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The phenazine pyocyanin is a terminal signalling factor in the quorum sensing network of *Pseudomonas aeruginosa*

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Summary

Certain members of the fluorescent pseudomonads produce and secrete phenazines. These heterocyclic, redox-active compounds are toxic to competing organisms, and the cause of these antibiotic effects has been the focus of intense research efforts. It is largely unknown, however, how pseudomonads themselves respond to – and survive in the presence of – these compounds. Using *Pseudomonas aeruginosa* DNA microarrays and quantitative RT-PCR, we demonstrate that the phenazine pyocyanin elicits the upregulation of genes/operons that function in transport [such as the resistance-nodulation-cell division (RND) efflux pump MexGHI-OpmD] and possibly in redox control (such as PA2274, a putative flavin-dependant monooxygenase), and downregulates genes involved in ferric iron acquisition. Strikingly, *mexGHI-opmD* and PA2274 were previously shown to be regulated by the PA14 quorum sensing network that controls the production of virulence factors (including phenazines). Through mutational analysis, we show that pyocyanin is the physiological signal for the upregulation of these quorum sensing-controlled genes during stationary phase and that the response is mediated by the transcription factor SoxR. Our results implicate phenazines as signalling molecules in both *P. aeruginosa* PA14 and PAO1.

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Introduction

Bacteria communicate with each other through secreted signalling factors. These are small, diffusible molecules that are specifically released and then recognized by adjacent cells, where they trigger a response in gene expression. This ensures the behavioural synchronization of cell populations (Lazdunski *et al*., 2004). Because these events require high cell densities (presumably to accumulate sufficient signal), this mode of bacterial communication has been termed quorum sensing (QS). The best-characterized QS system is the Lux system of *Vibrio fischeri*, a luminous Gram-negative bacterium. The *V. fischeri* QS mechanism consists of a synthase (LuxI) that produces the signal, an acyl homoserine lactone (AHL) (Eberhard *et al*., 1981), and a transcriptional activator (LuxR) that recognizes the signal, leading to the downstream activation of genes in the *lux* operon (Engebrecht *et al*., 1983). Homologues of these genes have been identified in a number of Gram-negative bacteria, demonstrating the generality of QS signalling (Fuqua *et al*., 1994).

The Gram-negative bacterium *Pseudomonas aeruginosa* contains two such systems, Las and Rhl. LasI and RhlI synthesize the AHLs 3-oxo-dodecanoyl-homoserine lactone (3O-C_{12}-HSL) and butanoylhomoserine lactone (C_4-HSL) respectively, which in turn induce gene expression by binding to their respective transcriptional activators, LasR and RhlR (Passador *et al*., 1993; Pearson *et al*., 1995). Recently, a third signal has been found to participate in the *P. aeruginosa* QS network, the quinolone 2-heptyl-3-hydroxyl-4-quinolone that has been named the *Pseudomonas* quinolone signal (PQS) (Pesci *et al*., 1999). The production of PQS is under the regime of the QS network (McGrath *et al*., 2004). Its structure is very similar to the so-called Pyo compounds, which had been identified as antibiotics in 1945 (Hays *et al*., 1945) and shown to belong to the family of 4-quinolones in 1952 (Wells, 1952). The realization that PQS itself functions as a QS signal was surprising, because it is not an AHL and the genes involved in its synthesis are not LuxI homologues. However, apart from these differences, PQS fits all the functional criteria of a QS signal: (i) cell density-dependent accumulation (Lepine *et al*., 2003) of (ii) a

small diffusible molecule that (iii) is recognized by adjacent cells, (iv) in which it triggers a specific transcriptional response (Camilli and Bassler, 2006). This underscores the importance of defining QS systems on a functional level rather than on the basis of homology to canonical QS molecules. The three QS signal/response systems in *P. aeruginosa* are arranged in a temporal cascade, with AHLs and PQS being released in early and late exponential phase respectively (Lepine *et al*., 2003). Until now, PQS has been thought to be the terminal signal in the *P. aeruginosa* QS cascade.

MvfR positively regulates the production of a number of virulence factors as well as expression of PA2274, a putative monooxygenase, and the *mexGHI-opmD* operon that encodes proton-driven efflux pumps of the resistance-nodulation-cell division (RND) transporter superfamily (Deziel *et al*., 2005). It is thought that this response is mediated through PqsE and the signal PQS (Deziel *et al*., 2005). Among the virulence factors that are produced in response to PQS signalling are phenazines (Deziel *et al*., 2004). These are heterocyclic, redox-active compounds that exert toxic effects on other prokaryotes and eukaryotes (Mazzola *et al*., 1992; Mahajan-Miklos *et al*., 1999). Like quinolones, phenazines are excreted from cells at specific points following exponential growth (Diggle *et al*., 2003). The antibiotic properties of phenazines have been the subject of intense research efforts, but little is known about their effect on the producing organisms (Hassett *et al*., 1992). How, for example, do pseudomonads respond to these toxic compounds, abundantly secreted by cells during stationary phase? Moreover, why are these compounds secreted at this late growth stage? Previously, we hypothesized that phenazines could be serving important physiological functions under these conditions, separate from being antibiotics, such as facilitating extracellular electron transfer by being cycled in and out of cells (Hernandez and Newman, 2001; Price-Whelan *et al*., 2006).

To gain insight into how phenazines might be functionally integrated into *P. aeruginosa* physiology, we analysed their effect on gene expression in the opportunistic pathogen *P. aeruginosa* (strains PA14 and PAO1). Here we report that the phenazine pyocyanin (PYO) is a terminal signalling molecule in the *P. aeruginosa* QS network, and suggest that phenazines may, in this capacity, control their own cycling.

Results

PA14 and PA01 excrete phenazines in early stationary phase

Pseudomonas aeruginosa releases at least four phenazines (Mavrodi *et al*., 2001). These are 1-hydroxyphenazine (1-OH-PHZ), phenazine-1-carboxamide (PCN) and PYO, which are all derived from the common precursor, phenazine-1-carboxylic acid (PCA) (Budzikiewicz, 1993; Mavrodi *et al*., 2001; Price-Whelan *et al*., 2006) (Fig. 1). To characterize phenazine release from *P. aeruginosa* strains PA14 and PAO1, we grew them in minimal medium, removed culture aliquots at different time points, and measured cell densities and the phenazine concentration in cell-free supernatants by high-performance liquid chromatography (HPLC). Under these conditions, phenazine release by PAO1 was below our detection limit, but PA14 released phenazines in early stationary phase (Fig. 2A). PYO, which is responsible for the blue colour of stationary phase cultures, and PCA were the predominant phenazines in cell-free supernatant by 10 h of growth, accumulating to approximately 30 μM. We were unable to unequivocally detect 1-OH-PHZ and PCN under these conditions.

When grown in Luria–Bertani (LB), we observed PYO to be released by both strains, at the same stage in cell growth, although the concentration of PYO was ~10 times greater for PA14 (~60 μM) than PAO1 (Fig. 2B).

Phenazines as signals: exogenous PYO elicits a specific genetic response in PA14

To gain insight into whether *P. aeruginosa* mounts a physiological response to phenazines, we investigated the effect of phenazines at the level of gene expression. Specifically, we used Affymetrix *P. aeruginosa* GeneChips to identify transcriptional changes in PA14 in response to exogenous phenazines. We restricted our focus to PYO, one of the predominant phenazines in the supernatant of

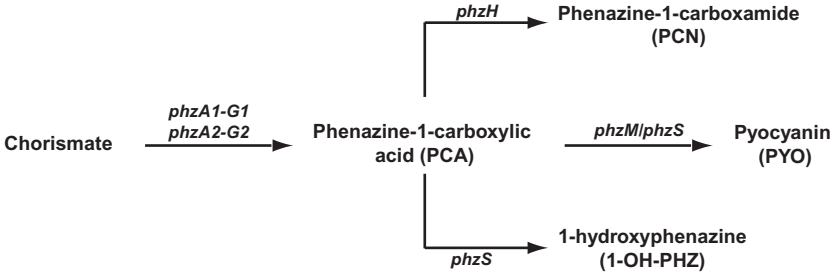


Fig. 1. Phenazine biosynthesis in *P. aeruginosa*. Genes encoding proteins involved in phenazine production are listed above the arrows.

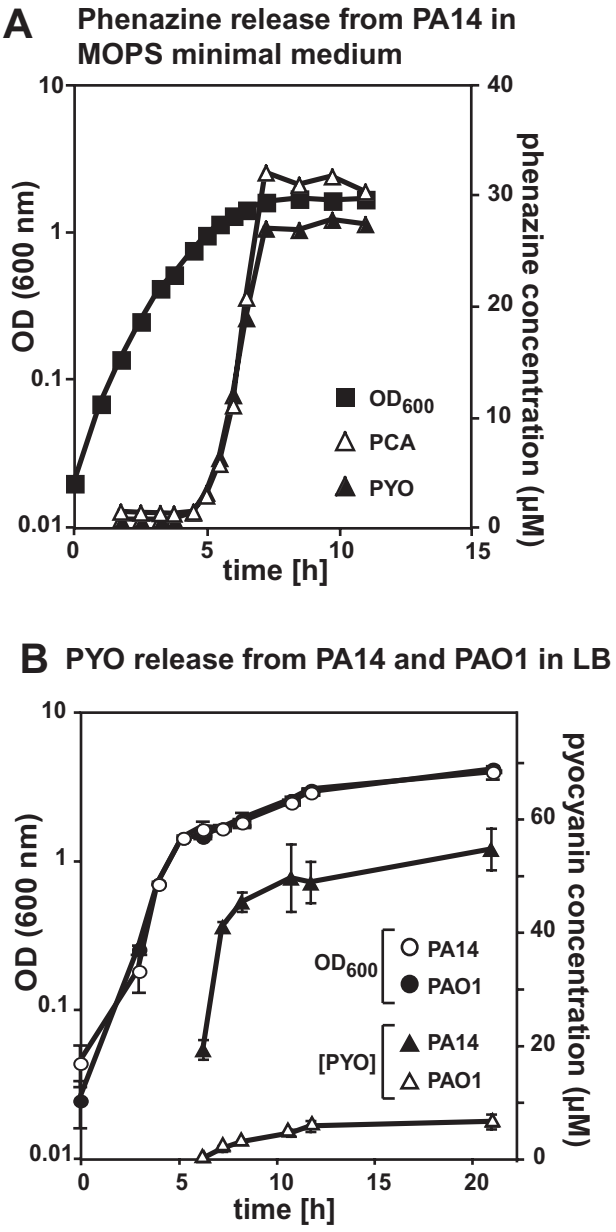
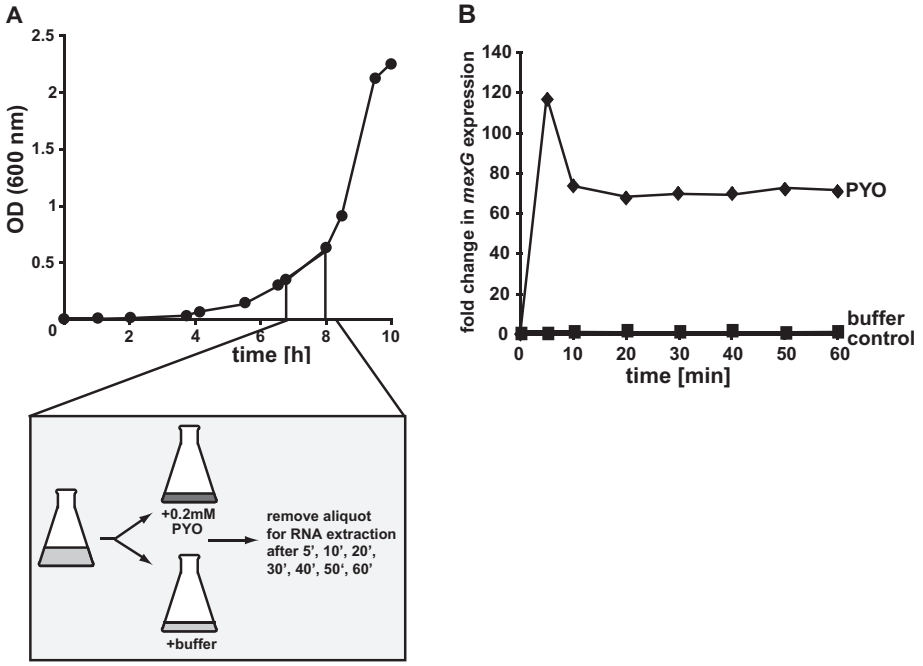


Fig. 2. PA14 and PAO1 release phenazines in early stationary phase.
A. PA14 grown in MOPS minimal medium releases phenazines in early stationary phase. PA14 was grown aerobically in a MOPS minimal medium with 20 mM succinate. Concentrations of phenazines were determined by HPLC analysis of filtrates from culture samples. Data are representative of at least three independent cultures.
B. PAO1 and PA14 grown in LB release PYO in early stationary phase. PA14 and PAO1 were grown aerobically in 50 ml of LB at 37°C. At indicated times, 1 ml was removed for measurement of optical density at 600 nm and PYO concentrations. Error bars represent standard deviations ($n = 3$).

stationary phase cultures. Purified PYO was added to PA14 cultures in early exponential phase ($OD_{600} = 0.4$), well before the release of endogenous phenazines (Fig. 3A). Following the addition of PYO, the culture was incubated for 1 h, and total RNA was extracted, labelled, and hybridized to Affymetrix GeneChips arrayed with *P. aeruginosa* DNA representing the total genome. Pairwise comparison of gene expression in the absence or presence of PYO was carried out using Affymetrix Microarray Suite software. Table 1 lists all genes that were up- or downregulated more than twofold in two independent experiments.

A number of features are striking: (i) PYO affects expression of only a limited number of genes. Twenty-two genes are upregulated, eight of which encode putative transporters [i.e. the RND transporters *mexGHI-opmD* (PA4205-4208), PA3923-3922 (*mexEF* homologues)-*opmE* and the putative Major Facilitator Superfamily (MFS) transporter PA3718]. Twenty-nine genes are downregulated, of which 16 are annotated as hypothetical proteins. Seven of the remaining 13 genes are implicated in ferric iron transport. (ii) The downregulated gene cluster PA5498-5500 encodes the transcriptional regulator np20 and proteins with similarity to zinc transporters. Interestingly, mutation of np20 was reported to abrogate PQS production, presumably due to a more than 10-fold decrease in *pqsH* expression (Gallagher *et al*., 2002). This suggests that PYO might inhibit PQS production. (iii) The most upregulated genes, PA2274 (a putative monooxygenase) and the *mexGHI-opmD* operon, had previously been found to be downregulated in an *mvfR* mutant in PA14 (Deziel *et al*., 2005). It is thought that MvfR controls gene expression by regulating the production of PQS and PqsE (Deziel *et al*., 2005). Among the genes controlled by MvfR are *phzM*, *phzS* and the operon *phzA1-G* (Deziel *et al*., 2005). The requirement of PQS and PqsE has been well established for expression of *phzA1-G1* (Diggle *et al*., 2003; Deziel *et al*., 2005). Based on these findings it could be assumed that *phzA1-G1* and *mexGHI-opmD* are part of the same, PQS/PqsE-responsive, regulon. However, it had also been noticed that while the *phz* operon was already expressed in (late) exponential phase, the expression of *mexGHI-opmD* occurred only in stationary phase of both PAO1 (Whiteley *et al*., 1999) and PA14 (Deziel *et al*., 2005). Our results may explain this finding: PQS signals phenazine production, which in turn upregulates PA2274 and *mexGHI-opmD*. This scenario predicts a fast gene expression response of *mexGHI-opmD* and PA2274 to PYO. To evaluate this possibility, we added PYO to an exponential phase culture of PA14 and followed *mexG* expression over 1 h by quantitative reverse transcription PCR (Q-RT-PCR) (Fig. 3B). In contrast to the significant lag between expression of *phz1* expression (which is controlled by PQS) and of *mexGHI-opmD* (Whiteley *et al*., 1999; Deziel *et al*., 2005), addition of



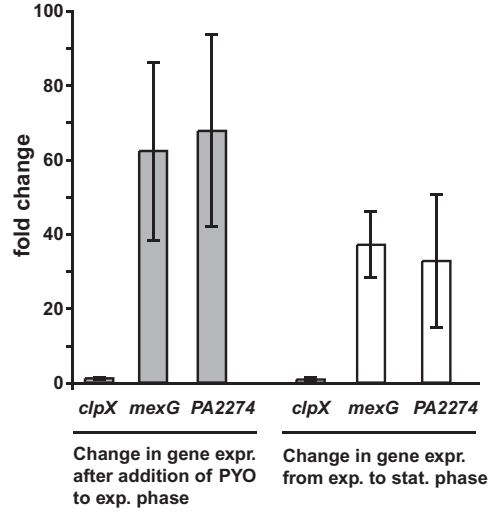


Fig. 3. A and B. PYO triggers a rapid response of mexG expression. PA14 was grown to OD_{600} = 0.4 (exponential phase) in 100 ml of MOPS minimal medium supplemented with 20 mM succinate and split into two flasks. To one flask 0.2 mM PYO was added, to the other flask just buffer. After incubation for 5, 10, 20, 30, 40, 50 and 60 min, aliquots (3 ml) were removed for RNA extraction. *mexG* and *clpX* (control) expression were monitored by Q-RT-PCR. *mexG* expression levels were normalized to the constitutively expressed *clpX*. Changes in *mexG* expression are shown. The same results were obtained after normalizing *mexG* expression to the constitutively expressed gene *recA* (not shown).
C. PYO-dependent upregulation of gene expression in PAO1. PAO1 was grown aerobically in 60 ml of LB at 37°C to OD_{600} = 0.3 (early exponential phase) and split into two flasks. To one flask 80 μM PYO were added, to the other flask just buffer. After incubation for 1 h at 37°C (OD_{600} = 0.5; exponential phase) 3 ml of aliquots was removed from both flasks for RNA extraction. Growth of the untreated culture was continued to an OD_{600} = 2.0 (stationary phase), then another 3 ml of aliquot was removed for RNA extraction. After preparation of cDNA, Q-RT-PCR was performed to analyse expression of *mexG*, PA2274 and the constitutively expressed *clpX* and *recA* (controls). All expression levels were normalized to *recA* expression. Changes in gene expression after addition of PYO to exponential phase cultures are shown by the grey bars. Changes in gene expression from exponential to stationary phase are shown by the white bars. PYO addition experiments to exponential phase cultures; white bars represent the difference. The data represent the average of biological triplicates and error bars indicate the standard deviation.

Table 1. Exogenous PYO elicits a specific gene response in PA14.

PA number	Fold change	Gene/annotation
Genes upregulated after addition of PYO to PA14 in exponential phase		
PA4205	36.8	*mexG*
PA4206	43.7	*mexH*
PA4207	30.9	*mexI*
PA4208	20.0	*opmD*
PA2274	30.4	Putative monooxygenase
PA3521	3.4	*opmE*
PA3522	6.3	76% similar to *mexF* (PA4294)
PA3523	6.2	60% similar to *mexE* (PA4293)
PA3515	5.3	Hypothetical protein
PA3516	5.4	Probable lyase
PA3517	5.5	Probable lyase
PA3518	6.6	Hypothetical protein
PA3519	5.9	Hypothetical protein
PA3920	4.2	Probable P-type ATPase
PA3718	3.9	Probable MFS transporter
PA3520	3.4	Hypothetical protein
PA3721	2.9	Probable transcriptional regulator
PA2275	2.8	Probable alcohol dehydrogenase
PA3720	2.7	Hypothetical protein
PA4878	2.3	Probable transcriptional regulator
PA3531	2.1	*bfrB* (bacterioferritin)
PA2847	2.1	Hypothetical protein
Genes downregulated after addition of PYO to PA14 in exponential phase		
PA3600	–20.4	Hypothetical protein
PA3601	–9.0	Hypothetical protein
PA4063	–7.6	Hypothetical protein
PA0781	–6.7	Putative haemin receptor
PA2386	–6.7	*pvdA* (L-ornithine N5-oxygenase)
PA2398	–6.7	*fpvA* (ferripyoverdine receptor)
PA2845	–6.7	Hypothetical protein
PA5532	–6.7	Hypothetical protein
PA4834	–6.6	Hypothetical protein
PA4835	–6.5	Hypothetical protein
PA4836	–6.2	Hypothetical protein
PA4837	–9.7	Putative ferrichrome iron receptor
PA4514	–5.8	*piuA*, probable receptor for iron transporter
PA1924	–5.1	Hypothetical protein
PA4064	–4.3	Probable component of ABC transporter
PA4065	–3.1	Hypothetical protein
PA4066	–3.0	Hypothetical protein
PA1921	–3.1	Hypothetical protein
PA1925	–3.0	Hypothetical protein
PA4133	–3.0	Cytochrome *c* oxidase subunit
PA5531	–3.0	*tonB*
PA4838	–2.7	Hypothetical protein
PA4134	–2.6	Hypothetical protein
PA4170	–2.6	Hypothetical protein
PA4515	–2.4	*piuC*, putative iron-uptake factor
PA1922	–2.4	Probable TonB-dependent receptor
PA5498	–2.0	*znuA* (ABC-type Zn^{2+} transport)
PA5499	–2.5	np20 (transcriptional regulator)
PA5500	–2.0	*znuC* (zinc transport protein ZnuC)

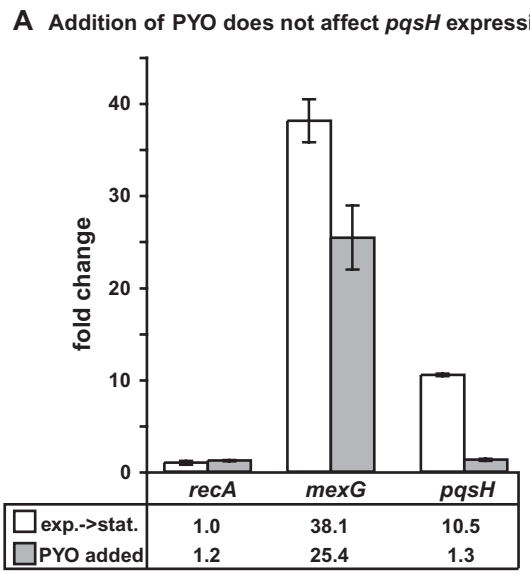
Cells were grown to $OD_{600} = 0.4$ (exponential phase) in MOPS minimal medium supplemented with 20 mM succinate and split into two flasks. PYO, purified from stationary phase cultures of PA14, was added to one flask to a final concentration of ~0.2 mM and buffer was added to the other as a control. The cells were incubated for 1 more hour at 37°C before RNA was harvested. The effects on gene expression were monitored by microarray analysis using Affymetrix GeneChips. Shown are genes that were up- or downregulated twofold or more in two independent experiments.

PYO induced a rapid response of *mexG* expression (< 5 min), suggesting that the corresponding operon is under the direct control of PYO. Similarly, addition of PYO to exponential phase cultures of PAO1 induced expression of *mexG* and PA2274 (Fig. 3C).

Because quinolones (such as PQS) are not made in early exponential phase (Diggle *et al*., 2003; Lepine *et al*., 2003) and our array did not reveal PYO-induced upregulation of upstream QS genes, our data suggested that PYO alone, rather than PQS, is a signal for *mexGHI-opmD* and PA2274 expression. To directly test this, we first ruled out the possibility that PQS production was induced by PYO by measuring the expression of *pqsH*, which encodes the last enzyme in the biosynthetic pathway for PQS (Gallagher *et al*., 2002), in the presence or absence of PYO in exponential phase. As expected, no significant difference in expression of *mexG* (one of the strongly upregulated genes from the array) was observed; in contrast *pqsH* expression significantly increased from exponential to stationary phase (Fig. 4A). Moreover, we still observed significant increases in *mexG* and PA2274 expression in a *pqsH* deletion mutant background when PYO was added to exponential phase cultures (Fig. 4B).

Physiological upregulation of genes in the PYO regulon in stationary phase is dependent on phenazine biosynthesis

If PYO were the physiological signal for the expression of genes identified in the array (Table 1), we would expect that these genes would be similarly regulated during stationary phase (when phenazines are released; Fig. 2) and that this response would require the production of phenazines. To test this, we deleted both *phz* operons in PA14 to prevent phenazine production (see *Experimental procedures*, Figs 1 and 5A). We verified the absence of phenazines by HPLC. The double mutant (Δ*phz1/2*) did not show a growth defect (Fig. 5B), ruling out any significant pleiotropic effects. We then used Q-RT-PCR to compare Δ*phz1/2* and wild-type cultures for their ability to regulate expression of PYO-controlled genes in stationary phase compared with exponential phase. For this we chose to follow the expression of genes that were strongly (> 35×) and weakly (<10 ×) upregulated (*mexGH* and PA2275 respectively) in the array (Table 1). We grew PA14 WT and PA14 Δ*phz1/2* in rich medium, removed aliquots during exponential and stationary phase, and extracted total RNA for Q-RT-PCR analysis. *mexG, mexH* and PA2275 were upregulated in stationary phase (Fig. 5C), and their relative expression levels corresponded to their response to exogenously added PYO (Table 1). Similar results were found for PAO1 (Fig. 3C). In contrast, absence of phenazine production (Δ*phz1/2*) prevented the increased expression of *mexG*, *mexH* and PA2275 in stationary phase. These



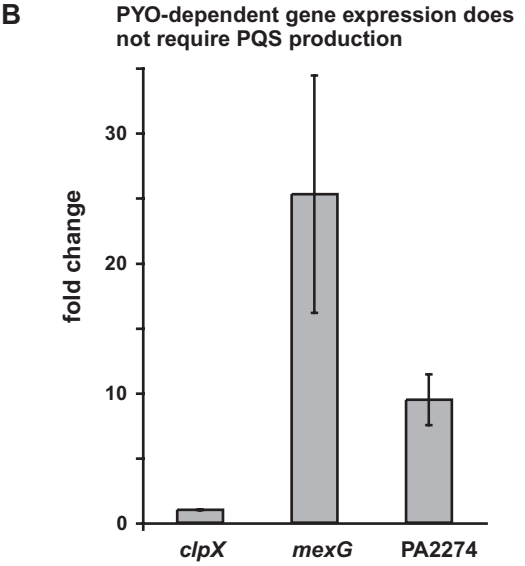


Fig. 4. PYO regulates gene expression in a PQS-independent manner.
A. PYO addition to PA14 does not affect *pqsH* expression. PA14 was grown and treated as described for Fig. 3C. Q-RT-PCRs were performed to analyse expression of *pqsH* and the constitutively expressed *clpX* and *recA* (controls). All expression levels were normalized to *clpX* expression. Changes in gene expression from exponential to stationary phase (white bars) and in response to PYO addition in exponential phase (grey bars) are shown. The data represent the average of biological triplicates and error bars indicate the standard deviation.
B. PYO-dependent gene expression does not require PQS production. PA14 Δ*pqsH* was grown aerobically in 60 ml of LB at 37°C to OD_{600} = 0.3 (early exponential phase) and split into two flasks. To one flask 80 μM PYO was added, to the other flask just buffer. After incubation for 40 min at 37°C (OD_{600} = 0.5; exponential phase) 3 ml were removed from both flasks for RNA extraction. Q-RT-PCRs were performed to analyse expression of *mexG*, PA2274 and the constitutively expressed *clpX* and *recA* (controls). All expression levels were normalized to *recA* expression. Changes in gene expression in response to PYO-addition (grey bars) are shown. The data represent the average of biological triplicates.

results were supported by similar experiments with a mutant defective in PhzM production [PhzM encodes an enzyme necessary for the biosynthesis of PYO (Mavrodi *et al*., 2001; 2006)] (Fig. S1). We conclude that phenazines are necessary for the physiological induction of downstream genes in the *P. aeruginosa* QS network.

PYO activates the transcription factor SoxR

It was recently demonstrated that *P. aeruginosa* PAO1 cells upregulate *mexGHI-opmD*, PA2274 and PA3718 upon exposure to paraquat (Kobayashi and Tagawa, 2004; Palma *et al*., 2005). All three genes/operons are also upregulated in the presence of PYO, suggesting that PYO and paraquat (a.k.a. 'methyl viologen', a small, redox active compound structurally similar to phenazines) affect the same transcriptional regulator. The response to paraquat is mediated through PA2273, which is homologous to *soxR* in *Escherichia coli* (Kobayashi and Tagawa, 2004). SoxR activates genes containing a specific upstream binding site (Sox box). In *P. aeruginosa* unequivocal Sox boxes are only found upstream of *mexGHI-opmD*, PA2274 and PA3718 (Kobayashi and Tagawa, 2004; Palma *et al*., 2005). To test if PYO-dependent upregulation of these genes is mediated by SoxR, we deleted PA2273 (*soxR*) and used Q-RT-PCR to determine changes in gene expression from exponential to stationary phase (Fig. 6). PYO-controlled genes lacking a Sox box, such as PA3920 and the *oprN* homologue *opmE* (PA3521) were not affected by the *soxR* deletion. In contrast, upregulation of *mexG* and PA2274 in stationary phase was lost in the absence of SoxR (Fig. 6). This differential effect on PYO-regulated genes not only establishes PYO as an endogenous activator of the SoxR regulon, but also demonstrates that additional transcription factor(s) must be involved in the PYO response for regulation of genes lacking a Sox box. The nature of these factors remains to be elucidated.

SoxR activation by PYO does not rely on superoxide generation

Previous work with *E. coli* has established that SoxR binds DNA as a homodimer, each monomer containing a [2Fe-2S] cluster (Gaudu *et al*., 1997; Hidalgo *et al*., 1997). These clusters are kept in a reduced state, rendering SoxR inactive. Only upon their oxidation does SoxR get activated (Ding *et al*., 1996; Gaudu and Weiss, 1996). A much-discussed mechanism of SoxR activation is the generation of superoxide, which can directly oxidize the [2Fe-2S] clusters. However, the necessity to generate superoxide for SoxR activation *in vivo* has been questioned. Given that PYO is a redox active compound with a standard redox-potential of –34 mV at pH 7 (Price-

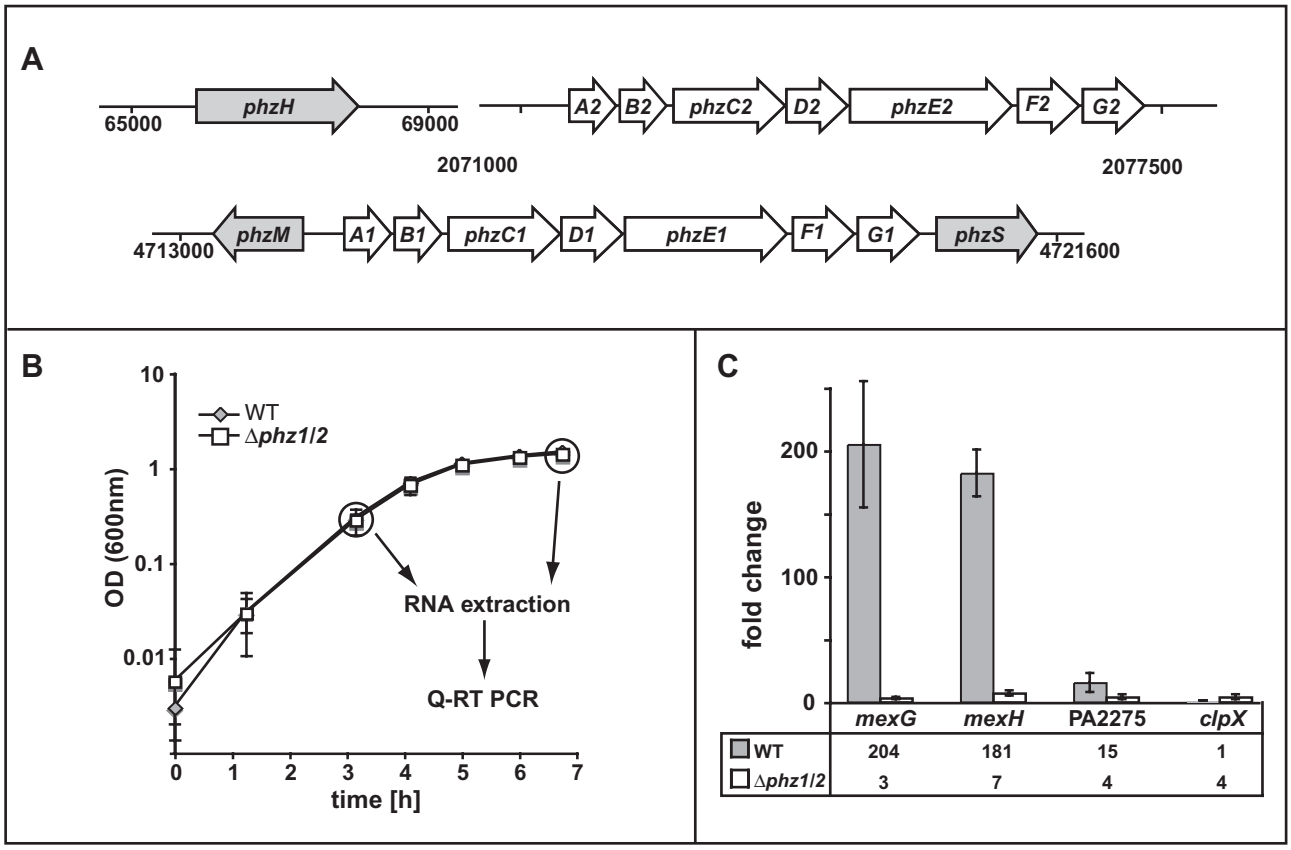


Fig. 5. Upregulation of the PYO regulon in stationary phase is dependent on phenazine production.
A. Genomic localization of *phz* genes in *P. aeruginosa*. White: the core operons *phzA1-G1* and *phzA2-G2*, which are responsible for PCA synthesis. Grey: monocistronic genes encoding for the phenazine-decorating enzymes PhzM, PhzS and PhzH.
B and C. PA14 WT and PA14 *ΔphzA1-G1/phzA2-G2* (*Δphz1/2*) were grown aerobically in 50 ml of LB at 37°C. At OD_{600} = 0.4 (exponential phase) or OD_{600} = 1.4 (stationary phase), 4 ml were removed for RNA extraction. After preparation of cDNA, Q-RT-PCR was performed to analyse expression of *mexG*, *mexH*, PA2275 and the constitutively expressed *clpX* and *recA* (controls). All expression levels were normalized to *recA* expression. Changes in gene expression from exponential to stationary phase are shown. The data represent the average of biological triplicates and error bars indicate the standard deviation.

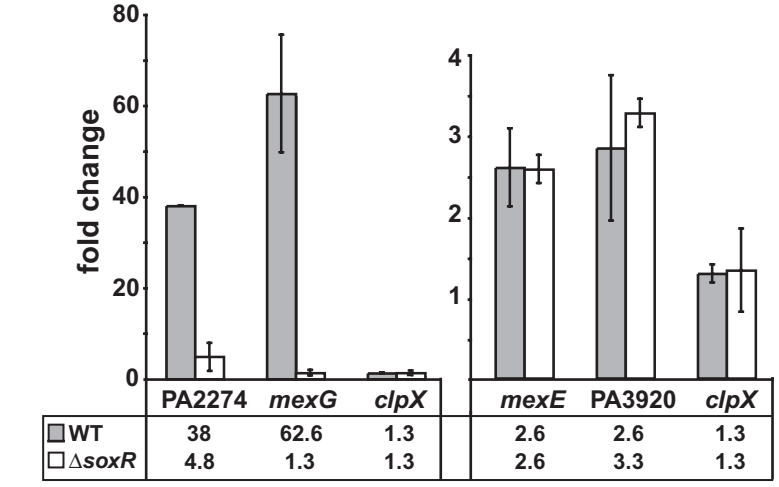


Fig. 6. Upregulation of a subset of the PYO-regulated genes is mediated by the transcription factor SoxR. PA14 and PA14 *ΔsoxR* were grown aerobically in LB to OD_{600} = 0.4 (exponential phase) or OD_{600} = 1.4 (stationary phase). RNA was extracted, cDNA was prepared, and Q-RT-PCR was performed to analyse expression of *mexG*, *mexH*, *mexE*, PA3920 and the constitutively expressed *clpX* and *recA* (controls). All expression levels were normalized to *recA* expression. Changes in gene expression from exponential to stationary phase are shown. The data represent the average of biological triplicates and error bars represent the standard deviation.

Whelan *et al*., 2006), we hypothesized that PYO might be able to activate SoxR [whose midpoint potential is −285 mV (Ding *et al*., 1996; Kobayashi and Tagawa, 2004)] in a superoxide-independent manner. To address this, we grew PA14 under strictly anaerobic conditions with nitrate as electron-acceptor. In exponential phase we added PYO that was dissolved in anoxic medium, incubated the culture for an additional hour, and determined expression of *mexG* and *mexH* (SoxR-regulated) and PA2275 (not SoxR-regulated) by Q-RT-PCR. As Fig. 7 shows, PYO was able to upregulate expression of *mexG* and *mexH*. In agreement with our results under aerobic conditions (Table 1, Fig. 5), upregulation of *mexGH* was still more than five times higher compared with changes in expression of PA2275. This strongly suggests that PYO can activate SoxR in a superoxide-independent manner, and implies that the SoxR regulon does not provide a defence against superoxide *per se* in *P. aeruginosa*. Consistent with this, we observe no growth defect of a *soxR* deletion mutant in aerobic PA14 cultures (Fig. S2), confirming the findings of Palma *et al*. (2005) in PAO1.

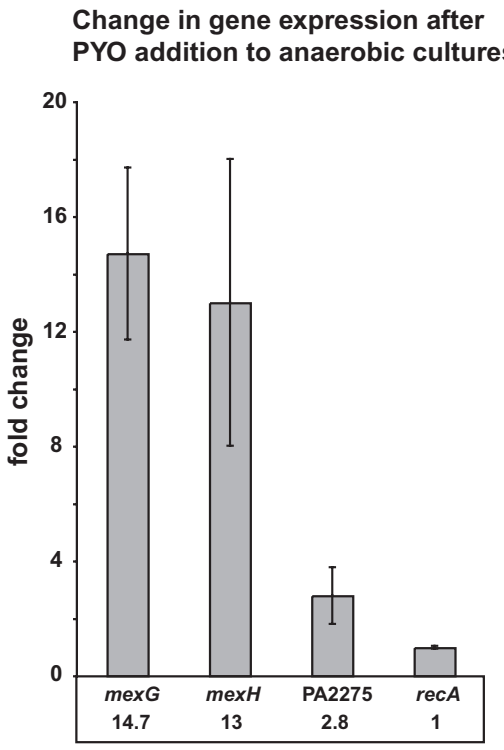


Fig. 7. PYO activates the SoxR regulon under anaerobic conditions. PA14 was grown anaerobically in minimal medium supplemented with 20 mM succinate (carbon source) and 100 mM potassium nitrate (electron acceptor) at 37°C. At OD_{600} = 0.4 (exponential phase) 0.2 mM PYO (in anaerobic minimal medium) were added. After 1 h incubation RNA was extracted, cDNA was prepared, and Q-RT-PCR was performed to analyse expression of *mexG*, *mexH*, PA2275 and the constitutively expressed *recA* and *clpX* (control), to which expression levels were correlated. The changes in gene expression from PYO-treated compared with buffer-treated cultures are shown. The data represent the average of biological triplicates and error bars indicate the standard deviation (except for PA2275 and *recA*; $n = 2$).

Discussion

In this study, we have shown that the secreted phenazine PYO, commonly thought of as a virulence factor, is a signalling molecule for *P. aeruginosa* strains PA14 and PAO1. PYO is both necessary (Fig. 5) and sufficient (Table 1, Fig. 3C) to upregulate the expression of a limited set of genes that we now designate the PYO stimulon. Within this stimulon are genes that appear to be involved in efflux and redox processes, as well as iron acquisition. *MexGHI-opmD* and PA2274, which are the most upregulated genes in the PYO stimulon, were previously found to be regulated by the QS signalling molecule PQS and PqsE (Deziel *et al*., 2005). PQS also controls expression of the *phz* operon (Gallagher *et al*., 2002; Deziel *et al*., 2004), which encodes the machinery responsible for phenazine synthesis (Mavrodi *et al*., 2001). Our results demonstrate that it is the production of phenazines (specifically PYO) that links PQS to *mexGHI*-opmD and PA2274 expression. This establishes PYO as a terminal physiological signal in the QS circuitry (Fig. 8).

The subset of the PYO stimulon that is most strongly upregulated maps directly to a set of genes whose expression had previously been shown to be induced by the redox-active compound paraquat (i.e. methyl viologen) (Palma *et al*., 2005). Our results provide genetic evidence that the transcription factor SoxR is responsible for activating this subset. Although the mechanism of PYO activation of SoxR remains to be elucidated, we find that such activation occurs under strictly anaerobic conditions. SoxR nitrosylation by nitric oxide has been demonstrated to be a mechanism for its activation (Touati, 2000), but this may not explain our findings because PYO is known to inactivate nitric oxide (Warren *et al*., 1990). Consistent with this, nitric oxide metabolite concentrations appear to be lower in the sputum of cystic fibrosis patients relative to the endotracheal secretions of healthy individuals (Grasemann *et al*., 1998); intriguingly, significant phenazine production is also known to occur in sputum (Palmer *et al*., 2005). Taken together, our data and these observations suggest that PYO can directly activate SoxR, and we are currently working to test this hypothesis.

Recognizing that PYO can activate SoxR under anaerobic conditions prompts us to re-evaluate the conventional wisdom that SoxR responds to superoxide stress. Historically, most studies of SoxR have been performed in *E. coli*. In this organism, SoxR has only one target gene, *soxS*. It is positioned next to *soxR* and encodes a transcription factor that turns on more than 40 genes, many of which are of crucial importance for *E. coli*'s oxidative stress response to superoxide (Pom-

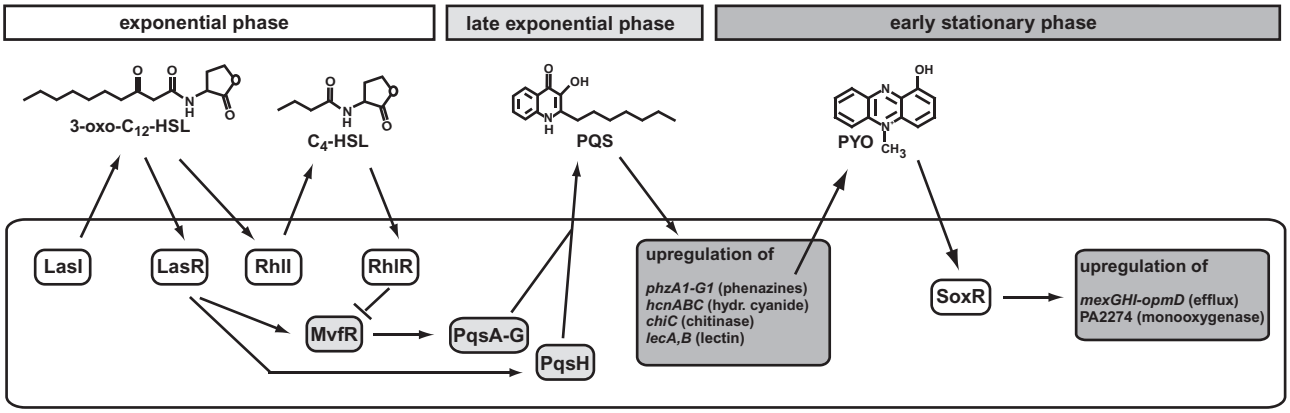


Fig. 8. Model of the QS network in *P. aeruginosa* PA14. The PA14 QS network comprises a cascade of three types of signalling molecules that function in a growth stage-dependent manner. In exponential phase LasI and LasR synthesize the AHLs 3O-C_{12}-HSL and butanoylhomoserine lactone (C_4-HSL) respectively, which in turn induce gene expression by binding to their respective transcriptional activators, LasR and RhlR. This results in the production and release of PQS, which is required for the synthesis of phenazines. The phenazine PYO activates the transcription factor SoxR, thereby upregulating the expression of *mexGHI-opmD* and PA2274 (modified from Price-Whelan *et al*., 2006).

posiello *et al*., 2001). As a consequence, mutations in *E. coli soxR* mutants confer increased sensitivity to superoxide generating compounds. *Pseudomonas* lacks a *soxS* homologue, however, and SoxR from *P. putida* and *P. aeruginosa* does not regulate genes involved in the oxidative stress response (Kobayashi and Tagawa, 2004; Palma *et al*., 2005; Park *et al*., 2006). Because deleting *soxR* does not significantly affect viability of *P. aeruginosa* PAO1 (Palma *et al*., 2005) and PA14 (Fig. S2), this argues for the existence of a SoxR-independent oxidative stress response. A recent observation by Demple and co-workers fits these findings (Park *et al*., 2006). After deleting *soxR* in *P. putida* KT2240 they found that this mutant remained as resistant to the superoxide-generating compound paraquat as the wild-type strain.

If SoxR does not induce an oxidative stress response in *P. aeruginosa*, then what is the function of its regulon? The six genes controlled by SoxR comprise the putative monooxygenase (PA2274) and two transporters, i.e. the RND efflux pump MexGHI-OpmD and the MFS transporter PA3718. Based on sequence homology, PA2774 groups with ActVA-Orf6 from *Streptomyces coelicolor*, a monooxygenase that binds to and oxygenates actinorhodin, an aromatic polyketide similar in structure to phenazines (Sciara *et al*., 2003). Interestingly, PhzS, the enzyme that participates in the conversion of PCA to PYO, is a monooxygenase as well (Mavrodi *et al*., 2001). We therefore hypothesize that PA2274 might recognize phenazines, allowing it to either (i) execute its putative enzymatic activity, (ii) act as a competitor against the monooxygenase PhzS, (iii) function as a chaperone, shielding the cellular environment from the toxic phenazines, or (iv) sense phenazines, thereby affecting gene expression. MexGHI-OpmD is one of 10 potential RND pumps in *P. aeruginosa* (Stover *et al*. 2000). It has recently been postulated that this efflux pump is required for the excretion of a PQS precursor (Aendekerk *et al*., 2002; 2005). In this respect it is striking that expression *of mexGHI-opmD* increases in response to phenazines (Table 3), which act downstream of PQS. We hypothesize that MexGHI-OpmD is not only implicated in the release of a toxic PQS precursor (Aendekerk *et al*., 2005), but potentially also in the efficient shuttling of phenazines (possibly in conjunction with the putative MFS transporter PA3718).

Phenazines and quinolones for a long time have been recognized for their antimicrobial activities. The fact that the quinolone PQS and now the phenazine PYO can act as intercellular signals underscores the possibility that 'secondary' metabolites may in fact be of primary importance in tuning the cell's response to particular physiological states. Indeed, a layered control network, such as the QS cascade in *P. aeruginosa*, appears to be optimally designed to permit this for a population. For example, phenazine production initiates under conditions of oxidant limitation. Once phenazines are produced, they can react with oxidized species other than molecular oxygen, such as ferric iron oxides, thus facilitating iron acquisition by reducing it to ferrous iron (Cox, 1986; Hernandez *et al*., 2004). It is therefore not surprising that a phenazine controls the expression of genes that appear to be involved in iron acquisition and redox homeostasis, as well as genes that likely control their own processing (*mexGHI-opmD).* We predict that this is but one of many cases where secondary metabolites will prove to have functions that transcend their antibiotic activities and enable the co-ordinated response of microbial communities to changes in their environment.

Table 2. Bacterial strains and plasmids used in this study.

Strains/plasmids	Characteristics	Source or reference
P. aeruginosa		
PA14	Clinical isolate UCBPP-PA14	Rahme *et al*. (1995)
PA14 Δ*phz1/2*	PA14 with deletions of operons *phzA1-G1* and *phzA2-G2*	This study
PA14 Δ*soxR*	PA14 with a deletion in *soxR*	This study
PA14 Δ*pqsH*	PA14 with an insertion in *pqsH*; Gm^R	Mashburn and Whiteley (2005)
PA14 *phzM*	PA14 mutant with a Gm^R cassette inserted into PA4209 (*phzM*)	Gift from P. Cornelis, Brussels, Belgium
PAO1	Wild-type	Holloway *et al*. (1979)
E. coli		
UQ950	*E. coli* DH5α λ(pir) host for cloning; F-Δ(*argF-lac)169 Φ80 dlacZ58*(ΔM15) *glnV44*(AS) *rfbD1 gyrA96*(Nal^R) *recA1 endA1 spoT1 thi-1 hsdR17 deoR* λ*pir*+	D. Lies, Caltech
WM3064	Donor strain for conjugation: *thrB*1004 *pro thi rpsL hsdS lacZ* ΔM15RP4–1360 Δ(*araBAD*)*567* Δ*dapA*1341::[*erm pir*(wt)]	W. Metcalf, University of Illinois
Plasmids		
pSMV10	9.1 kb mobilizable suicide vector; *oriR6K*, *mobRP4*, *sacB*, Km^R Gm^R	D. Lies, Caltech
pΔ*phzA1-G1*	2 kb fusion PCR fragment containing Δ*phzA1-G1* cloned into the SpeI site of pSMV10; used to make the strain PA14 Δ*phz1/2*	This study
pΔ*phzA2-G2*	2 kb fusion PCR fragment containing Δ*phzA2-G2* cloned into the SpeI site of pSMV10; used to make the strain PA14 Δ*phz1/2*	This study

Km, kanamycin; Gm, gentamicin.

Experimental procedures

Strains and plasmids

Bacterial strains and plasmids that were used or made in this study are listed in Table 2.

Growth conditions

All strains were grown aerobically at 37°C unless otherwise specified. LB medium was used for routine culturing. For certain experiments, *P. aeruginosa* was grown in morpholinepropanesulphonic acid (MOPS)-buffered medium [50 mM MOPS (pH 7.2), 93 mM NH_4Cl, 43 mM NaCl, 3.7 mM KH_2PO_4, 1 mM $MgSO_4$ and 3.5 μM $FeSO_4{\cdot}7H_2O$] with 20 mM succinate (Palmer *et al*., 2005).

Construction of mutants

We generated unmarked deletions of PA2273 (*soxR*) and of the two redundant phenazine biosynthetic operons *phzA1-G1* and *phzA2-G2* in PA14. To make the double mutant *ΔphzA1-G1*/Δ*phzA2-G2*, we first deleted *phzA1-G1* and then *phzA2-G2*. Here we describe the protocol for generating the unmarked deletion of *phzA1-G1*: The 5′ region (~1 kb in length) of the sequence flanking *phzA1* was amplified using the primer pair #1 and the 3′ region (~1 kb in length) of the sequence flanking *phzG1* with primer pair #2 (Table 2). These flanking DNA fragments were joined using overlap extension PCR. The resulting PCR product, containing a deletion of *phzA1-G1*, was cloned into a unique SpeI site in the mobilizable plasmid pSMV10. pSMV10 is a suicide plasmid for PA14 and contains an *oriR6K* origin of replication that does not function in PA14 but replicates in *E. coli* strains containing the *pir* gene; a gentamicin-resistance gene (*aacC1*); an *oriT* from RP4 that allows for mobilization by *E. coli* strains carrying RP4-derivatives on their chromosome (*E. coli* WM3064); and the counterselectable *sacB* gene. The resulting deletion plasmid was transformed into *E. coli* WM3064 and mobilized into PA14 using biparental conjugation (Whiteley *et al*., 1999). PA14 single recombinants (merodiploid containing the intact *phzA1-G1* operon and the deleted operon) were selected on LB agar containing gentamicin. Potential *phzA1-G1* deletion mutants were generated by selecting for a resolved merodiploid (double recombinant) by identifying strains that grew in the presence of 10% sucrose (these strains lost the *sacB*-containing plasmid because *sacB* is toxic in the presence of sucrose). Strains with properties of a double recombination were further analysed by PCR to determine if *phzA1-G1* has been deleted and one was selected. The deletion of *phzA2-G2* and *soxR* were made the same way, using primer pairs # 3 and 4, and #5 and 6 respectively (Table 3).

Quantification of phenazine derivatives

For cultures grown in MOPS medium, PYO and PCA concentrations were measured via HPLC analysis of culture filtrates (0.2 μm pore). Two hundred microlitres of filtrates from MOPS minimal medium cultures (aerobic cultures: 100 ml in a 500 ml Erlenmeyer flask) was loaded directly onto a Waters Symmetry C18 reverse-phase column (5 μm particle size; 4.6 × 250 mm) in a Beckman SystemGold set up with a photodiode array detector. Phenazines were separated in a gradient of water-0.01% TFA (solvent A) to acetonitrile-0.01% TFA (solvent B) at a flow rate of 1 ml min^{-1} in the following method: linear gradient from 0 to 15% solvent B from 0 to 2 min, linear gradient to 83% solvent B from 2 to 22 min, then a linear gradient to 0% solvent B from 22 to 24 min. The total method time was 25 min. Retention times for PYO and PCA averaged 10.933 and 19.918 respectively. Solutions of purified PYO and PCA were prepared at known concentrations based on their absorbance spectra at neutral pH [the extinction coefficient ε of PYO at 690 nm is 4130 M^{-1} cm^{-1} (Reszka *et al*., 2004); ε of PCA at 368 nm is 11 340 M^{-1} cm^{-1} (Trutko, 1989)].

Table 3. Primers used in this study.

	5′ primer	3′ primer
A. Primers used for *pSMV10* constructs		
pSMV10-ΔphzA1-G1		
# 1	GGactagtAGAACAGCACCATGTC	cccatccactaaatttaaataTGTACC
# 2	tatttaaatttagtggatgggCACCGCTACCTGCAAC	GCGactagtGGGT32TCTTCGATCACTAC
pSMV10-ΔphzA2-G2		
# 3	GCGactagtGCTGATCTGGAATGGCG	cccatccactaaatttaaataCAACCGTTGGTACTCTCG
# 4	tatttaaatttagtggatgggCACCGCTACCTGCAAC	GCGactagtGGGTTTCTTCGATCACTAC
pSMV10-ΔsoxR		
# 5	gcgACTAGTgatgcgcaggaattcttc	cccatccactaaatttaaataGGCCGCGAGCACGAC
# 6	tatttaaatttagtggatgggGGATGCGCAGGAATTCTTC	gcgACTAGTgcctcggtcacatgggc
B. Primers used for Q-RT-PCR		
Target gene		
PA1802 (*clpX*)	CCT GTG CAA TGA CAT CC	AGG ATG GTG CGG ATC TCT TT
PA2274	GAA CGC TGC TTC AGG AAC TG	GTG AAC GCC AGC AGG TGT AG
PA2275	GAC CAG GTG ATC CTT TCC AC	GTA GGG ATT GAG GTC GTG CT
PA2587 (*pqsH*)	CGG ATC GAG TTC ATC AGG A	CGA ACG AGG GTA TTC CTC AG
PA3617 (*recA*)	CTG CCT GGT CAT CTT CAT CA	ACC GAG GCG AGA ACT TCA G
PA3920	AGC GTC TAC CTC TGG CTG AC	AGG TAC TTG CCG AGG AGG AT
PA4205 (*mexG*)	AAC TCG CTC GAA AGC AAC TG	GCT GGC CTG ATA GTC GAA CA
PA4206 (*mexH*)	CAC CTC GGC CAG TAC CTC	GAC TGG TGC TTT CGT CCA G

All primers are written in 5′ to 3 ′ direction.

Aqueous solutions at approximately 0.1 mM phenazine were also loaded in 200 µl volumes onto the column and subjected to the same analysis as culture filtrates. System Gold 32 Karat Software was used to calculate the area under each peak in absorbance units in the 366 nm channel. Phenazine standards at known concentrations were used to calculate conversion factors for PYO and PCA and were 8×10^{-6} µM/AU and 9.5×10^{-6} µM/AU respectively.

For cultures grown in LB medium, cells were removed by centrifuging aliquots in spin columns (0.2 µm filter pore size) at 10 000 *g*. The concentration of PYO in the remaining filtrate was determined spectrophotometrically based on its absorbance at 690 nm.

Purification of PYO

Pyocyanin was prepared from late stationary phase cultures of PA14 grown in LB based on protocols established in Chang and Blackwood (1968) and Ingledew and Campbell (1969). Six hundred and fifty millilitres of cultures were grown in 2 l flasks, then split into two 500 ml centrifuge bottles and centrifuged for 10 min at 8000 *g*, at room temperature. Supernatants from PA14 cultures were then extracted twice with 250 ml chloroform. PYO was further purified by extraction into 2×250 ml 0.01 N HCl, and extracted again into 2×250 ml chloroform. The organic phase was collected and evaporated to obtain a volume of approximately 5 ml and loaded onto a Waters Sunfire C_{18} Prep column (5 micron particle size; 19×100 mm) in 1 ml of aliquots. Samples were subjected to the same solvent system and gradient described above, but at a flow rate of 10 ml min^{-1}, using the following method: 100% solvent A for 0–1 min, linear gradient to 70% solvent B from 1 to 10 min, then to 100% solvent B from 10 to 11 min, followed by a linear gradient to 100% solvent A from 11 to 12 min. The total method time was 15 min. Fractions containing PYO were collected using a Beckman SC100 fraction collector, and extracted into chloroform again as described above. They were then evaporated to dryness and resuspended in MOPS minimal medium to a final concentration of 5 mM.

Analysis of global gene expression using P. aeruginosa *Affymetrix GeneChips*

Pseudomonas aeruginosa was grown aerobically in MOPS minimal medium containing 20 mM succinate to an OD_{600} of 0.4, then 5 ml of culture was removed, mixed with 10 ml of Bacterial RNAProtect (Qiagen), and incubated for 5 min at room temperature before cells were pelleted (10 min, 5000 *g*). DNA-free RNA was isolated from the cell pellet using the RNAEasy Mini kit (Qiagen) including on-column DNase treatment. RNA integrity was monitored by gel electrophoresis of glyoxylated samples. Preparation of labelled cDNA and processing of *P. aeruginosa* GeneChip arrays were performed as previously described (Schuster *et al.*, 2003). Washing, staining and scanning of GeneChips were performed by the University of Iowa DNA Core Facility using an Affymetrix fluidics station. Gene-Chip arrays were performed in duplicate. Data were analysed using Microarray Suite software, and only genes exhibiting regulation levels of twofold or greater are reported. The GeneChip *P. aeruginosa* Genome Array (Affymetrix) contains probe sets for more than 5500 genes from *P. aeruginosa* PAO1, 199 probe sets corresponding to 100 intergenic sequences, and 117 additional genes from *P. aeruginosa* strains other than PAO1. Due to the strong similarity between strains PAO1 and PA14 the Array has been used for gene expression analysis of both strains (Whiteley *et al.*, 2001; Mashburn *et al.*, 2005). Verification of GeneChip data was performed with Q-RT-PCR. PA4205 (*mexG*), PA4206 (*mexH*) and PA2274 expression levels were examined using PA1802 (*clpX*) and PA3617 (*recA*) as constitutively expressed controls (primer sets given in Table 2).

Quantitative reverse transcriptase PCR

Pseudomonas aeruginosa was grown in LB medium or MOPS minimal medium containing 20 mM succinate to an OD_{600} of 0.4 (exponential phase) or 1.4–1.8 (stationary phase). One volume of culture was mixed with two volumes of Bacterial RNAProtect (Qiagen), incubated for 5 min at room temperature, and centrifuged for 10 min at 5000 *g*. Total RNA was extracted from the cell pellet using an RNAEasy Mini kit (Qiagen), according to the manufacturer's instructions, including the optional DNase treatment step. cDNA was generated using the extracted RNA as template for a Taqman (ABI Biosciences) random-primed reverse transcriptase reaction following the manufacturer's protocol. The cDNA was used as template for quantitative PCR (Real Time 7300 PCR Machine, Applied Biosystems) using the Sybr Green detection system (Applied Biosystems). Samples were assayed in triplicate. Signal was standardized to *recA* using the following equation: Relative expression $= 2^{(CT_{standard} - CT_{sample})}$, where CT (cycle time) was determined automatically by the Real Time 7300 PCR software (Applied Biosystems). Primers (Integrated DNA Technologies) for Q-RT-PCR were designed using Primer3 software (Rozen and Skaletsky, 2000). Criteria for primer design were a melting temperature of 60°C, primer length of 20 nucleotides, and an amplified PCR fragment of 100 base pairs.

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Supplementary material

The following supplementary material is available for this article online:

Figure S1. Characterization of PA14 *phzM*

Figure S2. Deletion of soxR does not affect planktonic PA14 growth.

This material is available as part of the online article from http://www.blackwell-synergy.com

