The *Pseudomonas aeruginosa* oxidative stress regulator OxyR influences production of pyocyanin and rhamnolipids: protective role of pyocyanin

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The LysR-type transcriptional regulator (LTTR) OxyR orchestrates the defence of the opportunistic pathogen *Pseudomonas aeruginosa* against reactive oxygen species. In previous work we also demonstrated that OxyR is needed for the utilization of the ferrisiderophore pyoverdine, stressing the importance of this regulator. Here, we show that an *oxyR* mutant is unable to swarm on agar plates, probably as a consequence of absence of production of rhamnolipid surfactant molecules. Another obvious phenotypic change was the increased production of the phenazine redox-active molecule pyocyanin in the *oxyR* mutant. As already described, the *oxyR* mutant could not grow in LB medium, unless high numbers of cells (>10⁸ ml⁻¹) were inoculated. However, its growth in *Pseudomonas* P agar (King’s A), a medium inducing pyocyanin production, was like that of the wild-type, suggesting a protective action of this redox-active phenazine compound. This was confirmed by the restoration of the capacity to grow in LB medium upon addition of pure pyocyanin. Although both rhamnolipid and pyocyanin production are controlled by quorum sensing, no obvious changes were observed in the production of N-acylhomoserine lactones or the *Pseudomonas* quinolone signal (PQS).

Complementation of rhamnolipid production and motility, and restoration of normal pyocyanin levels, was only possible when the *oxyR* gene was in single copy, while pyocyanin levels were increased when *oxyR* was present in a multicopy vector. Conversely, plating efficiency was increased only when the *oxyR* gene was present in multicopy, but not when in single copy in the chromosome, due to lower expression of *oxyR* compared with the wild-type, suggesting that some phenotypes are differently affected in function to the levels of OxyR molecules in the cell. Analysis of transcripts of oxidative stress-response enzymes showed a strong decrease of *katB*, *ahpC* and *ahpB* expression in the *oxyR* mutant grown in LB, but this was not the case when the mutant was grown on P agar, suggesting that the OxyR dependency for the transcription of these genes is not total.

**INTRODUCTION**

In aerobes the oxidative phosphorylation process is subjected to uncontrolled leakage of electrons from redox enzymes to oxygen, forming oxygen derivatives such as hydrogen peroxide (H₂O₂), the superoxide anion (O₂⁻⁻) and the hydroxyl radical (OH⁻⁻), resulting in protein carbonylation, cofactor degradation, lipid peroxidation and DNA damage (Storz & Imlay, 1999). Therefore, these micro-organisms need a battery of oxidative stress defences, including antioxidant enzymes (catalases, superoxide dismutases and peroxidases), and iron-sequestering proteins such as bacterioferritin and ferritin (Hassett et al., 1992).

*Pseudomonas aeruginosa* possesses two superoxide dismutases (Mn-cofactored SodA and Fe-cofactored SodB), which represent the first line of defence against the superoxide anion O₂⁻⁻, converting it to H₂O₂, while three catalases (KatA, KatB and KatE) protect the cell against H₂O₂. Finally, four alkylhydroperoxide reductases (AhpA, AhpB, AhpCF and Ohr) detoxify H₂O₂ and several organic peroxides (Ochsner et al., 2000). The *P. aeruginosa* 34 kDa Lys-R-transcriptional regulator OxyR is crucial for the upregulation of the antioxidant genes katB, ahpB and ahpCF after contact with H₂O₂ (Ochsner et al., 2000). As well as the crucial role in upregulating oxidative stress defence, OxyR contributes to the virulence of *P. aeruginosa*, as demonstrated in rodent and insect models, but also

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**Abbreviations:** AHL, N-acylhomoserine lactone; LTTR, LysR-type transcriptional regulator; PQS, *Pseudomonas* quinolone signal; qRT-PCR, quantitative real-time PCR.

†These authors contributed equally to this study.
increases resistance to human neutrophils (Lau et al., 2005).

Recently, we demonstrated that inactivation of the oxyR gene in both Pseudomonas fluorescens ATCC 17400 and P. aeruginosa PAO1 impairs pyoverdine-mediated iron uptake, but only after the FpvA ferripyoverdine receptor-binding step (Vinckx et al., 2008). In addition, growth of the oxyR mutant in nutrient-rich medium, in the presence of excess oxidants, or in either extreme iron-depleted or replete media was suppressed. However, this growth inhibition was not observed when cells were inoculated at high cell density. Growth inhibition in LB was found to be caused by H2O2 production since addition of bovine catalase restored growth to wild-type levels even at low cell density (Vinckx et al., 2008). This cell-density dependency of growth of the oxyR mutant could suggest an altered cell–cell communication (quorum sensing) to be partially responsible for the observed phenotype. Quorum sensing is mainly dependent on cell density. However, it has been demonstrated that quorum sensing is not only cell-density-dependent, but is also influenced by the presence of iron and/or oxygen (Bollinger et al., 2001; Kim et al., 2005). This could explain the observations in our study related to pyoverdine utilization and growth in the presence of oxidizing compounds, which was possible only at high inoculum sizes (Vinckx et al., 2008). When cell density is sufficiently high, specific genes are upregulated. For example, certain oxidative-stress-response genes, such as those encoding catalases and superoxide dismutases, are also regulated by quorum sensing (Hassett et al., 1999), which could partially compensate for the absence of OxyR-regulated oxidative stress defence mechanisms. In this paper we present results revealing an increase of pyocyanin production, strongly decreased swarming motility, and absence of rhamnolipid production in an oxyR mutant unlinked to obvious changes in production of quorum-sensing signal molecules. We also provide evidence that the amount of OxyR regulators has to be balanced in the cell for the restoration of wild-type phenotypes. Finally, we show that, in contrast to LB, growth of the oxyR mutant in King’s A (P agar) medium is unaffected, resulting in the production of large quantities of the phenazine pigment pyocyanin, which exerts a protective effect, while expression of oxidative stress-response genes is clearly less dependent on OxyR in this medium.

### METHODS

Bacterial strains, plasmids and growth conditions. Bacterial strains and plasmids used in this study are shown in Table 1. Both P.

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**Table 1. Strains and plasmids used in this study**

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Characteristics</th>
<th>Reference</th>
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<tbody>
<tr>
<td><strong>Pseudomonas aeruginosa</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAO1</td>
<td>Wild-type; ATCC 15692</td>
<td>Ochsner et al. (2000)</td>
</tr>
<tr>
<td>oxyR</td>
<td>Mutant in the oxyR gene, Gm'</td>
<td>Ochsner et al. (2000)</td>
</tr>
<tr>
<td>lecA::luxAapqA</td>
<td>Reporter strain for PQS detection</td>
<td>Fletcher et al. (2007)</td>
</tr>
<tr>
<td>PAO1/pBBR</td>
<td>PAO1 containing pBBR1MCS, Cm'</td>
<td>This study</td>
</tr>
<tr>
<td>PAO1/pBBR-oxyR</td>
<td>PAO1 containing pBBR-oxyR, Cm'</td>
<td>This study</td>
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<tr>
<td>oxyR/pBBR-oxyR</td>
<td>oxyR mutant containing pBBR1MCS-oxyR, Cm'</td>
<td>This study</td>
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<tr>
<td>oxyR::pUC-oxyR</td>
<td>oxyR mutant containing insertion of single copy oxyR in attTn7 site of P. aeruginosa</td>
<td>This study</td>
</tr>
<tr>
<td><strong>Escherichia coli</strong></td>
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<tr>
<td>DH5α</td>
<td>supE44 ΔlacU169 (Δ80 lacZΔM15) recA hsdR17 recA1 endA1 gyrA96 thi-1 relA1</td>
<td>Hanahan (1983)</td>
</tr>
<tr>
<td>SM10(lpir)</td>
<td>thi-1 thr leu tonA lacY supE recA::RP4-2 tc::Mu; λpir, Km'</td>
<td>Herrero et al. (1990)</td>
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<td>S17-1</td>
<td>thi pro hsdR recA; chromosomal RP4, Tra +</td>
<td>Simon et al. (1983)</td>
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<td>MH155</td>
<td>AHL biosensor with plasmid pUCP22NotI-Plac::gfp(ASV)Plac::lasR</td>
<td>Hentzer et al. (2002)</td>
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<tr>
<td><strong>Chromobacterium violaceum</strong></td>
<td></td>
<td></td>
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<tr>
<td>CV026</td>
<td>Mini-Tn5 mutant of ATCC 31532 deficient in AHL and violacein production</td>
<td>McClean et al. (1997)</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
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<td>pBluescript KS'</td>
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<td>Stratagene</td>
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<tr>
<td>pBBR-1MCS</td>
<td>Broad-host-range cloning vector, Cm'</td>
<td>Kovach et al. (1994)</td>
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<td>pUC18T-mini-Tn7</td>
<td></td>
<td>Choi &amp; Schweizer (2006)</td>
</tr>
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<td>pTNS2</td>
<td>Helper plasmid for integration of the expression cassette</td>
<td>Choi &amp; Schweizer (2006)</td>
</tr>
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<td>pBBR-oxyR</td>
<td>PAO1 chromosomal DNA fragment containing oxyR (PA5344) coding sequence in pBBR1MCS, Cm'</td>
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<tr>
<td>pUC-oxyR</td>
<td>PAO1 chromosomal DNA fragment containing oxyR (PA5344) coding sequence in pUC18T-mini-Tn7, Gm'</td>
<td>This study</td>
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**Table 2.** Primers used in this study

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<td>GTGAAGCTTTAGCATGATCCCGTCCGGAAAT</td>
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<tr>
<td>pBBRoxRyR-Rv-XbaI</td>
<td>GTGCTTAGACGTTTCCGGGATGGTTAGA</td>
</tr>
<tr>
<td>pUC18ToxyR-Fw</td>
<td>GTGAAGCTTTGCGCGACCCCGATGT</td>
</tr>
<tr>
<td>pUC18ToxyR-Rv</td>
<td>GTGAAGCTTTGCGCGACCCCGATGT</td>
</tr>
</tbody>
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**aeruginosa** and *Escherichia coli* strains were grown with aeration at 37 °C while the N-acylhomoserine lactone (AHL) indicator bacterium *Chromobacter violaceum* CV026 was grown at 28 °C (McClean *et al.*, 1997). When required, antibiotics were added to follow the concentrations: 200 μg spectinomycin (Spe) ml⁻¹, 50 μg gentamicin (Gm) ml⁻¹ and 300 μg chloramphenicol (Cm) ml⁻¹ for *P. aeruginosa*, 100 μg ampicillin (Amp) ml⁻¹ and 50 μg Cm ml⁻¹ for *E. coli*, and 25 μg kanamycin (Km) ml⁻¹ for *C. violaceum* CV026.

**Primer and oligonucleotides.** Primer and oligonucleotides used in this study are listed in Table 2.

**Measurement of pyocyanin and pyocyanin purification.** Pyocyanin production was visualized by plating the bacteria on *Pseudomonas* agar (P agar or King’s A medium), followed by 48 h incubation (Mavrodi *et al.*, 2001). Pyocyanin production resulted in a deep blue coloration of the medium. Pyocyanin was quantified from culture supernatants according to Mavrodi *et al.* (2001). Briefly, the agar was collected in a Falcon tube and 10 ml chloroform was added per 12.5 g agar medium. The phenazine pigment was extracted during 2 h incubation at 37 °C, after which 2 ml 0.5 M HCl was added and the mixture shaken vigorously. The pink top layer was removed and its absorbance was measured at 520 nm.

Pyocyanin was extracted from liquid cultures of *P. aeruginosa* as previously described (Dietrich *et al.*, 2006). *P. aeruginosa* PA01 was grown in glycerol alanine minimal (GA) medium (1% v/v glycerol, 6 g l-alanine, 2 g MgSO₄, 0.1 g K₂HPO₄, 0.018 g FeSO₄) for 24 h. After centrifugation (8000 g, 10 min, 4 °C), the bacterial pellet was removed and the supernatant was extracted three times with 1 vol. chloroform (Fluka). Further purification of pyocyanin consisted of five successive extractions of the red, acidified form of pyocyanin from the chloroform phase by 10 mM HCl followed by extractions of the blue pyocyanin from the neutralized water phase by adding 0.1 M NaOH. After these repeated extractions, pyocyanin was dissolved in 20 ml chloroform followed by drying with helium gas and concentrated in a final 2 ml volume of acidified water. The purity of the pyocyanin solution was confirmed by HPLC using a Waters 600 HPLC system and an Alltima C18 column (10 × 250 mm for semi-preparative analysis, 10 μm; Alltech). The gradient solvent system consisted of 0.1% trifluoroacetic acid (TFA) in water and 0.1% TFA in acetonitrile using 30 min runs. The pyocyanin fraction was collected, lyophilized, weighed, dissolved in absolute ethanol (Fluka) and stored at −20 °C.

**Growth complementation of the oxyR mutant by pyocyanin.** Bacterial strains were grown aerobically in LB broth at 37 °C overnight and 100 μl overnight culture was transferred to a 3 ml LB medium to reach an OD₆₀₀ of 1.0 (day culture, corresponding to approximately 10⁶ cells ml⁻¹). The fresh culture was series diluted in 24-well plates (made of polystyrene, BD Biosciences) and incubated aerobically at 37 °C for 12 h. For pyocyanin complementation, purified pyocyanin was added to the wells and mixed thoroughly. Absolute ethanol (Fluka) was added as control. The experiment was repeated independently three times and pictures were taken by a Canon digital camera and edited with Photoshop Elements.

**Extraction and detection of PQS.** PQS was extracted from cell-free supernatants of LB cultures grown overnight at 37 °C by adding ethyl acetate and 0.1 ml 1⁻¹ glacial acetic acid. After drying of the sample, the dried pellet was resuspended in methanol and loaded on a normal-phase silica F₂₅₄ (Merck) TLC plate, pretreated by soaking in 5% K₂HPO₄ for 30 min and activated at 100 °C for 1 h, using dichloromethane and methanol (95:5, v/v) as the mobile phase (Shaw *et al.*, 1997). PQS was visualized using UV and identified by comparison with a synthetic standard (5 μl of a 10 mM stock solution obtained from Dr Paul Williams, University of Nottingham, UK).

**Bioassay for AHLs.** To extract AHLs, supernatants of overnight cultures in LB grown at 37 °C were treated with dichloromethane as previously described (McCLean *et al.*, 1997). TLC was used to separate AHLs and overlaid with indicator strain. Samples (10 μl) were spotted onto reverse-phase silica RP-18 F₂₅₄S and separated using methanol and water (60:40, v/v) as the mobile phase. CV₃-HSL was assayed using *C. violaceum* CV026 as indicator (McCLean *et al.*, 1997). This strain is unable to produce the violet pigment violacein due to a Tn5 insertion in the cviD gene, encoding a homoserine lactone synthase. Violacein production is induced by AHLs with N-acyl side chains from C₄ to C₉ in length.

For the detection of 3-oxo-C₁₂-HSL, cell-free supernatants were applied in a well in LB medium onto which, after absorption of the supernatant, a top layer of soft-agar was applied containing *E. coli* MH155 harbouring the reporter plasmid pUCP22NotI-P₁₁₁₁::gfp(ASV)P₁₁₁₁::kasR (Hentzer *et al.*, 2002).

**Motility assay.** Three microlitres of cultures with an OD₆₀₀ of 0.5 were spotted on LB and Casamino acid (CAA) plates containing 0.6% agarose for swarming detection. For swimming, the cells were first washed twice with PBS, after which 3 μl was spotted on LB and CAA plates containing 0.3% agarose. The LB plates were incubated for 24 h at 37 °C, the CAA plates for 48 h.

Twitching motility, as described by McMichael (1992), was tested by injecting 3 μl cultures in LB with an OD₆₀₀ of 0.5 in LB containing 1.3% agarose, allowing the bacteria to spread on the synthetic bottom of the Petri dish. After 24 h incubation, cells were stained with crystal violet (Merck).

**Rhamnolipids.** To test rhamnolipid production, we used the blue plate assay of Siegmond & Wagner (1991). This assay is based on the formation of an insoluble ion pair between cetyl trimethylammonium bromide and methylene blue. Positive cultures are surrounded by a dark blue halo. Overnight cultures were spotted and plates were incubated at 28 °C for 48 h (Danese *et al.*, 2000).

For more accurate analysis, rhamnolipids were extracted from culture supernatants with ethyl acetate (1:2 v/v). The ethyl acetate phases were pooled and evaporated. Dried extracts were dissolved in 20 μl chloroform/methanol (9:1, v/v). A total volume of 10 μl was applied on a silica plate (Kieselgel 60 F₂₅₄, Merck). TLC was performed using chloroform/methanol/acetate (65:15:2, by vol.) as running buffer. After chromatography, the dried silica plate was briefly submerged in an ethanol/methanol/acetic acid (2:1:1) solution before air-drying. Rhamnolipid spots were detected under ultraviolet light (λ = 365 nm) and fluorescence was recorded using Microscopy In Imaging Data (MIID; ImageJ) software.
H$_2$SO$_4$ (15%, v/v, in ethanol) and dried again. The sugar moieties were stained by heating the plate to 100 °C.

Complementation in trans with wild-type oxyR

Multicopy vector pBBR1MCS (Kovach et al., 1994). The oxyR gene was amplified using primers pBBRoxR-Fw-HindIII and pBBRoxR-Rv-XbaI (Table 2), followed by a treatment with a PCR purification kit (Qiagen). This fragment, containing the oxyR gene, and the pBBR1MCS-vector were digested with restriction enzymes HindIII and XbaI (Fermentas) and ligated overnight with T4-DNA ligase (Fermentas). Afterwards, the ligation mixture was transformed to E. coli DH5α and positive, white clones were selected. The construct was confirmed with PCR and introduced into E. coli S17-1 in order to perform a conjugation with P. aeruginosa wild-type and oxyR mutant colonies containing pBBR-oxyR, and hence the oxyR gene in trans, were selected on media containing 300 μg Cm ml$^{-1}$.

Single-copy vector pUC18T mini-Tn7-Gm (Choi & Schweizer, 2006). The gene of interest was amplified with ProofStart Taq DNA polymerase (Qiagen) using primers pUC18T-oxyR-Fw and pUC18T-oxyR-Rv and cloned into the multiple cloning site of the mini-Tn7 vector (pUC18T-mini-Tn7-Gm). For mini-Tn7 delivery, electrocompetent cells of the P. aeruginosa oxyR mutant were prepared according to the described protocol (Choi et al., 2006). To 100 μl of electrocompetent cells in a 2 mm gap-width electroporation cuvette, 50 ng pUC18T-mini-Tn7-oxyR and 50 ng pTNS2 was added and mixed gently by stirring with the gel-loading tip to avoid air bubbles. Electroporation was carried out (25 μF, 200 Ω, 2.5 kV, <5 ms). Transformants were plated, after 1 h incubation in LB, on selective medium (LB supplemented with 50 μg Gm ml$^{-1}$) and confirmed by PCR amplification.

Quantitative real-time PCR (qRT-PCR). Bacterial cells were harvested in stationary phase, and bacterial RNA was extracted by using the High Pure RNA Isolation Kit (Roche) or RNeasy Midi kit (Qiagen). The purity and concentration of the RNA were determined by gel electrophoresis and spectrophotometry (NanoDrop). First-strand cDNA was reverse transcribed from 1 μg total RNA by using the First-strand cDNA Synthesis kit (Amersham Biosciences, GE Healthcare). qRT-PCR was performed in a Bio-Rad iCycler with Bio-Rad iQ SYBR Green Supermix. For all primer sets, the following cycling parameters were used: 94 °C for 3 min followed by 40 cycles of 94 °C for 60 s, 55 °C for 45 s and 72 °C for 60 s, followed by 72 °C for 7 min. oprI (housekeeping gene control, outer-membrane lipoprotein precursor) was used to normalize gene expression (Cornelis et al., 1989). Amplification products were electrophoresed on 0.8% agarose gels. For statistical analysis of relative gene expression, the 2$^{-ΔΔCt}$ method was used (Livak & Schmittgen, 2001). All experiments were carried out in duplicate. Results were analysed with the Sigma-Plot software. All experiments were done in triplicate.

RESULTS

Growth in LB medium and Pseudomonas P agar and pyocyanin production

Plating efficiency on LB (Fig. 1a) and CAA medium (not shown) was strongly reduced for the oxyR mutant compared to wild-type, an observation made previously (Hassett et al., 2000; Vinckx et al., 2008). Fig. 1(a) (lower panel) shows that the oxyR mutant grows even at low inoculum sizes on P agar medium, which is known to induce pyocyanin production. As shown in Fig. 1(b), purified pyocyanin restored the capacity of the mutant to grow in LB medium.

Complementation by oxyR is affected by the number of oxyR transcripts

The vector pBBR1MCS has been found to be stably maintained at about 10 copies per cell in Brucella melitensis (Elzer et al., 1995). Introduction of the oxyR gene cloned in pBBR1MCS restored the plating efficiency in LB medium (Fig. 1a) and growth under iron-restricted conditions (Vinckx et al., 2008). We also introduced the wild-type oxyR gene as a single copy in the oxyR mutant using the pUC18T-mini-Tn7-Gm vector, resulting in insertion of the oxyR gene in the chromosome (Choi & Schweizer, 2006). This time the plating efficiency was not restored, suggesting a lower expression of oxyR. Therefore we decided to check the levels of oxyR transcripts in wild-type and in the oxyR mutant complemented with pBBR-oxyR or with oxyR in the chromosome. Fig. 1(c) shows that, as expected, there is no oxyR expression in the mutant while the number of oxyR transcripts is much higher (about 50 times) in the pBBR-oxyR-complemented strain. Although there was oxyR expression in the mutant containing the chromosomal insertion of oxyR, the level of transcripts was clearly lower (14% of wild-type) compared to the wild-type, explaining why the restoration of plating efficiency on LB did not occur.

Effect of the oxyR copy number on pyocyanin production

Inactivation of oxyR in P. aeruginosa PA01 leads to a twofold increase of pyocyanin production in P agar medium (Figs 1 and 2). Surprisingly, complementation in trans with the multicopy vector pBBR1MCS containing the oxyR gene (pBBR-oxyR) in the oxyR mutant did not result in the expected decrease of pyocyanin production to wild-type amounts (Fig. 2), although it restored the plating efficiency almost to wild-type levels as already mentioned. In contrast, complementation with pBBR-oxyR led to a further increase of pyocyanin in both wild-type and mutant (Fig. 2). Conversely, when oxyR was reintroduced as single copy, pyocyanin production was this time restored to wild-type levels (Fig. 2).

OxyR is required for swarming motility and rhamnolipid production

Swarming is dependent on the combination of the polar flagellum, type IV pili and rhamnolipid production (Caiazza et al., 2005; Kohler et al., 2000). This type of motility is almost completely absent in the oxyR mutant grown on CAA medium (Fig. 3a). As for pyocyanin production, the wild-type swarming phenotype could only be restored when oxyR was reintroduced as single copy in the chromosome, but not when oxyR was present in
multiple copies (Fig. 3a). Identical results were obtained for cells grown on LB agar plates (results not shown). The chemotaxis-regulated motion mediated by the polar flagellum, termed swimming, and twitching, a movement depending on the activity of type IV pili, were not significantly affected in the \(\text{oxyR}\) mutant (results not shown).

In addition, we extracted rhamnolipids and separated all compounds present using TLC, and stained the sugar moieties. As shown in Fig. 3(b) there was a major decrease in production of both mono- and di-rhamnolipids in the \(\text{oxyR}\) mutant. Complementation with a single copy of \(\text{oxyR}\) could also restore the rhamnolipid production in the \(\text{oxyR}\) mutant (results not shown). Accordingly, we observed a reduced expression of the \(\text{rhlA}\) gene in the \(\text{oxyR}\) mutant (Fig. 3c). This result could be confirmed using the blue-plate assay (results not shown).

As rhamnolipids are believed to be responsible for the maintenance of channels in the biofilm architecture by inhibiting cell–cell interactions and by preventing biomass accumulation in the channels, we expected biofilm formation to be affected as well (Davey et al., 2003). However, inactivation of OxyR resulted only in a minor, negative effect on biofilm formation capacity (results not shown), confirming a recent report (Panmanee et al., 2008).

**Expression of oxidative stress defence enzymes in LB and P agar**

Since growth of the \(\text{oxyR}\) mutant was not affected in \(P\) agar medium, we decided to look at the expression of genes for some oxidative-stress-response enzymes, namely the catalase gene \(\text{katB}\), the alkyl-hydroperoxidase genes \(\text{ahpC}\) and \(\text{ahpB}\), and the superoxide dismutase genes \(\text{sodA}\) and \(\text{sodB}\). The first three genes are known to be under the
control of OxyR (Ochsner et al., 2000) and their expression is strongly decreased in LB medium (20 times for katB, 5 times for ahpB and ahpC), as shown in Fig. 4. The expression of sodA (coding for the Mn-dependent superoxide dismutase) and sodB (coding for the Fe-factored superoxide dismutase) is also decreased in the oxyR mutant grown in LB, although these two genes are not known to be under OxyR control. Surprisingly, there is only a two- to threefold decrease in the expression of katB, ahpC and ahpB in the oxyR mutant when it is grown in P agar medium. When oxyR mutant cells were grown in this last medium we observed opposite effects on the expression of sodA (25-fold decrease) and sodB (2–3-fold increase).

Production of signal molecules
Our results, concerning not only the cell density-dependent pyoverdine utilization and growth in oxidizing conditions, but also virulence, including production of pyocyanin and rhamnolipids, and motility, could be the consequence of an affected quorum-sensing network in the oxyR mutant. For this reason, we decided to analyse the production of quorum-sensing signal molecules. Two quorum-sensing

Fig. 2. Pyocyanin production of wild-type P. aeruginosa, the oxyR mutant and the complemented strain. Complementation with oxyR in single copy in the chromosome could restore wild-type levels of pyocyanin production in the oxyR mutant.

Fig. 3. (a) Swarming motility of wild-type P. aeruginosa, the oxyR mutant, and the oxyR complemented with the wild-type oxyR gene in multicopy (pBBR vector) or with the oxyR gene in single copy in the chromosome. Only the single-copy complementation could restore wild-type swarming. Results are shown for CAA medium but were similar for LB medium (results not shown). (b) Detection of rhamnolipids by TLC in P. aeruginosa wild-type, the oxyR mutant and the single-copy-complemented oxyR mutant. Rhamnolipid production could not be restored when oxyR was introduced as an insert in the multicopy vector pBBR (results not shown). (c) qRT-PCR determination of relative expression of rhlA in the oxyR mutant compared to wild-type in LB medium. The expression levels were adjusted to the expression of the housekeeping gene oprI. Results are the means ± se of three biological repeats.
systems, las and rhl, have been described in *P. aeruginosa*. Signal molecules participating in quorum sensing include *N*-acylhomoserine lactones (AHLs), such as *N*-(3-oxodecanoyl)-homoserine lactone (3-oxo-C12-HSL) and *N*-butyryl-1-homoserine lactone (C4-HSL), but also the *Pseudomonas* quinolone signal (PQS). These autoinducers regulate several virulence factors and are required for biofilm formation (Williams, 2007). As for AHLs, production of 3-oxo-C12 and C4-HSL by the *oxyR* mutant is also like that of the wild-type (Fig. 5a for C12 and Fig. 5b for C4). Despite the increased pyocyanin production, a PQS-dependent feature, the *oxyR* mutant of PAO1 produced equal amounts of this signal molecule (Fig. 5c).

**DISCUSSION**

The results presented here confirm that the loss of OxyR has a pleiotropic effect, affecting different phenotypes. Previously we showed that utilization of the siderophore pyoverdine for iron uptake was abolished in the *P. aeruginosa oxyR* mutant, but not the production of pyoverdine or its receptor (Vinckx et al., 2008). During our study we realized that other phenotypes were also affected, such as production of pyocyanin and rhamnolipids as well as swarming motility. Pyocyanin is a blue, redox-active phenazine that contributes to virulence by inhibiting the oxidative burst of phagocytic cells of the host and by inducing apoptosis in host cells. This respiratory pigment also participates in the reduction of iron and functions as an intracellular redox buffer (Price-Whelan et al., 2006, 2007). Pyocyanin has two redox states, resulting in electron cycling, reducing O2 to its dangerous derivatives O2− and H2O2. Recently, pyocyanin was shown to have a function in signalling, responsible for the upregulation of specific genes, such as the genes involved in ferric iron acquisition and of the operon encoding the efflux pump MexGHI-OpmD (Dietrich et al., 2006; Price-Whelan et al., 2006). Recently, it was shown that phenazine-defective mutants produce wrinkled colonies and accumulate NADH in the cells (Dietrich et al., 2008; Price-Whelan et al., 2007; Wangt & Newman, 2008). Pyocyanin is the phenazine that interacts most strongly with oxygen (Wangt & Newman, 2008) and it was proposed by Price-Whelan et al. (2007) that externally added pyocyanin could give electrons to molecular oxygen to reduce it to water by oxidizing internal NADH to NAD. We indeed found that growth of the *oxyR* mutant in LB medium is restored in the presence of pyocyanin or phenazine 1-carboxylic acid. Another possible explanation for this reduction of oxidative stress exemplified by overproduction of pyocyanin could lie in the fact that pyocyanin undergoes oxidation by H2O2 (Reszka et al., 2004) in the presence of a peroxidase that still remains to be identified.

One interesting observation is the absence of complementation of swarming motility, rhamnolipid production, and wild-type levels of pyocyanin when *oxyR* is present in multycopys while the converse is true for the restoration of growth on LB at low cell density. By qRT-PCR we found that the number of copies of *oxyR* transcript is lower in the chromosomal complementation than in the wild-type, which is probably too low for the restoration of growth in...
strongly oxidizing media. This could mean that the number of OxyR regulators in the cell is critical and has to be strictly controlled.

LysR-type transcriptional regulators (LTTRs) are known as transcriptional activators of a single gene or operon, transcribed divergently with respect to their own negatively autoregulated gene (Maddocks & Oyston, 2008). However, this is not an absolute rule and LTTRs may activate or repress distantly located genes and operons and have a more global role in regulation (Maddocks & Oyston, 2008). The LTTR-binding site generally consists of two subsites, an upstream regulatory-binding site (RBS) and an activation-binding site (ABS) that may overlap the promoter, each showing an imperfect palindromic sequence (T-N\(_{11}\)-A), which can vary in both base pair composition and length. Simultaneous binding of the LTTR to both subsites frequently induces an important DNA bending that may be in part released upon effector binding (Maddocks & Oyston, 2008). It could be that site occupation by the regulator varies in the function of the number of OxyR LTTR molecules, resulting in either activation or repression, explaining why the number of OxyR molecules in the cell is important. Control of some quorum-sensing regulated traits by OxyR seems to be independent of LasR, RhlR or MvrR (the PQS regulator) since the production of the two AHLs and PQS was unaffected by the oxyR mutation. Another unexpected finding is the observation that the loss of OxyR only partially affects the expression of katB, ahpC and ahpB when the cells are grown in P agar, meaning that the OxyR dependency for the expression of these genes is reduced in this medium.

In conclusion, this work reveals the pleiotropic effects of the oxyR mutation, and suggests that the OxyR regulon could comprise more target genes than those already described, and that regulatory factors other than OxyR may influence the oxidative stress response in different media. It also describes for the first time the protective effect of pyocyanin against H\(_{2}\)O\(_{2}\). More work will be needed to determine the entire OxyR-dependent regulon and to determine how OxyR binds its target sites and regulates transcription.

REFERENCES


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