due in part to the deficit of new antibiotics, which can be surpassed with the development of complementary or alternative non-antibiotic therapies [3]. Antimicrobial photodynamic inactivation (aPDI) therapy has gained a high relevance for the treatment of MDR-bacteria [8]. APDI is based on the use of photosensitizer (PS) compounds that produce light-activated local oxidative stress (photooxidative stress) [9]. Oxidative stress is produced by reactive oxygen species (ROS) that are generated when the light energy absorbed by the PS compounds is transferred to molecular oxygen found in aqueous solution [10]. The mode of action for ROS production can occur by Type I or Type II

* Corresponding author at: Laboratorio de Microbiología Celular, Facultad de Ciencias de la Salud, Universidad Central de Chile, Lord Cochrane 418, 3° piso Santiago, Post Cod: 8330546, Chile.
E-mail addresses: vanessa.bustamante@alumnos.ucentral.cl (V. Bustamante), ivan.gonzalez@ucentral.cl (I.A. González), paulina.dreyse@usm.cl (P. Dreyse), christian.palavecino@ucentral.cl (C.E. Palavecino).

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mechanisms, where the PS may transfer the energy accompanied by electrons producing superoxide anion radical ($O_2^{\bullet -}$), or without electrons producing singlet oxygen ($^1O_2$), respectively [10]. The $O_2^{\bullet -}$ may produce other ROS such as hydrogen peroxide (H_2O_2), and hydroxyl radical ($HO^{\bullet}$) [11–13]. The photooxidative stress induced by aPDI occurs mainly due to the action of the $^1O_2$ generated via the Type II effect. The photooxidative effect due to $^1O_2$ occurs by the production of concerted addition reactions of alkene groups on organic molecules close to the activated PS [14]. Those organic molecules such as proteins and lipids can be part of the bacterial envelope resulting in non-specific cell death [10,15]. For example, oxidative stress produces an increased permeability on the *K. pneumoniae* membrane [16].

In prokaryotes, the photooxidative stress induces multiple responses, such as direct oxidant detoxification enzymes like superoxide dismutase (SOD). In *Escherichia coli*, the response to the stress due to ROS of Type I effect is well known. For example, the stress produced by H_2O_2 induces the expression of genes controlled by the *oxy*R/S two-component system such as *kat*G, *ahp*CF, and *oxy*S genes [17]. Besides, the response by superoxide anion radical induces the expression of genes controlled by the *sox*R/*sox*S two-component system such as SOD (*sod*ABC genes) [18]. However, there are not sufficient studies explaining the cellular response of γ-proteobacteria to the photooxidative stress induced by ROS of Type II effect. Studies on the photosynthetic α-proteobacteria *Rhodobacter sphaeroides* point to the regulon controlled by the alternative sigma factor RpoE [3,19,20]. In *R. sphaeroides*, the RpoE activation is modulated by the expression of small non-coding RNAs (sRNA) that are regulated by the RNA chaperone Hfq [19,20]. RpoE protein is the extracytoplasmic stress-response sigma factor-24 (σ^{24}), which response to envelope damage such as protein misfolding, and contributes to the lipopolysaccharides (LPS) heterogeneity [21–23]. RpoE regulon controls the expression of extracytoplasmic genes, some of which are pathogenicity factors [5], such as fimbriae, capsule, and efflux pump [5,6]. The fimbriae production lays on pili factors encoded in the genome by the *mrk*ABCDF operon [24]. Besides, the mucoviscosity-associated gene A (*mag*A) and the regulator of the mucoid phenotype A gene (*rmp*A), are related to the polysaccharide capsule synthesis [25,26]. Finally, the *acr*RAB operon encodes a multi-drug efflux pump system [27].

Although only a few initiatives have explored the aPDI against *K. pneumoniae*, this could help resolve the lack of antibiotic therapy for MDR strains [3]. Previously, a PS compound based on a polipyridinic Ir (III) complex (PSIR-3, see Fig. 1A), demonstrated aPDI activity, inhibiting the bacterial growth of KPC^+ [28]. Due to limited knowledge about the response of Gram-negative bacteria to photooxidative stress, in this study, a possible mode of action was identified, evaluating the transcriptional response of *K. pneumoniae* treated with the PSIR-3 compound. RT-qPCR determined the gene expression of genes associated with the control of the response to oxidative stress and the membrane damage. The transcriptional response of genes encoding extracytoplasmic factors that, in turn, are associated with pathogenicity was also determined.

2. Materials and methods

2.1. Synthesis of the photosensitizer

We used a photosensitizer compound, which had previously demonstrated aPDI activity limiting the growth of sensitive and multidrug resistant strains of *Klebsiella pneumoniae* [28,29]. In particular, the PS compounds is based on Ir(III) with a polypyridine ancillary ligand and is described as $[Ir(ppy)_2(ppdh)]PF_6$ = PSIR-3 (ppy: 2-phenylpyridine and ppdh is pteridino[7,6–*f*][1,10]phenanthroline-1,13(10H,12*H*)-dihydroxy). The structure and purity of the compound were confirmed by nuclear magnetic resonance (NMR), Fourier-transform infrared spectroscopy (FT-IR), and mass spectroscopy (MALDI-MS) measurements. The absorption spectra were measured in acetonitrile

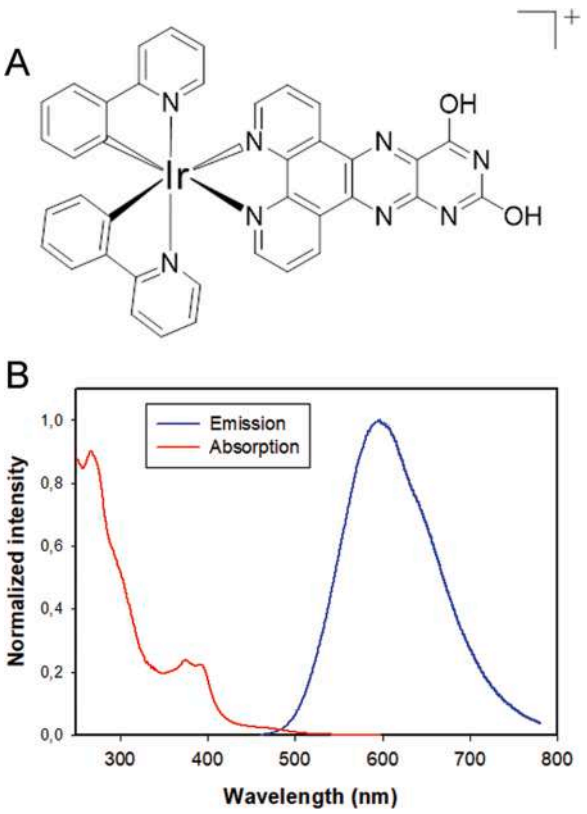

Fig. 1. The PSIR-3 structure and its photophysical properties. Chemical structure of Ir(III) complexes PSIR-3 ($[Ir(ppy)_2(ppdh)]PF_6$) **A**. The absorption and emission spectra of the compound in acetonitrile solution **B**.

(ACN) solution using a Shimadzu UV–vis Spectrophotometer UV-1900, and the photoluminescence spectra were taken on an Edinburgh Instrument spectrofluorimeter in ACN.

2.2. Antimicrobial activity of photosensitizers compounds

The PSIR-3 compound was solubilized as a stock solution in acetonitrile at 2 mg/mL and kept in the dark at 4 °C. This solution was diluted in distilled water at suitable concentrations previously to be used. For the antimicrobial assay, two *K. pneumoniae*, strains were used, the imipenem susceptible (KPC^-) sequenced strain KPPR1, and the imipenem resistant (KPC^+) strain ST258. All bacteria were growth as axenic culture in Luria Bertani broth or agar medium as convenient. For the photodynamic experiment, suspensions of 1×10^7 CFU/mL of each bacteria were mixed with 4 μg/mL of PSIR-3 in a final volume of 500 μL of cation –adjusted Muller Hinton (ca-MH) broth in triplicate in 24-well plates. For light exposure, a chamber with a white LED lamp of 17 μW/cm^2 photon flux was used. After the light exposure, bacteria were recovered, and CFU of viable bacteria was determined by broth-micro dilution and sub-cultured on ca-MH agar plates. Agar plates were incubated at 37 °C and colony counting was registered using a stereoscopic microscope after 16−20 h incubation in the dark [30].

2.3. Quantification of gene expression by real-time PCR

Total RNA from treated and untreated bacteria was extracted using the TRI Reagent (Sigma-Aldrich) and phenol-chloroform procedure. Genomic DNA was rid of incubating the total RNA with 1 U of RNAase free DNase (Promega) for 30 min at 37 °C. The cleaned total RNA was used to generate the cDNA by reverse transcription with the Impront II kit (Promega) using random hexamers. The gene expression profile of several bacterial genes was determined using specific primers (Table 1)

Table 1
Primers used for gene expression quantification.

Gene	Primers	Gene Type	Amplicon size
***oxy*R**	TCCCGAAGCTGGAAATGTAT GAGCATAATAAGGCGAAAGA	Oxidative and nitrosative stress transcriptional regulator	115
***mrk*D**	AAGCTATCGCTGTACTTCCGGCA GGCGTTGGCGCTCAGATAGG	Adhesin type 3 fimbriae	340
***mag*A**	GGTGCTCTTTACATCATTGC GCAATGGCCATTTGCGTTAG	Capsular serotype K1 and hypermucoviscosity phenotype	128
***rmp*A**	CATAAGAGTATTGGTTGACAG CTTGCATGAGCCATCTTTCA	Regulator of mucoid phenotype A	461
***acr*B**	GTAAACGTCGTTGGTTAGCC CTGTATGAGAGCTGGTCGAT	Acriflavine resistance protein B	108
***sod*A**	TTCCGGCTTCCCGATTATCGGCCT AGCTTCGTCCCAGTTCACTA	Superoxide dismutase	118
***rpo*E**	AACGGGTCCAGAAAGGAGAT CCTGAACAACGTCAGCGATA	Sigma factor 32,	120
Hfq	ATGGCTAAGGGGCAATCTTT GCTTGATACCATTCACCAAA	Post-transcriptional regulation	94
16S rRNA	ATTTGAAGAGGTTGCAAACGAT TTCACTCTGAAGTTTTCTTGTGTTC	Gene encoding the 16S ribosomal RNA	133

by qPCR in an OneStep Plus PCR machine (Applied Biosystems). There is no annotated gene sequence for the gene encoding the *K. pneumoniae* SOD enzyme. Therefore, we first use blast to search for a homolog of the *E. coli sod*A gene in the genome of the strain KPPR-1 of *K. pneumoniae* (accession No.: CP009208) and found it in position 75569–76184 with 89 % of identity. The variation of mRNA abundance of treated samples was determined using the relative quantification method of $2^{-\Delta\Delta Ct}$ comparing with the mRNA abundance of untreated controls.

2.4. Statistical analysis

The GraphPad Prism version 6.0 software was used to perform the statistical analyses. Statistical significance was assessed using one-way ANOVA with a posteriori Tukey test for the lethality curve or T-test for pairing groups.

3. Results

3.1. Experimental design for PSIR-3 compound evaluation

We have previously shown that Ir(III)-based compounds complexed with polypyridine ligand have photodynamic antimicrobial activity against imipenem-resistant *Klebsiella pneumoniae* [28,31]. The PSIR-3 compound has absorption processes at 375 and 392 nm, and emission with a maximum at 598 nm (Fig. 1B). In this study, we explore the transcriptional response activated by *K. pneumoniae* in the presence of the bactericidal effect of the PSIR-3 compound. For aPDI, we used a total of 1×10^7 CFU of each KPPR1, and the ST258 *K. pneumoniae* strains mixed with 4 μg/mL of PSIR-3 (its MEC). The mixtures were exposed for 1 h to white LED light of 17 μW/cm^{2,} and untreated control wells with bacteria culture without photosensitizer or with photosensitizer without exposure to light were also included. After the exposition time, an aliquot (1/10) of each bacterial strain, both treated and untreated, was used to determine viable bacteria by colony count. The rest of the bacterial inoculum was centrifuged at 10,000 rpm at 4 °C for 5 min. The supernatant was removed, and each pellet was homogenized in 1 mL of TRI reagent and frozen at −80 °C until use.

3.2. Antimicrobial photodynamic inhibition capacity of the PSIR-3

Photodynamic treatment was verified to produce the desired antimicrobial effect determining inhibition of bacterial growth of *K. pneumoniae* compared to untreated bacteria. The photodynamic activity of the PSIR-3 compound was compared to the activity of PS-Ru ($[Ru(bpy)_3](PF_6)_2$) reference compound, as a positive control [32–35]. As seen in Fig. 2, compared to the control of untreated bacteria (red bars), photodynamic treatment with 4 μg/mL PSIR-3 (yellow bars) inhibits bacterial growth > 3 $\log_{10}$ (> 99.9 %) of both *K. pneumoniae* strains (***$p < 0.001$; compared to untreated control). The results show that the bactericidal effect produced by PSIR-3 is light-dependent (orange bars) (ns = $p > 0.05$; compared to the untreated control). These results are comparable with those obtained with the positive control compound PS-Ru which also has shown the bacterial growth inhibition is light-dependent (**$p < 0.01$; compared to the untreated control). Then, the photodynamic treatment was efficient, producing the desired effect and, therefore, these bacteria were used to determine gene expression.

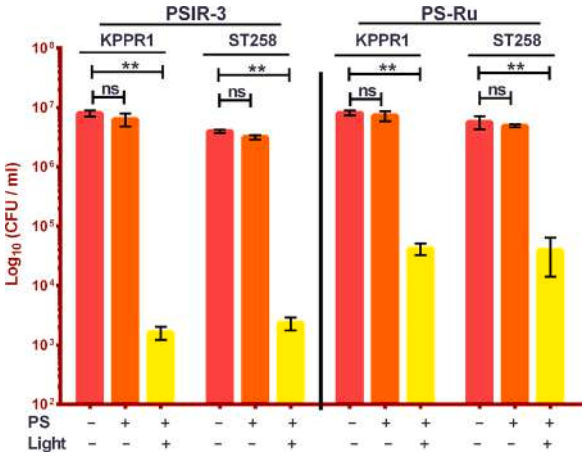

Fig. 2. Antimicrobial photodynamic inactivation capability of the PSIR-3 compound. Photodynamic antimicrobial capability of PSIR-3 compared to the PS-Ru control, determined by serial dilution of two strains of *K. pneumoniae*, the imipenem sensitive KPPR1, and the MDR-ST258. Viable bacteria were enumerated by colony count on ca-MH agar after serial microdilution. The CFU/mL values are presented as means ± SD, on a $\log_{10}$ scale. Not significant [ns] $p > 0.05$, ** $p < 0.01$ by Student's *t*-test among bacteria treated with PS exposed to light compared to untreated bacteria.

3.3. Evaluation of the transcriptional response to oxidative stress

The excited state of PSIR-3 exhibits a hight contribution of triplet charge-transfer transitions that could favor the energy transfer to molecular oxygen to yield mostly singlet oxygen [28]. Then, it suggests the PSIR-3 mode of action produces ROS mediated by the Type II mechanism rather than to transfer electrons to produce superoxide as occur by the Type I mechanism. In order to corroborate this possible behavior, the

transcriptional response to photooxidative stress of bacteria treated with aPDI was determined on total RNA extracted from the previously frozen sediment of treated and untreated bacteria. First, it was evaluated if PSIR-3 induces the typical bacterial response by Type I ROS producing H_2O_2 and superoxide. The *E. coli* responds to oxidative stress produced by H_2O_2, upregulates the expression of genes encoding the *oxy*R/S two-component system [17]. Furthermore, *E. coli* responds to superoxide upregulating the expression of the genes encoding SOD, *sod*ABC [18]. As shown in Fig. 3A, the treatment of *K. pneumoniae* with PSIR-3 poorly induced (~ 2-fold) the expression of the *oxy*R gene, and does not modify the expression of the *sod*A gene. Conversely, the exposure to the PS-Ru control compound produced a higher upregulation of the *oxy*R gene (~ 6-fold) and also did not modify the *sod*A expression (Fig. 3A). It suggests the *K. pneumoniae* does not respond to PSIR-3-induced photooxidative stress, through the two-component systems *oxy*R/S, or *sox*R/S, which are associated with the response to Type I ROS. Besides, it appears that bacteria exposed to the PS-Ru compound partially respond to the Type I effect when responding via *oxy*R/S but not via *sox*R/S.

Then, the response of *K. pneumoniae* to ROS of the Type II effect was assessed. Since the photooxidative stress induced by singlet oxygen is poorly understood in Gram-negative bacteria, we performed a search for genes described previously in the photosynthetic bacteria *Rhodobacter sphaeroides*. The response to photooxidative stress in *R. sphaeroides* involved the activation of the RpoE regulon by a complex mechanism that initiates with the activation of a series of sRNAs such as *chr*R anti-*rpo*E, the RSP_1090 and the RSP_1091 (a comprehensive review may be found in Valenzuela-Valderrama et al. 2019 [3]). A blast search did not show the *chr*R anti-*rpo*E nor RSP_1090 nor RSP_1091 sRNAs orthologous are present in any *K. pneumoniae* sequence available in GenBank. On the other hand, in *E. coli*, the RpoE protein is repressed anchored to the plasma membrane and can be released by the proteolytic action of an enzyme activated by the damage on cell envelope structures [36]. The release of RpoE activates the extracytoplasmic regulon that includes the upregulation of the gene encoding the RpoE regulator. In Fig. 3C, the results show that, compared to untreated bacteria, the photodynamic treatment with PSIR-3 strongly upregulated (>6 fold) the *rpo*E gene expression in both *K. pneumoniae* strains. In comparison, the treatment with the PS-Ru compound poorly induced the *rpo*E expression (~2.5 fold). These results suggest that the RpoE regulator is involved in response to photooxidative stress caused by the PSIR-3 compound. On the other hand, a blast search found that the RNA-binding Hfq gene is present in all available *K. pneumoniae* sequenced strains. The Hfq gene displays an essential role in the post-transcriptional control to photooxidative response presented by *R. sphaeroides* [19]. Similar to *rpo*E, the *hfq* gene expression was significantly upregulated in both strains of *K. pneumoniae* treated with PSIR-3, which was significantly stronger than bacteria treated with the PS-Ru control (Fig. 3D). These results suggest the participation of small RNAs regulation in response to photooxidative stress induced by the Type II mechanism.

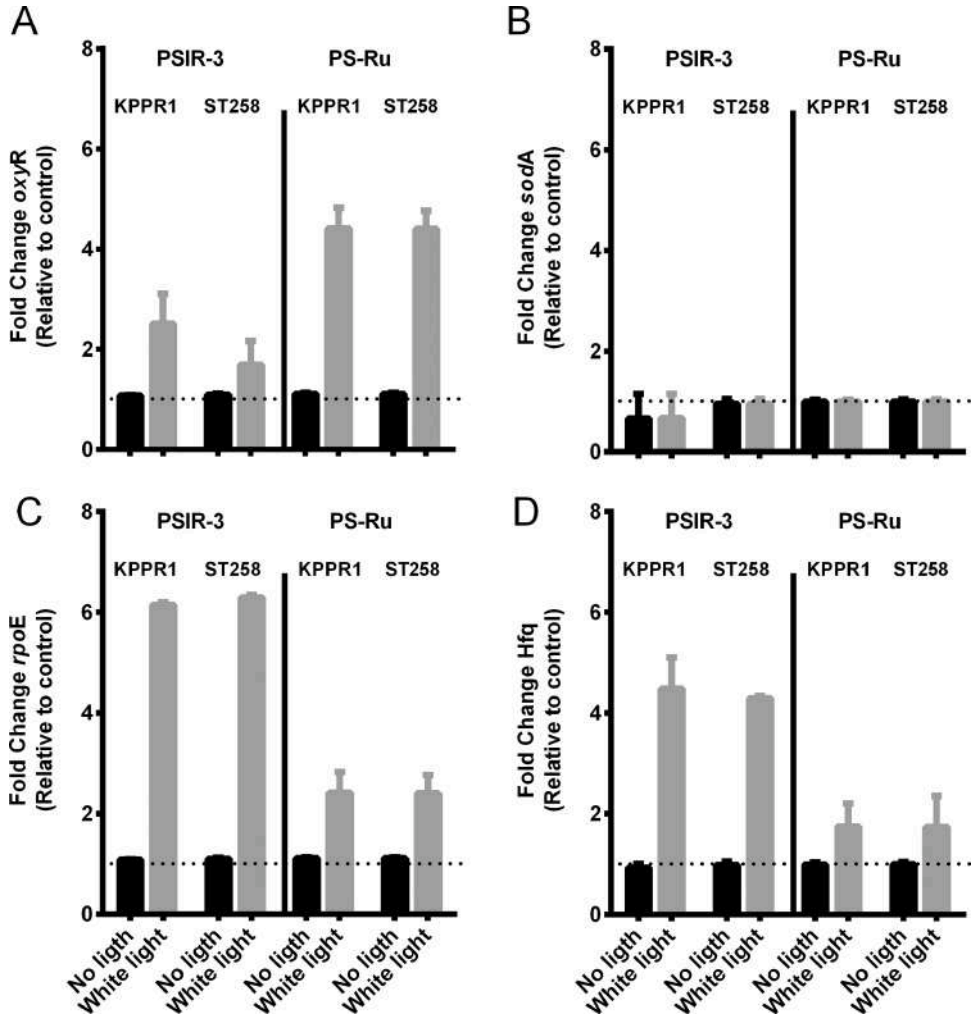

Fig. 3. Photodynamic treatment modifies the expression of oxidative stress response genes. Expression levels of *rpo*E, *oxy*R, *hfq,* and *sod*A genes determined by RT-qPCR from total RNA extracted for *K. pneumoniae* treated with PSIR-3 compound or Ps-Ru control. Values are expressed as fold change (means ± SD) of mRNA abundance compared to untreated bacteria.

3.4. Modulation of Virulence factors of Klebsiella pneumoniae during photooxidative stress

Although we did not conduct structural experiments to evaluate envelope damage, our results show that oxidative stress by PSIR-3 strongly induces expression of the *rpo*E gene, suggesting that the extracytoplasmic stress-response regulon may be activated. The RpoE sigma factor response controls in *E. coli* the expression of about 77–106 genes in response to envelope damage [21]. Then, the study was extended to determine the expression levels of genes that encode virulence factors related to the bacterial envelope maintenance, such as the *mrk*D, *acr*B, *mag*A, and *rmp*A genes. Fig. 4**A** shows that, compared to untreated bacteria, the photooxidative stress produced by PSIR-3 induced a strong upregulation (>6 fold) of the *mrk*D gene expression in both strains of *K. pneumoniae*. In comparison, the treatment with the PS-Ru control did not induce any change in its mRNA abundance (Fig. 4A). The *mrk*D gene is part of the operon that encodes the Type 3 pili [24]. Contrary to what was shown for the *mrk*D gene, photodynamic treatment with the PSIR-3 compound significantly decreased the expression of the *acr*B gene (Fig. 4B). Besides, treatment with the PS-Ru control did not induce a significant change in the expression of the *acr*B gene (Fig. 4B). The *acr*B gene encodes a membrane pump related to resistance to acriflavine [27]. It is noteworthy that the photodynamic treatment with the compound PSIR-3 induced a strong upregulation (> 6 times) in the expression of the capsule related genes *mag*A and *rmp*A (Fig. 4**C and D**). In contrast, the treatment with PS-Ru control did not induce a significant change in the expression of the *magA* or *rmpA* gene (Fig. 4**C and D**). Both the *mag*A and *rmp*A genes encode virulent factors related to the hypermucoviscous phenotype of *K. pneumoniae* [26].

Based on these results, a scheme to better understand how these different factors could be interacting was drawn up. As shown in Fig. 5A, organized extracytoplasmic proteins such as MrkD, MagA, and AcrB are found in the bacterial cell envelope. When light excites the PSIR-3 compound, the excess energy is transferred to oxygen, which changes its triplet ground state to a singlet excited state. The singlet oxygen may induce the degradation of those cell envelope structures (Fig. 5B). At the same time, the PS excitation induces the activation of the bacterial response to photooxidative stress. Our results suggest that PSIR-3 activates in *K. pneumoniae* the RpoE pathway, where the pre-formed regulator is bound to the RseA inhibitor [37]. The photooxidative stress may induce the enzymatic activity of DegA enzyme, which sense the envelope damage and degrade the RseA inhibitor releasing the RpoE regulator [22,37]. The released RpoE protein activates the expression of several genes under its control, for instance, the *mrk*D, *mag*A, and its encoding *rpo*E gene in an attempt to replace damaged structures (Fig. 5B).

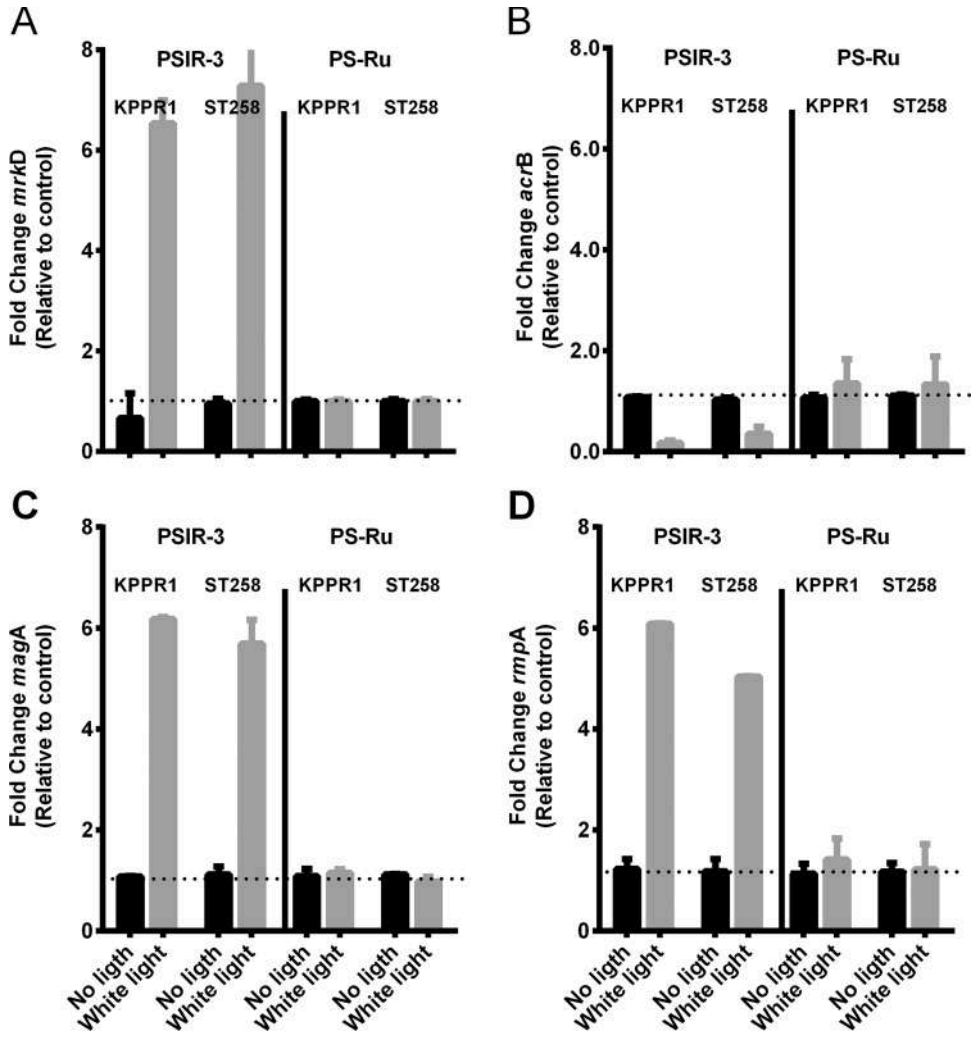

Fig. 4. Regulation of virulence-related genes by photodynamic treatment. Expression levels of *mrk*D, *acr*B, *mag*A, and *rmp*A genes determined by RT-qPCR from total RNA extracted for *K. pneumoniae* treated with PSIR-3 compound or Ps-Ru control. Values are expressed as fold change (means ± SD) of mRNA abundance compared to untreated bacteria.

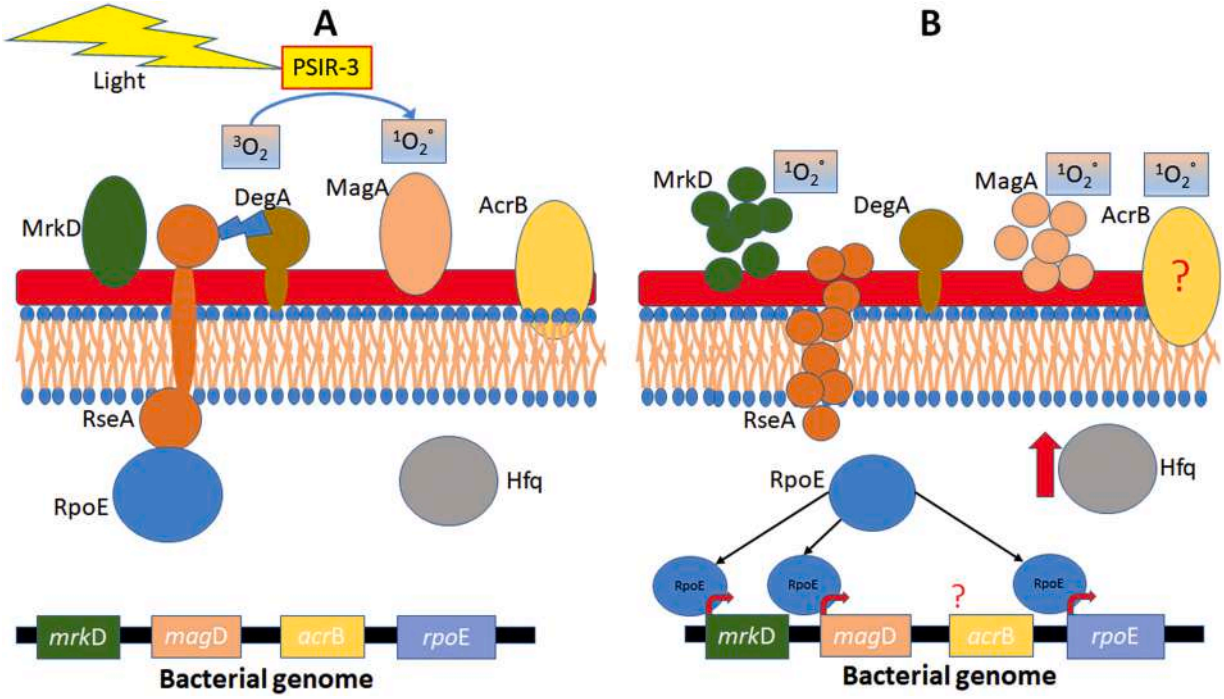

Fig. 5. Scheme of the possible interactions of regulatory genes and virulence factors in response to photooxidative stress. Extracytoplasmic proteins such as MrkD, MagA, and AcrB are found outer in the bacterial envelope. Light promotes the excited state of PSIR-3, and its energy excess is transferred to the oxygen produce singlet oxygen that may induce the degradation of this cell envelope structures **A**. At the same time, the PS excitation induces the activation of the bacterial response to photooxidative stress. The RpoE protein is negatively-regulated bound to the RseA inhibitor. The photooxidative stress may induce the enzymatic activity of DegA, which sense the envelope damage and degrade the RseA inhibitor releasing the RpoE regulator. The released RpoE protein activates the expression of several genes under its control, for instance, the *mrk*D, *mag*A, and its encoding *rpo*E gene in an attempt to replace damaged structures **B**.

4. Discussion

Since the depletion of therapeutic alternatives due to the proliferation of MDR-bacteria [1,2], the use of aPDI as complementary therapy not only becomes viable due to its usefulness as rescue therapy but also to reverse resistance to the antibiotics of choice minimize the induction of resistance [38]. Recently, we demonstrated the Ir(III)-based photosensitizer compound PSIR-3 has synergistic antimicrobial activity with imipenem against carbapenemase-producer *K. pneumoniae* [28]. *K. pneumoniae* is a flawless model because it is one of the most relevant MDR Gram-negative bacteria [2,3]. In this study, we confirm that the light-activation of PSIR-3 produced the expected antimicrobial photodynamic inactivation on *K. pneumoniae*. Then, this bacterial substrate was used to determine the bacterial transcriptional response to the photooxidative stress induced by the PSIR-3 compound.

Oxidative stress is a well-known strategy used by host cells to kill pathogenic bacteria. Bacteria are exposed to ROS compounds that affect multiple prokaryotic targets at once, for example, hydrogen peroxide and HO• kills or inhibits bacteria engulfed into the macrophages phagocytic vacuole [11]. Also, colonizing bacteria can be killed by exposition to oxidative stress out of cells into the luminal space of gut by H_2O_2 released by resident microbiota [12] or by intestinal cells [13]. The H_2O_2 may diffuse prokaryotic envelope and induce transcriptional stress and oxidation of amino acids [39]. Those Type I ROS activate the *oxy*R/S or the *sox*R/S two-component systems, and SOD upregulation [14]. Here, we observed that the PSIR-3 compound poorly induced the expression of the *oxy*R regulator, and produced no changes in the expression of the detoxifying enzyme SOD, suggesting that they are not involved in the bacterial response to PSIR-3 compounds. The results for the PS-Ru control suggest that induce a response to H_2O_2 but not to superoxide because it significantly upregulates *oxy*R expression but does not modify the expression of the *sod*A gene.

Then, the photooxidative action produced by PSIR-3 may be stimulated by Type II ROS. The highly reactive $^1O_2$ may be produced by photooxidative stress and also by macrophages respiratory burst [10]. Our results suggest that the mode of action to produce ROS by PSIR-3 compound is the Type II. This mode of action agrees with the triplet excited state of the PSIR-3 with hight contribution of charge-transfer transitions, that could favor the energy transfer to molecular oxygen, yield mostly singlet oxygen [28,40]. The bacterial response to the PSIR-3 compound activates the alternative sigma factor *rpo*E regulator, which response to envelope damage [20]. Although we do not have the results that confirm the damage of the bacterial envelope, the participation of the alternative sigma factor *rpoE* strongly suggests it [22]. The significant upregulation in the *rpo*E gene expression agrees with its activation since the RpoE protein induces its gene expression [22]. The damage to the bacterial envelope mediated by PSIR-3 should be detected by mechanisms that activate *rpo*E expression. This behavior coincides with the response of *R. sphaeroides* to Type II ROS [19,20]. In *E. coli*, the RpoE regulator is down-regulated by binding to the RseA membrane protein, which is degraded by the proteolytic activity of the DegA protein [22]. The DegA proteolytic activity initiates when sensing bacterial envelope damage, such as the accumulation of membrane proteins fragments or misfolded proteins [22]. In a blast search, we found both the *deg*A and the *rse*A genes in the *K. pneumoniae* KPPR1 genome. Moreover, in other γ-proteobacteria such as *Bordetella pertussis,* the *rpo*E activity-induced by envelope damage is also regulated by the RseA protein [41].

The gene composition of the *rpo*E regulon suggests its activation plays an important role in maintaining the integrity of the bacterial envelope [20,22]. For example, the bacterial membrane permeability is modified in *K. pneumoniae* exposed to oxidative stress [16]. Then, the activation of gene regulators, like *rpo*E and *hfq,* which participate in controlling extracytoplasmic factors, suggest the bacteria are responding to envelop stress and maintenance of LPS heterogeneity [23]. This response is consistent with previous reports in *K. pneumoniae,* where the orthologous of *oxy*R product was linked to resistance to H_2O_2 exposure

and for intestinal colonization through fimbrial synthesis and biofilm formation [42]. The OxyR regulates the expression of genes encoding the virulence factors fimbrial Types 1 (*fin*) and 3 (*mrk*D) during *K. pneumoniae* colonization of gut [42] and resistance-modulation-cell division *acr*B gene [43]. Similar to H_2O_2 [42], the photooxidative stress produced by PSIR-3 treatment induced a strong upregulation of *mrk*D gene expression that was higher than induced by PS-Ru control. In contrast to the H_2O_2 -induced oxidative response, the photooxidative response to PSIR-3 induces the downregulation of *acr*B gene expression [42]. Then, the activation of *rpo*E must result in a decrease in mRNA levels of *acr*B, which could be mediated by sRNA *rpo*E dependent repressors [36,44]. Remarkably only the photooxidative stress produced by the compound PSIR-3, and not by the control PS-Ru, upregulate the gene expression of the *mag*A and *rmp*A capsule-related genes. Those genes were upregulated distinctly than H_2O_2 via *oxy*R, or superoxide induction of SOD, suggesting they must be under the control of the *rpo*E regulon. As the sigma factor *rpo*E is activated upon damage occurs in the cell envelope, it is very likely that it promotes the expression of genes involved in the biosynthesis of the polysaccharide capsule, such as *mag*A and *rmp*A or the fimbria like *mrk*D genes. More research needs to be performed to demonstrate the control of RpoE over *mag*A and *rmp*A gene expression and identify an expression cascade that promotes other potentially undiscovered genes that protect bacteria against $^1O_2$ mediated stress.

In *E. coli*, the Hfq protein modulates the envelope stress response mediated by the *rpo*E regulator [36,44,45]. Besides, during $^1O_2$ stress, the specific response of sRNAs is significant for *R. sphaeroides*, which is consistent with the strong upregulation of *hfq* gene expression shown by *K. pneumoniae* during PSIR-3 aPDI. In both, the *hfq* upregulation suggests the post-transcriptional modulation of the *rpo*E regulon [46]. However, structural and functional studies are needed to fully characterize the envelope stress and the participation of the RpoE regulator. In summary, it identified that the mode of action of aPDI therapy with PSIR-3 to produce ROS must be through the Type II effect.

5. Conclusions

The mode of action of the compound PSIR-3 to produce photooxidative stress must be through the generation of the Type II ROS mechanism. This conclusion is based on the transcriptional response shown by *K. pneumoniae* treated with PSIR-3, which is not the canonical pathway through the two-component systems *oxy*R / S or *sox*R / S. Instead, the response of extracytoplasmic RpoE regulon occurs. This regulon induces a pleiotropic response, where we were able to verify the overexpression of several genes associated with bacterial envelope structures such as *mrk*D, *mag*A, and *rmp*A. The upregulation of these genes may represent an attempt by bacteria to maintain the structural integrity of their cell envelope.

Declaration of Competing Interest

The authors declare no conflict of interest.

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