The PqsE and RhlR proteins are an autoinducer synthase–receptor pair that control virulence and biofilm development in *Pseudomonas aeruginosa*

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Pseudomonas aeruginosa **is a leading cause of life-threatening nosocomial infections. Many virulence factors produced by *P. aeruginosa* are controlled by the cell-to-cell communication process called quorum sensing (QS). QS depends on the synthesis, release, and groupwide response to extracellular signaling molecules called autoinducers. *P. aeruginosa* possesses two canonical LuxI/R-type QS systems, LasI/R and RhlI/R, that produce and detect 3OC12-homoserine lactone and C4-homoserine lactone, respectively. Previously, we discovered that RhlR regulates both RhlI-dependent and RhlI-independent regulons, and we proposed that an alternative ligand functions together with RhlR to control the target genes in the absence of RhlI. Here, we report the identification of an enzyme, PqsE, which is the alternative-ligand synthase. Using biofilm analyses, reporter assays, site-directed mutagenesis, protein biochemistry, and animal infection studies, we show that the PqsE-produced alternative ligand is the key autoinducer that promotes virulence gene expression. Thus, PqsE can be targeted for therapeutic intervention. Furthermore, this work shows that PqsE and RhlR function as a QS-autoinducer synthase–receptor pair that drives group behaviors in *P. aeruginosa*.**

quorum sensing | biofilms | *Pseudomonas aeruginosa* | antimicrobial | virulence

Multidrug-resistant *Pseudomonas aeruginosa* is the leading cause of hospital-acquired infections in the United States (1). *P. aeruginosa* infections are difficult to eradicate because of antibiotic resistance and because *P. aeruginosa* forms communities called biofilms during infection (2). Essential for virulence and biofilm development in *P. aeruginosa* is a cell–cell communication process called quorum sensing (QS) (3, 4). QS allows bacteria to assess and collectively respond to changes in population density (reviewed in ref. 5). QS relies on the production, detection, and groupwide response to extracellular signaling molecules called autoinducers. In a typical Gram-negative bacterial QS system, an acyl-homoserine lactone autoinducer synthase, usually a LuxI homolog, produces an autoinducer that is bound by a partner transcription factor, usually a LuxR homolog. Most LuxR-type receptors function only when bound to their cognate autoinducers. Furthermore, autoinducer binding is required for some LuxR-type proteins to fold, and thus resist proteolysis (6).

The *P. aeruginosa* QS circuit consists of two LuxI/R type pairs: LasI/R and RhlI/R (7, 8). LasI produces and LasR responds to the autoinducer 3OC12-homoserine lactone (3OC12-HSL). The LasR:3OC12-HSL complex activates transcription of many genes including *rhlR*, encoding a second QS receptor. RhlR binds to the autoinducer C4-homoserine lactone (C4-HSL), the product of RhlI. RhlR:C4-HSL also directs a large regulon of genes, including those encoding secondary metabolites such as rhamnolipids (9–11) and pyocyanin (12, 13), that play crucial roles in virulence and biofilm formation. Typically, mutations in QS *luxI*-type and *luxR*-type genes (i.e., *lasI-lasR* and *rhlI-rhlR*) confer identical phenotypes because each component of the pair needs the other to function (5). However, we previously discovered that RhlR directs both RhlI-dependent and RhlI-independent regulons (14). Importantly, we showed that Δ*rhlI* mutant cell-free culture fluids contain an activity that stimulates RhlR-dependent gene expression, indicating that another autoinducer (an alternative ligand) must exist (14).

Here, we discover that PqsE is required for production of the alternative ligand that activates the RhlR QS receptor in the absence of the canonical autoinducer C4-HSL. PqsE is a thioesterase and residues in the active site are required for alternative-ligand synthesis. The PqsE-derived alternative ligand is sufficient to activate RhlR, and we identify residues in the RhlR ligand binding domain that are required for the response to the alternative ligand. Thus, PqsE and RhlR function as an autoinducer synthase–receptor pair that activates expression of genes specifying *P. aeruginosa* group behaviors. We demonstrate that PqsE is required and RhlI is dispensable for RhlR-directed virulence in animals.

Significance

The human pathogen *Pseudomonas aeruginosa* is the leading cause of hospital-acquired infections and, moreover, is resistant to commonly used antibiotics. *P. aeruginosa* uses the cell-to-cell communication process called quorum sensing (QS) to control virulence. QS relies on production and response to extracellular signaling molecules called autoinducers. Here, we identify the PqsE enzyme as the synthase of an autoinducer that activates the QS receptor RhlR. We show that the PqsE-derived autoinducer is the key molecule driving *P. aeruginosa* biofilm formation and virulence in animal models of infection. We propose that PqsE and RhlR constitute a QS synthase–receptor pair, and that this system can be targeted for antimicrobial development.

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Results

A Screen to Identify the Alternative-Ligand Synthase. We recently discovered that the *P. aeruginosa* QS receptor RhlR regulates hundreds of genes in the absence of its partner synthase RhlI, and thus in the absence of its canonical autoinducer C4-HSL (14). Specifically, on Congo red agar biofilm medium, although WT *P. aeruginosa* UCBPP-PA14 (hereafter called PA14) exhibits a rugose-center/smooth-periphery colony biofilm phenotype, the Δ*rhlR* mutant is hyper-rugose because it fails to produce phenazines (e.g., pyocyanin) (12, 13). In contrast, the Δ*rhlI* mutant is smooth because of the overproduction of phenazines (Fig. 1*A*). Thus, unlike most QS receptor–synthase pairs, the Δ*rhlR* and Δ*rhlI* mutants do not have identical phenotypes. We discovered that Δ*rhlI* mutant cell-free culture fluids contain an activity

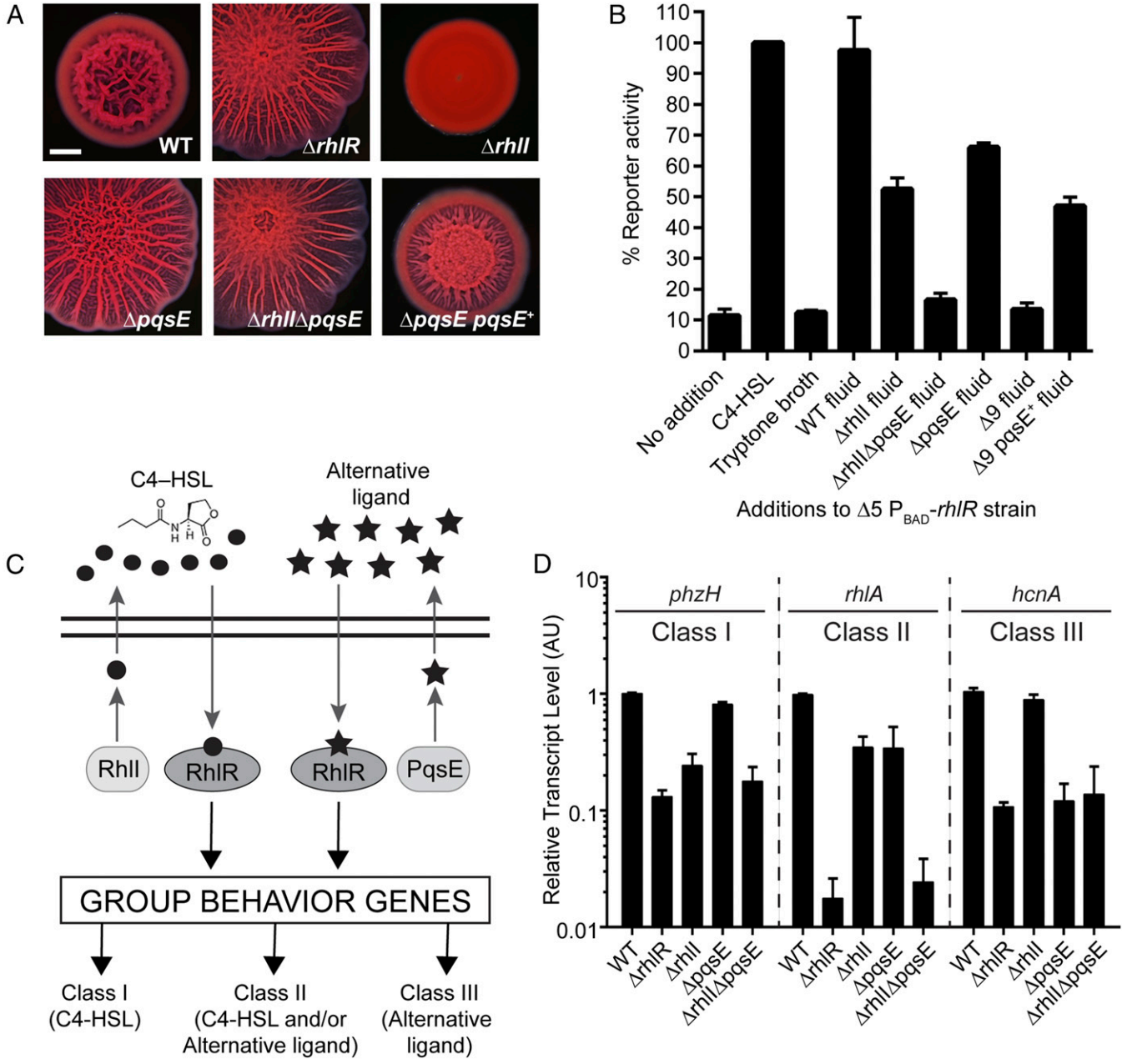

Fig. 1. Identification of *pqsE* as the alternative-ligand synthase. (*A*) Colony biofilm phenotypes of WT PA14 and the designated mutants on Congo red agar medium after 5 d of growth. *pqsE*$^+$ refers to complementation with *pqsE* under the P_{lac} promoter on the pUCP18 plasmid. (Scale bar, 2 mm.) (*B*) *rhlA* expression was measured using a chromosomally encoded P*rhlA-mNeonGreen* transcriptional reporter. The PA14 strain used in this analysis is Δ*lasR* Δ*lasI* Δ*rhlR* Δ*rhlI* Δ*pqsE* carrying P_{BAD}-*rhlR* on the chromosome; designated Δ5 P_{BAD}-*rhlR*. *rhlR* was induced with 0.1% L-arabinose. The *rhlA* reporter activity was set to 100% when 10 μM C4-HSL was added (second bar). For all other bars, either 30% (vol/vol) of broth or the indicated cell-free culture fluid was added. The bar designated Δ9 refers to culture fluid from the Δ*lasR* Δ*lasI* Δ*rhlR* Δ*rhlI* Δ*pqsABCDE* strain. The bar designated Δ9 *pqsE*$^+$ refers to culture fluid from the Δ*lasR* Δ*lasI* Δ*rhlR* Δ*rhlI* Δ*pqsABCDE* strain carrying *pqsE* on the pUCP18 plasmid under the P_{lac} promoter. (*C*) Schematic of the RhlR-dependent QS system: RhlI makes C4-HSL (circles), and here, PqsE is discovered to be required for alternative-ligand (stars) synthesis. The two black horizontal lines represent the cytoplasmic membrane. Class I, Class II, and Class III denote RhlI-dependent, partially dependent, and independent genes, respectively. Class II and III genes require the alternative ligand for full expression. (*D*) Relative expression of the representative RhlR-regulated genes *phzH*, *rhlA*, and *hcnA* measured by qRT-PCR in the WT and designated mutants grown planktonically to HCD (OD_{600} = 2.0). Data are normalized to 5S RNA levels. AU denotes arbitrary units. (*B* and *D*) Error bars represent SEM for three biological replicates.

(hereafter called the alternative ligand) that stimulates RhlR-dependent target gene expression (14).

In this study, our goal was to identify the gene or genes required for synthesis of the alternative ligand. We took a mutagenesis approach with the following rationale: in the Δ*rhlI* mutant that makes no C4-HSL autoinducer, disruption of the gene encoding the alternative-ligand synthase would eliminate production of the alternative ligand. As a consequence, RhlR would be rendered inactive because of the absence of both of its ligands. Thus, phenazine production would be abolished, which would confer the hyper-rugose colony biofilm phenotype to the strain. Such a mutant would also fail to make the alternative ligand that remains present in cell-free culture fluids of the Δ*rhlI* strain (14). Before screening, we first eliminated two obvious candidates: HdtS, a non-LuxI autoinducer synthase (15), and AmbBCDE, the enzymes that produce the Integrated Quorum Sensing Signal [2-(2-hydroxyphenyl)-thiazole-4-carbaldehyde] (16). We made single Δ*hdtS* and Δ*ambB* mutants and double Δ*rhlI* Δ*hdtS* and Δ*rhlI* Δ*ambB* mutants. None had the hyper-rugose colony biofilm phenotype (*SI Appendix*, Fig. S1*A*), and all possessed alternative-ligand activity in their cell-free culture fluids (*SI Appendix*, Fig. S1*B*). Thus, neither HdtS nor AmbB is involved in alternative-ligand synthesis, and the Integrated Quorum Sensing Signal cannot be the alternative ligand.

To discover the alternative-ligand synthase, we randomly mutagenized the Δ*rhlI* strain using the Tn*5* IS50L derivative IS*lacZ*/*hah* (17). We screened ∼10,000 colonies for those exhibiting the hyper-rugose colony biofilm phenotype. Transposon insertions were located in genes encoding hypothetical proteins, as well as proteins involved in motility and c-di-GMP production, which are known to affect the colony biofilm phenotype (*SI Appendix*, Table S1) (18, 19). We were particularly intrigued to identify multiple transposon insertions in *pqsA* and *pqsD* of the *pqsABCDE* operon, and we focused on these mutants here (*SI Appendix*, Fig. S2*A*). PqsABCD, but not PqsE, is required for the synthesis of PQS (2-heptyl-3-hydroxy-4-quinolone) (20) and other quinolones (*SI Appendix*, Fig. S2*B*) (21). This result was surprising, as we have previously shown that PQS is not the alternative ligand (14). To test whether another quinolone produced by PqsABCDE is the alternative ligand, *pqsA* and *pqsD* were deleted in the WT and Δ*rhlI* strains and the colony biofilm phenotypes assayed. The Δ*pqsA* and Δ*pqsD* mutants have approximately WT colony biofilm phenotypes, and the Δ*rhlI* Δ*pqsA* and Δ*rhlI* Δ*pqsD* double mutants form smooth colony biofilms (*SI Appendix*, Fig. S2*C*). We therefore infer that neither of these genes is responsible for alternative-ligand production. Because transposon insertions can be polar, and *pqsA* and *pqsD* lie upstream of *pqsE*, PqsE remained a candidate for the alternative-ligand synthase. We investigated this possibility by generating a deletion of *pqsE* in the WT and Δ*rhlI* strains. Indeed, the Δ*pqsE* mutant displays a hyper-rugose colony biofilm phenotype, and the Δ*rhlI* Δ*pqsE* double mutant has a colony biofilm phenotype indistinguishable from the Δ*rhlR* mutant (Fig. 1*A*). Introduction of a plasmid carrying *pqsE* restored the WT colony biofilm phenotype (Fig. 1*A*).

To examine whether PqsE is required for production of the alternative ligand, we constructed a PA14 RhlR-dependent reporter strain harboring deletions in the *lasR*, *lasI*, *rhlR*, *rhlI*, and *pqsE* genes that contains a chromosomal P*rhlA-mNeonGreen* transcriptional reporter fusion and chromosomal, arabinose-inducible *rhlR* (this strain is called Δ5 P_{BAD}-*rhlR*). Our results are shown in Fig. 1*B*. The addition of tryptone broth results in background levels of reporter activity similar to a no-addition control. Supplementation with synthetic C4-HSL or with WT cell-free culture fluids elicit maximal (∼ninefold) reporter activity. Addition of cell-free culture fluids from the Δ*rhlI* mutant fosters ∼fivefold induction of the reporter. Most important, in contrast to the Δ*rhlI* strain, cell-free culture fluids from the Δ*rhlI* Δ*pqsE* double mutant fail to stimulate the reporter. Last, cell-free culture fluids from the single Δ*pqsE* mutant show significant (∼sixfold) activity because they contain C4-HSL made by RhlI. To explore this finding further, we generated a nonuple mutant lacking the *lasR*, *lasI*, *rhlR*, *rhlI*, and *pqsABCDE* genes (we call this strain Δ9). Cell-free culture fluids from the Δ9 mutant elicit only background reporter activity. However, introduction of *pqsE* into the Δ9 strain restored activity, as shown by the ability of the fluids to stimulate RhlR-dependent reporter gene expression comparable to that elicited by the Δ*rhlI* strain (Fig. 1*B*). We conclude that PqsE is required for alternative-ligand synthesis.

The PqsE-Derived Ligand Activates Class II and III but Not Class I RhlR Target Genes. We previously showed that there exist three classes of RhlR-regulated genes based on whether C4-HSL, the alternative ligand, or both autoinducers are present (14). Class I genes, exemplified by *phzH*, require C4-HSL; class II genes, such as *rhlA*, depend on both C4-HSL and the alternative ligand; and class III genes, represented by *hcnA*, are activated by the alternative ligand independent of C4-HSL (Fig. 1*C*). These response patterns give us another means to assay for the presence/absence of the alternative ligand, and in turn, to pinpoint the role of PqsE in its production. We performed quantitative RT-PCR analyses on high-cell density (HCD; OD_{600} ∼ 2.0) planktonic cultures of WT, Δ*rhlR*, Δ*rhlI*, Δ*pqsE*, and Δ*rhlI* Δ*pqsE* mutants probing for *phzH*, *rhlA*, and *hcnA* (Fig. 1*D*). Expression of the class I gene, *phzH*, does not change when *pqsE* is deleted in either the WT or the Δ*rhlI* parent, whereas transcription of the class II gene, *rhlA*, declines in both the Δ*rhlI* and Δ*pqsE* strains, and even more so in the double mutant. Finally, consistent with PqsE being the alternative-ligand synthase, class III *hcnA* transcript levels decrease 10-fold in the Δ*pqsE* single and Δ*rhlI* Δ*pqsE* double mutants compared with the WT and Δ*rhlI* strains. Collectively, the data in Fig. 1 provide evidence that PqsE is the alternative-ligand synthase.

PqsE and RhlR Form an Autoinducer Synthase–Receptor Pair. A key feature of all QS circuits is that the autoinducer synthase produces a small molecule that is detected by the cognate receptor. Our present findings show that PqsE is required for synthesis of the alternative ligand that activates RhlR. To address whether the PqsE-derived alternative ligand is necessary and sufficient to stimulate RhlR-controlled group behaviors, we introduced *pqsE* and/or *rhlR* into the Δ9 strain and assessed whether or not RhlR-dependent target genes are expressed. Fig. 2*A* shows quantitative RT-PCR results for *phzH*, *rhlA*, and *hcnA*. Compared with WT, the Δ9 strain fails to express all three genes, and introduction of either *pqsE* or *rhlR* alone does not activate their expression. Consistent with our analyses showing that *phzH* is a RhlI-dependent class I gene, its expression is not activated when *pqsE* and *rhlR* are introduced into the Δ9 mutant. In contrast, introduction of *pqsE* together with *rhlR* restores the WT level of expression of *rhlA* and *hcnA*, which do depend on the alternative ligand.

Beyond regulating transcription, if RhlR and PqsE act as a receptor-synthase pair, together they should control group behaviors in vivo. To investigate this, we quantified pyocyanin production as a proxy for QS-controlled group behaviors in the Δ9 strain and in the Δ9 strain harboring *rhlR*, *pqsE*, or both genes, and we compared the output with that made by the WT. Indeed, introduction of both *rhlR* and *pqsE*, but neither gene alone, restored pyocyanin production to the maximum WT level (Fig. 2*B*). Thus, we conclude that PqsE and RhlR are an autoinducer synthase–receptor pair that drives group behaviors in *P. aeruginosa*.

The PqsE Enzyme Active Site Is Required for Alternative-Ligand Synthesis. PqsE is reported to be a thioesterase that plays a redundant role in converting 2-aminobenzoylacetyl-coenzymeA to 2-aminobenzoylacetate, a step in PQS synthesis (22). The PqsE

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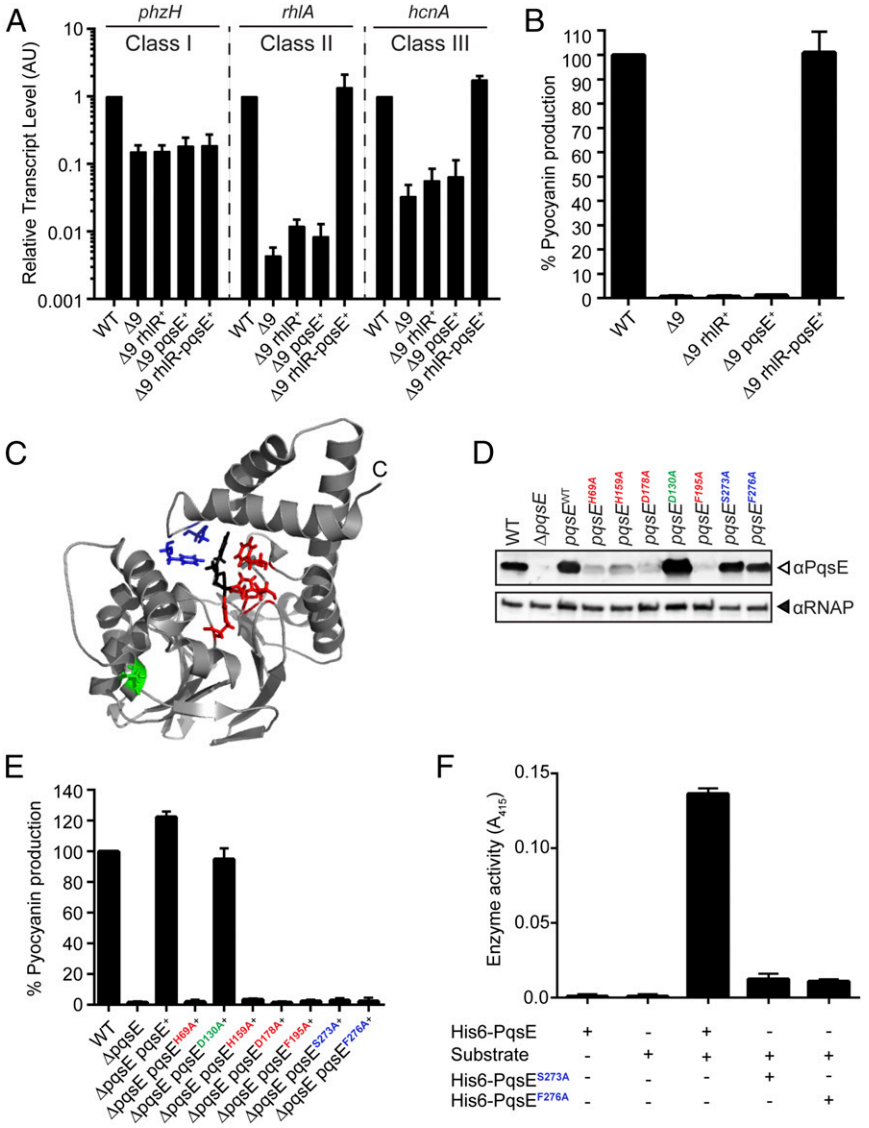

Fig. 2. PqsE and RhlR are an autoinducer synthase–receptor pair. (*A*) Relative expression of the RhlR-regulated genes *phzH*, *rhlA*, and *hcnA* measured by qRT-PCR in the WT and designated mutants grown planktonically to HCD. Class I, Class II, and Class III are as in Fig. 1. Data are normalized to 5S RNA levels. AU denotes arbitrary units. (*B*) Pyocyanin production (OD_{695}) was measured in WT PA14 and the designated mutants. (*C*) PqsE mutations mapped on the available structure of PqsE (gray) bound to 2-aminobenzoylacetate (black) (PDB ID: 5HIO) (24). Residues highlighted in red (H69, H159, D178, F195) form the metal ion center, S273 and F276 are required for alternative-ligand synthesis (blue), and the control residue for mutation is D130 (green). (*D*) Western blot analysis of lysates from WT PA14, the Δ*pqsE* mutant, and the Δ*pqsE* strain complemented with either WT *pqsE* or the indicated *pqsE* mutants. The blot was separately probed with anti-PqsE and anti-RNAP antibodies. (*E*) Pyocyanin production (OD_{695}) was measured in WT PA14 and the designated mutants. (*F*) In vitro thioesterase activity assay for WT PqsE and the designated mutant proteins using the artificial substrate S-(4-nitrobenzoyl) mercaptoethane. In *A*, *B*, *E*, and *F*, error bars represent SEM for three independent replicates.

crystal structure predicts that residues H69, H159, D178, F195, S273, and F276 are in the active site (Fig. 2*C*) (23, 24). We substituted the above residues with alanine and introduced the PqsE variants into the Δ*pqsE* PA14 strain to test their roles in alternative-ligand synthesis. We also substituted D130, a residue distal to the active site pocket, to serve as a WT control mutant. PqsE D130A produced a stable protein, and it restored pyocyanin production to the Δ*pqsE* strain (Fig. 2 *D* and *E*). Alanine substitution of H69, H159, D178, or F195, residues involved in coordination of two active-site metal ions required for enzyme function (23, 24), resulted in unstable protein, and therefore, these PqsE mutants failed to restore pyocyanin production to the Δ*pqsE* strain (Fig. 2 *D* and *E*). PqsE residues S273 and F276 are reported to be required for catalysis and for substrate binding, respectively (23, 25). Substitution of these residues to alanine resulted in stable but inactive PqsE protein, as they failed to complement the pyocyanin production defect of the Δ*pqsE* strain (Fig. 2 *D* and *E*). We tested the two stable PqsE proteins, $PqsE^{S273A}$ and $PqsE^{F276A}$, for defects in in vitro thioesterase activity, using a commercial substrate [S-(4-nitrobenzoyl) mercaptoethane] (23). Although the $PqsE^{WT}$ protein displays thioesterase activity, the $PqsE^{S273A}$ and $PqsE^{F276A}$ proteins do not (Fig. 2*F*). Thus, PqsE active site residues required for thioesterase activity are also required for alternative-ligand synthesis. We conclude that key PqsE active site residues S273 and F276 are crucial for alternative-ligand synthesis and group behavior.

PqsE Is Required for RhlR-Dependent Virulence in Animal Infection Models. We previously demonstrated that RhlR is required for *P. aeruginosa* virulence in nematode and murine infection models, whereas RhlI, and therefore C4-HSL, are dispensable (14). We

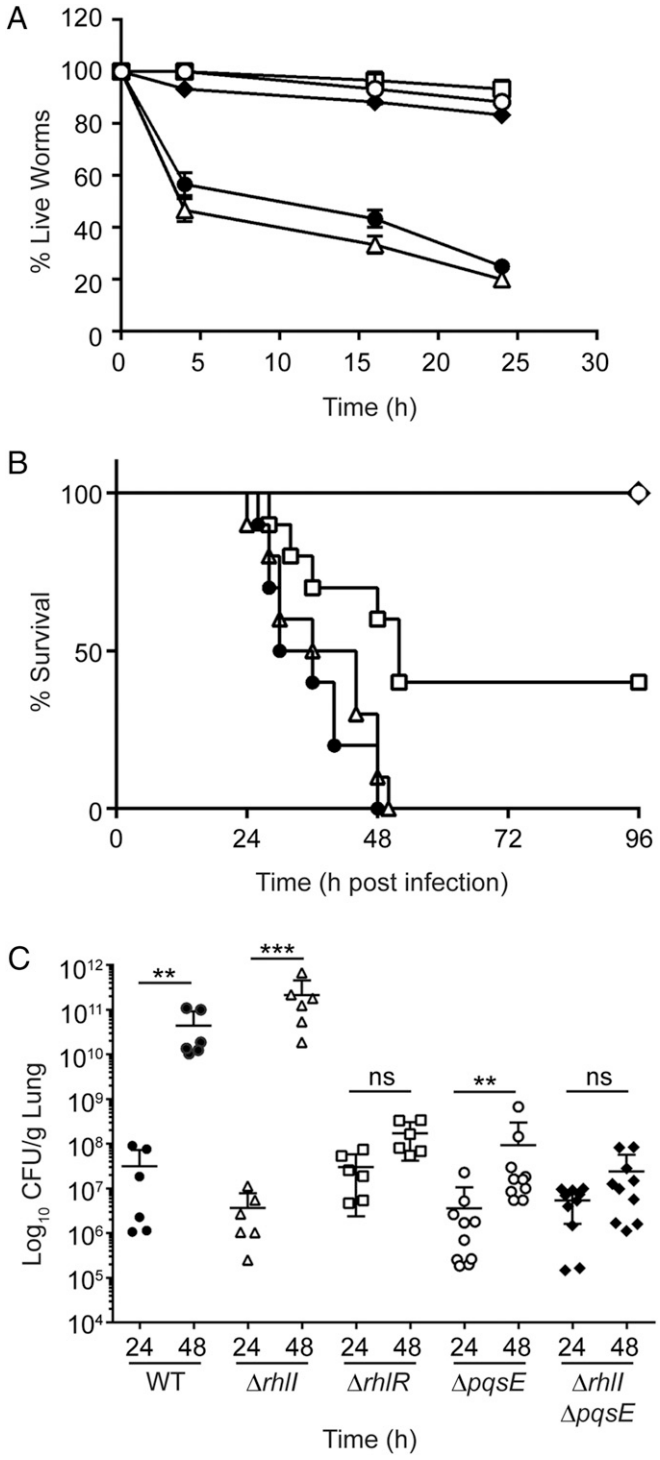

Fig. 3. PqsE is required for RhlR-dependent virulence in animal infection models. (*A*) *C. elegans* were applied to lawns of WT PA14 (closed circles), the Δ*rhlR* mutant (open squares), the Δ*rhlI* mutant (open triangles), the Δ*pqsE* mutant (open circles), and the Δ*rhlI* Δ*pqsE* double mutant (closed diamonds). Error bars represent SEM of three independent replicates. (*B*) For survival experiments, BALB/c mice were infected intratracheally with ~3×10^6 cfu of WT PA14 or the indicated mutants and were monitored for up to 4 d postinfection. Symbols as in *A*. Results are represented on Kaplan Meier curves and were compiled from two independent experiments; $n = 10$. Significant differences were calculated by log rank test by comparing each strain to the WT (Δ*rhlI*; $P = 0.374$, Δ*rhlR*; $P < 0.01$, Δ*pqsE*; $P < 0.0001$, Δ*rhlI* Δ*pqsE*; $P < 0.0001$). (*C*) Bacterial burden recovered from mice at 24 h and 48 h postinfection with WT PA14 or the indicated mutants. Results were analyzed by one-way ANOVA. Multiple comparisons were performed between the indicated points for each strain. ***$P < 0.001$; **$P < 0.05$; ns, not significant.

reasoned that it is the alternative ligand that promotes RhlR-dependent virulence in animals in the absence of RhlI. Discovering that PqsE is the alternative-ligand synthase gave us the means to test our hypothesis about the crucial role of the alternative ligand in RhlR-dependent virulence in animals. In a *Caenorhabditis elegans* fast-kill infection assay, WT *P. aeruginosa* is virulent, the Δ*rhlR* mutant is avirulent, and the Δ*rhlI* mutant that lacks C4-HSL but produces the alternative ligand is as virulent as the WT (Fig. 3*A*). Here we show that both the Δ*pqsE* and Δ*rhlI* Δ*pqsE* mutant strains are avirulent, displaying the same phenotype as the Δ*rhlR* mutant (Fig. 3*A*). We conclude that the PqsE-derived alternative ligand is the key autoinducer driving RhlR-dependent virulence in nematodes.

To determine whether the alternative ligand is the primary autoinducer that stimulates RhlR-dependent virulence in mammals, we examined pathogenicity of the above strains in a murine model of acute lung infection. We previously determined LD_{50} values for the WT, Δ*rhlR*, and Δ*rhlI* strains to be 1.9×10^6 cfu, 2.6×10^6 cfu, and 1.1×10^6 cfu, respectively (14). Here we show that the LD_{50} is >10–20 times higher for both the Δ*pqsE* (3.0×10^7 cfu) and Δ*rhlI* Δ*pqsE* (4.8×10^7 cfu) mutants than the WT. We compared the potential of each of these mutants to influence virulence in the lung infection model, using a single input dose, 3×10^6 cfu, corresponding to ~$1.5 \times LD_{50}$ of the WT strain. Fig. 3*B* shows that mice given the WT or the Δ*rhlI* mutant succumbed to infection by 48 h, whereas mice given the Δ*pqsE* or Δ*rhlI* Δ*pqsE* mutant all survived. Mice infected with the Δ*rhlR* mutant showed ~40% survival.

To determine the level of lung colonization achieved by infection, we administered sublethal doses (~0.5 LD_{50}) of these strains. At 24 h postinfection, the bacterial load was similar among all infected mice (Fig. 3*C*). However, at 48 h postinfection, the bacterial burden in mice infected with the WT and Δ*rhlI* strains increased by 10,000-fold (Fig. 3*C*). In contrast, the bacterial load in mice infected with the Δ*rhlR*, Δ*pqsE*, and Δ*rhlI* Δ*pqsE* strains did not change significantly (Fig. 3*C*). Thus, the Δ*rhlR*, Δ*pqsE*, and Δ*rhlI* Δ*pqsE* strains are highly attenuated, producing a four-order of magnitude lower burden of bacteria in the murine host than the WT and the Δ*rhlI* mutant. In sum, our results show that PqsE-derived alternative ligand is essential to activate RhlR to promote virulence gene expression in both the *C. elegans* and the murine animal models.

RhlR Does Not Require an Autoinducer for Solubility in *P. aeruginosa*. RhlR appears to be an atypical LuxR-type receptor, as it responds to two autoinducers: C4-HSL and the alternative ligand. We wondered how each autoinducer regulates RhlR function. One possibility could be autoinducer control of RhlR protein stability, a common mechanism for LuxI-LuxR type partners. Western blot analyses were used to assess in vivo RhlR levels in cell lysates prepared from the WT, Δ*rhlI*, Δ*pqsE*, and Δ*rhlI* Δ*pqsE* PA14 strains. Compared with WT, only a modest decrease in RhlR levels could be detected in the mutant strains (Fig. 4*A*). We speculate that this decrease occurs because, in the absence of one or both autoinducers, RhlR cannot properly feedback to activate its own transcription.

Our finding that RhlR is stable in the PA14 mutants was surprising, given that this is not the case for other studied LuxR-type proteins (6, 26, 27). Indeed, RhlR, when produced in *Escherichia coli*, is not soluble even in the presence of saturating C4-HSL (28). To further explore this result, we deleted *rhlR* in the WT, Δ*rhlI*, Δ*pqsE*, and Δ*rhlI* Δ*pqsE* backgrounds and reintroduced *rhlR* on a plasmid under a constitutive promoter to eliminate possible changes in RhlR transcription because of the absence of RhlI and/or PqsE.

Soluble RhlR protein is present in all four strains (Fig. 4*B*). We conclude that in PA14, neither C4-HSL nor the alternative ligand is required for RhlR to fold and become soluble. One formal possibility is that there exists a third autoinducer that is capable of solubilizing RhlR in the absence of C4-HSL and the alternative ligand. However, our evidence suggests this is not the case, as cell-free culture fluids prepared from the Δ*rhlI* Δ*pqsE* double mutant do not contain any activity that elicits RhlR-dependent gene expression (Fig. 1 *B* and *D*). We therefore infer that autoinducers are required only to activate RhlR as a transcription factor.

The RhlR Ligand-Binding Domain Is Crucial for Sensing the Alternative Ligand. RhlR contains an N-terminal ligand-binding domain (LBD) and a C-terminal DNA-binding domain (5). There is currently no structure of RhlR. Moreover, RhlR shows significant sequence divergence from TraR, the prototype for which a structure is solved (*SI Appendix*, Fig. S3*A*) (23). Thus, how RhlR binds C4-HSL and/or the alternative ligand is unknown. To explore how RhlR selects its autoinducers, we generated a homology model of RhlR based on the *E. coli* SdiA structure, the closest homolog of RhlR (47% sequence identity; Fig. 4*C* and *SI Appendix*, Fig. S3 *A* and *B*). In SdiA and other LuxR-type proteins, the highly conserved amino acids W68 and D81 (positions refer to RhlR) interact with the amide group-oxygen and the amide group-nitrogen, respectively, of the cognate HSL autoinducers (29, 30). Other conserved residues, such as Y72 and W96, are required for hydrophobic and van der Waals interactions with the ligands (Fig. 4*C* and *SI Appendix*, Fig. S3*C*). We substituted W68, Y72, D81, and W96 with alanine and introduced these RhlR variants into the Δ*rhlR* strain carrying WT *rhlI* and *pqsE*, which is therefore capable of producing both C4-HSL and the alternative ligand. Western blot shows that these RhlR mutant proteins are stable (Fig. 4*D*). To determine how the RhlR mutations affect autoinducer response, we measured transcription of the C4-HSL-dependent class I gene *phzH* and the alternative-ligand–dependent-class III gene *hcnA*. RhlR-driven *phzH* and *hcnA* expression was abolished in every case (Fig. 4 *E* and *F*). We conclude that all four residues are required for sensing both C4-HSL and the alternative ligand.

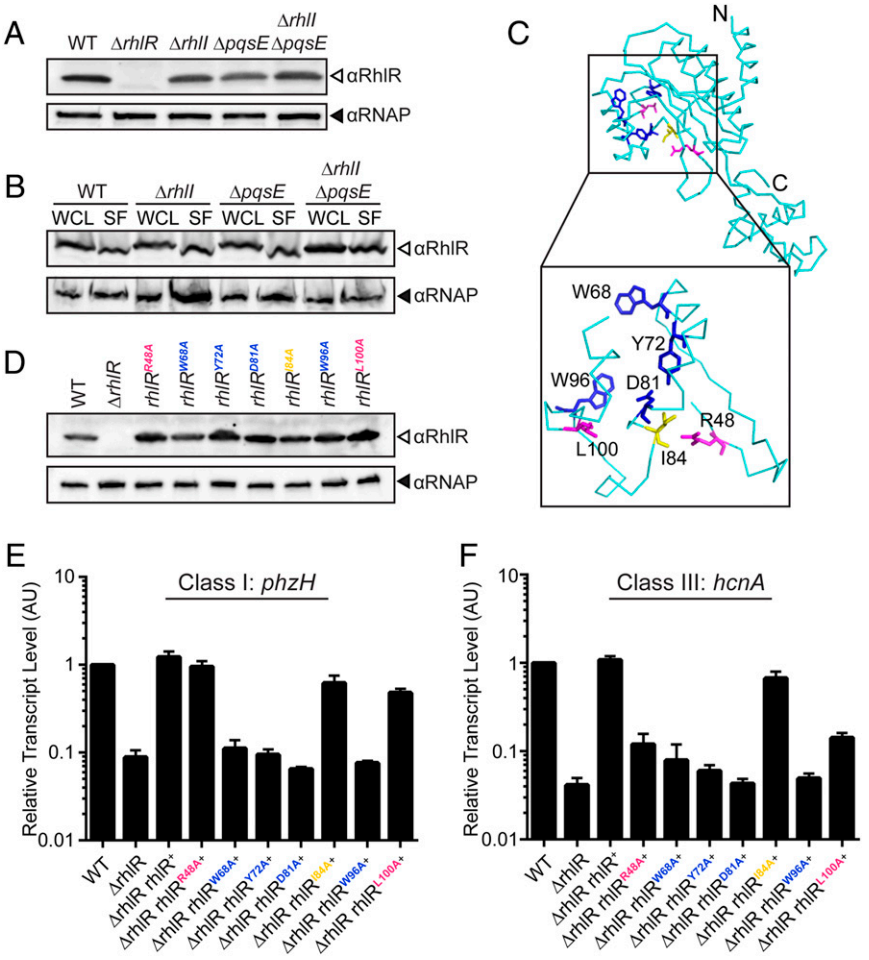

Fig. 4. The RhlR ligand-binding domain is required for sensing the alternative ligand. (*A*) Western blot analysis of lysates from WT, Δ*rhlR*, Δ*rhlI*, Δ*pqsE*, Δ*rhlI*, Δ*pqsE* PA14 strains. RhlR levels were detected using anti-RhlR antibody, and RNAP was probed as the loading control using anti-RNAP antibody. (*B*) Western blot analysis of whole-cell lysates (WCL) and soluble fractions (SF) from the indicated strains; all were additionally deleted for *rhlR* and carry *rhlR* on the pUCP18 plasmid under the P_{lac} promoter. (*C*) The predicted ribbon structure of the RhlR monomer (cyan) based on Phyre2 threading and comparison with the crystal structure of the closest homolog, SdiA, from *E. coli* (PDB ID: 4Y15) (30, 38). (*Inset*) Amino acids lining the putative RhlR ligand-binding pocket that were mutated in the present work. Residues in blue (W68, Y72, D81, W96) are required for response to both C4-HSL and the alternative ligand; residues in magenta (R48, L100) are required for sensing the alternative ligand but not C4-HSL, whereas reside I84 (yellow) is dispensable for detection of both ligands. (*D*) Western blot analysis of lysates from WT PA14, the Δ*rhlR* mutant, and the Δ*rhlR* mutant complemented with either WT *rhlR* or the indicated *rhlR* point mutants. (*E*) Relative expression of the RhlI-dependent *phzH* gene measured by qRT-PCR in the WT and mutant strains grown planktonically to HCD. Data are normalized to 5S RNA levels. Error bars represent SEM of three biological replicates. AU denotes arbitrary units. (*F*) As in *E* showing expression of the PqsE-dependent *hcnA* gene.

Guided by the putative RhlR LBD tertiary structure, we identified amino acids R48, I84, and L100, as predicted to face the interior of the LBD (Fig. 4*C*). Again, we generated alanine substitutions and introduced the RhlR variants into the Δ*rhlR* strain. All the variants produce stable protein (Fig. 4*D*). The RhlR R48A and L100A substitutions eliminated *hcnA* expression without affecting *phzH* expression. The RhlR I84A mutant behaved similar to WT (Fig. 4 *E* and *F*). These data indicate that W68, Y72, D81, and W96 are required for the response to C4-HSL and the alternative ligand. However, the R48 and L100 residues are dispensable for C4-HSL detection, but are necessary for the RhlR response to the alternative ligand. We did not discover any residue that was required exclusively for C4-HSL detection.

Discussion

The PqsE enzyme is widely distributed in *P. aeruginosa* strains and is essential for *P. aeruginosa* QS-dependent group behaviors (21, 31–33). Initially thought to be required for synthesis of the PQS autoinducer, based on its location in the *pqsABCDE* PQS biosynthetic operon, it is now known that PqsE, unlike the other genes in the operon, is dispensable for PQS biosynthesis (*SI Appendix*, Fig. S2*B*) (21, 31). Here, we define the role of PqsE: PqsE catalyzes the synthesis of the alternative ligand, a ligand that is necessary and sufficient to activate RhlR-dependent group behaviors in in vivo and in vitro assays and during animal infection. We are currently working to identify this alternative ligand. Our identification of PqsE as the alternative-ligand synthase explains several previously reported puzzling observations. First, PqsE was reported to require RhlR to enhance Rhl-directed QS (21). Our results show that the PqsE-derived alternative ligand functions together with RhlR to activate RhlR transcriptional activity (Fig. 1). Second, PqsE overexpression/induction was reported to lower PQS levels by an unknown mechanism (31, 34). Our current study, combined with earlier results, provides the mechanism: the PqsE-derived alternative ligand drives RhlR-dependent repression of *pqsA* transcription, thereby reducing PQS production (14). PqsE was also proposed to exert its effect on QS via protein–protein interaction (24). Here, we show that mutating PqsE S273 and F276, putative nonsurface exposed active site residues, eliminate RhlR-directed QS group behaviors, suggesting that the PqsE effect on Rhl QS is not mediated by direct protein–protein interaction with RhlR. Rather, we interpret these earlier results to be a consequence of interaction of the PqsE product, the alternative ligand, with RhlR, but not PqsE itself.

RhlR belongs to the LuxR family of proteins and, similar to its homologs, possesses conserved amino acids in the LBD that, in other receptors, are required for recognition of HSL autoinducers (29, 30, 35). Our finding that mutation of these conserved residues abrogates both C4-HSL and alternative-ligand detection/response suggests that the binding surface for the alternative ligand overlaps with that of the canonical autoinducer C4-HSL. Importantly, RhlR residues R48 and L100, required for response to the PqsE-derived alternative ligand, are distinct from those typically used for HSL recognition. Thus, RhlR LBD has overlapping but not identical binding sites for its autoinducers.

P. aeruginosa is a pathogen of high clinical relevance (36, 37). The key finding in the present work is that PqsE and the alternative ligand are essential, whereas RhlI and the canonical C4-HSL autoinducer are dispensable for RhlR-driven virulence in animal models of infection. Unlike in the *C. elegans* infection assay, in the murine model of acute lung infection, the Δ*pqsE* and Δ*rhlI*Δ*pqsE* mutants were more attenuated than the Δ*rhlR* mutant. We speculate that, beyond virulence, PqsE has a role in PA14 survival in the complex murine lung environment because the alternative ligand controls factors that contribute to bacterial fitness in this niche. Together, these results provide a set of unanticipated targets for therapeutic intervention. PqsE enzyme function, PqsE stability, RhlR interaction with the alternative ligand, RhlR:alternative-ligand dimerization, RhlR:alternative-ligand DNA binding, and RhlR:alternative-ligand RNA-Polymerase engagement are all now revealed as candidates for inhibition in pursuit of new therapeutics. Moreover, these same biocomponents are revealed to function together as a QS pathway in one of the most actively studied QS pathogens, *P. aeruginosa*.

Materials and Methods

Detailed experimental procedures are described in the *SI Appendix*, *Extended Experimental Procedures*. Strains, and plasmids used in this study are listed in *SI Appendix*, Table S2.

Pyocyanin Assay. PA14 strains were grown overnight in LB liquid medium at 37 °C with shaking. Cultures were back diluted 1:1,000 into fresh medium and grown for 18 h. The cells were pelleted by centrifugation, and the culture fluids were passed through 0.22 μm filters into clear plastic cuvettes. The OD_{695} of each sample was measured on a spectrophotometer (Beckman Coulter DV 730).

Colony Biofilm Assay. The procedure for enabling colony biofilm formation has been described (12, 14). Briefly, 1 μL of overnight PA14 cultures was spotted onto 60 × 15 mm Petri plates containing 10 mL 1% TB medium fortified with 40 mg/L Congo red and 20 mg/L Coomassie brilliant blue dyes and solidified with 1% agar. Biofilms were grown at 25 °C, and images were acquired after 120 h, using a Leica stereomicroscope M125 mounted with a Leica MC170 HD camera at 7.78× zoom.

qRT-PCR. PA14 strains were harvested from planktonic cultures (OD_{600} = 2.0), and RNA was purified using Qiagen kits, and subsequently, DNase treated (TURBO DNA-free; Thermo Fisher). cDNA was synthesized using SuperScript III Reverse Transcriptase (Invitrogen) and quantified using PerfeCTa SYBR Green FastMix Low ROX (Quanta Biociences).

Animal Infection Models. *C. elegans* fast-killing assay and murine infection assays were performed as described previously (9). Detailed procedure can be found in *SI Appendix*. All animal procedures were conducted according to the guidelines of the Emory University Institutional Animal Care and Use Committee, under approved protocol number DAR-2003421–042219BN. The study was carried out in strict accordance with established guidelines and policies at Emory University School of Medicine, and recommendations in the Guide for Care and Use of Laboratory Animals of the National Institute of Health, as well as local, state, and federal laws.

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