

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 ($>10^8$ 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.

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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 LysR-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.

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.*

Table 1. Strains and plasmids used in this study

Strain/plasmid	Characteristics	Reference
<i>Pseudomonas aeruginosa</i>		
PAO1	Wild-type; ATCC 15692	Ochsner <i>et al.</i> (2000)
<i>oxyR</i>	Mutant in the <i>oxyR</i> gene, Gm ^r	Ochsner <i>et al.</i> (2000)
<i>lecA::luxΔpqsA</i>	Reporter strain for PQS detection	Fletcher <i>et al.</i> (2007)
PAO1/pBBR	PAO1 containing pBBR1MCS, Cm ^r	This study
PAO1/pBBR- <i>oxyR</i>	PAO1 containing pBBR- <i>oxyR</i> , Cm ^r	This study
<i>oxyR</i> /pBBR- <i>oxyR</i>	<i>oxyR</i> mutant containing pBBR1MCS- <i>oxyR</i> , Cm ^r	This study
<i>oxyR::pUC-oxrR</i>	<i>oxyR</i> mutant containing insertion of single copy <i>oxrR</i> in <i>attTn7</i> site of <i>P. aeruginosa</i>	This study
<i>Escherichia coli</i>		
DH5α	<i>supE44 ΔlacU169 (φ80 lacZΔM15) recA hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i>	Hanahan (1983)
SM10(λpir)	<i>thi-1 thr leu tonA lacY supE recA::RP4-2 tc::Mu; λpir</i> , Km ^r	Herrero <i>et al.</i> (1990)
S17-1	<i>thi pro hsdR recA</i> ; chromosomal RP4, Tra ⁺	Simon <i>et al.</i> (1983)
MH155	AHL biosensor with plasmid pUCP22NotI-P _{lasB} :: <i>gfp</i> (ASV)P _{lac} :: <i>lasR</i> used for the detection of 3-oxo-C ₁₂ -HSL	Hentzer <i>et al.</i> (2002)
<i>Chromobacterium violaceum</i>		
CV026	Mini-Tn5 mutant of ATCC 31532 deficient in AHL and violacein production	McClellan <i>et al.</i> (1997)
Plasmids		
pBluescript KS ⁺	Cloning vector, Ap ^r	Stratagene
pBBR-1MCS	Broad-host-range cloning vector, Cm ^r	Kovach <i>et al.</i> (1994)
pUC18T-mini-Tn7	Gm ^r on mini-Tn7	Choi & Schweizer (2006)
pTNS2	Helper plasmid for integration of the expression cassette	Choi & Schweizer (2006)
pBBR- <i>oxyR</i>	PAO1 chromosomal DNA fragment containing <i>oxyR</i> (PA5344) coding sequence in pBBR1MCS, Cm ^r	This study
pUC- <i>oxrR</i>	PAO1 chromosomal DNA fragment containing <i>oxrR</i> (PA5344) coding sequence in pUC18T-mini-Tn7, Gm ^r	This study

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 (McClellan *et al.*, 1997). When required, antibiotics were added at the following 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.

Primers 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* PAO1 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 consecutive 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 10⁸ cells ml⁻¹). The fresh culture was series diluted in 24-well plates (made of polystyrene, BD Bioscience) 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 l⁻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 (McClellan *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. C₄-HSL was assayed using *C. violaceum* CV026 as indicator (McClellan *et al.*, 1997). This strain is unable to produce the violet pigment violacein due to a Tn5 insertion in the *cvil* gene, encoding a homoserine lactone synthase. Violacein production is induced by AHLs with *N*-acyl side chains from C₄ to C₈ 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-P_{lasB}::*gfp*(ASV)P_{lac}::*lasR* (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 Siegmund & 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

Table 2. Primers used in this study

Name	Sequence (5'-3')
pBBRoxyRFw-HindIII	GTGAAGCTTAGCATGATCCGTCGGGAAT
pBBRoxyRRv-XbaI	GTGTCTAGACGGTATTCGGATGGTTAGA
pUC18ToxyR-Fw	GTGAAGCTTCGTCGTCGACACCGATGT
pUC18ToxyR-Rv	GTGAAGCTTGGTATTCGGATGGTAGATCT

H₂SO₄ (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 pBBRoxyRFw-*Hind*III and pBBRoxyRRv-*Xba*I (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 *Hind*III and *Xba*I (Fermentas) and ligated overnight with T4-DNA ligase (Fermentas). Afterwards, the ligation mixture was transformed to *E. coli* DH5 α 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 μ g 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 μ l 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 μ F, 200 Ω , 2.5 kV, <5 ms). Transformants were plated, after 1 h incubation in LB, on selective medium (LB supplemented with 50 μ g 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 μ g 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^{- $\Delta\Delta$ C_t} 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* PAO1 leads to a two- to threefold 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

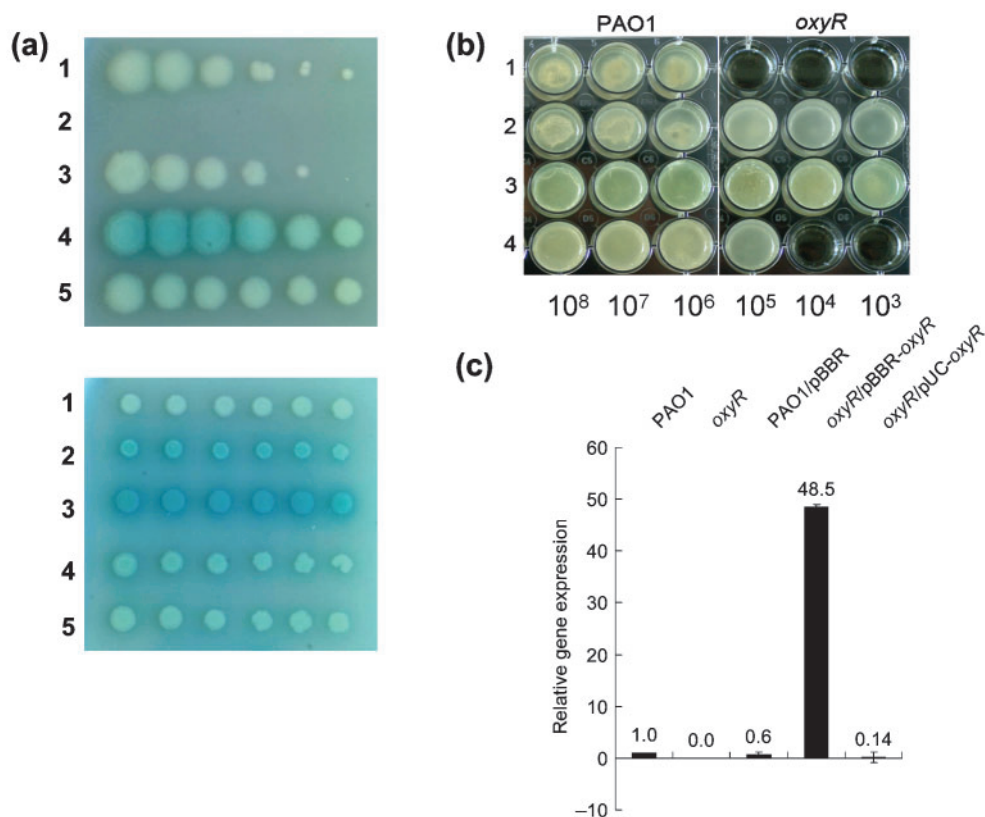


Fig. 1. (a) Plating efficiency of wild-type *P. aeruginosa* (1), *oxyR* mutant (2), *oxyR* complemented with pBBR-*oxyR* (3), PAO1 containing pBBR-*oxyR* (4) and PAO1 containing empty vector pBBR (5). The top picture corresponds to LB medium, the bottom one to Pseudomonas P agar; 10^8 – 10^3 cells ml⁻¹ were spotted from left to right in 10-fold dilutions. (b) Growth of wild-type *P. aeruginosa* and *oxyR* mutant in liquid LB medium without addition (row 1), with 1 µl pure pyocyanin (25 mM, row 2), 10 µl pure pyocyanin (250 mM, row 3), and 10 µl ethanol, used to dissolve pyocyanin (row 4). The number of inoculated cells is indicated below each well. (c) qRT-PCR measurement of the level of expression of *oxyR* (normalized to wild-type) in the wild-type, the *oxyR* mutant (zero expression), the wild-type with empty vector pBBR-1MCS, the *oxyR* mutant with pBBR-*oxyR*, and the *oxyR* mutant with the chromosomal insertion of *oxyR*. Except for the *oxyR* mutant, bands corresponding to the *oxyR* transcript could be visualized on gel (results not shown). Values relative to the wild-type are indicated above the bars.

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 *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 *oxyR* mutant. Complementation with a single copy of *oxyR* could also restore the rhamnolipid production in the *oxyR* mutant (Fig. 3b), but not when *oxyR* was in multicopy (results not shown). Accordingly, we observed a reduced expression of the *rhlA* gene in the *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 *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 *katB*, the alkyl-hydroperoxidase genes *ahpC* and *ahpB*, and the superoxide dismutase genes *sodA* and *sodB*. The first three genes are known to be under the

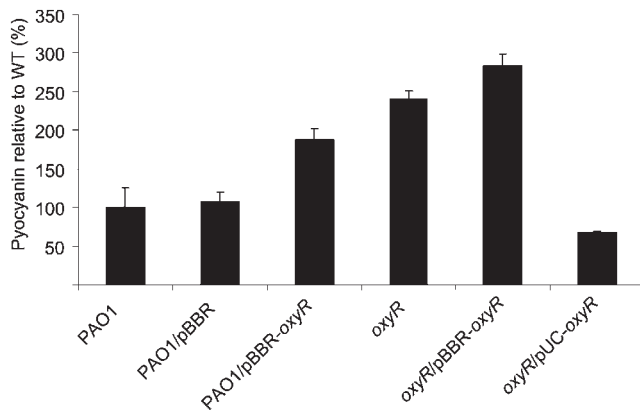


Fig. 2. Pyocyanin production of wild-type *P. aeruginosa*, the *oxyR* mutant and the complemented strain. Complementation with *oxyR* in single copy in the chromosome could restore wild-type levels of pyocyanin production in the *oxyR* mutant.

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

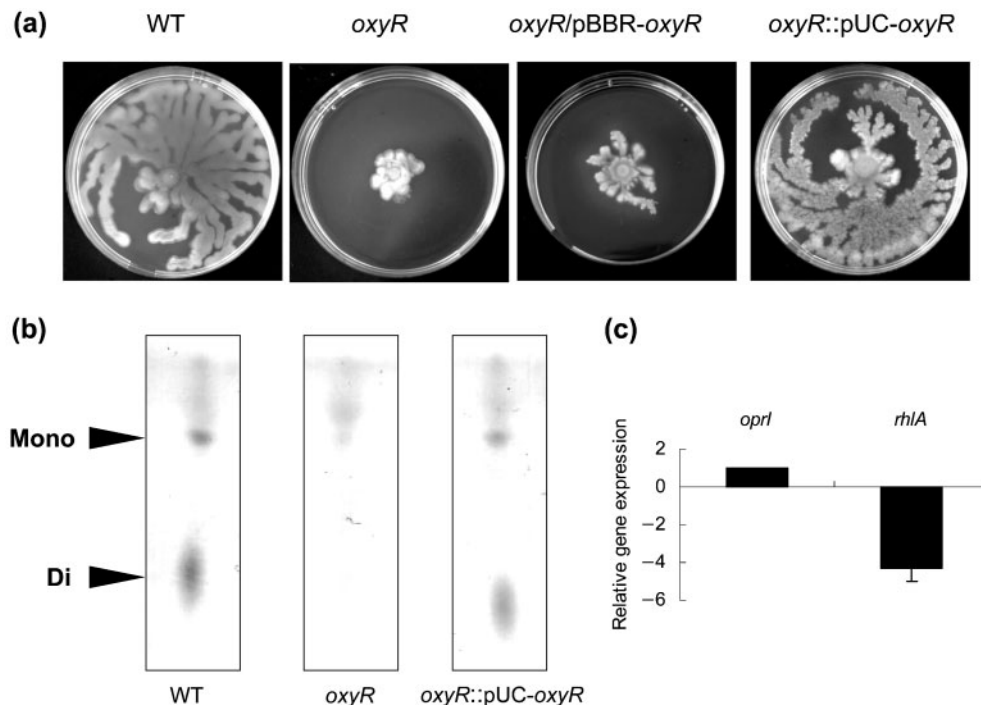


Fig. 3. (a) Swarming motility of wild-type *P. aeruginosa*, the *oxyR* mutant, and the *oxyR* complemented with the wild-type *oxyR* gene in multicopy (pBBR vector) or with the *oxyR* gene in single copy in the chromosome. Only the single-copy complementation could restore wild-type swarming. Results 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 \pm SE of three biological repeats.

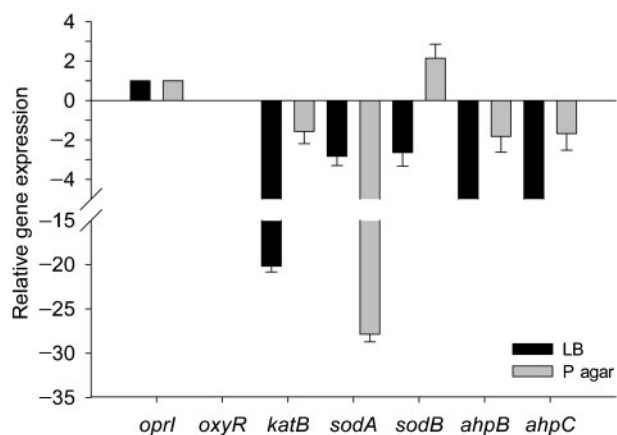


Fig. 4. qRT-PCR analysis of transcription of *oxyR*, *katB*, *sodA*, *sodB*, *ahpB* and *ahpC* in the *oxyR* mutant compared to wild-type, in LB medium and in extracted P agar medium. The expression levels were adjusted to the expression of the housekeeping gene *oprI*. Results are the means \pm SE of 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-oxododecanoyl)-homoserine lactone (3-oxo-C₁₂-HSL) and *N*-butyryl-L-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

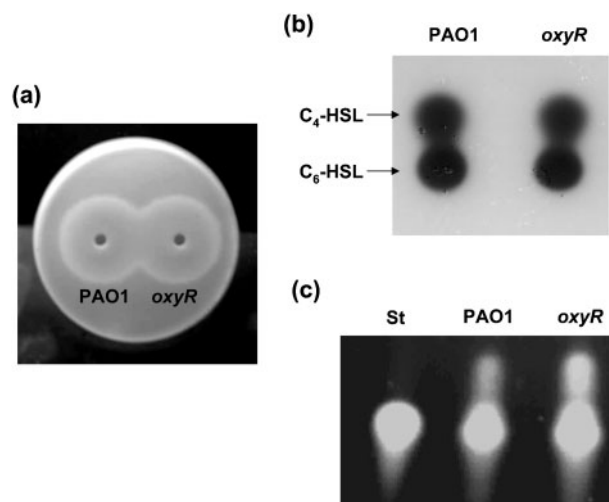


Fig. 5. (a) Production of 3-oxo-C₁₂-HSL by the wild-type (left) and the *oxyR* mutant (right). Ten microlitres of filtered culture supernatant was deposited in wells in the agar plate, which was inoculated with the reporter strain producing GFP (see Methods). (b) Production of C₄-HSL by wild-type (left) and *oxyR* mutant *P. aeruginosa* cells (right) detected after separation of supernatants extracted with dichloromethane as described in Methods. (c) PQS was extracted from the supernatant of *P. aeruginosa* wild-type and *oxyR* mutant and analysed on a TLC plate under UV light. Left, PQS standard; middle, PAO1 PQS extract from LB; right, *oxyR* mutant PQS extract from LB.

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 multicopy 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-N₁₁-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 MvfR (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 H₂O₂. More work will be needed to determine the entire OxyR-dependent regulon and to determine how OxyR binds its target sites and regulates transcription.

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