Response to photo-oxidative stress of *Pseudomonas aeruginosa* PA01 mutants impaired in different functions

Viviana Teresa Orlandi,¹* Fabrizio Bolognese,¹ Eleonora Martegani,¹ Vincenzo Cantaluppi,² Claudio Medana³ and Paola Barbieri¹

**Abstract**

Clinicians often have to deal with infections that are difficult to control because they are caused by superbugs resistant to many antibiotics. Alternatives to antibiotic treatment include antimicrobial photodynamic therapy (aPDT). The photodynamic process causes bacterial death, inducing oxidative stress through the photoactivation of photosensitizer molecules in the presence of oxygen. No PDT-resistant bacteria have been selected to date, thus the response to photo-oxidative stress in non-phototrophic bacteria needs further investigation. The opportunistic pathogen *Pseudomonas aeruginosa*, in particular, has been shown to be more tolerant to PDT than other micro-organisms. In order to find any genetic determinants involved in PDT-tolerance, a panel of transposon mutants of *P. aeruginosa* PA01 involved in the quorum sensing signalling system and membrane cytoplasmic transport were photoinactivated as part of this study. Two pseudomonas quinolone signalling (PQS) knock-out mutants, *pqsH* and *pqsC*, were as PDT-sensitive as the PA01 wild-type strains. Two PQS hyperproducer variants, *pqsA<sup>+</sup>* and *rsal<sup>-</sup>* were shown to be more tolerant to photo-oxidative stress than the wild-type strain. In the *pqsA<sup>+</sup>* mutant, the hyperpigmentation due to the presence of phenazines could protect cells against PDT stress, while in *rsal<sup>-</sup>* no pigmentation was detectable. Furthermore, a mutant impaired in an ATP-binding cassette transport involved in maintaining the asymmetry of the outer membrane was significantly more tolerant to photo-oxidative stress than the wild-type strain. These observations support the involvement of quorum sensing and the importance of the bacterial cell envelope when dealing with photo-oxidative stress induced by photodynamic treatment.

**INTRODUCTION**

The development and spread of resistance to antibiotics is an important issue that makes clinical infections difficult and sometimes impossible to eradicate [1, 2]. The scientific community is searching for new drugs and alternative therapies to adopt alongside traditional chemotherapy. These include antimicrobial photodynamic therapy (aPDT), which is considered a promising tool [3, 4].

PDT is an approach that requires the simultaneous presence of visible light, oxygen and a photosensitizer (PS) [5]. A PS, solubilized in the cytoplasm or bound to the bacterial cell envelope, absorbs light and passes from the ground state (S<sub>0</sub>) to the excited triplet state (T<sub>1</sub>), before returning to the ground state and transferring electrons (Type I reaction) or energy (Type II reaction) mainly to oxygen. The resulting development of reactive oxygen species such as superoxide anion (O<sub>2</sub><sup>-</sup>), hydroxyl radical (OH·), hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and singlet oxygen (¹O<sub>2</sub>), causes bacterial damage by destroying biomolecules such as lipids, nucleic acids and proteins [6].

The role of pigments in counteracting photo-oxidative stress has been known for about 60 years when Griffith reported the damage occurring to carotenoid-deficient *Rhodobacter sphaeroides* during photosynthesis in an aerobic atmosphere. Regarding this bacterium, carotenoids play an important role in protecting cells against photo-oxidative stress, directly quenching ¹O<sub>2</sub> through both physical and chemical mechanisms [7].

Only a few reports have investigated the response of non-phototrophic bacteria to photodynamically induced stress.
Indeed, pigments were found to play a role also in PDT tolerance. Lipovsky observed that the sensitivity of different Staphylococcus aureus strains was related to the endogenous porphyrin and carotenoid levels [8]. In Pseudomonas aeruginosa, which is particularly tolerant to photodynamically induced oxidative stress [9, 10], pyomelanin appears to be particularly efficient in counteracting radicals and singlet oxygen produced by two different PSs, while phenazine pigments also provide a certain degree of protection [11].

Different kinds of responses to photo-oxidative stress were investigated in other micro-organisms. St. Denis investigated the possible role of heat shock proteins in conferring tolerance to PDT stress in Escherichia coli and demonstrated that, upon photo-oxidative stress, the expression of GroEL and DnaK increased sevenfold and threefold respectively [12]. Park analysed the effect of PDT treatment on the transcriptome of S. aureus. As a whole, photodynamic treatment seems to switch cell physiology from growth to survival. In greater detail, in S. aureus many genes that are upregulated or downregulated during photoactivation belong to the Agr regulon which operates as a quorum sensing (QS) system, suggesting its possible role in photo-oxidative stress tolerance [13].

In P. aeruginosa one of the interconnected QS systems, pseudomonas quinolone signalling (PQS), was shown to play a role in sensitizing cells to solar ultraviolet-A radiation [14]. Haussler reported that PQS could act as a pro-oxidant, sensitizing the bacteria to oxidative stress, but also as an anti-oxidant, inducing a protective response [15]. As PQS provides a link between las and rhl systems, being activated by las and repressed by rhl [16], a QS network, taken as a whole, could contribute to the response to oxidative/photo-oxidative stress.

Furthermore, in P. aeruginosa, the impermeable outer cell membrane prevents the cellular uptake of dyes, making the micro-organism more tolerant than Gram-positive bacteria to photodynamic treatment [5]. In addition, it has been suggested that PSs are substrates of the efflux pump MexAB-OprM [17]. Thus, the chemical composition of a bacterial envelope can influence the outcome of the photodynamic process, making a micro-organism more or less tolerant to PDT treatment.

The aim of this study was to find genetic determinants in P. aeruginosa involved in PDT tolerance and not correlated to bacterial pigmentation. Since the photodynamic screening of a transposon library of PAO1 strains was unsuccessful, a group of transposon mutants underwent PDT inactivation and were compared to the wild-type strain. We focused on four mutant strains impaired in the QS signalling system and two mutants impaired in cytoplasmic membrane transport. Both the signalling network and the bacterial envelope could play a role in responding to photo-oxidative stress.

**METHODS**

**Photosensitizer**

Stock 1 mM water solutions of toluidine blue O (TBO) (Sigma Aldrich) was diluted in PBS (KH$_2$PO$_4$ /K$_2$HPO$_4$ 10 mM pH 7.4) to the final working concentration 5 µM.

**Bacteria, plasmids and media**

P. aeruginosa PAO1 strain [18] was used to construct the transposon library and E. coli S17 Apir was used as the transposon donor [19]. P. aeruginosa derivatives (Table 1) were obtained in this study using a transposon mutagenesis approach. pBT20 was used as the mariner-based transposon delivery plasmid [20].

P. aeruginosa wild-type and E. coli were routinely grown in Luria–Bertani (LB) broth and on LB agar plates under aerobic conditions at 37 °C. P. aeruginosa wild-type and transposon mutants were also grown in mineral medium M9 supplemented with 10 mM glucose. Gentamicin and ampicillin were added, when necessary, to a final concentration of 100 µg ml$^{-1}$.

**Construction of the transposon insertion library**

Transposon insertions in the PAO1 chromosome were generated by following the protocol of Kulasakera [20]. Briefly, the donor strain E. coli S17 Apir carrying pBT20 containing the transposon Tn mariner, was suspended in LB to a final OD$_{600}$ of 40 and the recipient, P. aeruginosa PAO1 to a final OD$_{600}$ of 20. 10 µl droplets of 1 : 1 conjugation mix were spotted on LB agar and incubated at 37 °C for 2 h. P. aeruginosa PAO1 transconjugants were selected on mineral medium M9 added with 10 mM glucose and gentamicin 100 µg ml$^{-1}$; the donor strain E. coli S17 Apir, auxotrophic for proline and thymine, is counterselected. Single colonies were picked and inoculated into 96-well plates, each well containing 200 µl LB supplemented with gentamicin. Bacteria were incubated for 48 h at 37 °C.

**Transposon library screenings**

A photodynamic-based screening was attempted: clones were treated in the dark with TBO, and after a replica plating on LB agar were irradiated.

For the other screenings, a replica plating was performed on 1.5 % LB agar, 1.5 % LB agar supplemented with Congo red agar (CRA) and 0.5 % LB agar. After overnight growth at 37 °C, the colony variants were checked as follows. Pyoverdine is a fluorescent iron-chelator and its production may be easily checked by UV lamp (365 nm) irradiation [21, 22]. The fluorescence of colony growth on 1.5 % LB agar was compared to PAO1 fluorescence. Reddish clones grown on CRA were selected for their different ability to form biofilms [23]. Swarming motility mutants were selected and their morphology and dimension colony on 0.5 % LB agar with PAO1 strain were compared [24].
CV staining

*P. aeruginosa* PAO1 wild-type and mutant strains grown overnight in medium M9 supplemented with glucose 10 mM were diluted 100-fold in a fresh medium to inoculate a 12-well plate. After 24 h at 37°C, the cell suspensions were removed and their OD was measured at 600 nm. The biofilms were stained with crystal-violet (CV) 0.1 % for 15 min, washed twice with PBS, dried at 37°C for 2 h and then the CV was dissolved in acetic acid 30 % for 10 min. The amount of solubilized dye was measured spectrophotometrically at 590 nm. The biofilm-forming index (BFI) was determined by applying the formula BFI=(AB-CW)/G, in which AB is the OD of the stained attached micro-organisms, CW is the OD of the stained control wells containing micro-organism-free medium only and G is the OD of the cell growth in suspended culture [25].

Identification of interrupted sequences by arbitrary primed PCR

The DNA sequences flanking the transposon inserts were determined using arbitrary PCR [26]. Briefly, in the first round of PCR, 100 ng of purified chromosomal DNA, obtained using Genomic DNA purification KIT (Promega Corporation, Madison, WI), were amplified with the random tagged primer Rnd1-ARB0-Pa paired with Rnd1-TnM20 primer. A nested PCR was then performed on the first PCR amplification product, with Rnd2-ARB and Rnd2-BT20 primers. Amplification products were then sequenced (Primm Biotech, Italy) by the use of the sequencing primer. Insertion sites were identified with BLASTN searches, against the annotated sequence of the *P. aeruginosa* PAO1 genome, using software provided by NCBI.

| Table 1. Strains, plasmids and primers used in this work |
|---------------------------|-----------------------------|
| **Strain, plasmid, primer** | **Relevant characteristics** | **Reference(s) or source** |
| **P. aeruginosa strains** | | |
| PAO1 | Wild-type strain | [18] |
| R8 | PAO1 derivative GmR pqs A | This study |
| 2/14 | PAO1 derivative GmR rsaL | This study |
| BB8 | PAO1 derivative GmR pqs C | This study |
| N13 | PAO1 derivative GmR pqs H | This study |
| Mar 94 | PAO1 derivative GmR PA4455 | This study |
| Y18 | PAO1 derivative GmR shaA | This study |
| B13 | PAO1 derivative GmR retS | This study |
| O19 | PAO1 derivative GmR PA3806 | This study |
| T9 | PAO1 derivative GmR fleN | This study |
| **E. coli** | | |
| S17pir | Δ araD araB lacX74 galE galK phoA20 thi-1 rpsE argE(Am) recA1 λpir phage lysogen | [19] |
| JM109 | recA1 hsd17 thi Δ lac-proAB (F traD36 proAB lacIq ZAM15) | Yanish Peron et al., 1985 |
| **Plasmids** | | |
| pBT20 | ApR; GmR; mariner transposon plasmid | [20] |
| **Primers** | | |
| Rnd1-ARBUniv | 5'-GGCCACGCGTCGACTAGTACNNNNNNNNNNNNNN-3' | |
| pTac | 5'-CATCGCCTGATATAATGGTG-3' | |
| Rnd2-ARB | 5'-GGCCACGCGTCGACTAGTAC-3' | |
| p8T20 | 5'-CGGCTGTATAATGTTGGAATGTTG-3' | |
| TNM20 | 5'-ACAGAAAAACAGGTACTAGAGG-3' | |
| pqsA1178FW | 5'-GGACCTACATTCTCTCCCGGAC-3 | |
| pqsA1718RW | 5'-CATGCGATGCGCGAAGGATCACTTTG-3 | |
| pqsCFW | 5'-TGAAGGCCGGAAGAATGCTGG-3 | |
| pqsCRW | 5'-CATCGGCTGATGCGGATG-3 | |
| pqsHFW | 5'-ACTCCCTGAGGCTGTTAGT-3 | |
| pqsHRW | 5'-TACGGAGGGGCGGATGAG-3 | |
| rsaLFw | 5'-CGACGGGAAAGCGGAGA-3 | |
| rsaLRW | 5'-TTACTCTCGATGCTGGCTCTC-3 | |
| PA4453RW | 5'-GTTCATGATGTCAGTGGCGGCCCG-3' | |
| shaRW | 5'-CTCCTGCTGGCGGGGATGAG-3 | |
| recAFW | 5'-CAGACGGGAAAAGCGGAG-3 | |
| recARW | 5'-GTGATGGAAGATCGCAAGGG-3' | |
Reverse transcription PCR and PCR analyses

Reverse transcriptase (RT)-PCR was used to evaluate the transcriptional expression of pqsC, pqsH, rsaL. Total bacterial RNA was isolated from cultures grown to an OD₆₀₀ of 0.5 using a TRIzol (R) MAX (TM) (Invitrogen, Carlsbad, CA) method. Overall, 5 µg of total RNA were then treated with RNase-free DNase (Promega Corporation, Madison, WI) (1.5 U g⁻¹ of RNA) for 35 min. The DNase-treated RNA was extracted with phenol and precipitated using 100 % ethanol. Altogether, 250 ng of DNase-treated RNA were reverse-transcribed with VILO mastermix (Invitrogen) according to the manufacturer’s instructions. cDNAs were then PCR-amplified using pqsCFW and pqsCRW primers for the pqsC gene; pqsHFW and pqsHRW for the pqsH gene; rsaLFW and rsaLRW for the rsaL gene, recAFW and recARW for the housekeeping gene recA. Primers were designed by Software free source, Ape-a plasmid editor (by M. Wayne Davies). PCR reactions were performed as follows: 94 °C for 2 min and 35 cycles at 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 1 min, 72 °C for 5 min. The removal of contaminating DNA from each RNA sample was verified by a PCR reaction in the absence of reverse transcription.

A semi-quantitative RT-PCR was used to evaluate the relative amount of pqsA and pqsC mRNAs from PAO1 and R8 transposon mutants with respect to the housekeeping gene recA. The PCR was performed as described, and samples were collected after 22, 25, 29, 31, 33 and 35 cycles and analyzed by gel electrophoresis.

PCR reactions were performed on genomic DNA from Mar94 and Y18 clones, using pFAC primer and PA4453RW or shaRW, respectively.

Photoinactivation experiments

P. aeruginosa PAO1 and transposon mutants were grown overnight at 37 °C in M9 medium supplemented with glucose 10 mM. Overnight cultures were inoculated in a 24-well microplate. Cell suspensions were treated with TBO 5 µM and incubated for 10 min in the dark without shaking. Then samples were irradiated with a 500W halogen-tungsten lamp (fluence rate 48 mW cm⁻², considering the 400 nm of the entire width of the lamp emission spectrum) for 25, 50 and 75 min. The lamp was placed 20 cm above the sample and a 1.5 cm thick circulating water/glass filter was interposed to avoid overheating. A panel of controls was set for each experiment: PS untreated and dark incubated (−PS, −light); PS treated and dark incubated (+PS, −light); PS untreated and irradiated (−PS, +light) control samples. The experiments were repeated at least three times. The PDT effect on cellular viability was evaluated after irradiation by means of a plate count technique and expressed as c.f.u. ml⁻¹.

In order to evaluate the effect of phenazines on N13 and BB8 clones’ photoinactivation, pyocyanin (PYO) and phenazine-1-carboxylic acid (PCA) (50 µg ml⁻¹) were added to mutant cells grown in M9 medium (glu 10 mM). Cells were incubated in the dark for 10 min with TBO 5 µM and then irradiated (210 J cm⁻²) and cellular viability was checked.

Spectrophotometric analysis

P. aeruginosa PAO1 and mutant strains were grown in LB medium or in M9 medium supplemented with 10 mM glucose and were centrifuged (13 000 r.p.m. for 10 min). The supernatants were analysed spectrophotometrically (Perkin–Elmer Lambda 10 instrument). The obtained UV–vis absorption spectra were compared to those of PCA and PYO reported by Mavrodi [27].

Liquid chromatography mass spectrometry analysis

Chemicals: HPLC grade acetonitrile (purity ≥99.9 %), formic acid and standard 2-heptyl-3-hydroxy-4-quinolone (PQS) were obtained by Sigma-Aldrich (Milan, Italy) and used as received.

Sample preparation: bacterial cultures of P. aeruginosa PAO1 and chosen mutants were grown overnight at 37 °C in M9 supplemented with glucose 10 mM. They were centrifuged (5000 r.p.m. for 5 min). The supernatants were treated with ethyl acetate supplemented with 1 % acetic acid. The extracts were centrifuged (10 000 r.p.m. for 10 min) and dried [28].

A Nexera LC-30AD (Shimadzu, Milan, Italy) instrument equipped with a Gemini NX C18 150×2.1 mm, 3 µm particle size column (Phenomenex, Milan, Italy) was used to carry out chromatography analysis. The eluents were acetonitrile (A) and 0.05 % formic acid (B) in the following gradient conditions: from 15 to 100 % of solvent A in 32 min, up to and re-equilibration. The injection volume was 10 µl, the flow rate was 200 µl min⁻¹ and the column was maintained at a temperature of 45 °C.

A QTrap-5500 (ABSciex, Milan, Italy) instrument, dressed in a Turbo Ion Spray source, was utilized to analyse samples. The source parameters were: curtain gas 30 (arbitrary unit, a.u.), gas1 40 (a.u.), gas2 55 (a.u.), temperature 500 °C, ion spray voltage 5500 V, declustering potential 200 (a.u.) and entrance potential 11 (a.u.). The detector was used in MRM mode and the transitions for PQS detection were: 260–75 m z⁻¹, 260–188 m z⁻¹ and 260–147 m z⁻¹ for CL27C (collision energy 35, 42 and 49 V respectively). A calibration curve was created using standard solutions of the analyte at a concentration of 0.5÷200 ppb, finding LLOQ=0.5 ppb.

Statistical analysis

The experiments were repeated at least three times on separate dates. Mean and SD calculations were performed using Microsoft Excel 2003. Data were analysed by means of one-way ANOVA (Origin_7.0 SR0; Origin lab). Significant treatment effects were estimated (P<0.05 and P<0.01).
RESULTS

Isolation of transposon mutants

The aim of this study was to obtain isogenic variants of *P. aeruginosa* PAO1 that were more or less sensitive to photodynamic treatment than the wild-type in order to evaluate the possible role of bacterial genetic determinants in response to photo-oxidative stress.

A transposon insertion library of the model micro-organism *P. aeruginosa* PAO1 was obtained by conjugation between the *E. coli* S17 Apir carrying pBT20 plasmid as the donor strain and *P. aeruginosa* PAO1 as the recipient strain. Approximately 2000 transconjugant clones resistant to gentamicin were obtained.

Some attempts to select more PDT-sensitive or PDT-tolerant mutants, performed using a photodynamic-based screening, were unsuccessful. We thus considered alternative strategies.

As QS systems are involved in the response to different stresses [29, 30], a QS network could thus also influence the response to photo-oxidative stress. Library screenings based on easily detectable QS-related phenotypes were set up to enrich QS mutants [23]. Two clones, B13 and R8, were exploited for this purpose.

The UV-based screening made it possible to select two clones, BB8 and N13, both of which were more fluorescent than the PAO1 strain, and one clone, 2/14, that was not fluorescent (Fig. 1). The Congo red assay enabled us to select biofilm mutants [23, 24] were exploited for this purpose.

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As summarised in Table 2, mariner transposase inserted gentamicin cassettes in or near genes involved in the bacterial communication system (*pqxA, pqxC, pqxH*, *rsaL*), in genes codifying membrane proteins (*shaA, PA4455*), in protein involved in motility and attachment (*fleN*), in a two-component regulatory system (*retS*) and in an unknown protein (*PA3806*).

In this work we focused our attention on clones carrying mutations directly or indirectly related to the PQS network and in membrane proteins.

PQS mutant characterization

The mutations in three transposon mutants, R8, BB8 and N13, were found to be directly involved in the PQS intracellular system (Fig. 2). The R8 mutant displayed the gentamicin cassette upstream from *pqxA* (Fig. 3). PqsA is an anthranilate-coenzyme A ligase that activates anthranilate to form anthraniloyl-coenzyme A, initiating the first step of PQS mediator, 2-heptyl-3-hydroxy-4-quinolone, biosynthesis [31, 32]. BB8 displayed transposon insertion into the

![Table 2. Transposon mutants and gene localization obtained by the sequencing](https://www.microbiologyresearch.org/content/163/1557-1567-fig1.png)

**Table 2.** Transposon mutants and gene localization obtained by the sequencing

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Locus tag</th>
<th>Gene Functional classification</th>
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<tbody>
<tr>
<td>B13</td>
<td>PA4856</td>
<td><em>retS</em> Two-component regulatory systems</td>
</tr>
<tr>
<td>R8</td>
<td>PA0996</td>
<td><em>pqxA</em> Biosynthesis of cofactors, prosthetic groups and carriers</td>
</tr>
<tr>
<td>2/14</td>
<td>PA1431</td>
<td><em>rsaL</em> Transcriptional regulator</td>
</tr>
<tr>
<td>BB8</td>
<td>PA0998</td>
<td><em>pqxC</em> Adaptation, protection</td>
</tr>
<tr>
<td>N13</td>
<td>PA2587</td>
<td><em>pqxH</em> Biosynthesis of cofactors, prosthetic groups and carriers</td>
</tr>
<tr>
<td>T9</td>
<td>PA1454</td>
<td><em>fleN</em> Motility and attachment</td>
</tr>
<tr>
<td>O19</td>
<td>PA3806</td>
<td>Gene for conserved hypothetical protein</td>
</tr>
<tr>
<td>Mar94</td>
<td>PA4455</td>
<td><em>yrbE</em> Probable permease of ABC transporter</td>
</tr>
<tr>
<td>Y18</td>
<td>PA1054</td>
<td><em>shaA</em> Membrane proteins Transport of small molecules</td>
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![Fig. 1. Transposon screenings. The transposon library was screened by three assays. The UV assay selected two clones more fluorescent (N13 and BB8) and one less fluorescent (2/14) than PAO1. The Congo red assay selected two clones more reddish than PAO1 (B13 and R8), their biofilm were stained with CV. The swarm motility assay selected four clones different from PAO1.](https://www.microbiologyresearch.org/content/163/1557-1567-fig1.png)
The pqsC gene (Fig. 3). PqsC, together with PqsB and PqsD, are probable 3-oxoacyl-(acyl carrier protein) synthases, mediating the conversion of anthranilate into 2-heptyl-4-quinolone (HHQ), the precursor of 2-heptyl-3-hydroxy-4-quinolone [32, 33]. In the N13 clone, the gentamicin cassette was inserted in the pqsH gene (Fig. 3). PqsH is a putative flavin-dependent monooxygenase that should hydroxylate HHQ, converting it into 2-heptyl-3-hydroxy-4-quinolone [34]. Among the transposon mutants, the mutation in the 2/14 clone was indirectly connected to the PQS system (Fig. 2). The gentamicin cassette was inserted in the rsaL gene (Fig. 3), which codifies a transcriptional repressor of lasI codifying N-3-oxo-dodecanoyl homoserine lactone synthase enzyme. 3OC₁₂-HSL activates lasR which induces transcriptional activation of pqsABCDE, pqsR and pqsH. In R8, BB8 and N13 mutants, the transposon insertion directly influence the synthesis of the PQS mediator, in the 2/14 mutant the influence is indirect.

RT-PCR analyses confirmed the gene expression knock-out of pqsC, pqsH and rsaL in BB8, N13 and 2/14 mutants respectively (data not shown). As in the R8 clone, the gentamicin cassette was upstream from the pqsA gene and no interruption could be observed in the corresponding gene. The semi-quantitative RT-PCR showed that pqsA transcription levels were higher in R8 compared to the PAO1 strain (Fig. S1, available in the online Supplementary Material). Since pqsC expression was also increased, the hyperexpression effect was polar. As mariner transposon harbours an outward-directed Ptac promoter and a gentamicin resistance promoter, it could be hypothesized that one of these could cause the hyperexpression of the pqsA gene.

The amount of 2-heptyl-3-hydroxy-4-quinolone, the most important signal molecule in the PQS system [35], was evaluated in each strain by means of mass-spectrometric analyses. The amount of 2-heptyl-3-hydroxy-4-quinolone, expressed as ng ml⁻¹ and detected in cells grown in minimal conditions, differed among the investigated strains. The R8 and 2/14 clones respectively produced around eleven and eight times more PQS signal molecule than the PAO1 strain. In N13 and BB8 no signal molecule was detected (Fig. 4).

Photodynamic inactivation of PQS mutants

P. aeruginosa PAO1 and the isogenic PQS mutant strains were photoinactivated by means of TBO. Treating the PAO1 strain with TBO caused a decrease in cellular viability proportional to the light energy absorbed: a 4-log units decrease following 75 min of irradiation was observed (Fig. 5). Both PQS hyperproducer mutants, R8 and 2/14, displayed a certain degree of tolerance, which was statistically significant (P<0.05) compared to the PAO1 strain (Fig. 5a). On the other hand, the N13 and BB8 clones, PQS-deficient mutants, showed a sensitivity to PDT treatment comparable to that of PAO1 (Fig. 5b).
Since previous studies highlighted the protective role of *P. aeruginosa* phenazine with regards to photo-oxidative stress [11], the production of PYO and PCA was checked in the transposon mutants. *P. aeruginosa* PAO1, 2/14, N13 and BB8 clones grown in minimal medium did not show any visible pigmentation, while the R8 clone showed blue pigmentation and its UV-visible spectrum (Fig. 6) displayed two main peaks at 310 and 370 nm compatible with phenazine pigments, PYO and PCA, respectively [27]. While the observed tolerance in R8 could be partially or completely due to the phenazine presence, the significant tolerance in the 2/14 clone could not be ascribed to pigmentation. The N13 and BB8 mutants increased their tolerance to photo-oxidative stress when PYO and PCA 50 mg ml\(^{-1}\) were administered to cells (data not shown).

**Photodynamic inactivation of the membrane transport system mutants**

The transposon mutants Y18 and Mar94, knocked out in genes codifying transporter proteins in the cytoplasmic membrane, were both chosen to be photoinactivated.

In Mar94, PCR analyses (data not shown) confirmed that the PA4455 gene (*yrbE*) codifying a protein of a putative ATP-binding cassette (ABC) transporter permease was knocked out. This transport system seems to be involved in maintaining lipid asymmetry in the outer membrane [36]. The growth in minimal medium of Mar94 differed compared to the PAO1 strain, as cellular clusters were visible after overnight incubation (Fig. 7a).

In Y18, the gentamicin cassette, as confirmed by PCR (data not shown), interrupted the *shaA* gene (PA1054) belonging to the *sha* (sodium hydrogen antiporter) operon codifying a multi-subunit transport complex. The *sha* system is found at cytoplasmic membrane level and is important for the homeostasis of H\(^+\), Na\(^+\) and other monovalent cations [37].

When Y18 and Mar94 were photoinactivated by means of TBO upon irradiation, a different response was observed. The higher PDT-sensitivity shown by Y18 compared to the PAO1 strain was not statistically significant (Fig. 7b). On the other hand, the tolerance to photoinactivation shown by Mar94 was significant (*P*<0.05) compared to the PAO1 strain upon increasing irradiation times (Fig. 7a).

**DISCUSSION**

The aim of this study was to isolate *P. aeruginosa* transposon mutants able to respond to the photo-oxidative stress...
photodynamically induced in a different manner compared to the wild-type.

The QS network of *P. aeruginosa* consists of at least four interconnected signalling systems (Las, Rhl, PQS, IQS) hierarchically organized. In particular, the production of the siderophore pyoverdine is regulated by QS. The activation of *pvd* genes coding for enzymes involved in pyoverdine synthesis is induced by LasR [38] and PQS [35], that in turn are connected among them and with the other systems [30]. As pyoverdine production at nanomolar concentration can be detected by UV fluorescence [21, 22], a UV-based screen was set up. As QS showed to exert control of biofilm development through regulation of swarming motility, motility and biofilm formation were also investigated by easy screenings [23, 24]. The screens permitted the selection of a heterogeneous subpopulation of mutants directly or indirectly related to QS to submit to PDT treatment.

The PDT was applied to two groups of selected transposon mutants. The first group of mutants was chosen because the transposon insertion directly or indirectly influenced the production of the signal molecule of the PQS system, while the mutants in the second group were knocked out in cytoplasmic membrane transport complexes. The

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**Fig. 6.** Representative UV-VIS spectra of the supernatant of *P. aeruginosa* and transposon derivatives 2/14 and R8 grown in M9 medium supplemented with glucose 10 mM. Images of corresponding cultures are shown.

**Fig. 7.** Photoinactivation by TBO 5 µM of *P. aeruginosa* PAO1 and MAR94 (a), PAO1 and Y18n (b). After 10 min of incubation in the dark and increasing irradiation times, the cellular viability was checked. The data are the means of at least three independent experiments±sd. *P*<0.05 for mutant derivatives versus PAO1 strain.
characterization and the response to PDT of the other mutants isolated in the present work is under analysis.

PQS is an intracellular network associated with membrane vesicles in *P. aeruginosa* and plays an important role in the expression of several virulence factors as well as in inducing a protective stress response to deteriorating environmental conditions [15, 39]. The signalling molecule 2-heptyl-3-hydroxy-4-quinolone exhibited both beneficial and deleterious activities, depending on the specific conditions, such as oxygen and transition metal availability. Haussler and Becker observed that the PQS mediator may act as a pro-oxidant compound, initiating radical mediated processes, and as an antioxidant, contributing to UV tolerance as the *pqsA* mutant was hypersensitive to UV irradiation [15]. Since 2-heptyl-3-hydroxy-4-quinolone was toxic to cells exposed to UVA radiation, Pezzoni proposed the role of an endogenous PS [14].

This study evaluated whether PQS mutant derivatives could cope with photo-oxidative stress in a different manner compared to the PAO1 wild-type strain. Under minimal growth conditions, the selected mutants produced different yields of 2-heptyl-3-hydroxy-4-quinolone compared to the wild-type strain: R8 and 2/14 derivatives were PQS mediator hyperproducers, while N13 and BB8 were PQS-deficient. The PQS amounts detected in each mutant are compatible with the corresponding genetic background. In R8, the high level of PQS could be due to the upregulation of the *pqsABCDE* regulon. In 2/14, the knock-out of *rsaL* could switch off the PQS *rsaL*-dependent derepression with an increase in the 2-heptyl-3-hydroxy-4-quinolone level. The interruption of PQS genes in N13 and BB8 clones is compatible with PQS default.

When the PQS mediator levels were correlated to PDT sensitivity, it could be inferred that a certain degree of statistically significant tolerance towards photo-oxidative stress was linked to a high level of PQS signal molecules. The PQS-deficient strains, N13 and BB8, were as sensitive to PDT as the PAO1 strain that produced a very low amount of signalling molecule. In the R8 mutant, which was a 2-heptyl-3-hydroxy-4-quinolone hyperproducer, the presence of phenazines may contribute partially or wholly to defending the cells against stress elicited by TBO. On the other hand, in the 2/14 mutant, also a 2-heptyl-3-hydroxy-4-quinolone hyperproducer, pigment defence may not be considered. No pigment was detectable in this mutant and the high levels of PQS molecule favour PDT tolerance. PQS could play a role in regulating the expression of genes involved in the response to photo-oxidative stress and/or PQS itself could quench singlet oxygen or scavenger radicals.

Furthermore, Haussler and Becker observed that the HHQ did not show any anti-oxidant activity and hypothesized that the lack of the 3-OH group makes HHQ a weaker electron-donor than PQS [15]. In the N13 mutant, the interruption of *pqsH* codifying a putative flavin-dependent monoxygenase that hydroxylates HHQ at the third-position, converting HHQ into a PQS compound [34], is conceivable with HHQ accumulation. The PDT-sensitive phenotype of the N13 mutant is in accordance with the lack of any anti-oxidant activity linked to HHQ accumulation.

Furthermore, this study investigated the response to PDT of two strains with impaired cytoplasmic membrane transport. The knock-out of the *sha* gene codifying the sodium/hydrogen antiporter did not influence the response to photo-oxidative stress in test conditions. The disruption of PA4455, encoding a putative cytoplasmic membrane-spanning ABC transporter permease, increased the bacterium’s tolerance to TBO-induced photo-oxidative stress. This ABC transport system is highly conserved and prevents phospholipid accumulation in the outer leaflet of the outer membrane. The presence of phospholipids in the outer membrane may increase permeability by hydrophobic molecules and the mutant Mar94 should be less permeable to cationic PS such as TBO. Recently, Chen demonstrated that this ABC transporter, besides the import of phospholipids, small molecules including nutrients, antibiotics and drugs, also plays a role in tetracycline expulsion [36, 40]. As the steric hindrance of the three rings of TBO is less than the four ring of tetracycline, it could be hypothesized that the ABC transporter drives the PS out of the cell and causes the observed tolerance in PDT experiments. Furthermore, the Mar94 mutant forms aggregates that resemble biofilm clusters, and the observed tolerance could also be due to the well-known higher tolerance of biofilms to photodynamic treatment [41].

In conclusion this study made it possible to isolate *P. aeruginosa* variants impaired in different functions and tolerant to photodynamic stress, suggesting a multifactorial basis for PDT tolerance. Under the PDT experimental conditions, PQS seems to both directly and/or indirectly hinder the oxidative stress elicited by PDT. Since polymorphonuclear neutrophils (PMN) are committed to bacterial killing by oxidative burst [42], it could be investigated whether the resulting PQS mutants are better able to deal with PMN oxidative stress than the wild-type. Further mutants impaired in the bacterial envelope could contribute to lighten the action site of different PSs, at intracellular and/or extracellular level.

The obtained PDT-tolerant strains could be used as tester micro-organisms, which are more difficult to photoinactivate than the wild-type, so as to evaluate the efficiency of new PSs and/or PDT protocols. Since the photodynamic approach seems very promising against *P. aeruginosa* biofilms [43], mutant strains grown as adherent forms will be assayed.

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**Conflicts of interest**
The authors declare that there are no conflicts of interest.

**References**


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