Pigments influence the tolerance of *Pseudomonas aeruginosa* PAO1 to photodynamically induced oxidative stress

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*Pseudomonas aeruginosa* is an opportunistic pathogen known to be resistant to different classes of antibiotics and disinfectants. *P. aeruginosa* also displays a certain degree of tolerance to photodynamic therapy (PDT), an alternative antimicrobial approach exploiting a photo-oxidative stress induced by exogenous photosensitizers and visible light. To evaluate whether *P. aeruginosa* pigments can contribute to its relative tolerance to PDT, we analysed the response to this treatment of isogenic transposon mutants of *P. aeruginosa* PAO1 with altered pigmentation. In general, in the presence of pigments a higher tolerance to PDT-induced photo-oxidative stress was observed. Hyperproduction of pyomelanin makes the cells much more tolerant to stress caused by either radicals or singlet oxygen generated by different photosensitizers upon photoactivation. Phenazines, pyocyanin and phenazine-1-carboxylic acid, produced in different amounts depending on the cultural conditions, are able to counteract both types of PDT-elicited reactive oxygen species. Hyperproduction of pyoverdine, caused by a mutation in a quorum-sensing gene, rendered *P. aeruginosa* more tolerant to a photosensitizer that generates mainly singlet oxygen, although in this case the observed tolerance to photo-oxidative stress cannot be exclusively attributed to the presence of the pigment.

**INTRODUCTION**

Photodynamic therapy (PDT) exploits the photo-oxidative stress elicited by exogenously administered photosensitizers (PSs) that absorb visible light and cause the production of reactive oxygen species (ROS) by energy transfer or electron flow to oxygen, which in turn cause oxidative damage to bacterial cells (Wainwright, 1998). Different classes of PSs are known that produce ROS by distinct mechanisms. When energy is transferred to oxygen via a type II mechanism, singlet oxygen \( \text{O}_2 (^1\text{A}_g) \) arises. When electrons are transferred to oxygen via a type I mechanism, radicals such as the superoxide radical anion \( \text{O}_2^- \), hydrogen peroxide \( \text{H}_2\text{O}_2 \) and hydroxyl radical \( \text{OH}^- \) arise, and these may trigger the development of inorganic or organic radicals depending on the microenvironment (Huang et al., 2012). When ROS are generated near the outer side of the bacterial envelope, the barrier functions are altered and cellular integrity is compromised. When the PSs pass through the cell wall, ROS are generated in the cytoplasm causing macromolecule degradation and bacterial killing (Nitzan & Kauffman, 1999; Caminos et al., 2008). Thus, PDT is regarded as a new and promising approach to disinfection, either alone or in combination with conventional chemotherapy.

*Pseudomonas aeruginosa* is able to survive in different ecological niches, such as soils, plants, water and animals, owing to its catabolic versatility and genetic plasticity. *P. aeruginosa* is also a well-known opportunistic pathogen that may negatively affect infected patients due to its ability to produce many virulence factors. Among the *P. aeruginosa* virulence factors, pigments such as pyocyanin, a water-soluble blue and non-fluorescent phenazine (Lau et al., 2004; Jayaseelan et al., 2014), pyoverdine, a fluorescent siderophore playing an important role in iron uptake (Visca et al., 2007), pyorubin and pyomelanin, non-fluorescent red and brown pigments (Rodríguez-Rojas et al., 2009), play an important role. Concern

**Abbreviations:** C4-HSL, N-(butanoyl)-homoserine lactone; FT-IR, Fourier transform infrared; PCA, phenazine-1-carboxylic acid; PDT, photodynamic therapy; PS, photosensitizer; QS, quorum sensing; ROS, reactive oxygen species; RT-PCR, reverse transcriptase PCR; TBO, Toluidine Blue-O; TMPyP, 5,10,15,20Tetrakis-(1-methyl-4-pyridyl)-21H,23porphine,tetra-p-tosylate.

Four supplementary figures are available with the online Supplementary Material.
regarding \textit{P. aeruginosa} is mainly due to its resistance to many antibiotics and its tolerance to disinfectants and antimicrobial treatments (Jimenez et al., 2012; Morita et al., 2014). Thus, new systems to control the growth of this micro-organism are urgently required.

In recent years, reports about the efficacy of PDT against \textit{P. aeruginosa} have been published, and it has become clear that \textit{P. aeruginosa} is particularly tolerant to photo-dynamically induced oxidative stress (Philippova et al., 2003; Tegos et al., 2006), although no studies have investigated this in detail.

Photo-oxidative stress has been widely studied in photo-synthetic bacteria (Glaeser & Klug, 2005; Ziegelhofer & Donohue, 2009), in which the role of pigments was highlighted about 60 years ago with reported damage to carotenoid-deficient \textit{Rhodobacter sphaeroides} during photosynthesis in an aerobic atmosphere (Griffiths et al., 1955). In \textit{R. sphaeroides}, during solar energy capture, the bacteriochlorophyll \textit{a} (Bchla) reaches a triplet excited state and reacts with oxygen generating singlet oxygen ($^1\text{O}_2$) (Glaeser & Klug, 2005). In this bacterium, carotenoids play an important role in protecting cells from photo-oxidative stress, because of their capacity to directly quench $^1\text{O}_2$ and the excited form of Bchla ($^3\text{Bchl}^*$) through both physical and chemical mechanisms (Ramel et al., 2012).

The ability of \textit{P. aeruginosa} to produce pigments, combined with the ability to elicit an oxidative stress response (Ochsner et al., 2000), may thus contribute to survival to PDT-induced oxidative stress. Here we investigated the role of pigments in \textit{P. aeruginosa} tolerance to PDT-induced oxidative stress. To this end, differently pigmented transposon mutants of \textit{P. aeruginosa} PAO1 were isolated and submitted to photodynamic treatment with two different PSs, a phenothiazinium dye, Toluidine Blue-O (TBO), that acts mainly via a type I mechanism, and a porphyrin dye, 5\textit{,10,15,20Tetakis-(1-methyl-4-pyridyl)-2H,23porphine,tetra-p-tosylate (TMPyP)}, that acts mainly via a type II mechanism.

**METHODS**

**Bacteria, plasmids and media.** \textit{P. aeruginosa} strain PAO1 (Stover et al., 2000) was used to construct the transposon library and \textit{Escherichia coli} S17 $^\text{Ap}$ir was used as transposon donor (Herrero et al., 2005). pBT20 was used as the Mariner-based transposon delivery plasmid (Kulasekara et al., 2005). \textit{P. aeruginosa} and the mutants were screened for different pigmentation on Luria–Bertani (LB) agar plates. BL (blue), RE (red) and YE (yellow) clones were mutated in the \textit{phzA1}, \textit{hmngA} and \textit{rhl} genes, respectively.

\textit{P. aeruginosa} wild-type and \textit{E. coli} were routinely grown in LB broth and on LB agar plates under aerobic conditions at 37 °C. \textit{P. aeruginosa} wild-type and transposon mutants were grown also in mineral medium M9 (Kahn et al., 1979) supplemented with 10 mM glucose, in the differential medium \textit{Pseudomonas} P $^\text{agar}$ and \textit{Pseudomonas} F $^\text{agar}$ (King et al., 1954). Gentamicin and ampicillin were added, when necessary, to a final concentration of 100 $\mu$g ml$^{-1}$.

**Construction of the transposon insertion library.** Transposon insertions in the PAO1 chromosome were generated by following the protocol of Kulasekara et al. (2005). Briefly, the donor strain \textit{E. coli} S17 $^\text{Ap}$ir carrying pBT20 containing the transposon Tn Mariner was suspended in LB broth to a final OD$_{600}$ of 40 and the recipient, \textit{P. aeruginosa} PAO1, to a final OD$_{600}$ of 20. Then, 10 $\mu$l droplets of a 1 : 1 conjugation mix were spotted on LB agar and incubated at 37 °C for 2 h. \textit{P. aeruginosa} PAO1 transconjugants were selected on minimal medium M9 supplemented with 10 mM glucose and gentamicin at 100 $\mu$g ml$^{-1}$; the donor strain \textit{E. coli} S17 $^\text{Ap}$ir, auxotrophic for proline and thymine, was counterselected. Colonies were selected and inoculated into 96-well plates, each well containing 200 $\mu$l LB medium supplemented with gentamicin. Plates were incubated for 48 h at 37 °C and pigmented mutants were isolated.

**Identification of interrupted sequences by arbitrary primed PCR.** The DNA sequences flanking the transposon inserts were determined using arbitrary PCR (O’Toole & Kolter, 1998). Briefly, in the first round of PCR, 100 ng of purified chromosomal DNA, obtained using a genomic DNA purification kit (Promega), was amplified with the random tagged primer Rnd1-ARB0p (5’GGC CACGGGCTCGA TGCACGN non-specific 3’-3’ pair) paired with Rnd1-TnM20 primer 5’-CATCGGCTG TGAATAA GTGTTG-3’. A nested PCR was then performed on the first PCR amplification product, with primers Rnd2-ARB (5’GGCCACGGCGTCGACTA GTAC-3’) and Rnd2-BT20 (5’-ACAGGAAA ACAGGAC TCTAGAGG-3’). Amplification products were then sequenced (Promm Biotech) using the primer 5’-ACAGGAAA ACAGGACTCTAGAGG-3’. Insertion sites were identified with BLAST searches, against the annotated sequence of the \textit{P. aeruginosa} PAO1 genome, using software provided by NCBI.

**Semi-quantitative reverse transcriptase PCR (RT-PCR).** RT-PCR was used to evaluate the transcriptional expression of \textit{phzB}. Total bacterial RNA was isolated from cultures grown to an OD$_{600}$ of 0.5 using a TRIzol Max (Invitrogen) isolation kit. Five micrograms of total RNA was then treated with RNase-free DNase (Promega) (1.5 U per gram of RNA) for 30 min. The DNase-treated RNA was extracted with phenol and precipitated using 100 % ethanol. Then, 250 ng of DNase-treated RNA was reverse-transcribed with Vilo mastermix (Invitrogen) according to the manufacturer’s instructions. cDNAs were then PCR-amplified using phzB1 FW (5’-AATGTACGTA AATCCAAA GGCAG CAGG-3’) and phzB1 RW (5’-AATTCCGGGT TTTCC GCTTGA CTTG-3’ for the \textit{phzB} gene, and 5’-CAGATCGAA CCAT TTCGCGAAG-3’ and 5’-GTTGATGAA GTGACGCG CAGG-3’ for the housekeeping gene \textit{recA}). PCRs were performed as follows: 94 °C for 2 min and 35 cycles of 94 °C for 30 s, 50 °C for 30 s and 72 °C for 1 min. The removal of contaminating DNA from each RNA sample was verified by a PCR in the absence of reverse transcriptase. PCR samples were collected at cycles 22, 25, 29, 31, 33 and 35 and 10 $\mu$l of each reaction was controlled by agarose gel electrophoresis (1.5 %, w/v).

**Phenazine purification and analysis.** \textit{P. aeruginosa} PAO1 and \textit{P. aeruginosa} BL were grown overnight at 37 °C under aerobic conditions in mineral medium M9 supplemented with 10 mM glucose or in LB medium. After centrifugation (10 min at 12 000 r.p.m., 50 ml supernatants were filtered (0.22 $\mu$m pore diameter) and extracted with a 1 : 5 volume of chloroform and analysed by TLC. The samples were plated on silica gel plates and developed in methanol. The $R_t$ value of each fraction was calculated.

The chloroform phase was further treated to purify pyocyanin as described by Chehuappa (2014). In a separation funnel the chloroform phase was acidified with 100 $\mu$l of 1 M HCl and added to 10 ml H$_2$O. The lower organic phase (yellowish) was vacuum dried. The acidified pink upper aqueous phase was further purified: after

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neutralization with a 1:100 volume of 1 M NaOH the solution turned blue and the pigment could be extracted again with 1 volume of chloroform. Five repetitions of the above described purification steps (acidification, neutralization and chloroform extraction) were performed before vacuum-drying in order to analyse the purified pigment.

After suspension in methanol the dried samples were filtered (Minisart RC15 @ 0.20 μm; Sartorius) and analysed by GC-MS (series DSQ II, dual stage quadrupole GC-MS; Thermo Scientific). A polysilphenylene-siloxane GC capillary column (s.n. 260F298P, CP-Sil5) was used to separate the compounds according to the following programme: injection temperature 220 °C, carrying gas He (flow rate 1.5 ml min⁻¹), temperature programme 40 °C for 1 min, gradient of 30 °C min⁻¹ to 250 and 250 °C for 10 min (Mavrodi et al., 2001). The data were processed using Xcalibur software (Thermo Scientific). Identification of bioconversion products was made by comparison of their mass spectra to those collected in the meta-library supplied with the equipment (NIST).

**Fourier transform infrared (FT-IR) spectrophotometry.** FT-IR spectroscopy is most useful for identifying the types of chemical bonds (functional groups) and therefore can be used to elucidate some components of an unknown mixture. Molecular characterization was performed using supernatant of *P. aeruginosa* RE grown overnight in minimal medium, supplemented with 10 mM glucose and 5 mM tyrosine. IR absorption spectra were recorded on a Thermo Nicolet, AVATAR 330 FT-IR system with a spectral resolution and wave number accuracy of 4 and 0.01 cm⁻¹, respectively. All measurements consisted of 500 scans in anhydrous KBr (200 °C for 2 h) (Vasanthabharathi et al., 2011).

**Spectrophotometric analysis.** PAO1 and mutants strains were grown in either LB or M9 medium supplemented with 10 mM glucose and then centrifuged (13 000 r.p.m. for 10 min). The supernatants were spectrophotometrically analysed (Perkin-Elmer Lambda 10 instrument). The UV–vis absorption spectra obtained were compared to spectra of pigments already reported in the literature. The spectrum for pyomelanin was reported by Kurian et al. (2014), those for phenazine-1-carboxylic acid (PCA) and pyocyanin by Mavrodi et al. (2013) and that for pyoverdine by Braud et al. (2009).

With the aim of evaluating the pigments’ sensitivity to light stress or PDT stress, UV–vis absorption spectra were obtained as follows. *P. aeruginosa* RE and YE strains were grown for 48 h in LB medium and the supernatant was diluted fivefold in phosphate buffer (KH₂PO₄/K₂HPO₄, 10 mM, pH 7.4), and purified phenazines were diluted in phosphate buffer at 50 μg ml⁻¹. The supernatant and purified phenazine were treated with PSs at 10 μM and incubated in the dark or irradiated with a 500 W halogen–tungsten lamp [fluence rate 48 mW cm⁻²; considering the whole width (400 nm) of the lamp emission spectrum] for 50 min (energy density 140 J cm⁻²). The spectra were registered in the wavelength range from 300 to 800 nm.

**Spectrofluorometric analysis.** *P. aeruginosa* PAO1 wild-type and YE transposon mutant cultures were grown overnight in LB and centrifuged (13 000 r.p.m. for 10 min). To evaluate the presence of pyoverdine in LB-grown cells, the supernatant was excited at 380 nm and fluorescence absorption intensity was recorded at 470 nm (Gaonkar et al., 2012) using an FP750 spectrofluorometer (Jasco Analytical Instruments).

**Growth curves.** *P. aeruginosa* PAO1 and *P. aeruginosa* PAO1 Mariner transposon derivatives were grown overnight in LB medium at 37 °C and diluted to an OD₆₀₀ of 0.1 in LB medium. Bacterial growth was monitored by measuring OD₆₀₀ at 1 h intervals.

**Photoinactivation experiments.** To verify the ROS elicited by PSSs, *P. aeruginosa* PAO1 was grown overnight at 37 °C in mineral medium M9 supplemented with 10 mM glucose. Diluted samples were treated with 5 μM TnPyP or TBO with or without 50 mM sodium azide. After 10 min of incubation in the dark without shaking, the samples were irradiated using a 500 W halogen–tungsten lamp for 25, 50 and 75 min (energy density 70, 140 and 210 J cm⁻², respectively). 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 second series of PDT experiments were aimed at comparing the photoinactivation yields of the strains grown under different conditions. *P. aeruginosa* PAO1 and mutants were grown overnight in M9 medium supplemented with 10 mM glucose and treated with 5 μM TnPyP or TBO. After 10 min of incubation in the dark, irradiation (210 J cm⁻²) was started (Orlandi et al., 2014).

For LB-grown cultures, a tenfold dilution was used and the PS concentration was increased to 25 μM to obtain satisfactory photoinactivation results. After 10 min of incubation in the dark, the samples were irradiated with 140 J cm⁻² to evaluate the different responses of the mutants. To evaluate the effect of pyomelanin on the photoinactivation of the PAO1 and RE strains, cells were grown overnight in M9 supplemented with 10 mM glucose and 0.1 mM l-tyrosine. After 10 min of incubation in the dark with 5 μM PSSs, cells were irradiated (210 J cm⁻²). To evaluate the effect of phenazines on PAO1 photoinactivation, pyocyanin and PCA (50 μg ml⁻¹) were added to PAO1 cells grown in M9 medium (with 10 mM glucose). Cells were incubated in the dark for 10 min with 5 μM PSSs and then irradiated (210 J cm⁻²).

Viable counts (expressed as c.f.u. ml⁻¹) were estimated after incubation in the dark and after irradiation in each experiment by a plate count technique: a volume (0.01 ml) of undiluted or serially diluted samples was plated on LB agar plates and incubated for 24 h at 37 °C.

**Statistics.** 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 effects of treatments were estimated (*P*<0.05 and *P*<0.01).

**RESULTS**

**Isolation and characterization of differently pigmented *P. aeruginosa* PAO1 mutants**

To evaluate the influence of pigmentation on sensitivity to bacterial photoinactivation, isogenic variants of *P. aeruginosa* PAO1 characterized by different pigmentation with respect to the wild-type strain were isolated. A transposon bank was constructed in the model microorganism *P. aeruginosa* PAO1 as described in Methods, and among ~2000 mutants screened on LB agar, three were differently pigmented from the wild-type strain. One transposon mutant showed red–brown colonies, a second one deep blue colonies and a third clone showed colonies more yellowish than the wild-type (Fig. 1).

In the mutant showing red–brown pigmentation (*P. aeruginosa* RE), the gentamicin cassette was mapped in the *hmgA* gene (Fig. 1), which encodes the enzyme homogentisate-1,2-dioxygenase involved in the catabolism of aromatic amino acids such as tyrosine (Fig. 2). It was
previously reported that inactivation of \textit{hmgA} results in the secretion of homogentisic acid, which auto-oxidises and self-polymerizes to form pyomelanin (Rodríguez-Rojas et al., 2009). To verify the possible hyperproduction of pyomelanin in the mutant strain, \textit{P. aeruginosa} PAO1 and RE were grown in minimal medium M9 supplemented with 10 mM glucose as carbon source and increasing concentrations of tyrosine (0.5, 1, 5 and 10 mM). The red–brown pigmentation detected after 24 h of incubation at 37°C increased in a dose-dependent manner in the mutant strain, but not in \textit{P. aeruginosa} PAO1 (Fig. 2a). The supernatant of the RE culture grown on minimal medium supplemented with 5 mM tyrosine was then subjected to FT-IR spectroscopy. In agreement with the FT-IR of pyomelanin reported by Vasanthabharathi et al. (2011), two regions, one (3400–3100) rich in OH stretch and one (1100) rich in NH, were detected (Fig. S1, available in the online Supplementary Material).

In the chromosome of the blue mutant (\textit{P. aeruginosa} BL) the transposon was mapped in \textit{phzA1} (Fig. 1), a gene belonging to one of the two homologous phenazine gene clusters involved in pyocyanin production, \textit{phzA1B1C1-D1E1F1G1} and \textit{phzA2B2C2D2E2F2G}, respectively. When plated on \textit{Pseudomonas} agar P, a differential medium that enhances pyocyanin production (Forbes et al., 2007), \textit{P. aeruginosa} BL showed a blue colour more intense than that of the wild-type (Fig. 1), in accordance with the hypothesis of this mutant being a pyocyanin hyperproducer. The UV–Vis spectrum of the supernatant of an LB liquid culture displayed two main peaks, at 310 and 364 nm (Fig. S2), compatible with pyocyanin and PCA, respectively (Mavrodi et al., 2013). TLC analyses of extracted supernatant of LB-grown cells showed an intense blue spot and a less intense orange–yellow spot (\(R_f\) 0.4 and 0.53, respectively). To identify the two pigments, the supernatant of LB-grown cells was chloroform-extracted, resulting in an organic blue fraction and an aqueous orange–yellow fraction. GC-MS analyses of both the organic and aqueous fraction confirmed the two pigments as pyocyanin and PCA, respectively (Fig. S3). Based on spectrophotometric analyses, the mutant strain produced an amount of pyocyanin approximately three to four times higher than the wild-type.

<table>
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<tr>
<th>Phenotype</th>
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<td>LB agar</td>
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\begin{figure}
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\includegraphics[width=\textwidth]{fig1}
\caption{Summary of phenotypic and genotypic characterization of transposon mutant strains described in the text. In the first column the growth of mutant strains on LB agar and on selective medium (agar P for pyocyanin producer and agar F for pyoverdine producer). In the second column insertion of the Mariner transposon is represented for each mutant. The third column gives the shortened name used in the text for the identification of the corresponding strain.}
\end{figure}
The Mariner transposon has a strong outward-pointing promoter, and insertion of the transposon in the *phzA1* gene (see Fig. 1) probably enhances expression of the *phzB1* gene, which is located immediately downstream of the *phzA1* gene (in the same operon).

When the BL strain was grown in M9 medium supplemented with 10 mM glucose, TLC analyses of extracted supernatant enabled the separation of two spots with the same *R*<sub>F</sub> as calculated for spots of extracted supernatant of LB grown cells (*R*<sub>F</sub> 0.4 and 0.53, respectively). However, in this case the yellow–orange spot was more intense than the blue one (data not shown).

In the yellow variant of PAO1, *P. aeruginosa* YE, the Mariner transposon was inserted in the *rhlI* gene (see Fig. 1) such that the pigment pyoverdine is overproduced. The *rhlI* gene directs the synthesis of N-(butanoyl)-homoserine lactone (C4-HSL), the signalling molecule of one of the *P. aeruginosa* quorum-sensing (QS) systems which then interacts with the cognate RhlR, influencing transcription of target genes (Whiteley *et al.*, 1999; Lee & Zhang, 2015). On *Pseudomonas* agar F, a differential medium formulated to enhance pyoverdine production, the typical *P. aeruginosa* fluorescent yellow pigmentation was more intense in the YE mutant colonies than in those of the wild-type strain PAO1 (Fig. 1). The soluble fluorescent pigment produced by overnight LB cultures showed an absorbance peak at 400 nm (Fig. S2) compatible with pyoverdine (Braud *et al.*, 2009). Upon excitation at 380 nm the supernatant of YE cultures showed a fluorescence at 470 nm, 70-fold higher than that of *P. aeruginosa* PAO1 cultures (Fig. 3). In accordance with the published effects of metals on pyoverdine fluorescence (Braud *et al.*, 2009), a hypochromic effect was observed when FeCl₃ (100 μM) was added to the samples, whereas the addition of CuSO₄ (100 μM) caused a hyperchromic effect (data not shown).

None of the mutants described above showed altered growth rate and yield compared with the wild-type, either in rich (LB) or minimal (M9 supplemented with 10 mM glucose) medium. In the latter medium, only the BL mutant accumulated a visible amount of pigment (Fig. S2).
Photodynamic inactivation of \textit{P. aeruginosa} PAO1 and derivatives

To evaluate the sensitivity of the differently pigmented variants of \textit{P. aeruginosa} to photo-oxidative stress, two PSs, TBO and TMPyP, belonging to two different classes and eliciting photo-oxidative stress via a type I and type II mechanism, respectively, were chosen. In PAO1 photoinactivation experiments with TMPyP confirmed its ability to generate mainly singlet oxygen, as demonstrated by the protection effect exerted by sodium azide, which, by contrast, enhanced the photosensitization effects induced by TBO, as expected for radical inducing PSs (Fig. 4) (Tavares et al., 2011; Kasimova et al., 2014).

As the chemical composition of the growth medium influences not only the pigmentation but also PDT efficiency (Orlandi et al., 2012), the photoinactivation experiments were performed with cells grown both in both rich and minimal medium.

The protocol adopted for M9-grown cells (Orlandi et al., 2014) was, as expected, inefficient compared with LB-grown cells. A new optimized protocol was thus developed as described in Methods. \textit{P. aeruginosa} PAO1 and the three isolated mutants showed a certain degree of tolerance to TBO, while the response to TMPyP was strain-dependent, \textit{P. aeruginosa} PAO1 and BL being more sensitive than the \textit{P. aeruginosa} YE and RE mutants (Fig. 5a). It thus appears that, under these conditions, pyomelanin could protect cells from singlet oxygen-induced damage.

When photoinactivation experiments were performed against M9/glucose-grown cells, the PSs were administered at 5 $\mu$M and irradiated at 210 J cm$^{-2}$. \textit{P. aeruginosa} BL showed tolerance to both TMPyP and TBO, while the

\begin{figure}[h]
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\caption{Fluorescence spectra of undiluted supernatant of PAO1 and 25-fold diluted supernatant of YE strains grown in LB medium recorded in the range 390–700 nm. The maximal fluorescence intensity at 470 nm is ascribable to pyoverdine.}
\end{figure}

\begin{figure}[h]
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\includegraphics[width=\textwidth]{fig4.png}
\caption{Photoinactivation of \textit{P. aeruginosa} PAO1 by TMPyP (a) and TBO (b). Sodium azide was supplemented as an antioxidant. After 10 min of incubation in the dark, the cells were irradiated with increasing energy densities. The data are the means of at least three independent experiments $\pm$ SD: $^{*}P<0.05$ and $^{**}P<0.01$ for $+$ PS + light versus (PS + light samples).}
\end{figure}

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other strains were successfully photoinactivated (Fig. 5b). Thus, under these conditions, the phenazine pigments, pyocyanin and/or PCA, can act as a defence against both singlet oxygen and radicals.

Based on these results, we further investigated the role of pyomelanin and phenazines. As demonstrated above, the addition of tyrosine to the growth medium enhanced the production of pyomelanin in *P. aeruginosa* RE (Fig. 2a). When *P. aeruginosa* PAO1 and RE were grown in minimal medium supplemented with 0.1 mM tyrosine, they showed a very different sensitivity to PDT. *P. aeruginosa* PAO1 was sensitive to photoinactivation, suggesting that under these conditions tyrosine cannot act as an antioxidant, whereas the mutant strain, in contrast to observations in the absence of the pigment (Fig. 5b), was tolerant to the photo-oxidative stress elicited by both PSs (Fig. 6).

The addition of either purified pyocyanin or purified PCA to the medium at the same time as the administration of the PS effectively protected M9-grown PAO1 cells from oxidative stress induced by either TMPyP or TBO (Fig. 7).

**Sensitivity of pigments to photo-oxidative stress**

To obtain more information about the potential protective role of the pigments, we evaluated their sensitivity to irradiation and/or photodynamic photo-oxidative stress elicited by PSs. Supernatants enriched in pigments (pyomelanin and pyoverdine) or purified pigments (pyocyanin and PCA) were thus irradiated in either the absence or presence of either TMPyP or TBO, and analysed spectrophotometrically.

The absorbance spectrum in the range 300–700 nm of the supernatant of *P. aeruginosa* RE grown for 48 h in LB medium and enriched in pyomelanin did not change following irradiation (140 J cm$^{-2}$) or photoinactivation by both tested PSs at a concentration of 10 μM (Fig. 8).

Following irradiation (140 J cm$^{-2}$), the $A_{400}$ of pyoverdine in the supernatant of *P. aeruginosa* YE grown for 48 h in LB medium was decreased ~30% compared with the sample kept in the dark. The absorbance spectrum changed upon photoinactivation by TMPyP and TBO, as shown in Fig. 8. The sensitivity of pyoverdine to photo-oxidative stress was further investigated by measuring fluorescence.

![Fig. 5. Photoinactivation of *P. aeruginosa* PAO1 and transposon mutant strains grown in LB medium (a) or minimal medium (b) and treated with TMPyP and TBO. The data are the means of at least three independent experiments $\pm$ SD: *$P<0.05$ and **$P<0.01$.](image1)

![Fig. 6. Protective effect of pyomelanin on RE strain. *P. aeruginosa* PAO1 and RE strains grown in minimal medium with tyrosine (0.1 mM) were photoinactivated by TMPyP and TBO. The data are the means of three independent experiments $\pm$ SD: *$P<0.05$ and **$P<0.01$.](image2)
emission. The intensity of fluorescence ($A_{468}$) following 380 nm excitation of irradiated (140 J cm$^{-2}$) YE supernatant decreased by 44% compared with the corresponding samples kept in the dark. The fluorescence intensity ($A_{468}$) following photo-oxidative stress elicited by 10 µM TMPyP and 10 µM TBO decreased by ~10 and ~20%, respectively, compared with the untreated and irradiated samples.

Purified pyocyanin at 50 µg ml$^{-1}$ was not sensitive to irradiation, but was sensitive to PDT by 10 µM TBO and TMPyP (Fig. 9). Purified PCA at 50 µg ml$^{-1}$ was not sensitive to irradiation or to PDT by TBO and TMPyP (Fig. 9).

**DISCUSSION**

The pigmentation of *P. aeruginosa* is the result of the production of different pigments such as pyocyanin and other phenazines, as well as pyoverdine, pyomelanin and pyorubin (Lau et al., 2004; Visca et al., 2007; Rodrı́guez-Rojas et al., 2009; Jayaseelan et al., 2014).

The biosynthesis of each pigment can be influenced by genetic arrangement, physiological cellular state and environmental conditions. The pigmentation of *P. aeruginosa* PAO1 cultures changes depending upon medium composition, inoculum volume, temperature, rate of aeration and agitation, and incubation time. For example, the medium composition greatly affects the pigmentation of *P. aeruginosa* PAO1: cells grown overnight in M9 medium did not show any pigmentation, while cells grown overnight in LB medium appeared green, compatible with the combination of blue pyocyanin and yellow LB medium (Rada & Leto, 2013). Under vigorous shaking cultures are green, but when the shaking is stopped a green–yellow gradient close to the medium–air interface forms, depending on the loss of oxygen tension and consequent decrease of the blue oxidized form of pyocyanin (Rada & Leto, 2013). As reported by Rada & Leto (2013), pyocyanin changes colour upon its oxidation state: it is blue when completely oxidized and colourless when reduced.

**Fig. 7.** Protective effect of purified 50 µg pyocyanin ml$^{-1}$ and 50 µg PCA ml$^{-1}$ on photoinactivation of *P. aeruginosa* PAO1 by TMPyP and TBO. The data are the means of three independent experiments ± SD: **$P < 0.01$.

**Fig. 8.** Effect of photo-oxidative stress on supernatant enriched in pyomelanin and pyoverdine. Representative UV–Vis spectra of the supernatant of *P. aeruginosa* RE and YE strains grown in LB untreated and treated with TMPyP or TBO. The supernatants were incubated in the dark (blue lines) or irradiated (red lines).
The isolation of variants of *P. aeruginosa* able to overproduce pigments, compared with the wild-type strain PAO1, was done with the aim of evaluating the role of pigment background in the response to photo-oxidative stress. In addition, the choice to induce photo-oxidative stress by means of two different PSs, TMPyP generating mainly singlet oxygen and TBO generating mainly radicals (see Fig. 4), could help in demonstrating a different sensitivity of the pigments under investigation.

In general, PDT was remarkably efficient against non-pigment-producing M9-grown cells (Fig. S2), regardless of the PS used, suggesting that in the absence of pigments, the stress induced by either singlet oxygen or radicals was comparably damaging. By contrast, TMPyP and TBO displayed a very different efficacy when used against pigment-producing LB-grown cells, singlet oxygen appearing much more destructive. Under these conditions, it seems that even the small amount of pigments produced by the wild-type strain PAO1 (Fig. S2) can be sufficient to counteract the effects of the radicals generated by photoactivated TBO (Fig. 5a).

In *P. aeruginosa* RE the production of a red–brown pigment, identified as pyomelanin, is due to the inactivation of the homogentisate-1,2-dioxygenase *hmgA* gene that leads to the accumulation of homogentisate, which, in turn, oxidizes and polymerizes to give pyomelanin (Fig. 1), a dark-brown/black pigment that is one of the many forms of melanin produced by a wide variety of organisms (Ketelboeter et al., 2014). The *P. aeruginosa* RE strain was tolerant to singlet oxygen elicited by TMPyP and to radicals elicited by TBO, but only when pyomelanin was visible.

When the *P. aeruginosa* RE strain was grown in minimal medium, pigmentation was not visible and the strain was sensitive to photo-oxidative stress elicited by both TMPyP and TBO. Rodríguez-Rojas et al. (2009) reported that in the pyomelanogenic *P. aeruginosa* PA14 *hmgA* strain, pyomelanin was responsible for H$_2$O$_2$ protection, and Zughai et al. (1999) observed that melanin in *Burkholderia cepacia* scavenged host-generated free radicals. Our photoinactivation data suggest that pyomelanin not only scavenges radicals, but also quenches singlet oxygen, even being able to protect cells grown in mineral medium M9 (see Fig. 6). In addition, neither irradiation nor photo-oxidative stress caused oxidation of the pigment, at least as regards alterations relievable by a modification of its absorbance spectrum, suggesting that its polymeric structure, composed of a variable amount of homogentisic acid units, was not compromised. The observed tolerance to singlet oxygen of pyomelanin emphasizes its role as a virulence factor in a clinical background. Indeed pyomelanin production has been reported in *P. aeruginosa* isolates from chronically infected cystic fibrosis patients (Hunter & Newman, 2010), so the observed tolerance to singlet oxygen stress could contribute to their surviving in alveolar areas where macrophages and neutrophils generate both radicals and singlet oxygen (du Bois, 1985).

Among pigments produced by *P. aeruginosa*, pyocyanin represents the most important pigment belonging to the class of phenazines. In the PAO1 strain two operons, *PhzA1B1C1D1E1F1G1* and *PhzA2B2C2D2E2F2G2*, have been identified as responsible for the synthesis of PCA; subsequently the methyltransferase encoded by *phzM* and the monooxygenase encoded by *phzS* convert PCA to

![Figure 9](image-url) **Fig. 9.** Effect of photo-oxidative stress on purified phenazines. Representative UV–Vis spectra of 50 µg pyocyanin ml$^{-1}$ and 50 µg PCA ml$^{-1}$ untreated and treated with TMPyP or TBO. The supernatants were incubated in the dark (red lines) or irradiated (blue lines).
pyocyanin (Mavrodi et al., 2001). In P. aeruginosa BL, the transposon disrupts the phzA1 gene, whose product is involved in more than one step of phenazine biosynthesis (Ahuja et al., 2008). The Mariner transposon harbours an outward-directed Ptac promoter and it is reasonable to assume that the phenotype of the BL strain is due to hyper-expression of the phzB1 gene, which is located downstream of phzA1 in the same operon. This assumption is supported by the results of a semi-quantitative RT-PCR that showed that the gene phzB was expressed more in the mutant strain than in the wild-type strain PAO1 (Fig. S4). It was not possible to distinguish phzB1 from phzB2, as they share an identical sequence.

Hyperproduction of pyocyanin does not seem to improve the tolerance of LB-grown cells to singlet oxygen elicited by TMPyP, as both PAO1 and the BL strains were successfully photoinactivated. By contrast and surprisingly, the accumulation of the phenazine pigments effectively protects M9-grown cells from photo-oxidative stress induced by either TMPyP or TBO. However, we observed that, when grown in LB medium, the BL strain produced an amount of pyocyanin larger than that of PCA, whereas the opposite ratio was observed in M9-grown cultures (Fig. S2). Due to the large amount of pyocyanin produced by BL cells grown in LB medium, we cannot exclude that a fraction of the compound is in the reduced form. The reduced form of pyocyanin is an unstable free radical that reacts rapidly with molecular oxygen, favouring the generation of ROS (O$_2^•$ and/or H$_2$O$_2$) (Hassett et al., 1992), and thus the accumulation of high concentrations of pyocyanin could also favour the presence of large amounts of reduced pigment toxic to cells. We also observed that purified PCA was not modified by photo-oxidative stress, whereas pyocyanin was (see Fig. 9). This is in agreement with the observation that the synthetic 1-methoxy-5-methylphenazinium methyl sulfate, with a methoxy substituent at C1, was more resistant to oxidation than pyocyanin, where a phenolate moiety could be important for its oxidation (Reszka et al., 2006). Similarly the carboxylic substituent at C1 in PCA could make it more resistant to photo-oxidation than pyocyanin. Reszka et al. (2006) also suggested that pyocyanin was largely oxidized by singlet oxygen rather than by hydroxyl radicals, as also observed in our experiments with purified pyocyanin (see Fig. 7). On the basis of these observations, it is reasonable to assume that the greater tolerance of M9-grown cells to TMPyP- or TBO-induced photo-oxidative stress could be attributed more to PCA than to pyocyanin. The protective effect of phenazine pigments was also demonstrated by the addition of purified pyocyanin or PCA to M9-grown PAO1 cells, although in this case, probably due to the concentrations used, we could not observe any difference between the effect of pyocyanin and that of PCA.

In the YE strain, the transposon disrupted the rhl~ gene involved in the synthesis of QS signal molecule C4-HSL, and YE cells did not show the typical green pigmentation due to the presence of pyocyanin, in accordance with the observation of a C4-HSL positively regulated synthesis of this pigment (Brint & Ohman, 1995; Whiteley et al., 1999). When grown in LB medium, YE instead produced more pyoverdine than the wild-type strain PAO1. To the best of our knowledge, there are no reports regarding a direct correlation between the rhl QS system and pyoverdine production, which has been instead related to the las and the P. aeruginosa QS systems (Stintzi et al., 1998; Diggle et al., 2007). On the other hand, it is well known that the P. aeruginosa QS systems are interconnected (Lee & Zhang, 2015), and thus an influence of the rhl system on the production of this pigment cannot be ruled out. With respect to the wild-type PAO1, LB-grown YE cells showed a certain degree of tolerance to TMPyP, although the pigment itself was sensitive to irradiation both in the presence and in the absence of the Ps. The role of pyoverdine in photo-oxidative stress defence is thus uncertain, as it cannot be ruled out that other C4-HSL-controlled factors may be involved. However, Huang & Shih (2000) observed that P. aeruginosa PAO-JP2, in which both the lasl and the rhlII QS signal systems had been silenced, was more tolerant to H$_2$O$_2$ treatment with respect to isogenic P. aeruginosa PAO1 wild-type, but the precise relationship between QS cascade and response to oxidative stress has not been investigated in depth.

Overall, it seems that at least some of the pigments synthesized by P. aeruginosa PAO1 may contribute to protection against PDT-induced photo-oxidative stress. In particular, pyomelanin appeared to be particularly efficient in counteracting radicals and singlet oxygen produced by the two Ps without compromising its overall structure, independently of the physiological status of the cells. Phenazine pigments also provide a certain degree of protection, although our data do not allow us to fully discriminate the role of pyocyanin and PCA in this phenomenon.

The role of the pigments secreted by P. aeruginosa can be relevant for in vivo PDT applications, as P. aeruginosa mutants altered in pigment production can spontaneously arise in infected tissues, decreasing PDT efficiency not only against P. aeruginosa itself, but probably also against other bacterial species present at the infection site.

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