Differential Regulation of the Phenazine Biosynthetic Operons by Quorum Sensing in \textit{Pseudomonas aeruginosa} PAO1-N

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The \textit{Pseudomonas aeruginosa} quorum sensing (QS) network plays a key role in the adaptation to environmental changes and the control of virulence factor production in this opportunistic human pathogen. Three interlinked QS systems, namely \textit{las}, \textit{rhl}, and \textit{pqs}, are central to the production of pyocyanin, a phenazine virulence factor which is typically used as phenotypic marker for analysing QS. Pyocyanin production in \textit{P. aeruginosa} is a complex process involving two almost identical operons termed \textit{phzA}_1\textit{B}_1\textit{C}_1\textit{D}_1\textit{E}_1\textit{F}_1\textit{G}_1 (\textit{phz1}) and \textit{phzA}_2\textit{B}_2\textit{C}_2\textit{D}_2\textit{E}_2\textit{F}_2\textit{G}_2 (\textit{phz2}), which drive the production of phenazine-1-carboxylic acid (PCA) which is further converted to pyocyanin by two modifying enzymes PhzM and PhzS. Due to the high sequence conservation between the \textit{phz1} and \textit{phz2} operons (nucleotide identity > 98%), analysis of their individual expression by RNA hybridization, qRT-PCR or transcriptomics is challenging. To overcome this difficulty, we utilized luminescence based promoter fusions of each phenazine operon to measure in planktonic cultures their transcriptional activity in \textit{P. aeruginosa} PAO1-N genetic backgrounds impaired in different components of the \textit{las}, \textit{rhl}, and \textit{pqs} QS systems, in the presence or absence of different QS signal molecules. Using this approach, we found that all three QS systems play a role in differentially regulating the \textit{phz1} and \textit{phz2} phenazine operons, thus uncovering a higher level of complexity to the QS regulation of PCA biosynthesis in \textit{P. aeruginosa} than previously appreciated.

\textbf{IMPORTANCE}

The way the \textit{P. aeruginosa} QS regulatory networks are intertwined creates a challenge when analysing the mechanisms governing specific QS-regulated traits. Multiple QS regulators and signals have been associated with the production of phenazine virulence factors. In this work we designed experiments where we dissected the contribution of specific QS switches using individual mutations and complementation strategies to gain further understanding of the specific roles of these QS elements in controlling expression of the two \textit{P. aeruginosa} phenazine operons. Using this approach we have teased out which QS regulators have either indirect or...
direct effects on the regulation of the two phenazine biosynthetic operons. The data obtained highlight the sophistication of the QS cascade in *P. aeruginosa* and the challenges in analysing the control of phenazine secondary metabolites.

**Keywords:** *Pseudomonas aeruginosa*, phenazines, pyocyanin, quorum sensing, LasR, RhlR, RsaL, PqsE

**INTRODUCTION**

*Pseudomonas aeruginosa* is a highly adaptable bacterium, which can be found in a range of challenging environments, including the human host. This is achieved in great part by the ability of this opportunistic pathogen to finely control the expression of a wide range of genes, including those involved in the production of virulence determinants, in response to environmental and metabolic stimuli (Lee et al., 2006; Balasubramanian et al., 2013; Sun et al., 2016). The expression of many virulence genes in *P. aeruginosa* is also controlled in a cell density dependent manner by quorum sensing (QS) (Smith and Iglewski, 2003; Bjarnsholt and Givskov, 2007).

*P. aeruginosa* has a sophisticated QS network consisting of three separate but interwoven systems, namely *las, rhl*, and *pq* and their cognate QS signal molecules (QSMs). The QSMs *N*-3-oxo-dodecanoyl-homoserine lactone (*3OC_{12}-HSL*) produced by *LasI*, and *N*-butanoyl-homoserine lactone (*C_{4}-HSL*) produced by *RhlH* interact with their cognate transcriptional regulators *LasR* and *RhlR* respectively, leading to the activation or repression of multiple genes including the genes coding for their cognate signal synthases (Schuster et al., 2013). The *LasR/3OC_{12}-HSL* complex also induces the transcription of *rsal*, a gene integrated in the *las* QS system coding for the global transcriptional regulator *RsaL* (de Kievit et al., 1999). This protein directly represses the transcription of multiple genes, including *lasI*, hence exerting a homeostatic effect on *3OC_{12}-HSL* production, and conferring robustness to the expression of a sub-set of genes of the *las regulon* with respect to fluctuations in *LasR* levels (Rampioni et al., 2006, 2007; Bertani et al., 2007).

The *pq* QS system is more complex than the *las* and *rhl* systems, since multiple enzymes encoded by the *pqA-B-C-D-E-F* operon are required for the synthesis of 2-alkyl-4(1H)-quinolones (AQs) including the QSMs 2-heptyl-4-hydroxyquinoline (HHQ), which in turn is converted to 2-heptyl-3-hydroxy-4-quinolone (PQS) by the monoxygenase PqsH. Both HHQ and PQS can bind to and activate the transcriptional regulator PqsR (also known as MvfR). The PqsR/HHQ and PqsR/PQS complexes bind the PqsaA promoter region and increase the transcription of the *pqsA-B-C-D-E* operon, thus generating a feedback loop that accelerates AQ biosynthesis and increasing production of PqsE, coded by the last gene of the *pqsA-B-C-D-E* operon (Heeb et al., 2011; Dulcey et al., 2013). PqsE is a thioesterase involved in AQ biosynthesis (Drees and Fetzner, 2015) but this protein also controls indirectly the expression of multiple virulence factors even in the absence of AQs. The molecular mechanism by which PqsE impacts on QS target gene expression remains unknown (Hazar et al., 2010; Rampioni et al., 2010, 2016).

The QS circuit of *P. aeruginosa* has been widely reported to have a hierarchical structure. Under growth conditions using rich media, it is generally accepted that the *las* QS system is the first to become active leading to the activation of the *rhl* and *pq* systems (Pesci et al., 1997; de Kievit et al., 2002; Gallagher et al., 2002; Xiao et al., 2006). However it has been reported that *RhlR* can in part overcome the absence of the *las* system in late stationary phase (Dekimpe and Deziel, 2009). *RhlR* is required for production of certain virulence factors but has a negative impact on the *pq* system by repressing PQS signal production through interference with the expression of *pqsR* and *pqA-B-C-D-E* (McKnight et al., 2000; Wade et al., 2005; Xiao et al., 2006; Brouwer et al., 2014). In turn the *pq* system has a positive effect upon the *rhl* system, as addition of PQS to a *P. aeruginosa* culture has been shown to increase the levels of *RhlR* and the *rhl* QS signal *C_{4}-HSL* (McKnight et al., 2000; Diggle et al., 2003). The interactions of the QS systems are detailed in Figure S1.

QS has been shown to affect the transcription of hundreds of downstream genes (Schuster et al., 2003; Wagner et al., 2003; Rampioni et al., 2007, 2010) with some of these specifically controlled by distinct QS systems, while others are induced or repressed by multiple QS regulators (Schuster and Greenberg, 2007; Farrow et al., 2008; Cornforth et al., 2014; Rampioni et al., 2016).

The production of pyocyanin (PYO), a key virulence factor produced by *P. aeruginosa*, has been linked to multiple QS systems. This particular phenazine is often used as a marker to assess QS behavior as it is easily measurable and contributes significantly toward the green color of *P. aeruginosa* cultures (Frank and Demoss, 1959). Although PYO is the most studied phenazine in *P. aeruginosa*, this organism is capable of producing up to 5 different phenazine derivatives (Mavrodi et al., 2001, 2010). Phenazine biosynthesis begins with the conversion of chorismic acid to phenazine-1-carboxylic acid (PCA) by the action of the enzymes encoded by the biosynthetic operon *pzhA-B-C-D-E-F-G*, which is conserved across the fluorescent Pseudomonad species (Mavrodi et al., 2006, 2010). Interestingly *P. aeruginosa* has 2 functional copies of this operon designated *pz1* and *pz2*. Both operons produce PCA, which can be further converted to phenazine-1-carboxamide by the action of PhzH and to 1-hydroxyphenazine by PhzS. The action of PhzM is to convert PCA to 5-methylphenazine-1-carboxylic acid betaine, which can be further converted to PYO by PhzS (Mavrodi et al., 2001, 2006, 2010).

PYO production has been linked to QS in many reported studies and to date *LasR, RhlR, RsaL, PqsE*, *PqsR* and both AQ signal molecules HHQ and PQS have been found to play a role in the control of its production (Whiteley and Greenberg, 2001; Gallagher et al., 2002; Diggle et al., 2003; Schuster et al., 2003; Wagner et al., 2003; Rampioni et al., 2007, 2010; Farrow et al., 2007).
et al., 2008; Lu et al., 2009; Liang et al., 2011; Recinos et al., 2012; Cabeen, 2014; Sun et al., 2017). Although QS controls PYO production, the high sequence conservation between the two phenazine producing operons phz1 and phz2 have made analysing their individual expression by DNA hybridization techniques challenging (Schuster et al., 2003; Wagner et al., 2003; Rampioni et al., 2007, 2010).

It is unlikely that both phenazine biosynthesis operons are controlled in the same manner as they are located some distance apart on the PAO1 chromosome and have very different promoter regions (Mavrodi et al., 2001; Whiteley and Greenberg, 2001; Rampioni et al., 2007; Winsor et al., 2011). The phz1 operon (from PA4210 to PA4216) is flanked by phzM upstream (PA4209) and phzS downstream (PA4217), both of which are required to produce PYO. The phz2 operon (from PA1899 to PA1905) is flanked upstream by the qscR gene (PA1898), coding for the orphan QS receptor QscR, and downstream by the PA1906 gene, coding for a hypothetical protein of unknown function. The phzH1 gene (PA0051) is unlinked to the other phenazine biosynthetic genes (Figure S2). It would appear by looking at the positions of the operons on the chromosome of P. aeruginosa PAO1 that the phz1 operon is clustered with the genes required to produce PYO, and hence could be more closely associated with PYO production than phz2. That said, the phz2 operon has been shown to contribute significantly to the production of PYO, especially under non-planktonic growth conditions (Recinos et al., 2012; Dietrich et al., 2013).

There is a greater quantity of available information about the control of phz1 than phz2, and a lux box, for LasR or RhlR binding, has been predicted upstream of the −10 region of the phz1 promoter (PphzA1) (Whiteley and Greenberg, 2001). The QS repressor RsaL has also been shown to bind to this promoter in an electrophoretic mobility shift assay (EMSA) at the downstream end of the −10 promoter region, thus acting as a repressor of phz1 transcription (Rampioni et al., 2007). Moreover, RsaL exerts an indirect negative effect on phz1 transcription by increasing the production of the phz1 repressor protein CdpR (Sun et al., 2017).

Less is known about the regulation of the phz2 promoter (PphzA2). The intergenic region between qscR and phz2 was probed for RsaL binding by different groups with negative results (Rampioni et al., 2007; Sun et al., 2017), hence RsaL appears to control PphzA1 but not PphzA2. Recinos and colleagues found that phz2 transcription is induced by HHQ under anaerobic conditions (Recinos et al., 2012). Identification of a predicted ANR/DNR binding site within the PphzA2 promoter supports the notion that phz2 transcription is increased in anaerobic environments (Trunk et al., 2010). The orphan luxR QS regulator, QscR, which is encoded directly upstream of phzA2 has been reported to be a repressor of phzA2 (Ledgham et al., 2003; Lequette et al., 2006). The qscR and phz2 intergenic region was probed for QscR binding with a negative result (Lee et al., 2006) suggesting the effect of QscR on phzA2 is indirect due to the ability of QscR to form inactive heterodimers with LasR and RhlR (Chugani et al., 2001).

To gain a further understanding of the control of each phenazine biosynthesis operon by QS, lux-based promoter fusions for each operon were created and tested in a range of QS mutants. We identified an RsaL dependent switch, which can move PCA production from one operon to the other, and vice versa. This switching mechanism was confirmed by modification of the QS network activity in selected mutants with the addition of QS signal molecules and specific QS regulator genes expressed from plasmids. This allowed us to confirm the hierarchal structure of QS in rich media under planktonic conditions and to develop a more in depth model of how QS controls phz1 and phz2 transcription in P. aeruginosa.

**MATERIALS AND METHODS**

**Bacterial Strains and Growth Conditions**

The bacterial strains and plasmids used in this study are detailed in Table S1. They were routinely grown in Lysogeny Broth (LB) at 37°C with shaking at 200 rpm, with the exception of P. aeruginosa conjugation recipient strains, which were incubated at 42°C. When required, LB was supplemented with the following antibiotics: for *E. coli*, 10 µg ml⁻¹ tetracycline (Tc), 30 µg ml⁻¹ chloramphenicol (Cm), or 100 µg ml⁻¹ ampicillin (Ap); for *P. aeruginosa*, 150 µg ml⁻¹ Tc, 375 µg ml⁻¹ Cm or 800 µg ml⁻¹ streptomycin (Sm). Media were supplemented with 1 mM (final concentration) isopropyl β-D-1-thiogalactopyranoside (IPTG) for inducible strains where required, unless otherwise stated. Synthetic signal molecules PQS and 2-methyl-3-hydroxy-4-quinolone (mPQS) were added to cultures at a final concentration of 100 µM where required. To select for *P. aeruginosa* after mating experiments LB agar plates were supplemented with 15 µg ml⁻¹ nalidixic acid (Nal).

**DNA Manupulations**

All plasmids generated and/or used in this study are listed in Table S1. Routine DNA manipulations including extraction, restriction, ligation, electroporation, conjugation and agarose gel electrophoresis were performed using standard molecular methods (Sambrook and Russell, 2001). Plasmid extraction was completed using a Qiagen™ QiaQuick miniprep kit following the manufacturer’s instructions. The TcR marker of pMINI-CTX1 derived constructs integrated into the chromosome of *P. aeruginosa* was removed using the Flp recombinase system as previously described (Huang et al., 1998, 2000). All primers used for DNA amplification by PCR are detailed in Table S2. DNA sequencing was conducted at the University of Nottingham’s DNA sequencing facility.

**Generation of pPphzA1-lux, pPphzA2-lux, and pRsal Plasmids**

pMINI-lux was generated by cloning the BamHI-EcoRI fragment of pBluelux (Atkinson et al., 2008), containing the luxCDABE operon, into similarly digested mini-CTX1, using standard molecular methods (Sambrook and Russell, 2001). The PphzA1 and PphzA2 promoter regions were PCR amplified from *P. aeruginosa* PAO1 chromosomal DNA using primer pairs FWPPhza1-RVPPhza1, and FWPPhza2-RVPPhza2, respectively (Table S2). The PCR products were independently cloned into the pMINI-lux construct between the EcoRI and

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XhoI restriction sites, resulting in the plasmids pPphzA1-lux and PphzA2-lux, respectively.

The rsaL coding region was amplified by PCR from P. aeruginosa PAO1 chromosomal DNA using primers FWrsaL and RVrsaL (Table S2). The resulting PCR product was cloned into pME6032 between the EcoRI and XhoI restriction sites using standard molecular techniques. This plasmid was introduced to P. aeruginosa strains by electroporation (Choi et al., 2006).

All cloned fragments obtained by PCR were verified by DNA sequencing to match the reference sequences (Winsor et al., 2011).

### Generation of P. aeruginosa Mutant Strains

To generate the double mutant strain P. aeruginosa pqsE ΔlasR, the lasR gene was deleted from the chromosome of the PAO1 pqsEind strain (Rampioni et al., 2010) by using the pME3087-ΔlasR plasmid (Harrison et al., 2014).

Briefly, the pME3087-ΔlasR plasmid was mobilized by conjugation into the P. aeruginosa pqsEind recipient strain using E. coli S17.1 Δpir as a donor. Exconjugants were selected on LB plates supplemented with 150 μg ml⁻¹ Tc and 15 μg ml⁻¹ Nal. Strains were re-streaked twice on LB lacking antibiotic and then subjected to 1 round of Tc sensitivity enrichment to select for double cross-over events (Voisard et al., 1994). Five colonies which were Tc^S were then tested by PCR for loss of the lasR coding region.

To generate a P. aeruginosa PAO1 mutant strain with an rsaL deletion (ArsaL), allelic exchange was obtained by using the pDM4-ArsaL plasmid, derived from the suicide vector pDM4 (Milton et al., 1996). The upstream and downstream DNA regions of rsaL were PCR amplified from P. aeruginosa PAO1 chromosomal DNA using primer pairs FWrsaLUP + RVrsaLUP and FWrsaLDOWN + RVrsaLDOWN, respectively (Table S2). The upstream and downstream PCR fragments were subsequently cloned in pDM4 by XhoI-BamHI and BamHI-XbaI restriction, respectively. The resulting pDM4-ArsaL plasmid was verified by restriction analysis and sequencing. Allelic exchange in P. aeruginosa PAO1 following conjugal mating with the E. coli S17.1 Δpir (pDM4-ArsaL) donor strain and sucrose counter selection was performed as previously described (Westfall et al., 2004). The resulting PAO1 ΔarsaL mutant strain was confirmed by PCR.

### Gene Expression Assays

Three independent single colonies of P. aeruginosa strains carrying reporter constructs were grown overnight in LB (separate tubes) at 37°C with shaking at 200 rpm. One milliliter of overnight culture was washed in 1 ml of fresh LB to remove secreted bacterial products and QS signal molecules. Twenty-microliter aliquots were inoculated into 1 ml of fresh LB, and 300 μl of the resulting cultures were dispensed into wells of a 96-well black flat-transparent-bottom microtiter plate. When needed, strains with inducible genes were grown with or without 1 mM IPTG unless otherwise stated. Microtiter plates were incubated at 37°C in a TECAN GENios automated luminometer-spectrophotometer with which luminescence and turbidity were recorded every 30 min. Promoter activity per cell is given as relative light units divided by absorbance at 600 nm wavelength (A_600).

### Statistical Tests

Standard deviation of the mean of the three biological replicates is reported. A paired t-test was used to compare each mutant with the relevant control for each experiment. A P-value of ≤0.05 was considered significant.

### RESULTS

#### QS Control of phz1 and phz2 Expression

To ascertain how the phenazine operons phz1 and phz2 are regulated by the different elements of the QS circuit in P. aeruginosa PAO1-N, the pPphzA1-lux and pPphzA2-lux reporter plasmids, containing transcriptional fusions between the PphzA1 and PphzA2 promoter regions and the luxCDABE operon for bioluminescence, respectively, were generated and inserted in the chromosome of strain PAO1-N and different mutants derived from it. In detail, in the pPphzA1-lux plasmid a 727-bp DNA fragment comprising the entire intergenic region between phzM and phzA1 was cloned upstream of the luxCDABE operon, while in pPphzA2-lux a 497-bp DNA fragment including the entire intergenic region between qseB and phzA2 was cloned instead. By including these relatively large promoter regions the likelihood of missing any key regulatory element upstream of the known unique transcription start sites of PphzA1 and PphzA2 (Dötsch et al., 2012) was minimized. Cloned promoter regions include the first two codons of phzA1 and phzA2, respectively. Since the vast majority of studies on the QS circuit of P. aeruginosa have been undertaken in rich media, LB was used in this work so that predictions about the behavior of the QS network in specific QS mutants could be made and the results obtained compared with previous studies.

Firstly, the impact of QS elements, previously identified as key players in the regulation of PYO production, on PphzA1 and PphzA2 activity was investigated in PAO1-N, since there have been some strain-specific differences shown in the regulation of these operons by QS (Sun et al., 2016). Figure 1, shows that under the planktonic conditions studied the activity of PphzA1 is several fold higher than that of PphzA2 which is in line with what has been detected in P. aeruginosa PA14 (Recinos et al., 2012). LasR, RhlR, and PqsE showed a positive effect on the activation of the PphzA1 and PphzA2 promoters. PphzA1 activity was completely abrogated in the ΔlasR and ΔrhlR mutants, and strongly decreased (90% reduction) in the non-induced pqsE conditional mutant strain, pqsEind (P < 0.01). The effect of LasR, RhlR, and PqsE on PphzA2 appear to be milder although still significant (P < 0.05), with reporter activities reduced by 78, 71, and 52% in the ΔlasR, ΔrhlR and non-induced pqsEind strains, respectively. When PqsE was fully induced with 1 mM IPTG in the pqsEind strain, a 3.5-fold increase in promoter activity of both PphzA1 and PphzA2 was observed relative to the PAO1-N wild type (P < 0.01). Conversely, RsaL had an opposite effect on the two promoters, since PphzA1 activity is significantly increased (298% increase) and PphzA2 activity decreased (80%
Moreover, a study by McKnight et al. (2000) showed that addition of exogenous PQS positively regulates the PphzA2 activity in the wild type PAO1-N and the ral mutant background, with and without complementation. Promoter activities are normalized by cell density (A600).

Figure S3. Effect of pRsaL on PphzA2 activity. Maximal promoter activity of PphzA2-lux in the wild type PAO1-N and the ral, mutant background, with and without complementation. Promoter activities are normalized by cell density (A600).

FIGURE 2 | Effect of pRsaL on PphzA2 activity. Maximal promoter activity of PphzA2-lux in the wild type PAO1-N and the ral, mutant background, with and without complementation. Promoter activities are normalized by cell density (A600).

not been shown to directly bind to PphzA2 in EMSA studies (Rampioni et al., 2007; Sun et al., 2017). The growth data for this experiment is shown in Figure S4.

Detailed Analysis of the Impact of the QS Cascade on PphzA1 Activity

High levels of PqsE resulted in an increase in promoter activity for both phenazine biosynthesis operons (Figure 1). Since a lux-box is present in the PphzA1 promoter region and PqsE does not act as a transcriptional regulator, it can be hypothesized that PqsE exerts a positive effect on PphzA1 activity via the LasR and/or RhlR transcriptional regulators. This hypothesis was tested by analysing PphzA1 activity in the double mutants pqsEind ΔlasR and pqsEind ΔrhlR respectively in which pqsE expression can be restored in the presence of IPTG. Figure 3 reveals that while PqsE induction with IPTG resulted in high PphzA1 activity in the pqsEind strain, the activity of this promoter under induced conditions was reduced by 80% in the pqsEind ΔlasR and ΔrhlR strains and a 2 h delay in activation of PphzA1 relative to the pqsEind strain induced with IPTG was observed. PphzA1 activity was completely abrogated in the pqsEind ΔlasR and ΔrhlR background. The growth data for this experiment is shown in Figure S5.

The las QS system has a positive effect on the activity of both the rhl and pqs QS systems (Pesci et al., 1997; Medina et al., 2003; Xiao et al., 2006). Moreover, a study by McKnight et al. (2000) showed that addition of exogenous PQS positively regulates the rhl system, and Diggie et al. (2003) showed that addition of exogenous PQS advances and enhances pyocyanin production and increases RhlR levels. We therefore hypothesized that the reduction of transcriptional activity of PphzA1 in the pqsEind ΔlasR mutant could be caused by reduced activity of the rhl and pqs systems in this mutant background, and hence exogenous provision of PQS should compensate for a las mutation. To test this, PphzA1 activity was analyzed in the ΔlasR and ΔrhlR strains of P. aeruginosa wild type. These results are in accordance with published data for other P. aeruginosa strains, showing a positive effect of LasR, RhlR and PqsE on PCA biosynthesis in the human pathogen P. aeruginosa PA14 and in the rhizosphere bacterium P. aeruginosa PA1201 (Recinos et al., 2012; Sun et al., 2016, 2017). Also the dual effect of RsaL on PphzA1 and PphzA2 is in line with what was previously observed in P. aeruginosa PA1201 (Sun et al., 2017). The growth data for this experiment is shown in Figure S5.

To further validate the regulation of PphzA2 by RsaL, the rsaL deletion was complemented via the IPTG inducible pRsaL plasmid. Some partial restoration of PphzA2 activity was observed in the ΔrsaL strain in the presence of pRsaL, likely as a consequence of basal rsaL transcription from the tac promoter (Guzman et al., 1995), while in the presence of 0.1 mM IPTG PphzA2 activity was restored to wild type levels (P < 0.05) (Figure 2). Overall, these data confirm that in PAO1-N RsaL is a represor of phz1 transcription and has a positive effect upon PphzA2, the latter likely mediated by an ancillary PphzA2-regulator under the control of RsaL, since purified RsaL has

reduction) (P < 0.01), in the ΔrsaL mutant compared to PAO1-N wild type. These results are in accordance with published data for other P. aeruginosa strains, showing a positive effect of LasR, RhlR and PqsE on PCA biosynthesis in the human pathogen P. aeruginosa PA14 and in the rhizosphere bacterium P. aeruginosa PA1201 (Recinos et al., 2012; Sun et al., 2016, 2017). Also the dual effect of RsaL on PphzA1 and PphzA2 is in line with what was previously observed in P. aeruginosa PA1201 (Sun et al., 2017). The growth data for this experiment is shown in Figure S5.
in the presence of 100 µM exogenous PQS. To discard any effects related to the iron chelating properties of PQS, the non-signaling quinolone molecule methyl PQS (mPQS) was used as a control, since this molecule is capable of binding iron like PQS, but is unable to trigger gene expression via PqsR (Diggle et al., 2007). Addition of PQS was found to compensate for a lasR deletion (P < 0.05), while an rhlR deletion resulted in no activation of the PphzA1 promoter, irrespective of the absence or presence of PQS (Figure 4). The addition of PQS also impacted on the timing of PphzA1 gene expression in both wild type and lasR deletion strains with the activation of this promoter triggered 1 h earlier than in the absence of this signal molecule (data not shown). The growth data for this experiment is shown in Figure S6.

These data suggest that RhlR is a direct positive regulator of the phz1 operon, as loss of this element abolishes PphzA1 activity, while LasR acts as an indirect PphzA1 regulator.

Addition of PQS in the previous experiment would be expected to increase the expression of the pqsABCDE operon and hence PqsE production through the activation of PqsR. The data presented here suggests that PqsE is required to activate PphzA1 and PqsE would be present in high levels after the addition of exogenous PQS to the culture. To further investigate the importance of RhlR and LasR in the PqsE-mediated activation of PphzA1 the above experiments were repeated using the pqsEInd conditional mutant with additional lasR and rhlR mutations.

Addition of PQS to the un-induced pqsEInd strain resulted in no significant increase in PphzA1 activity, compared to the un-induced pqsEInd strain (P < 0.05). This result confirms that PqsE rather than PQS on its own or through PqsR activation is required to reach high levels of PphzA1 transcription (Figure 5). Since PqsR has been reported to directly bind to rhlI/R resulting in some increased expression of these genes (Maura et al., 2016) this result suggests that activated RhlR in combination with PqsR are unable to activate PphzA1 transcription. In the pqsEInd strain the expression of pqsE is decoupled from that of pqsABCD, due to a transcriptional terminator introduced downstream of pqsD, hence PqsE production is not under the control of PqsR (Rampioni et al., 2010). Addition of PQS when pqsEInd was induced by IPTG slightly increased PphzA1 activity compared with the non-induced pqsEInd without PQS, this may be due to some PqsR direct activation of rhlI/R. Again PQS was unable to trigger reporter gene expression in the pqsEInd ΔlasR strain when pqsE was not induced. Interestingly, when pqsE was fully induced and PQS was added to the pqsEInd ΔlasR mutant, a significant increase in PphzA1 activity (P < 0.05) of approximately 50% was observed compared with the fully induced pqsEInd mutant. No PphzA1 expression was detected in the pqsEInd ΔrhlR strain under any of the conditions tested confirming the importance of RhlR in activating phz1 transcription (Figure 5). The growth data for this experiment is shown in Figure S6.

The high levels of PphzA1 activity observed when both, PQS is added and PqsE expression is induced with IPTG in the pqsEInd ΔlasR strain, could be due to low levels of Rsal, which is a repressor of PphzA1 (Figure 1). Since LasR activates the rsaL promoter (PrsaL), the ΔlasR mutant is expected to express low
levels of RsaL. Overall, these data are consistent with PqsE and RhlR as the key activators of the phz1 operon with RsaL acting as a repressor.

**Detailed Analysis of the Impact of the QS Cascade on PphzA2 Activity**

In Figure 1 we show that both PqsE and RsaL exert positive control over the expression of PphzA2 but the influence the las and rhl systems may have on this regulation is not clear. To investigate this further, the expression of PphzA2 was studied in pqsEind and pqsEind with a lasR or a rhlR deletion. When pqsE was induced in either strain, PphzA2 activity could only achieve 5% and 10% of the pqsEind strain (Figure 6), growth data Figure S7. This differed from the result obtained for PphzA1, as a lasR deletion in the pqsEind induced strain decreased but did not abrogate PphzA1 activity (Figure 5). To ascertain whether addition of exogenous PQS could compensate for lasR and rhlR deletions, PphzA2 activity was evaluated in the ΔlasR and ΔrhlR mutants supplemented with exogenous PQS or mPQS, the latter molecule used as an iron-binding negative control as before.
Unlike PphzA1, where PQS restored promoter activity in the ΔlasR mutant, no significant increase (P < 0.05) in PphzA2 activity was observed when PQS was added to the ΔlasR and ΔrhlR strains (Figure 7), growth data Figure S8.

These results suggest that both LasR and RhlR are required to activate PphzA2. Since addition of exogenous PQS increases the levels of PqsE (Heeb et al., 2011) we next investigated whether PqsE or PqsR were responsible for the increase in PphzA2 activity. The same experimental strategy as for PphzA1 analysis was used and activity of PphzA2 was assayed in the pqsEind mutant strain and the pqsEind strains with additional lasR and rhlR deletions, in the presence of exogenously added PQS and IPTG.

The result of this experiment showed that addition of PQS to the un-induced pqsEind strain resulted in no significant increase in PphzA2 activity, compared to the un-induced pqsEind strain (P < 0.05). When pqsE was induced with IPTG, PphzA2 activity was triggered and further increased by addition of exogenous PQS (P < 0.05) (Figure 6). These data suggest that PqsE rather than PqsR is required to positively regulate PphzA2, as it was for PphzA1. Hardly any increase in the expression of the PphzA2 promoter was observed in the pqsEind strains carrying additional lasR and

![Figure 6](image6.png)

**FIGURE 6** | PQS is unable to induce PphzA2 transcription in the absence of PqsE, LasR, and RhlR. Maximal promoter activity of PphzA1-lux in the pqsEind strain and pqsEind strain carrying additional lasR or rhlR mutations in the presence or absence of IPTG and PQS. Promoter activities are normalized by cell density (A600).

![Figure 7](image7.png)

**FIGURE 7** | PphzA2 activity cannot be restored by exogenous PQS in lasR or rhlR mutants. Maximal promoter activity of PphzA2-lux in the wild type PAO1-N, lasR and rhlR deletion backgrounds in the presence of PQS or methyl PQS (mPQS). Promoter activities are normalized by cell density (A600).
rhIR deletions, either in the absence or presence of IPTG and/or PQS, suggesting that LasR, RhlR and PqsE are all key for PphzA2 transcription.

We have shown that RsaL has a positive effect on PphzA2 (Figures 1, 2). The rsaL promoter is positively regulated by LasR, hence in the lasR deletion mutant low levels of RsaL would be expected, which in turn should have a negative impact on PphzA2 activity. Therefore, we hypothesized that LasR is an indirect activator of PphzA2 acting via RsaL, and to test this we transformed the lasR mutant strain with the inducible pRsaL plasmid. As LasR affects the activity of the rhl and pqs QS systems, exogenous PQS was also added to increase the activity of the rhl and pqs QS systems.

The result of these experiments suggest that LasR is an indirect activator of PphzA2, since an increase in PphzA2 activity in the lasR mutant carrying pRsaL was observed, compared with the lasR mutant. Addition of exogenous PQS further increased PphzA2 activity to the level of the wild type PAO1-N level when pRsaL was induced with IPTG ($P < 0.05$) (Figure 8), growth data Figure S9.

These data suggest that RsaL, alone is unable to induce the PphzA2 promoter to wild type levels and must be working in conjunction with other regulatory elements. To gain further evidence that LasR is an indirect activator of PphzA2 and investigate the requirement of PqsE and RhlR to activate PphzA2, we introduced pRsaL in the pqsEInd and pqsEInd strains with additional lasR and rhlR deletions. When RsaL and PqsE expression was induced in these strains with IPTG, PphzA2 activity of the pqsEInd ΔlasR strain carrying pRsaL was significantly increased ($P < 0.05$) compared to the pqsEInd ΔlasR strain and PphzA2 activity was comparable to the induced pqsEInd strain. No PphzA2 activity was observed in the pqsEInd strain carrying an additional rhlR deletion, confirming that RsaL, RhlR, and PqsE are all required to trigger transcription of the phz2 operon (Figure 9). The growth data for this experiment is shown in Figure S10.

**DISCUSSION**

**New Model of Phenazine Production Control by QS**

Here it has been demonstrated that the QS regulators LasR, RhlR, RsaL and the enzyme PqsE are all involved in controlling the expression of both phenazine operons phz1 and phz2 in *P. aeruginosa* with some differences. Initially it was unclear which regulators played a direct role in activating each operon and which were indirect because of the hierarchal structure of the QS network in rich media (Figure 1). A combination of the deletion of specific genes, the inducible expression of specific QS regulators and/or exogenous provision of QS signal molecules has allowed us to tease out which regulators are direct activators and which can be considered indirect because of their effect upon the QS network (Figures 3–9). Although rich media is not representative of the natural environment in which *P. aeruginosa* is found our experiments have closed an unanswered question of which QS regulators directly control each phz operon.

The results obtained allow us to postulate a model by which the QS cascade interacts and controls phenazine production in planktonic cultures (Figure 10).

Evidence has been presented showing that RhlR is a positive regulator for both operons and also that PqsE must be present to induce each operon. This is not surprising as it has been previously reported that PqsE and RhlR are both required for pyocyanin production (Farrow et al., 2008). It could be the case that all genes in the rhl regulon may be co-dependent upon PqsE as the production of the RhlR controlled genes lasB and rhlA are
enhanced in the presence of PqsE (Farrow et al., 2008; Rampioni et al., 2010). The data presented show that PqsE rather than PqsR is required to activate the transcription of both phenazine biosynthesis operons. It was demonstrated by Recinos et al. (2012) that HHQ plays a role in activation of PhzA2 under anaerobic conditions. HHQ would inevitably increase the levels of PqsE as pqsABCDE is a direct target for PqsR when bound to either HHQ or PQS (Fletcher et al., 2007; Rampioni et al., 2016). As molecular oxygen is required to convert HHQ to PQS (Schertzer et al., 2010) it would appear that under anaerobic conditions PqsE can be produced when the signal HHQ binds PqsR, emphasizing that PqsE is important for activating the phenazine operons under both aerobic and anaerobic conditions.

In rich media, LasR drives expression of the rhl and pqs systems, which then interact through RhlR and PqsE to activate the phenazine operon promoters. A deletion of lasR caused a reduction in PhzA1, which further demonstrates that in rich media the QS cascade has a hierarchical structure. This is in accordance with the work of others who have demonstrated that the loss of LasR results in a delay in production of pyocyanin (Dekimpe and Deziel, 2009; Cabeen, 2014). LasR also drives expression from PrsaL and in turn RsaL represses PhzA1, Plasl and its own production. In vitro protein-DNA interaction experiments revealed that RsaL binds to the PhzA1 promoter at a region encompassing the −10 sequence (Rampioni et al., 2007; Sun et al., 2017), hence RsaL directly exerts a repressive effect on Phz1 expression. Moreover, RsaL was shown to exert an indirect repressive effect on PhzA1 by increasing the expression of the PhzA1-repressor CdpR (Sun et al., 2017). It has been hypothesized by Rampioni et al. (2007) that RsaL maintains signal homeostasis by repressing PlasI and in the context of phenazine production could provide a similar feature (Bondi et al., 2017). RsaL could act to keep PhzA1 inactive until the rhl and pqs systems are interacting before commencing transcription, thereby creating a checkpoint in the system. The QS signal molecule PQS has multiple functions as it can bind iron and also act as an anti-oxidant (Diggle et al., 2007; Häussler and Becker, 2008). In the presence of oxygen, pyocyanin generates reactive oxygen species (ROS) (Rada and Leto, 2013). Hence this checkpoint could function to allow adequate PQS to be produced and reduce deleterious effects of ROS produced by pyocyanin before triggering transcription of phz1.

It is thought that RsaL has a secondary function and strong evidence that RsaL can repress PhzA1 but indirectly induce PhzA2 has been presented. Previous studies have failed to demonstrate an interaction between RsaL and a DNA probe encompassing the PhzA2 promoter region (Rampioni et al., 2007; Sun et al., 2017), suggesting that the positive effect exerted by RsaL on phz2 expression is not direct. RsaL increases the expression of the PhzA1-repressor CdpR, but a ChIP-seq assay did not show any interaction between CdpR and the PhzA2 promoter region in strain PA1201 (Zhao et al., 2016), suggesting that CdpR is not involved in the RsaL-mediated activation of PhzA2. The positive effect of RsaL on PhzA2 is probably achieved via an unidentified phenazine biosynthesis gene regulator, which we termed Unidentified Phenazine Regulator of phzA2 (Upr2). Although this regulator has not been identified, data presented thus far strongly imply the existence of this additional regulator, which in turn is controlled by the QS repressor RsaL.

We hypothesize that Upr2 is a PhzA2 repressor and its expression could be repressed by RsaL. If Upr2 had a positive effect upon PhzA2 we would expect that in an rhlR mutant some PhzA2 activity would be observed. We show that when RsaL and PqsE are present but rhlR is mutated, no PhzA2 activity was observed (Figure 9) making it unlikely that Upr2 is a positive regulator. It is likely that when the QS network is activated by LasR, that RsaL represses the promoter of upr2 and as Upr2 is turned over and diluted through cell division that PhzA2 can be triggered by RhlR in conjunction with PqsE, since both the rhl and pqs systems are positively regulated by LasR. In these experiments we observed significantly less activity from PhzA2 compared with PhzA1, further suggesting that the PhzA2 promoter is tightly controlled by a repressor. Upr2 could have its own regulon which may have significant overlap with that of the las regulon. We found that phz2 transcription can be triggered in a lasR mutant when the lasR deletion is complemented and PQS added exogenously to stimulate RhlR and PqsE production (Figure 8). Hence it is likely that some of the genes identified as lasR- or rsaL- specific in comparative transcriptome studies could belong to the upr2 regulon.

We hypothesize that Pseudomonas aeruginosa can switch PCA production from phz1 to phz2 when RsaL levels are elevated and from phz2 to phz1 when RsaL is absent. This switch could be related to a reduction in oxygen availability and an increase in oxidative
stress as the population size increases, however, this remains to be investigated.

PCA is converted to PYO via the action of PhzM and PhzS. The phzM gene is located directly upstream of phzA1 and the intergenic region between these has two predicted lux boxes (Whiteley and Greenberg, 2001). The lux boxes are flanked by two rsaL binding sites (Rampioni et al., 2007) which suggests that phz1 and phzM are controlled in a similar manner. In the study by Rampioni et al. (2007) a microarray assay was used to identify the rsaL regulon. In that work it was discovered that both phzM and phzS were up regulated in the rsaL mutant compared with the wild type, suggesting that RsaL represses both genes. Here we present evidence that RsaL also represses PphzA1. As phzM, phzS, and phz1 are all required to produce PYO, which in turn contributes toward oxidative stress. It is conceivable that when oxidative stress is high, RsaL can repress phz1, phzM and phzS but maintain PCA production by indirectly activating phz2. Evidence to support this hypothesis was provided when the oxidative stress response regulator OxyR was shown to bind the promoter of rsaL (Wei et al., 2012). A previous study of the OxyR regulon showed that when this regulator is mutated, pyocyanin levels increase, suggesting that OxyR can repress pyocyanin production and this could be achieved through rsaL (Vinckx et al., 2010).

One of the proposed main functions of phenazines is to cycle electrons which allows P. aeruginosa to continue respiration in microaerophilic environments by controlling the intracellular redox state (Dietrich et al., 2013). Switching off PYO production would therefore cause a problem under these conditions. Unlike PYO, PCA can donate electrons to iron (III) rather than oxygen, hence maintaining redox homeostasis without producing ROS (Wang and Newman, 2008; Wang et al., 2011). PYO may be used in addition to PCA to cycle electrons as O2 is a better electron acceptor than iron. Through this switch, phenazine production may continue while lowering oxidative stress on the bacterium and maintaining redox balance.

**AUTHOR CONTRIBUTIONS**

SHi, SHe, GR, MF, PW, and MC designed the study and analyzed the data. SHi, GR, and MF conducted the experiments. SHi, SHe, GR, and MC wrote the manuscript. All authors reviewed the manuscript.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2018.00252/full#supplementary-material

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