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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 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.

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 ΔrhlR mutant is hyper-rugose because it fails to produce phenazines (e.g., pyocyanin) (12, 13). In contrast, the ΔrhlI mutant is smooth because of the overproduction of phenazines (Fig. 1A). Thus, unlike most QS receptor–synthase pairs, the ΔrhlR and ΔrhlI mutants do not have identical phenotypes. We discovered that ΔrhlI mutant cell-free culture fluids contain an activity...
(hereafter called the alternative ligand) that stimulates RhIR-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 ΔrhlI 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, RhIR 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 ΔrhlI 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 ΔhdtS and ΔambB mutants and double ΔrhlI ΔhdtS and ΔrhlI ΔambB 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 ΔrhlI strain using the Tn5 IS909 derivative ISlacZ/ohu (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 PqsE is not the alternative ligand (14). To test whether another quinoline produced by PqsABCD is the alternative ligand, pqsE and pqsD were deleted in the WT and ΔrhlI strains and the colony biofilm phenotypes assessed. The ΔpqsA and ΔpqsD mutants have approximately WT colony biofilm phenotypes, and the ΔrhlI ΔpqsA and ΔrhlI ΔpqsD 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 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 ΔrhlI strains. Indeed, the ΔpqsE mutant displays a hyper-rugose colony biofilm phenotype, and the ΔrhlI ΔpqsE double mutant has a colony biofilm phenotype indistinguishable from the ΔrhlI mutant (Fig. L4). Introduction of a plasmid carrying pqsE restored the WT colony biofilm phenotype (Fig. L4).

To examine whether PqsE is required for production of the alternative ligand, we constructed a PA14 RhIR-dependent reporter strain harboring deletions in the lasR, lasI, rhlR, rhlI, and pqsABCDE genes that contains a chromosomal Phla-inNeoGreen transcriptional reporter fusion and chromosomal, arabinosinducible rhIR (this strain is called Δ5 P_{lasR>Phla}). Our results are shown in Fig. 1B. 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 ΔrhlI mutant fosters ~fivefold induction of the reporter. Most important, in contrast to the ΔrhlI strain, cell-free culture fluids from the 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 RhIR. 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 RhIR-dependent reporter gene expression comparable to that elicited by the ΔrhlI strain (Fig. 1B). We conclude that PqsE is required for alternative-ligand synthesis.

The PqsE-Derived Ligand Activates Class II and III but Not Class I RhIR Target Genes. We previously showed that there exist three classes of RhIR-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. 1C). 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, ΔrhlI, ΔrhlI ΔpqsE, and ΔrhlI ΔpqsE mutants probing for phzH, rhlA, and hcnA (Fig. 1D). Expression of the class I gene, phzH, does not change when pqsE is deleted in either the WT or the ΔrhlI parent, whereas transcription of the class II gene, rhlA, declines in both the ΔrhlI and ΔpqsE 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 ΔpqsE single and ΔrhlI ΔpqsE double mutants compared with the WT and ΔrhlI strains. Collectively, the data in Fig. 1 provide evidence that PqsE is the alternative-ligand synthase.

PqsE and RhIR 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 RhIR. To address whether the PqsE-derived alternative ligand is necessary and sufficient to stimulate RhIR-controlled group behaviors, we introduced pqsE and or rhIR into the Δ9 strain and assessed whether or not RhIR-dependent target genes are expressed. Fig. 2A 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 rhIR alone does not activate their expression. Consistent with our analyses showing that phzH is a RhIR-dependent class I gene, its expression is not activated when pqsE and rhIR are introduced into the Δ9 mutant. In contrast, introduction of pqsE together with rhIR restores the WT level of expression of rhlA and hcnA, which do depend on the alternative ligand.

Beyond regulating transcription, if RhIR 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 rhIR, pqsE, or both genes, and we compared the output with that made by the WT. Indeed, introduction of both rhIR and pqsE, but neither gene alone, restored pyocyanin production to the maximum WT level (Fig. 2B). Thus, we conclude that PqsE and RhIR 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-aminobenzoyleacetyl-coenzymeA to 2-aminobenzoyleactate, a step in PQS synthesis (22). The PqsE
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
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 *Caenorhabditis elegans* fast-kill infection assay, WT *P. aeruginosa* is virulent, the ΔrhlR mutant is avirulent, and the Δhll mutant that lacks C4-HSL but produces the alternative ligand is as virulent as the WT (Fig. 3A). Here we show that both the ΔpqsE and Δhll ΔpqsE mutant strains are avirulent, displaying the same phenotype as the ΔrhlR mutant (Fig. 3A). 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, ΔrhlR, and Δhll 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 ΔpqsE (3.0 × 10^5 cfu) and Δhll ΔpqsE (4.8 × 10^5 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 × LD_{50} of the WT strain. Fig. 3B shows that mice given the WT or the Δhll mutant succumbed to infection by 48 h, whereas mice given the ΔpqsE or Δhll ΔpqsE mutant all survived. Mice infected with the ΔrhlR 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. 3C). However, at 48 h postinfection, the bacterial burden in mice infected with the WT and Δhll strains increased by 10,000-fold (Fig. 3C). In contrast, the bacterial load in mice infected with the ΔrhlR, ΔpqsE, and Δhll ΔpqsE strains did not change significantly (Fig. 3C). Thus, the ΔrhlR, ΔpqsE, and Δhll ΔpqsE strains are highly attenuated, producing a four-order of magnitude lower burden of bacteria in the murine host than the WT and the Δhll 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, Δhll, ΔpqsE, and Δhll ΔpqsE 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 *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, Δhll, ΔpqsE, and Δhll 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.

*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 ΔrhlR mutant (open squares), the Δhll mutant (open triangles), the ΔpqsE mutant (open circles), and the Δhll ΔpqsE 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 (Δhll; *P* = 0.374, ΔhllR; *P* < 0.01, ΔpqsE; *P* < 0.0001, Δhll ΔpqsE; *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.**
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.

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 Plac 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 rhlI 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 hcnA expression without affecting phcH 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 auto-inducer, based on its location in the pqsABCDE 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 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 the RhlR effects 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. 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.

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.

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-20150124-002198N. 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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