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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 \textit{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. \textit{P. aeruginosa} possesses two canonical LuxI/R-type QS systems, LasI/R and RhlI/R, that produce and detect 30C12-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 Rhl. 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 \textit{P. aeruginosa}.


The human pathogen \textit{Pseudomonas aeruginosa} is the leading cause of hospital-acquired infections and, moreover, is resistant to commonly used antibiotics. \textit{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 \textit{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.

Significance

The human pathogen \textit{Pseudomonas aeruginosa} is the leading cause of hospital-acquired infections and, moreover, is resistant to commonly used antibiotics. \textit{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 \textit{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.


Reviewer: J.B.X., Memorial Sloan-Kettering; and D.E.O., Virginia Commonwealth University Medical Center.

The authors declare no conflict of interest.

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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. 1A). 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...
(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 \( \Delta hhiI \) 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 \( \Delta hhiI \) 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-carboxaldehyde] (16). We made single \( \Delta hhiS \) and \( \Delta ambB \) mutants and double \( \Delta hhiI \Delta hhiS \) and \( \Delta hhiI \Delta ambB \) mutants. None had the hyper-rugose colony biofilm phenotype (SI Appendix, Fig. S1A), and all possessed alternative-ligand activity in their cell-free culture fluids (SI Appendix, Fig. S1B). 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 \( \Delta hhiI \) strain using the Tn5 IS50L derivative IslacZ/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. S2A). PqsABCD, but not PqsE, is required for the synthesis of PQS (2-heptyl-3-hydroxy-4-quinolone) (20) and other quinolones (SI Appendix, Fig. S2B) (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 PqsABCD is the alternative ligand, pqsA and pqsD were deleted in the WT and \( \Delta hhiI \) strains and the colony biofilm phenotypes assessed. The \( \Delta pqsA \) and \( \Delta pqsD \) mutants have approximately WT colony biofilm phenotypes, and the \( \Delta hhiI \Delta pqsA \) and \( \Delta hhiI \Delta pqsD \) double mutants form smooth colony biofilms (SI Appendix, Fig. S2C). We therefore infer that neither of these genes is responsible for alternative-ligand production. Because transposon insertions can be polar, and neither of these genes is responsible for alternative-ligand production, we performed quantitative RT-PCR analyses of the alternative-ligand synthase, 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, \( \Delta hhiI \), \( \Delta pqsE \), and \( \Delta hhiI \Delta pqsE \) mutants probing for pzhH, rhlA, and hcnA (Fig. 1D). Expression of the class I gene, pzhH, does not change when pqsE is deleted in either the WT or the \( \Delta hhiI \) parent, whereas transcription of the class II gene, rhlA, declines in both the \( \Delta hhiI \) and \( \Delta pqsE \) 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 \( \Delta pqsE \) single and \( \Delta hhiI \Delta pqsE \) double mutants compared with the WT and \( \Delta hhiI \) 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 \( \Delta 9 \) strain and assessed whether or not RhlR-dependent target genes are expressed. Fig. 2A shows quantitative RT-PCR results for pzhH, rhlA, and \( hcnA \). Compared with WT, the \( \Delta 9 \) 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 pzhH is a RhlR-dependent class I gene, its expression is not activated when pqsE and \( rhlR \) are introduced into the \( \Delta 9 \) 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 \( \Delta 9 \) strain and in the \( \Delta 9 \) 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. 2B). 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-amino-6-benzoylacetamide-3-coenzymeA to 2-amino-5-benzoylacetate, a step in QS synthesis (22). The PqsE ΔpqsE double mutant fail to stimulate the reporter. Last, cell-free culture fluids from the single ΔpqsE mutant show significant (~sixfold) activity because they contain C4-HSL made by RhlI. To explore this further, we generated a nonpolar mutant lacking the \( lasR \), \( lasI \), \( rhlR \), and pqsABCDE genes (we call this strain \( \Delta 9 \)). Cell-free culture fluids from the \( \Delta 9 \) mutant elicit only background reporter activity. However, introduction of pqsE into the \( \Delta 9 \) strain restored activity, as shown by the ability of the fluids to stimulate RhlR-dependent reporter gene expression comparable to that elicited by the \( \Delta hhiI \) strain (Fig. 1B). We conclude that PqsE is required for alternative-ligand synthesis.
crystal structure predicts that residues H69, H159, D178, F195, S273, and F276 are in the active site (Fig. 2C) (23, 24). We substituted the above residues with alanine and introduced the PqsE variants into the ΔpqsE 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 ΔpqsE strain (Fig. 2D 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 ΔpqsE strain (Fig. 2D 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 ΔpqsE strain (Fig. 2D 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. 2F). 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 RhlII, and therefore C4-HSL, are dispensable (14). We
mutant strains are Δ values for mutant succumbed to infection by 48 h, 10 strains are highly attenuated, ∆ (3.0 \times 10^3) cfu, and 11.1 \times 10^6 cfu, respectively (14). Here we show that both the ∆pqse and ∆rhlI ∆pqse mutant strains are avirulent, displaying the same phenotype as the ∆rhlI mutant (Fig. 34). 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, ∆rhlI, and ∆rhlI strains to be 1.9 \times 10^6 cfu, 2.6 \times 10^6 cfu, and 1.1 \times 10^6 cfu, respectively (14). Here we show that the LD_{50} is 10–20 times higher for both the ∆pqse (3.0 \times 10^3 cfu) and ∆rhlI ∆pqse (4.8 \times 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 \times 10^4 cfu, corresponding to \sim 1.5 \times LD_{50} of the WT strain. Fig. 38 shows that mice given the WT or the ∆rhlI mutant succumbed to infection by 48 h, whereas mice given the ∆pqse or ∆rhlI ∆pqse mutant all survived. Mice infected with the ∆rhlI mutant showed \sim 40% survival.

To determine the level of lung colonization achieved by infection, we administered sublethal doses (\sim 0.5 LD_{50}) of these strains. At 24 h postinfection, the bacterial load was similar among all infected mice (Fig. 3C). However, at 48 h postinfection, the bacterial burden in mice infected with the WT and ∆rhlI strains increased by 10,000-fold (Fig. 3C). In contrast, the bacterial load in mice infected with the ∆rhlI, ∆pqse, and ∆rhlI ∆pqse strains did not change significantly (Fig. 3C). Thus, the ∆rhlI, ∆pqse, and ∆rhlI ∆pqse strains are highly attenuated, producing a four-order of magnitude lower burden of bacteria in the murine host than the WT and the ∆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 ΔrhlR, Δpqse, and ∆rhlI Δpqse P. aeruginosa PA14 strains. Compared with WT, only a modest decrease in RhlR levels could be detected in the mutant strains (Fig. 4A). 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 E. coli, is not soluble even in the presence of saturating C4-HSL (28). To further explore this result, we deleted rhlR in the WT, ∆rhlI, Δpqse, and ∆rhlI Δpqse backgrounds and reintroduced rhlR on a plasmid under a constitutive promoter to eliminate possible changes in RhlR transcription because of the absence of RhlR and/or PqsE.

**Mukherjee et al.** 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.
Soluble RhlR protein is present in all four strains (Fig. 4B). 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 ΔrhlI ΔpqsE double mutant do not contain any activity that elicits RhlR-dependent gene expression (Fig. 1B 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. S3A) (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. 4C and SI Appendix, Fig. S3A 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. 4C and SI Appendix, Fig. S3C). We substituted W68, Y72, D81, and W96 with alanine and introduced these RhlR variants into the ΔrhlR 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. 4D). 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. 4E and F). We conclude that all four residues are required for sensing both C4-HSL and the alternative ligand.

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**Fig. 4.** The RhlR ligand-binding domain is required for sensing the alternative ligand. (A) Western blot analysis of lysates from WT, ΔrhlR, ΔrhlI, ΔpqsE, ΔrhlI ΔpqsE, 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 residue I84 (yellow) is dispensable for detection of both ligands. (D) Western blot analysis of lysates from WT PA14, the ΔrhlR mutant, and the ΔrhlR 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. 4C). Again, we generated alanine substitutions and introduced the RhlR variants into the ΔrhlR strain. All the variants produce stable protein (Fig. 4D). The RhlR R48A and L100A substitutions eliminated hcn expression without affecting pheH 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. S2B) (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 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 QS 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 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 proteines 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 ΔpqsE and ΔrhlΔpqsE mutants were more attenuated than the ΔrhlR 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 dimmerization, 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 1:100 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_{600} of each sample was measured on a spectrophotometer (Beckman Coulter DU 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/mL 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 Bichromes).

**Animal Infection Models.** *C. elegans* fast-killing assay andmurine 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-2003421–0422198N. 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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