

RhlR Expression in *Pseudomonas aeruginosa* Is Modulated by the *Pseudomonas* Quinolone Signal via PhoB-Dependent and -Independent Pathways^{∇†}

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The expression of virulence determinants in *Pseudomonas aeruginosa* is coordinately regulated in response to both the social environment—commonly referred to as quorum sensing—and to environmental cues. In this study we have dissected the various independent regulation levels for pyocyanin production, which is influenced by the homoserine lactone- and *Pseudomonas* quinolone signal (PQS)-mediated quorum-sensing systems as well as by iron and phosphate availability. We demonstrate that the phosphate regulon is involved in the transcriptional activation of *rhlR* and the augmentation of PQS and pyocyanin production under phosphate limitation. However, we also observed an enhancement of *rhlR* transcription under low-iron medium conditions and after the addition of PQS that was independent of the phosphate regulon. These results highlight the complexity of secondary metabolite production in *P. aeruginosa* via environmental cues and the quorum-sensing system.

Bacterial organisms that elaborate traits tailored to their surroundings have better chances of surviving the pressures of unfavorable environmental conditions and host defenses. The outstanding capability of *Pseudomonas aeruginosa* for adaptation is reflected by the large number of putative transcriptional regulators (53), as bacterial differentiation is often controlled by transcription factors whose activity is influenced by local cues. Moreover, it has been recognized that environmental signals (33) as well as the social surrounding control bacterial virulence factor production. Cell-density-dependent gene regulation is commonly referred to as quorum sensing (QS) (20). QS is based on the release of soluble communicator molecules that trigger the transcription of QS-dependent genes when the bacterial population has reached a certain cell density. Many of these genes are involved in bacterial pathogenicity (9, 19, 39, 40, 50). Three chemically distinct signal molecules have been identified so far in *P. aeruginosa*. Two of these are acyl-homoserine lactones (AHL): a butyryl-homoserine lactone and a 3-oxo-dodecanoyl homoserine lactone, which together with their corresponding transcriptional activator proteins (R proteins) comprise the two hierarchically organized QS systems *las* and *rhl* (8, 27, 38, 41, 44) and control the expression of over 200 genes (23, 48, 57). The third signal molecule is 2-heptyl-3-hydroxy-4-quinolone (43). This *Pseudomonas* quinolone signal (PQS) interacts with the *las* and the *rhl* systems. While the *las*

system seems to induce the production of PQS, exogenous PQS up-regulates the expression of the *rhl* system (15, 16, 31). In *P. aeruginosa* the impact of the *rhl* QS on the biosynthesis of the secondary metabolites pyocyanin and rhamnolipids is well documented (42). However, the production of these secondary metabolites also seems to be dependent on environmental cues (3, 62). A link between QS and iron homeostasis was suggested previously (5, 11, 21, 24, 25, 52, 60). Moreover, PQS was shown to exhibit an iron-chelating activity, and PQS-dependent *rhlR* induction seems to be at least in part a consequence of iron depletion (6).

Recent studies on the transcriptional regulation of RhlR revealed that the *rhlR* promoter region harbors four different transcription start sites (32). Whereas under rich medium conditions the expression of RhlR is dependent on LasR, under phosphate-limiting conditions various transcriptional activators, including Vfr, RhlR, and the sigma factor σ^{54} , participate in the expression of RhlR from multiple promoters. Moreover, it was demonstrated that *rhlR* expression can be enhanced under low-phosphate medium conditions, albeit phosphate limitation was shown to reduce AHL levels while allowing rhamnolipid production (3). These results indicate that QS involves a very complex genetic regulatory circuit, and its fine-tuning is regulated by both environmental and cell-density-dependent cues, which seems to be important not only for the pathogenesis but also for the adaptation of the pathogen to given environmental conditions (32). In this study we demonstrate that the physiological sensing of phosphate as an environmental factor is important in *rhlR* gene transcription and in PQS and pyocyanin production. Moreover, our results suggest that PhoB links environmental and cell-density-dependent cues to secondary metabolite production in *P. aeruginosa*, thus

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establishing a molecular genetic basis that couples the Pho regulon and quorum sensing.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The clinical *P. aeruginosa* strain SCV 20265, isolated from the respiratory tract of a cystic fibrosis patient who attended the Cystic Fibrosis Clinic at Hanover Medical School, Hanover, Germany, was used in this study (22). This *P. aeruginosa* strain produced large amounts of hydroxy-alkyl-quinolones (HAQs), including PQS (7), was an efficient pyocyanin producer, and therefore was especially suitable for monitoring pyocyanin and PQS expression under various medium conditions. *P. aeruginosa* was routinely cultured at 37°C on Columbia or Luria-Bertani (LB) agar. A transposon mutant of SCV 20265 (*phoB* mutant) that was generated using the transposon construction vector EZ:TN pMOD-2 (Epicenter) was grown in LB medium supplemented with 50 µg/ml gentamicin. This mutant had been previously identified within an (unpublished) screen for low pyocyanin production and harbored a transposon insertion within the *phoB* gene located upstream of *phoR* on the same operon. The expression of *rhIR* was studied by the determination of β-galactosidase activity in bacteria harboring the plasmid pMAL.V, which contains a *lacZ* transcriptional fusion of *rhIR* and was provided by courtesy of A. Lazdunski (28). For the complementation of the *phoB* gene locus, *phoB* was amplified from chromosomal DNA by using flanking primers (5'-AAA AAA GCT TAT GGT TGG CAA GAC AAT CCT CA-3' and 5'-AAA AGG ATC CTC AGC TCT TGG TGG AGA AAC G-3') designed against the 5' upstream and 3' downstream regions adjacent to the relevant coding regions. The amplicons were cloned into the XhoI-BamHI sites of pUCP20T (54) to construct pUCP20:*phoB*.

Bacteria were cultured at 37°C and 180 rpm in 100-ml Erlenmeyer flasks, wide neck, in 20 ml LB medium and in 250-ml Erlenmeyer flasks, wide neck, in 40 ml of a synthetic medium according to the protocol of Frank and DeMoss (18). The medium contained either 0.8 mM or 4.0 mM dipotassium hydrogen phosphate and was supplemented with 2.5 µM or 50 µM ferric citrate, respectively. Where indicated, synthetic PQS (7) was added at a concentration of 40 µM.

Measurement of β-galactosidase activity. Miller assays were carried out as described previously (34). Briefly, 100- to 200-µl samples of the bacterial culture grown to an optical density at 600 nm of 0.6 to 1 were added to the reaction mix and vortexed. The reaction mix consisted of 800 to 900 µl Z buffer, 10 µl 0.1% sodium dodecyl sulfate, and 10 µl of chloroform. A 200-µl volume of *o*-nitrophenyl-β-D-galactopyranoside (4 mg/ml 0.1 M K₂HPO₄) was added to the reaction mixture and incubated until there was a color change or for 30 min if there was no obvious color change.

Extraction of extracellular *P. aeruginosa* HAQ metabolites and TLC. HAQ metabolites were extracted from *P. aeruginosa* broth cultures with dichloromethane as described previously (7). Briefly, the bacterial cultures were extracted with 2 volumes of dichloromethane by vigorous shaking. After centrifugation at 3,500 × g for 10 min, the lower organic layer was evaporated. Thin-layer chromatography (TLC) was performed using a silica gel 60 F254 TLC plate which had been previously soaked for 30 min in 5% KH₂PO₄ and activated at 85°C for 1.5 h. The extracted *P. aeruginosa* material was dissolved in dichloromethane and separated by TLC using 95:5 dichloromethane-methanol as solvent. Fluorescent spots were visualized under UV light and photographed. Synthesized PQS and 2-heptyl-4-hydroxy-quinolin (HHQ) were used as standards.

Assay for pyocyanin production. Pyocyanin production was determined as described previously (17). Briefly, the bacterial supernatant was extracted with chloroform and then reextracted with 0.2 N HCl to give a pink solution. The absorbance was measured at 520 nm, and the pyocyanin content was calculated as described elsewhere (17).

Search for putative PHO box sequences in the *P. aeruginosa* genome. A position weight matrix (PWM) model was built from 33 aligned heptameric PHO half-sites using the data from the PRODORIC database (35). The PHO box was afterwards modeled using this PWM for a bipartite pattern with a variable spacer length between 2 and 5 bp. The scoring was done by using the information theoretical approach (46) and by summing up the individual scores of the two half-site PWMs to an overall score. We additionally refined the method by a linear correction of noise (47) due to the high GC content of the *P. aeruginosa* genome. The genome-wide search was performed using the Virtual Footprint software (36). Sequence logos from the matches derived were created using WebLogo (12).

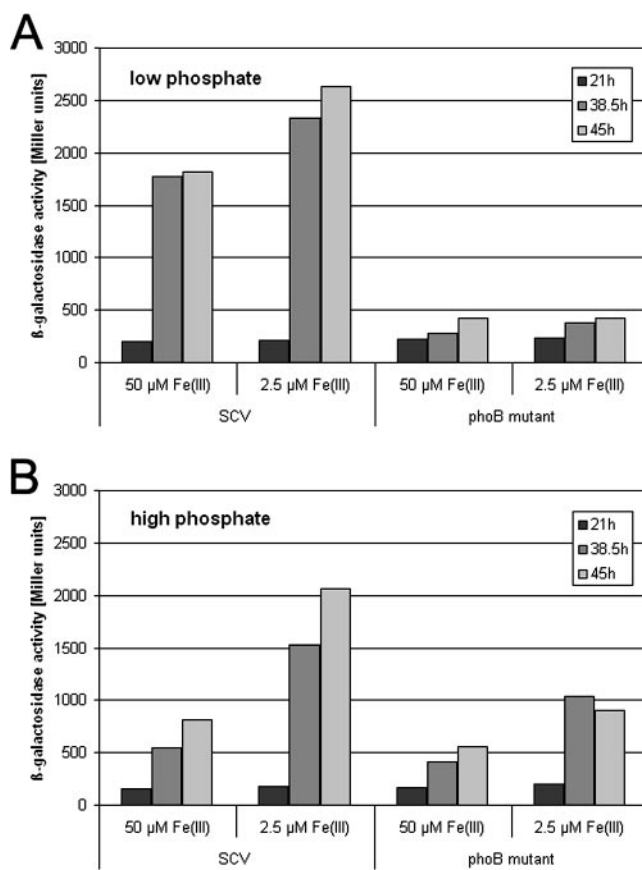


FIG. 1. *rhIR* promoter activity at three different time points in SCV 20265 and the *phoB* mutant cultivated in low-phosphate (0.8 mM) (A) and high-phosphate (4 mM) (B) medium supplemented with 50 µM and 2.5 µM ferric citrate, respectively. This graph is representative of at least three independent experiments.

RESULTS AND DISCUSSION

The role of iron in bacterial pathogenicity is well established, as the low concentration of iron present in the host is an important signal to enhance the expression of a wide variety of bacterial toxins and other virulence determinants (29, 33). More recently, phosphate has also been recognized as an important nutrient and environmental signal that regulates virulence in bacteria, and a cross talk between the phosphate regulons and virulence has been suggested in various bacterial species (2, 45, 49, 56, 61).

Phosphate limitation stimulates the expression of *rhIR* in *P. aeruginosa* in a *phoB*-dependent manner. With the aim to re-evaluate *rhIR* expression in *P. aeruginosa* under defined medium conditions, we introduced a *rhIR-lacZ* transcriptional fusion in the low-copy-number plasmid pMP220 (pMAL.V) into *P. aeruginosa* SCV 20265 and a *phoB* mutant background and determined the β-galactosidase activity in bacteria grown in low-phosphate (0.8 mM phosphate) or high-phosphate (4 mM phosphate) medium supplemented with 2.5 µM or 50 µM ferric citrate, respectively. As demonstrated in Fig. 1, under low-phosphate medium conditions (50 µM ferric citrate) we found an increased β-galactosidase activity in the wild-type cultures compared to the controls grown under high-phosphate



FIG. 2. PhoB sequence logo derived from genome-wide matches with the preferred spacer length of 4 bp.

medium conditions (paired *t* test, $P = 0.0036$). The highest β -galactosidase activity was found under low-phosphate and low-iron medium conditions. In contrast to the increased *rhIR* expression of the wild type under low-phosphate conditions, we did not observe an induction of *rhIR* transcription in the *phoB* mutant (Fig. 1). However, low-iron medium induced *rhIR* expression even in the *phoB* mutant under phosphate-replete conditions (Fig. 1), albeit not significantly (paired *t* test, $P = 0.24$).

Under high-phosphate medium conditions, the overall levels of β -galactosidase activity of the *phoB* mutant did not reach the level of those detected in the wild-type background. This PhoB/PhoR dependency of *rhIR* transcription even under high-phosphate medium conditions implicates that PhoB activation might not be restricted to low-phosphate medium conditions. A functional basis for a concept of cross-regulation in the PHO regulon has been proposed before in *Escherichia coli*, where a regulatory coupling has been suggested to exist between the PHO regulon and genes for enzymes in central metabolism for incorporation of phosphate into ATP (59).

Search for putative PHO box sequences in the *P. aeruginosa* genome. Our data showing a *phoB/phoR*-dependent *rhIR* induction under phosphate depletion led us to survey the intergenic regions of the *P. aeruginosa* genome for consensus sequences with similarities to the *Escherichia coli* PHO box. In *E. coli*, the phosphate-dependent regulation of the phosphate

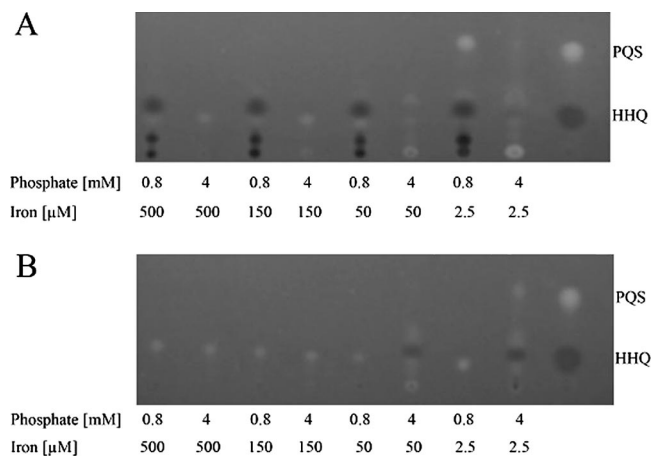


FIG. 3. Thin-layer chromatography of dichloromethane extracts of the SCV 20265 (A) and the isogenic *phoB* mutant (B) grown to stationary phase. Bacterial cultures were extracted after cultivation in low-phosphate (0.8 mM) and high-phosphate (4 mM) medium supplemented with 500 μ M, 150 μ M, 50 μ M, and 2.5 μ M ferric citrate. Synthetic PQS and HHQ served as controls. This TLC is representative of at least three independent experiments.

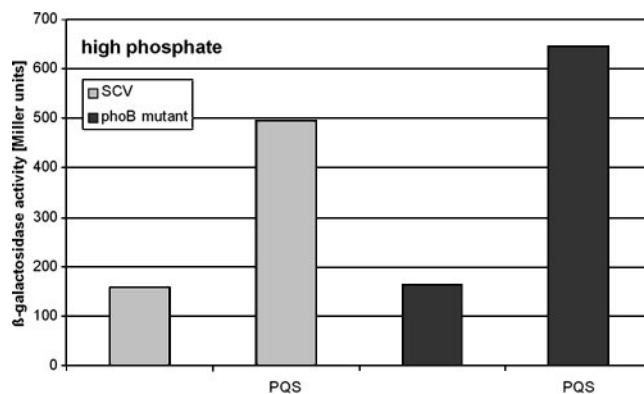


FIG. 4. *rhIR* promoter activity in SCV 20265 and the isogenic *phoB* mutant grown in high-phosphate (4 mM) medium supplemented with 50 μ M ferric citrate with or without the addition of 40 μ M PQS. This graph is representative of at least three independent experiments.

regulon is controlled by the two-component regulatory system PhoR-PhoB (58), comprising the response regulator PhoB and its partner sensor kinase, PhoR. Each phosphate-regulated Pho regulon promoter is preceded by an upstream activation site with a consensus PHO box sequence for transcriptional activation by phosphorylated PhoB. The PHO box consists of two 7-bp direct repeats separated by a 4-bp segment: CTGTCAT-A(A/T)A(T/A)-CTGT(C/A)A(C/T) (4, 30, 55). However, the spacer length between the two heptamers seems to be variable (26). In *P. aeruginosa* a very similar Pho regulon system exists (1). Thereby, PhoB and PhoR constitute an operon with PhoB being promoter proximal. A well-conserved putative PHO box was previously identified in the regulatory region of *phoB* (1) and upstream of the *P. aeruginosa* ABC phosphate transporter (*pstC*, *pstA*, *pstB*, and *phoU*) (37). In this study we applied a global approach and scanned the intergenic regions extending 300 bp upstream of the translation start site of the whole *P. aeruginosa* genome. We identified 237 putative PHO boxes, including the two published PHO boxes preceding *pstC* (37) and the *phoB* gene (1). This number represents 417 putative downstream genes without consideration of operon structures. Among the genes preceded by putative PHO boxes we identified *phzA2/phzA1* and *rhIR*, *rhII*, and *lasR* as well as *pqsR*. The complete list of genes preceded by a putative PHO box is shown in Table S1 in the supplemental material. The most frequent category (apart from hypothetical genes) consists of genes known or predicted to be involved in transcriptional regulation. Genes in the category membrane proteins, transport of small molecules, and putative enzymes are also numerous. A graphical representation of aligned sequences with a 4-bp spacer is depicted in Fig. 2 as a sequence logo. The match found most frequently exhibited a spacer length of 4 bp and showed the highest information content.

HAQ production is enhanced under low-phosphate medium conditions. Apart from the two AHL-mediated QS systems (*las* and *rhI*), a third interbacterial communication system, the PQS system, is important in *P. aeruginosa* pathogenesis (10). PQS signaling seems to be independent of the *rhI* QS regulon (13) and has been described to influence secondary metabolite production in *P. aeruginosa* (16, 51). The putative PHO boxes preceding *rhIR/II*, *lasR*, and *pqsR* suggest that phosphate avail-

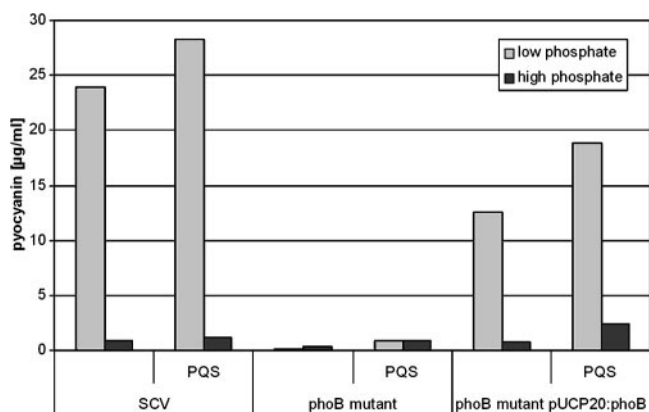


FIG. 5. Pyocyanin production of SCV 20265 grown for 96 h under high- and low-phosphate medium conditions and supplemented with 50 μM ferric citrate with or without the addition of 40 μM PQS. This graph is representative of at least three independent experiments.

ability has an influence on all three QS systems, including PQS signaling. Thus, we analyzed the impact of low-phosphate medium conditions on HAQ biosynthesis. As shown in Fig. 3, HAQ production was enhanced under low-phosphate medium conditions, and this induced HAQ production was abolished in a *phoB* mutant background. Moreover, decreasing iron concentrations induced PQS/HAQ production in the wild type and the *phoB* mutant background (Fig. 3). We also detected an increased pyochelin production (bright fluorescent spot at the place of depot [6]) under low-iron and high-phosphate concentrations.

PQS activates *rhIR* transcription in a *phoB*-independent manner and enhances pyocyanin production in a *phoB*-dependent manner. While PQS appears to act as an intercellular signal, the mechanism by which PQS controls gene expression remains elusive. However, it is clear that a PQS-controlled regulatory pathway must act at several different levels (16).

The effect of PQS has been shown previously to be partially mediated via up-regulation of the *rhl* system (16). However, a *rhl*-independent system has been suggested to exist, as PQS induces the *rhl* QS system but it does not mediate its regulatory activity through *lasRI*, *rhlRI*, or the production of AHL. RhIR and butyryl-homoserine lactone levels during entry into stationary phase were shown not to be significantly altered in mutants defective in PQS production or response, and yet these mutants failed to induce pyocyanin production (16).

To further dissect the various independent regulation levels for *rhl* QS- and PQS-induced genes, we tested whether the enhancement of pyocyanin production by the addition of PQS is indirect via an induction of the *rhl* QS system or whether there are direct effects that are independent from the *rhl* system. PQS has previously been described to induce the *rhl* QS system, probably by depleting iron from the growth medium (6). Since in a *phoB* mutant *rhIR* expression is induced under low-iron medium conditions, we were interested in whether PQS activates *rhIR* transcription in *P. aeruginosa* in a *phoB*-independent manner. Indeed, as demonstrated in Fig. 4, PQS addition to the wild type and the *phoB* mutant enhanced *rhIR* expression under high-phosphate medium conditions. In addition, as shown in Fig. 5, PQS stimulates the production of pyocyanin in *P. aeruginosa* SCV 20265. However, this effect cannot be attributed to the direct induction of the *rhl* system alone, since the addition of PQS did not induce high pyocyanin levels under high-phosphate medium conditions or in a *phoB* mutant background (Fig. 5). Thus, although PQS activates *rhIR* transcription in *P. aeruginosa* in a *PhoB*-independent manner, *phoB* mutants cannot be stimulated by PQS to produce high levels of pyocyanin. Since a putative PHO box was also identified preceding the two phenazine biosynthetic operons (*phzA2/phzA1*), it seems that pyocyanin production is, apart from the regulatory influence of the *rhl* system, under the strong

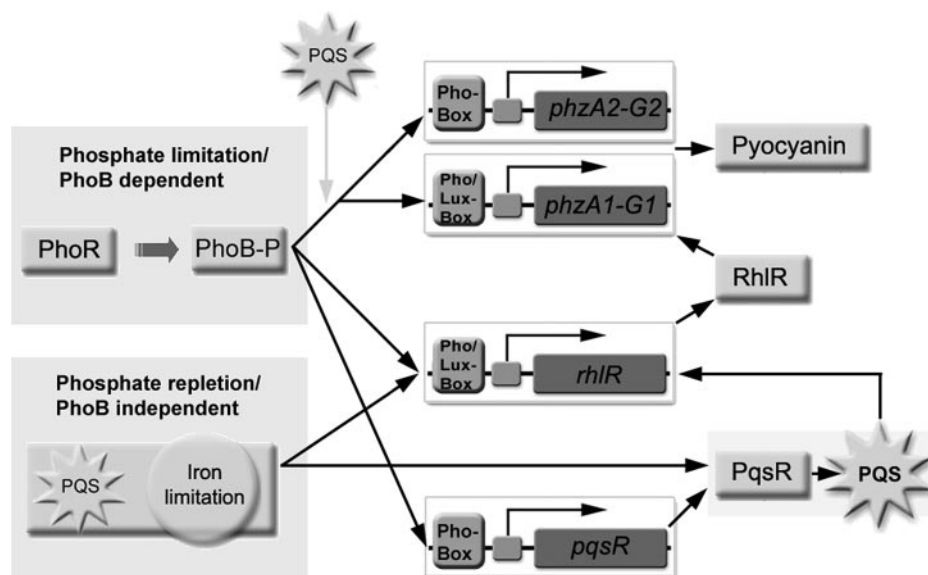


FIG. 6. Model of the complex regulatory circuit of secondary metabolite production in *P. aeruginosa*.

regulatory influence of PhoB and that this influence is enhanced in the presence of PQS. Recent work of Dietrich et al. (14) provided evidence that pyocyanin itself functions as a signal molecule and up-regulates genes encoding the MexGHI-OmpD pump. Interestingly, we found a putative PHO box preceding *mexG*, implicating that the pump is also under the regulatory influence of PhoB. To further confirm our suggestion that pyocyanin production is influenced by PhoB, we monitored pyocyanin production in the *phoB* mutant background after complementation with *phoB* in *trans* with and without the addition of PQS (Fig. 5). Under low-phosphate medium conditions, complementation of *phoB* could partially restore pyocyanin production. The complemented mutant probably did not reach the pyocyanin levels of the wild type due to a lack of a (fully) functional PhoR. Under high-phosphate medium conditions the *phoB* mutant, the *phoB*-complemented strain, and the wild type did not produce high levels of pyocyanin, albeit in all three strains the expression level could be slightly enhanced (significant induction could only be observed for the *phoB* mutant [paired *t* test, $P = 0.02$]) by the addition of PQS probably via a PQS-dependent *rhlR* activation.

Conclusion. Our results amount to the elucidation of the complex relationship between the phosphate regulon and QS in *P. aeruginosa* (Fig. 6): in response to low phosphate and possibly yet-to-be-determined other (environmental) factors, PhoB is activated, and phosphorylated PhoB subsequently enhances PhoB-dependent gene transcription. We found an enhanced pyocyanin production probably directly by a *phoB*-dependent transcriptional activation of *phzA1/phzA2* and indirectly via an augmentation of *rhlR* and *pqsR* expression, both of which positively influence pyocyanin production. PQS (possibly due to its iron-chelating capability) and low-iron medium conditions also stimulate *rhlR* transcription and the expression of HAQs in a *phoB*-independent manner under high-phosphate medium conditions.

It appears that QS in *P. aeruginosa* is a very complex and fine-tuned regulatory circuit important for the pathogenesis of *P. aeruginosa* (32). The identification of PhoB as a response regulator that links environmental and cell-density-dependent cues sheds light on the molecular mechanisms by which environmental signals trigger the regulatory events required for QS. Research in this area and molecular details on how PQS signaling is dependent on PhoB will contribute significantly to the understanding of the molecular mechanism underlying bacterial adaptation involving both perception of the environment and cell density.

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