PqsE Is Essential for RhlR-Dependent Quorum Sensing Regulation in Pseudomonas aeruginosa

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ABSTRACT The bacterium Pseudomonas aeruginosa has emerged as a central threat in health care settings and can cause a large variety of infections. It expresses an arsenal of virulence factors and a diversity of survival functions, many of which are finely and tightly regulated by an intricate circuitry of three quorum sensing (QS) systems. The las system is considered at the top of the QS hierarchy and activates the rhl and pqs systems. It is composed of the LasR transcriptional regulator and the LasI autoinducer synthase, which produces 3-oxo-C12-homoserine lactone (3-oxo-C12-HSL), the ligand of LasR. RhlR is the transcriptional regulator for the rhl system and is associated with RhlI, which produces its cognate autoinducer C4-HSL. The third QS system is composed of the pqsABCDE operon and the MvfR (PqsR) regulator. Pqs-ABCD synthetize 4-hydroxy-2-alkylquinolines (HAQs), which include ligands activating MvfR. PqsE is not required for HAQ production and instead is associated with the expression of genes controlled by the rhl system. While RhlR is often considered the main regulator of rhlI, we confirmed that LasR is in fact the principal regulator of C4-HSL production and that RhlR regulates rhlI and production of C4-HSL essentially only in the absence of LasR by using liquid chromatography-mass spectrometry quantifications and gene expression reporters. Investigating the expression of RhlR targets also clarified that activation of RhlR-dependent QS relies on PqsE, especially when LasR is not functional. This work positions RhlR as the key QS regulator and points to PqsE as an essential effector for full activation of this regulation.

IMPORTANCE Pseudomonas aeruginosa is a versatile bacterium found in various environments. It can cause severe infections in immunocompromised patients and naturally resists many antibiotics. The World Health Organization listed it among the top priority pathogens for research and development of new antimicrobial compounds. Quorum sensing (QS) is a mechanism that relies on the release of small signaling molecules as a way to regulate the expression of genes controlled by the QS circuit. Here, we validate the central role of the PqsE protein in QS particularly by its impact on the regulator RhlR. This study challenges the traditional dogmas of QS regulation in P. aeruginosa and ties loose ends in our understanding of the QS circuit by confirming RhlR to be the main QS regulator in P. aeruginosa. PqsE could represent an ideal target for the development of new control methods against the virulence of P. aeruginosa. This is especially important when considering that LasR-defective mutants frequently arise, e.g., in chronic infections.

KEYWORDS cell-cell communication, gene regulation, pyocyanin, virulence factors

Pseudomonas aeruginosa, a bacterium found in a large variety of environments, is most closely associated with human activities (1). This opportunistic human pathogen can cause infections in diverse animals and plants. Its ability to adapt to various conditions has been linked to the many layers of regulation allowing it to control the expression of virulence factors and optimize survival. Quorum sensing (QS) is a mechanism that relies on the release of small signaling molecules as a way to regulate the
expression of several genes in a population density-dependent manner. In *P. aeruginosa*, three QS systems are hierarchically organized (Fig. 1). The las system, which is composed of the transcriptional regulator LasR and the acyl-homoserine lactone (AHL) synthase LasI, is generally considered to be at the top of the regulatory hierarchy. LasR is activated by 3-oxo-C12-homoserine lactone (3-oxo-C12-HSL), the autoinducing signal produced by LasI. This system regulates several virulence functions such as elastase (LasB) and phospholipase C (PlcB) but also the gene encoding the LasI synthase (*lasI*). LasR also activates the transcription of the *rhlI* and *rhlR* genes, which code for the AHL synthase RhlI and the transcriptional regulator RhlR (5, 7). In this second AHL-mediated QS system of *P. aeruginosa*, RhlR associates with C4-HSL, produced by RhlI, and activates the transcription of genes implicated in several functions, such as the biosynthesis of rhamnolipids (*rhlAB*), hydrogen cyanide (*hcnABC*), and phenazines (two orthologous *phzABCDEFG* operons) as well as genes encoding lectins (*lecA* and *lecB*) (2, 5, 8–13). The third QS system relies on signaling molecules of the 4-hydroxy-2-alkylquinoline (HAQ) family. The transcriptional regulator MvfR (PqsR) responds to dual ligands 4-hydroxy-2-heptylquinoline (HHQ) and with higher affinity to the *Pseudomonas* quinolone signal (PQS; 3,4-dihydroxy-2-alkylquinoline) (14). While LasR activates the transcription of the *mvfR* gene and the *pqs* operon, RhlR has a negative effect on the transcription of *pqsABCDE* (15–17).

LasR-defective mutants frequently arise in various environments (18–22). It could be expected that these mutants would be unable to regulate QS-dependent genes; however, we have shown that RhlR is also able to activate the transcription of LasR target genes when the latter is nonfunctional (23). Indeed, LasR-defective strains expressing RhlR-regulated functions are found (22, 24, 25), implying that QS is not abolished in the absence of LasR. In recent work, a *lasR* mutant isolated from the lungs of an individual with cystic fibrosis expressed a *rhl* system that acted independently of the *las* system (26). It allowed this strain to produce factors essential for its growth...
under a specific condition that would normally require a functional LasR. When evolved under controlled conditions, this strain gained a mutation in MvfR (PqsR) making it unable to produce PQS and to activate the RhlR-dependent genes, highlighting the link between the pqs operon and RhlR.

Although a thioesterase activity of PqsE could participate in the biosynthesis of HAQs (27), the protein encoded by the last gene of the pqs operon is not required, since a pqsE mutant shows no defect in HAQ production (14). On the other hand, PqsE is implicated in the regulation of genes that include many of the RhlR-dependent targets, such as the phz and hcn operons and the lecA gene, through an unknown mechanism (28–33). An impact of PqsE on the RhlR-dependent regulon was proposed; for instance, PqsE could enhance the affinity of RhlR for C4-HSL (28) or even synthesize an alternative ligand for RhlR (34). Importantly, such function is independent of its thioesterase function, as inhibitors of this activity had no impact on the regulatory functions of PqsE (27, 28).

In this study, we validate that activation of RhlR-dependent QS strongly relies on the presence of a functional PqsE and reveal that this is especially important for activation of the rhl system in cases where LasR is not functional. This makes RhlR the key QS regulator and points to PqsE as an essential effector for full activation of this regulation. These findings thus strengthen the position of RhlR as the master regulator of QS and place PqsE at the center of QS regulatory circuitry in P. aeruginosa.

RESULTS AND DISCUSSION

RhlR is not the main activator of C4-HSL production. Quorum sensing regulation is typically described as a partnership between a LuxI-type AHL synthase and a LuxR-type transcriptional regulator. The LuxR-type regulator is activated by a cognate AHL and then regulates the transcription of target genes as well as the gene encoding the synthase, which upregulates AHL production, resulting in an autoinducing loop. In P. aeruginosa, the 3-oxo-C12-HSL synthase LasI is associated with the LasR regulator and the C4-HSL synthase RhlI with the RhlR regulator. Interestingly, LasR regulates the transcription of both rhlI and rhlR genes (2, 5, 7, 35); actually, it has been argued that LasR, and not RhlR, is the primary regulator of rhlI (35). Accordingly, we previously reported that C4-HSL production is decreased in a lasR mutant (23, 26). Indeed, a study in strain 148 showed that LasR binds the lux box found in the promoter region of rhlI but that RhlR does not (36), while other studies showing a direct regulation of rhlI by RhlR were actually performed in a heterologous host, in the absence of LasR (7, 35). Together, these reports would suggest that RhlR mostly activates the transcription of rhlI when LasR is unable to.

To verify that RhlR is not the main regulator of C4-HSL production in a LasR-positive background, we measured concentrations of this AHL in cultures using liquid chromatography-tandem mass spectrometry (LC-MS/MS). The production of C4-HSL is only detectable at the stationary phase in a lasR mutant, while in a rhlR mutant, the production is only slightly delayed compared to that of wild-type (WT) P. aeruginosa strain PA14 (Fig. 2). This concurs with the often-overlooked idea (e.g. see reference 37) that it is LasR, rather than RhlR, that is primarily responsible for activating the transcription of rhlI and thus the production of C4-HSL, the ligand of RhlR. Interestingly, production is even more diminished in a double lasR pqsE mutant, while it is not affected at all in the ΔpqsE mutant, indicating PqsE has a role in LasR-independent activation of C4-HSL production (Fig. 2).

PqsE is important for LasR-independent quorum sensing. A plausible explanation for the results presented in Fig. 2 is that RhlR is a secondary regulator of rhlI, mostly important in the absence of LasR only, and that the absence of PqsE negatively affects the activity of RhlR only when LasR is not functional. To verify this hypothesis, we needed to investigate the activity of RhlR through one of its primary targets. Phenazines are redox-active metabolites produced by P. aeruginosa and are synthetized via two redundant operons: phzA1-G1 (phz1) and phzA2-G2 (phz2). These operons are almost identical and encode proteins that catalyze the synthesis of phenazine-1-
carboxylic acid (PCA). PCA converts into derivatives such as pyocyanin, the blue pigment characteristic of *P. aeruginosa* cultures (38). The *phz* operons are differentially regulated depending on conditions, but the *phz1* operon shows higher expression than *phz2* in planktonic cultures of strain PA14 (39). The promoter of the *phz1* operon contains a *las* box which can be recognized by both LasR and RhlR (40). We measured the activity of a chromosomal *phzA1-lux* reporter in both *lasR* and *rhlR* mutants to verify their involvement in the regulation of the transcription of the *phz1* operon (Fig. 3). The transcription of *phz1* is completely abolished in a *rhlR* mutant but it is still observed in a *lasR* mutant, although it starts much later than for the WT (after an optical density at 600 nm [OD$_{600}$] of 4.0). This is consistent with the delayed production of pyocyanin (23, 41) and C$_4$-HSL (Fig. 2) observed in cultures of a *lasR* mutant. Since we know that

![Graph showing C$_4$-HSL production and transcription of the *phz1* operon](image1)

**Fig 2** C$_4$-HSL production depends mostly on LasR. C$_4$-HSL production was measured in cultures of PA14 and Δ*pqsE, lasR::Gm, lasR::Gm Δ*pqsE*, and *rhlR::MrT7* mutants at different time points during growth. The values are means ± standard deviations (error bars) from three replicates.

![Graph showing transcription of the *phz1* operon](image2)

**Fig 3** Transcription of the *phz1* operon absolutely requires RhlR and PqsE in a *lasR*-negative background. Luminescence of a *phzA1-lux* chromosomal reporter was measured in *P. aeruginosa* PA14 and various isogenic mutants at different time points during growth. The values are means ± standard deviations (error bars) from three replicates.
transcription of \textit{phz1} and production of pyocyanin are abrogated in a double \textit{lasR} \textit{rhlR} mutant (23, 41), these results indicate that \textit{RhlR}, but not \textit{LasR}, regulates the transcription of \textit{phzA1} and that \textit{RhlR} is responsible for the late activation of \textit{phzA1} expression in a \textit{lasR}-negative background. We used transcription of the \textit{phz1} operon to further study the influence of \textit{PqsE} on \textit{RhlR}-dependent regulation. Even if cultures of a \textit{pqsE} mutant do not show any visible pyocyanin, we still observe clear expression of \textit{phz1} (Fig. 3). Since there is no pyocyanin produced in the \textit{WT} until an \textit{OD}_{600} of around 2.5 even if there is expression from the \textit{phzA1} promoter, there seems to be a minimal level of expression of \textit{phz} genes for detectable pyocyanin. Also, pyocyanin is not a direct product of the \textit{phz} operons and it is possible that other enzymes (e.g., PhzM or PhzS) implicated in the conversion of PCA to pyocyanin do not follow the same pattern of expression in this background (29). The transcription of \textit{phzA1} is completely abolished in a double \textit{lasR pqsE} mutant. Many studies report an impact of PQS-dependent QS on the regulation of the \textit{phz} operons or pyocyanin production (28, 31, 39, 41, 42). More specifically, this effect necessitates a functional \textit{PqsE} (28, 42).

Because \textit{LasR} regulates the expression of \textit{rhlI} (5, 7, 23), we performed a \textit{\beta}-galactosidase assay using a \textit{rhlI-lacZ} reporter to verify the impact of \textit{PqsE} on the transcription of \textit{rhlI}. As expected, transcription of \textit{rhlI} is much delayed in a \textit{lasR} mutant (Fig. 4). This is compatible with the late activation of \textit{phz1} we observed (Fig. 3) and is apparently occurring because \textit{RhlR} takes the relay in activating the transcription of \textit{rhlI} following the initial activation by \textit{LasR}. When the \textit{pqsE} gene is inactivated in a \textit{lasR} background, very low transcription of \textit{rhlI} is observed (Fig. 4) which concurs with the production of \textit{C}_{4}-\textit{HSL} in this background (Fig. 2) and which agrees with a \textit{PqsE}-dependent activity of \textit{RhlR}. Again, since \textit{RhlR} takes over regulating the production of \textit{C}_{4}-\textit{HSL} following the initial activation by \textit{LasR}, the transcription of \textit{rhlI} slows down in \textit{rhlI} and \textit{rhlR} mutants after an \textit{OD}_{600} of 2.0, when \textit{LasR} main activity is decreasing (the levels of 3-oxo-\textit{C}_{12}-\textit{HSL} are rapidly declining) (23, 31). Together, these data point to a role for \textit{PqsE} in \textit{LasR}-independent regulation of the \textit{rhl} system.

\textbf{\textit{PqsE}/\textit{RhlR}/\textit{C}_{4}-\textit{HSL} collude to activate \textit{LasR}-independent quorum sensing.} Since \textit{C}_{4}-\textit{HSL} has an effect on \textit{RhlR} activity (2, 7, 28), we needed to better understand the functional complementarity of \textit{C}_{4}-\textit{HSL} with \textit{PqsE} in modulating the activity of \textit{RhlR}. We measured the activity of the \textit{phzA1-lux} reporter in a \textit{rhlI} mutant as well as in a double \textit{rhlI pqsE} mutant. Transcription of \textit{phzA1} in the \textit{rhlI} mutant was delayed, but not
abolished, suggesting that RhlR utilizes its AHL ligand to activate the phz1 operon but that its presence is not essential (Fig. 5A). However, when both C4-HSL and PqsE are absent (rhlR pqsE double-negative background), there is no residual transcription of phz1 (Fig. 5A), like in the rhlR-negative background (Fig. 3). The profile of expression of phz1 significantly differs between pqsE and rhlI mutants (P values of <0.05 from OD600s of 3.0 to 3.6). In the pqsE mutant, the expression starts at an OD600 of around 2.0, while in the rhlI mutant, it starts later (OD600 of around 3.5) and keeps augmenting through the rest of the growth curve. This suggests that both elements increase the activity of RhlR through different mechanisms.

Since the absence of LasR seems to impose the requirement for PqsE to achieve efficient RhlR activity, we overexpressed pqsE in a lasR-null background. As previously shown (43), the constitutive expression of PqsE augments and advances the transcription of phzA1 (Fig. 5B). When we added exogenous C4-HSL in the lasR mutant bearing a plasmid-borne pqsE, the transcription of phz1 started even earlier and reached higher
levels than with either one separately (P values of 0.046 and 0.002, respectively). Farrow et al. (28) proposed that PqsE acts by enhancing the affinity of RhlR for C4-HSL. However, we see that PqsE increases the activity of RhlR even in the absence of RhlI (Fig. 4 and 5A), thus not supporting this hypothesis; our data suggest that RhlR full activity depends on both C4-HSL and PqsE and that their impact is cumulative.

The induction of RhlR activity by PqsE in the absence of rhlI could be explained by the proposed PqsE-dependent production of a putative alternative RhlR ligand. Indeed, Mujurkhee and colleagues (13) observed activation of rhlA transcription by adding culture-free fluids from a ΔrhlI mutant to a Q5 mutant expressing rhlR under the control of an arabinose-inducible promoter. They proposed in a subsequent study that this activity was PqsE dependent (34). We thus tested the effect of pqsE, rhlI, and rhlI pqsE mutants cell-free culture fluids on the activation of phzA1-lux in the rhlI pqsE double-negative background. As expected, the activity of the reporter is strongly induced by culture supernatants from PA14 or a pqsE mutant (which both contain C4-HSL). On the other hand, there is no activation by supernatants from rhlI and rhlI pqsE mutants (see Fig. S1 in the supplemental material), even when combined with an overexpression of rhlR (data not shown). This argues against an unknown RhlR inducer whose production would require PqsE. The same results were obtained when using an hcnA-lacZ reporter (data not shown).

To validate our model, we looked at the regulation of the hcnABC operon, a dual target of both LasR and RhlR (12, 41), and obtained results similar to what we observed for the phz1 operon and the rhlI gene (see Fig. S2). Taken altogether, our data highlight a possible homeostatic loop between RhlR-RhlI-PqsE and demonstrate that PqsE is essential for maintaining control of RhlR-dependent QS functions in a LasR-independent way.

**Excess RhlR, but not C4-HSL, can overcome a PqsE deficiency.** We then sought to better understand how C4-HSL and PqsE both contribute to RhlR activity. First, we verified if overproduction of C4-HSL could counterbalance a lack of PqsE. It was already shown that adding C4-HSL alone could not restore pyocyanin production in a triple ΔlasR ΔrhlI ΔpqsA mutant, but that adding PQS and C4-HSL together could (41). We thus used a plasmid-borne plac-rhlI for constitutive C4-HSL production and measured its effects on the transcription of phz1 and on pyocyanin production in various backgrounds. Overexpression of rhlI complements the transcription of phz1 in a lasR mutant enough to show pyocyanin production at the stationary phase (Fig. 6A; see also Fig. S3). As expected, this complementation was not as efficient when a pqsE mutation was added to the lasR-negative background, as there was even less transcription of phz1 (P values of <0.05 at all growth phases) (Fig. 6A). Taken together, these results confirm that C4-HSL cannot counterbalance the absence of PqsE and highlight an important role for PqsE in regulating RhlR-dependent genes; this is especially striking in the absence of LasR.

We then looked at the overexpression of RhlR, since it partially restores pyocyanin production in a ΔpqsE background (30). We observed an augmentation in both the transcription of phzA1 and pyocyanin production (Fig. 6B and S3). Figure S3 shows that when RhlR is overexpressed, both lasR and lasR pqsE mutants produce higher levels of pyocyanin, coupled with strong activation of phzA1-lux expression in both backgrounds. This is the first ever report of restoration of phz1 transcription and pyocyanin production in the absence of PqsE. Surprisingly, we observed a discrepancy between the transcription from the phzA1 promoter and pyocyanin production, which indicates that the transcription of the target genes shows a more realistic portrait of the activity of RhlR than only looking at pyocyanin production.

Further supporting our model, the transcription of phzA1 and the production of pyocyanin when rhlR was overexpressed were higher in the lasR mutant than in the lasR pqsE mutant (P value of <0.05 at OD600s of 2.0 to 4.0), and these results again confirm an effect of PqsE on RhlR activity.

**PqsE affects RhlR regulatory activity on its targets, including itself, in the absence of LasR.** The very late activity of phz1 in lasR-negative backgrounds can be
explained by low levels of RhlR, whose initial transcription also requires LasR (2, 5–7, 35). When measuring the activity of an rhlR-lacZ reporter, there was indeed a lower transcription of rhlR in a lasR mutant (Fig. 7). Since overexpression of rhlI did not lead to full activation of the phz genes in a double lasR pqsE mutant background (Fig. 6A), we hypothesized that this was instead caused by low transcription of the rhlR gene. Interestingly, the level of rhlR transcription was even lower in the double lasR pqsE mutant background than in the single lasR mutant. This result is unexpected since the transcription of rhlR is weakly affected in a pqsE-null background (30). Because RhlR can activate the target genes of LasR when the latter is absent (23), we hypothesized that RhlR could therefore regulate itself, explaining the impact of PqsE only in the absence of LasR. Transcription of rhlR-lacZ was accordingly lower in a double lasR rhlR mutant.
to levels similar to those in the lasR pqsE mutant (nonsignificant, $P > 0.05$ at all growth phases) (Fig. 7). This indicates that RhlR directs its own transcription only in the absence of LasR and that PqsE is important for this activity. These data confirm that PqsE is an essential element in RhlR activity when LasR is not functional.

**Conclusion.** The complex quorum sensing circuitry of *P. aeruginosa* has been extensively studied, and we know all three systems are intimately intertwined (44, 45). Although RhlR is often believed to form a traditional autoinducing pair with rhlI, we confirm here that LasR really is the main activator of C4-HSL production and that RhlR activation of rhlI is mainly observed in the absence of a functional LasR. LasR is also an activator of the pqs operon and thus of PqsE. However, production of C4-HSL and PQS are not completely abolished in a lasR mutant, only delayed. In a lasR-null background, the importance of RhlR and PqsE on the activation of phzA1, rhlI, or hcnA is higher than in the WT, since LasR is at the top of the regulation cascade. This allowed us to observe that RhlR is able to fully activate target genes only if PqsE is present. The function of PqsE has been a subject of many studies but is still enigmatic (32). In this work, we show that PqsE most likely promotes the function of RhlR and that this effect seems independent of the presence of C4-HSL or another putative ligand, as previously proposed.

Under laboratory conditions, *P. aeruginosa* can afford a late activation of QS or even no activation of QS at all. In a more competitive environment, it is likely there is pressure to control these genes and to activate their transcription independently of LasR when necessary. PqsE could thus be important as a trigger for stronger and/or earlier RhlR activity. A growing number of studies report on the presence of LasR-deficient variants in chronic infections settings (18, 19, 22). With the absence of a functional LasR in these strains, the traditional QS hierarchy is altered and independent expression of RhlR becomes necessary for the bacteria to activate functions important for survival in hosts, such as virulence factors (like exoproteases and HCN) or biofilm formation (rhamnolipids and lectins).

Importantly, among LasR-deficient *P. aeruginosa* strains isolated from clinical settings, some still express a functional quorum sensing response through the activity of RhlR, independently of LasR (22, 26). Since this study was limited to the prototypical strain PA14, it will be important to extend our findings and investigate the implication of PqsE in the activation of the RhlR regulon in diverse clinical and environmental isolates in order to better understand its role in QS gene regulation in *P. aeruginosa*.
TABLE 1 Strains used in this study

| Strain     | Description | Reference or source |
|------------|-------------|---------------------|
| E. coli    |             |                     |
| DH5α       | F−, Δ80dlacZAM15 Δ(lacZYA-argF)U169 deoR recA1 endA1 hsdR17(rK- mK+) phoA supE44 λ− thi-1 gyrA96 relA1 | Lab collection |
| χ7213      | thr-1 leuB6 thiA21 lacY1 ginV44 recA1 ΔasdA4 Δ(zsf-2::Tn10) thi-1 RP4-2-Tc::Mu [λ pir] | Lab collection |
| P. aeruginosa |           |                     |
| ED14/PA14  | Clinical isolate UCBPP-PA14 | 50 |
| ED36       | ΔppqE      | 14                  |
| ED69       | lasR::Gm   | 14                  |
| ED247      | lasR::Gm ΔppqE | This study |
| ED503      | rhlR::Gm   | 30                  |
| ED297      | rhl::MrT7  | 51                  |
| ED3579     | rhl::MrT7 ΔppqE | This study |
| ED266      | lasR::Gm rhlR::Tc | 23 |

MATERIALS AND METHODS

Strains, plasmids, and growth conditions. Bacterial strains are listed in Table 1. Plasmids used in this study are listed in Table 2. Unless otherwise stated, bacteria were routinely grown in tryptic soy broth (TSB; BD Difco, Canada) at 37°C in a TC-7 roller drum (NB, Canada) at 240 rpm or on lysogeny broth (LB) agar plates. When antibiotics were needed, the following concentrations were used: for Escherichia coli, 15 μg/ml tetracycline and 100 μg/ml carbenicillin, for P. aeruginosa, 100 μg/ml gentamicin, tetracycline at 125 μg/ml (solid) or 75 μg/ml (liquid), and 250 μg/ml carbenicillin. Diaminopimelic acid (DAP) was added to cultures of the auxotroph E. coli χ7213 at 62.5 μg/ml. All plasmids were transformed in bacteria by electroporation (46).

All experiments presented in this work were performed with three biological replicates and repeated at least twice.

Construction of the double ΔppqE mutants. A knockout in both rhl and ppqE was constructed by transfer between chromosomes (46). The genomic DNA (gDNA) of strain ED297 rhl::MrT7 was extracted using the EasyPure bacteria genomic kit (Trans Gen Biotech, China). Three milliliters of an overnight culture of ΔppqE was centrifuged (16,000 × g, 2 min) in separate microtubes. Pellets were washed twice with 300 mM sucrose. Five hundred nanograms of gDNA was added to the bacterial suspension, and the mixture was transferred to a 0.2-mm electroporation cuvette. The cells were electroporated at 2,500 V, immediately transferred to 1 ml LB, and incubated at 37°C for 1 h. Selection was performed on LB agar containing gentamicin. Clones were selected and verified by PCR. The lasR::Gm mutation was introduced in the ΔppqE background by allelic exchange using pSB219.9A as described (14, 47).

Construction of phz1-lux chromosomal reporter strains. The mini-CTX-phz1-lux construct was integrated into the chromosomes of PA14 WT and mutants by conjugation on LB agar plates containing DAP with E. coli χ7213 containing the pCDS101 plasmid. Selection was performed on LB agar plates containing tetracycline.

β-Galactosidase activity assays and luminescence reporter measurements. Strains containing the reporter fusions were grown overnight in TSB with appropriate antibiotics and diluted at an OD600 of 0.05 in TSB. For lacZ reporter assays, culture samples were regularly taken for determination of growth (OD600) and β-galactosidase activity (48). For lux reporter assays, luminescence was measured using a Cytation 3 multimode microplate reader (BioTek Instruments, USA). When mentioned, C6-HSL was added at a final concentration of 20 μM from a stock solution prepared in high-performance liquid chromatography (HPLC)-grade acetonitrile. Acetonitrile only was added in controls. All OD600 measurements were performed with a NanoDrop ND100 spectrophotometer (Thermo Fisher Scientific, Canada).

TABLE 2 Plasmids used in this study

| Plasmid     | Description                                   | Reference or source |
|-------------|-----------------------------------------------|---------------------|
| pCDS101     | Promoter of phz1 in mini-CTX-lux, Tet*        | 52                  |
| pPCS1002    | rhl-lacZ reporter, Carb*                     | 2                   |
| pSB219.9A   | pRIC380 carrying lasR::Gm                     | 47                  |
| pME3846     | rhl-lacZ translational reporter, Tet*         | 53                  |
| pME3826     | hcnA-lacZ translational reporter, Tet*        | 54                  |
| pUCPSK      | Pseudomonas and Escherichia shuttle vector, Carb* | 55 |
| pMIC62      | rhlR gene under control of the lac promoter in pUCPSK | John Mattick |
| pUCPhh      | rhlR gene under control of the lac promoter in pUCPSK | 47 |
| pUCP20      | Pseudomonas and Escherichia shuttle vector, Carb* | 56 |
| pUCP20-ppqE | ppqE gene under control of the lac promoter in pUCP20, Carb* | 57 |
**Pyocyanin quantification.** Overnight cultures of PA14 and mutants were diluted to an OD₆₀₀ of 0.05 in TSB and grown until an OD₆₀₀ of 4 to 5 was reached. Cells were resuspended at 13,000 × g for 5 min, and the cleared supernatant was transferred to 96-well microplates. The absorbance at 695 nm was measured using a Cytation 3 multimode microplate reader. Pyocyanin production was determined by dividing the OD₆₉₅ by the OD₆₀₀.

**Quantification of AHLs.** Analyses were performed by liquid chromatography-mass spectrometry (LC-MS) as described before with 5,6,7,8-tetradetero-4-hydroxy-2-heptylquinoline (HHQ-d₄) as an internal standard. (49).

**Data analysis.** Statistical analyses were performed using R software version 3.6.3 (http://www.R-project.org) using one-way analysis of variance (ANOVA) with Tukey post hoc tests at different stages of growth. All conclusions discussed in this paper were based on significant differences. Probability (P) values of less than 0.05 were considered significant.

**SUPPLEMENTAL MATERIAL**
Supplemental material is available online only.

**FIG S1**
PDF file, 0.1 MB.

**FIG S2**
PDF file, 0.2 MB.

**FIG S3**
PDF file, 0.1 MB.

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