Non-canonical pattern recognition of a pathogen-derived metabolite by a nuclear hormone receptor identifies virulent bacteria in *C. elegans*

**Highlights**
- PCN, a metabolite secreted by *P. aeruginosa*, activates innate immunity in *C. elegans*
- The *C. elegans* nuclear hormone receptor NHR-86 is the sensor for PCN
- PCN binds to NHR-86 and activates its anti-pathogen transcriptional program
- PCN is surveilled by *C. elegans* to assess the relative threat of virulent *P. aeruginosa*

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**In brief**
Immune sensing of infectious microorganisms is essential for animal health. Peterson and Tse et al. characterize a non-canonical pattern recognition system that intercepts pathogen-derived signals of growth and virulence to assess the relative threat of virulent bacteria. A *C. elegans* nuclear hormone receptor senses phenazine-1-carboxamide (PCN), a toxic metabolite produced by pathogenic strains of *Pseudomonas aeruginosa*, to activate innate immunity.
Non-canonical pattern recognition of a pathogen-derived metabolite by a nuclear hormone receptor identifies virulent bacteria in *C. elegans*

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SUMMARY

Distinguishing infectious pathogens from harmless microorganisms is essential for animal health. The mechanisms used to identify infectious microbes are not fully understood, particularly in metazoan hosts that eat bacteria as their food source. Here, we characterized a non-canonical pattern-recognition system in *Caenorhabditis elegans* (*C. elegans*) that assesses the relative threat of virulent *Pseudomonas aeruginosa* (*P. aeruginosa*) to activate innate immunity. We discovered that the innate immune response in *C. elegans* was triggered by phenazine-1-carboxamide (PCN), a toxic metabolite produced by pathogenic strains of *P. aeruginosa*. We identified the nuclear hormone receptor NHR-86/HNF4 as the PCN sensor in *C. elegans* and validated that PCN bound to the ligand-binding domain of NHR-86/HNF4. Activation of NHR-86/HNF4 by PCN directly engaged a transcriptional program in intestinal epithelial cells that protected against *P. aeruginosa*. Thus, a bacterial metabolite is a pattern of pathogenesis surveilled by nematodes to identify a pathogen in its bacterial diet.

INTRODUCTION

The ability to discriminate pathogens from beneficial microorganisms is essential for the health of all metazoan animals. This problem is particularly challenging for organisms, such as free-living nematodes, that eat bacteria as their food source and are thus constantly exposed to bacterial features that activate immune defenses in other metazoans (i.e., microbe- or pathogen-associated molecular patterns [MAMPs/PAMPs]). Indeed, nematodes lost the classical mechanisms of pattern recognition for the detection of pathogens during evolution. *Caenorhabditis elegans* (*C. elegans*), for example, do not utilize pattern recognition receptors, such as members of the Toll-like receptor (TLR) or nucleotide-binding and oligomerization domain (NOD)-like receptor (NLR) protein families, to detect microbial infection and yet are still able to mount pathogen-specific immune defenses.

The innate immune response in *C. elegans* requires the function of conserved signaling regulators, such as the p38 PMK-1 immune pathway, to maintain the constitutive or tonic expression of immune effector genes. Dietary cues, inputs from sensory neurons, and changes in the availability of essential host metabolites, such as cholesterol, adjust the basal activity of the p38 PMK-1 pathway to prime immune effector expression during periods of relative vulnerability to infection. *C. elegans* also evolved mechanisms to sense pathogens indirectly to target host defenses toward invading pathogens or secreted toxins. For example, the G protein-coupled receptor DCAR-1 in the *C. elegans* hypodermis recognizes a host ligand or damage-associated molecular pattern that is elaborated as a sequela of fungal infection. *C. elegans* also activates immune defenses in response to perturbations in host physiology that accompany infection with pathogenic microbes or the effects of their secreted toxins, a process that is often called surveillance immunity. In addition, bloating of the *C. elegans* intestinal lumen induced by microbial colonization activates a behavioral avoidance response and the transcription of immune effector genes. However, whether *C. elegans* has evolved mechanisms for direct detection of pathogens, akin to the classical mechanisms of pattern recognition present in other metazoan animals, remains unknown.

Bacteria produce a wide array of metabolites that regulate growth, virulence, and intra- and inter-species interactions. Thus, these molecules may readout the virulence potential of pathogens and be intercepted by hosts to program adaptive defenses. Phenazine metabolites produced by *Pseudomonas*
aeruginosa (P. aeruginosa), for example, are sensed by chemo-sensory neurons in C. elegans, which activates the transcription of a transforming growth factor β (TGF-β) family member daf-7. Neuroendocrine signaling controlled by DAF-7 is necessary for C. elegans to induce protective avoidance behavior in the presence of P. aeruginosa.21 However, individual phenazines produced by P. aeruginosa do not elicit C. elegans avoidance behavior, and wild-type nematodes still readily avoid pseudomonal mutants that are unable to make phenazines.21 Thus, the behavioral responses of C. elegans to P. aeruginosa in this context are likely multi-factorial.

Nuclear hormone receptors are a large family of transcription factors that are regulated by small molecule ligand binding. Compared with other metazoans, C. elegans express an expanded family of nuclear hormone receptors—274 are present in C. elegans, whereas Drosophila and humans have only 21 and 48, respectively.27-30 The marked expansion of this protein family suggests that these transcription factors have important roles in nematode physiology, potentially as direct sensors of bacterial metabolites. However, very few C. elegans nuclear hormone receptors have been characterized in detail and the ligands for only four have been determined, none of which are produced by bacteria.31-36

Here, we demonstrated that a C. elegans nuclear hormone receptor, which is a homolog of mammalian HNF4, is a bacterial pattern recognition receptor that senses a pathogen-derived metabolite to activate anti-pathogen defenses. We discovered that phenazine-1-carboxamide (PCN), a toxic phenazine metabolite produced by P. aeruginosa, bound to and activated the C. elegans nuclear hormone receptor NHR-86/HNF4. We showed that activated NHR-86/HNF4 trafficked to the promoters of infection-response genes, independent of intermediary signaling pathways, to engage a transcriptional program that provided protection from bacterial killing. We also showed that PCN specifically marked P. aeruginosa in a disease-causing state. Thus, PCN is a pattern of pathogenesis37 sensed by C. elegans, rather than canonical MAMPs, to identify an infectious bacterial pathogen from among its bacterial food and to activate innate immunity.

RESULTS

The pathogen-derived metabolite phenazine-1-carboxamide activates anti-pathogen defenses in the C. elegans intestine

To determine how C. elegans senses infection by the bacterial pathogen P. aeruginosa, we examined P. aeruginosa strains with mutations in key transcriptional regulators that control pathogen virulence (Figures 1A and S1A–S1C).38 For these studies, a transgenic C. elegans strain that carries a GFP-based transcriptional reporter for infection-response gene (irg)-4, a secreted immune effector that is transcriptionally induced in the intestine during bacterial infection, was used as an *in vivo* sensor of immune activation.7,9,39-44 Mutations in three of the 17 P. aeruginosa transcriptional regulators eliminated the induction of C. elegans *irg-4p::gfp* during infection: pseudomonal mutants in *rhlR*, *pqsR*, and *lasR* (Figures 1A and S1A–S1C). P. aeruginosa RhlR, PqsR, and LasR are each transcription factors that function in bacterial quorum-sensing pathways and together control the expression of so-called group behavior genes, which include virulence effectors.45,46 Thus, we undertook a secondary screen of 152 P. aeruginosa strains with mutations in genes known to be regulated by one of these transcription factors, RhlR,47 to identify individual pseudomonal effectors that drive C. elegans immune activation (Figure S1D). We identified only three hits in this screen (phzA2, phzB2, and phzH4), all of which contained mutations in phenazine biosynthesis genes (Figure S1E). C. elegans *irg-4p::gfp* immune reporter animals infected with a P. aeruginosa strain containing clean deletions in both phenazine biosynthesis operons (P. aeruginosa ΔphzM mutant) failed to upregulate *irg-4p::gfp* in the intestine during infection (Figure 1A). RNA sequencing (RNA-seq) confirmed that P. aeruginosa phenazine biosynthesis is required for C. elegans innate immune activation (Figure 1B). Importantly, this experiment identified a group of C. elegans genes whose induction during P. aeruginosa infection was entirely dependent on the production of phenazines (Figure 1B). Of these 27 genes, 22 are C. elegans innate immune effectors or detoxification genes (Figure 1B; Table S1A). Examination of transcriptional reporters for the anti-pathogen gene *irg-5* (Figure S1F) and the cytochrome p450 gene *cyp-35C1* (Figure S1G) confirmed that the induction of these genes in the intestine was abrogated during infection with the P. aeruginosa ΔphzM mutant.

*P. aeruginosa* produces four major phenazine metabolites: phenazine-1-carboxylic acid (PCA), phenazine-1-carboxamide (PCN), pyocyanin (PYO), and 1-hydroxyphenazine (1-HP) (Figure 1C).38 Importantly, supplementation with PCN, but not the three other secreted phenazine metabolites, was sufficient to restore both C. elegans *irg-4p::gfp* (Figure 1D) and cyp-35C1p::gfp (Figure S1H) activation in the P. aeruginosa ΔphzM mutant. Additionally, in the absence of infection, supplementing PCN, but not the three other phenazines, drove the dose-dependent activation of C. elegans *irg-4p::gfp* (Figures 1E and S1I) and cyp-35C1p::gfp expression (Figure S1J)—a finding that was confirmed by qRT-PCR analysis for these and other innate immune effectors (Figure 1F).

Consistent with the role of PCN in inducing C. elegans innate immune defenses, infection with a *P. aeruginosa* strain containing a mutation in *phzH*, a glutamate amidotransferase that synthesizes PCN (Figure 1C), abrogated the induction of C. elegans *irg-4p::gfp* (Figure 1G), irg-5p::gfp (Figure S1K), and cyp-35C1p::gfp expression (Figure S1L). We used liquid chromatography-tandem mass spectrometry (LC-MS/MS) to confirm that the *P. aeruginosa* *phzH* mutant is deficient in the production of PCN but not the other phenazine molecules (Figure S1M). Notably, C. elegans infected with *P. aeruginosa* strains with mutations in either *phzM* or *phzS*, the enzymes that synthesize PYO and 1-HP (Figure 1C), did not affect the induction of these immune effectors (Figures 1G, S1K, and S1L). Moreover, we found that the transcriptional signature of C. elegans exposed to PCN mimics that of animals infected with *P. aeruginosa* (Figure 1H; Table S1B). Thus, the *P. aeruginosa* metabolite PCN specifically and robustly activates *C. elegans* intestinal innate immune defenses.

The anti-pathogen transcriptional program induced by PCN requires the C. elegans nuclear hormone receptor *nhr-86*

To identify the C. elegans receptor for PCN, we focused our analysis on nuclear hormone receptors, given their function as...
ligand-gated transcription factors that can potentially sense bacterial metabolites. We used RNAi to screen 271 of 274 nuclear hormone receptor genes in the C. elegans genome and identified one hit that strongly suppressed C. elegans \textit{ird-4}:\textit{gfp} immune reporter induction by PCN: \textit{nhr-86} (Figure S2A; Table S5). Knockdown of \textit{nhr-86} abrogated the induction of \textit{C. elegans ird-4}:\textit{gfp} (Figure 2A) and \textit{cyp-35C1}:\textit{gfp} (Figure 2B) by PCN treatment and during \textit{P. aeruginosa} infection. Two \textit{nhr-86} loss-of-function alleles \textit{tm2590} and \textit{ums12} fully suppressed the induction of \textit{ird-4}:\textit{gfp} (Figure 2C) and \textit{ird-5}:\textit{gfp} (Figure S2B) under these conditions. We used CRISPR-Cas9 to tag \textit{nhr-86} with an auxin-inducible degron (AID) at its endogenous locus. Treatment with the phytohormone auxin in a transgenic \textit{C. elegans} strain expressing the auxin-binding receptor transduction by the proteasome in all tissues.\textsuperscript{51} We confirmed that auxin treatment induced the degradation of \textit{NHR-86::AID} protein in this strain (Figure S2C). The depletion of \textit{NHR-86} abrogated
Figure 2. The anti-pathogen transcriptional program induced by PCN requires the C. elegans nuclear hormone receptor nhr-86
(A and B) Images of C. elegans irg-4p::gfp (A) and cyp-35C1p::gfp (B) transcriptional reporters with indicated RNAi conditions either exposed to PCN in the absence of infection or during P. aeruginosa infection (scale bar, 200 μm).
(C) Images of C. elegans irg-4p::gfp transcriptional reporters with indicated genotypes and conditions (scale bar, 200 μm).
(D–K) qRT-PCR analysis of the indicated innate immune genes in wild-type and NHR-86::AID animals exposed to either PCN in the absence of infection (n = 3) or during infection with (D–K) qRT-PCR analysis of the indicated innate immune genes in wild-type and NHR-86::AID animals exposed to either PCN in the absence of infection (n = 3).
(L) Data from mRNA-sequencing experiments comparing genes differentially regulated in wild-type and NHR-86::AID animals exposed to either PCN in the absence of infection (n = 3) (D–K) or during infection with P. aeruginosa (n = 4) (H–K). All conditions are in the presence of 50 μM auxin. Data are the mean of biological replicates with error bars giving SEM. *equals p < 0.05 (two-way ANOVA with Tukey’s multiple comparisons test).
(M–R) ChIP-qPCR analysis of NHR-86 binding to the indicated DNA regions in wild-type and NHR-86::GFP animals exposed to solvent control or PCN. Protein-DNA complexes were immunoprecipitated with an anti-GFP antibody. Data are the mean of biological replicates with error bars giving SEM (n = 3). *equals p < 0.05 (two-way ANOVA with Tukey’s multiple comparisons test). Source data for this figure is in Table S1C. See also Figure S2.
the induction of irg-4 following exposure to PCN (Figure 2D) and during P. aeruginosa infection (Figure 2H), findings that are consistent with our prior study.\textsuperscript{38} We also found that the PCN- and P. aeruginosa-mediated induction of irg-5 (Figures 2E and 2I), cyp-35C1 (Figures 2F and 2J), and ugt-13 (Figures 2G and 2K) was attenuated in NHR-86-depleted animals. Consistent with these data, RNA-seq revealed that nhr-86 is required for the induction of C. elegans genes following exposure to PCN (Figures 2L and S2D; Table S1C). In this experiment, the transcripts of wild-type and nhr-86(RNAi) C. elegans animals, each exposed to solvent control or PCN, were compared. These data revealed that 63 of the 133 genes upregulated by PCN in wild-type worms (q < 0.05) required nhr-86 for their induction (Figures 2L and S2D; Table S1C).

We performed chromatin immunoprecipitation to characterize the promoter occupancy of NHR-86 at baseline and during PCN treatment using GFP-tagged NHR-86 protein (NHR-86::GFP) and an anti-GFP antibody. NHR-86 was enriched at the promoters of the four representative anti-pathogen effector genes during PCN treatment but not in untreated controls (Figures 2M–2P). Importantly, there was no enrichment of NHR-86 at these promoter regions in wild-type animals (which do not express NHR-86::GFP) that were exposed to PCN (Figures 2M–2P). Furthermore, PCN exposure did not cause the enrichment of NHR-86 at two intergenic regions in chromosome IV (Figures 2Q and 2R). The p38 mitogen-activated protein (MAP) kinase PMK-1 pathway is a central regulator of anti-pathogen defenses in C. elegans that controls the basal expression of immune effector genes, including irg-4 and irg-5. Consistent with our NHR-86 promoter occupancy data, we found that PCN did not induce the phosphorylation of the p38 MAP kinase PMK-1, as measured in a western blot experiment using antibodies that specifically recognize the doubly phosphorylated TGY motif of activated PMK-1 and the total PMK-1 protein (Figures S2E and S2F). Together, these data demonstrated that PCN caused NHR-86 to traffic directly to the promoters of innate immune effector genes to activate their transcription, independent of the p38 PMK-1 pathway.

In a previous study, we showed that NHR-86 activates the transcription of intestinal immune defense genes in the presence of a synthetic immunostimulatory molecule (R24).\textsuperscript{39} Indeed, we found that, upon activation by R24, NHR-86 traffics to the promoters of immune effectors that we also identified as NHR-86 targets following PCN treatment.\textsuperscript{39} Consistent with these findings, PCN and R24 induced similar transcriptional signatures (Figure S2G; Table S1D). Furthermore, the nhr-86-dependent genes that were induced during PCN treatment and those that were upregulated by nhr-86 following R24 treatment were also tightly correlated (Figure S2H; Table S1E). These data suggest that the bacterial metabolite PCN and the xenobiotic R24 each activate NHR-86 to induce anti-pathogen defenses.

NHR-86 principally localizes to the nuclei of intestinal epithelial cells and several neurons.\textsuperscript{50} NHR-86 directly regulates the transcription of innate immune effector genes, such as irg-4, irg-5, and cyp-35C1, that are expressed in intestinal epithelial cells (Figures 1 and 2). Consistent with this observation, the knockdown of nhr-86 only in intestinal epithelial cells, using a transgenic C. elegans strain engineered to perform RNAi only in this tissue, suppressed the induction of irg-4 by PCN (Figure S2J).

These data suggest that NHR-86 functions in intestinal epithelial cells to activate the transcription of anti-pathogen defenses.

Chemosensation of P. aeruginosa secondary metabolites, including PCN, induces the transcription of the TGF-β family member daf-7 in ASJ chemosensory neurons.\textsuperscript{26} Although daf-7 is required for C. elegans to avoid P. aeruginosa,\textsuperscript{56} individual phenazines, including PCN, do not induce avoidance behavior in C. elegans.\textsuperscript{21} In addition, wild-type nematodes still readily avoid P. aeruginosa with mutations in the genes that make phenazines, including phzH mutants.\textsuperscript{21} Importantly, the induction of C. elegans irg-4::gfp by PCN occurs independently of daf-7 (Figure S2J). In addition, auxin-induced degradation of C. elegans NHR-86::AID does not alter the avoidance response to P. aeruginosa (Figure S2K). Together, these data indicate that C. elegans behavioral responses to P. aeruginosa occur independently of PCN sensing by NHR-86.

Individual phenazines produced by P. aeruginosa also activate the mitochondrial unfolded protein response (UPR)\textsuperscript{56} in a manner that requires the transcription factor ATFS-1.\textsuperscript{52,53} However, knockdown of atfs-1 by RNAi did not suppress irg-4::gfp induction during P. aeruginosa infection (Figure S2L).

Additional, induction of mitochondrial stress by either treatment with mitochondrial poisons (Figure S2M) or knockdown of a key mitochondrial protease, spg-7 (Figure S2N), did not lead to irg-4::gfp induction. Likewise, gene set enrichment analysis of genes differentially expressed in wild-type animals following treatment with PCN did not reveal a signature of a mitochondrial stress response induced by either spg-7(RNAi) (Figure S2O) or in the atfs-1(et18) gain-of-function mutant (Figure S2P). Collectively, these data demonstrated that the activation of innate immune defenses by PCN occurred through nhr-86 and not via previously characterized responses to P. aeruginosa phenazines.

The bacterial metabolite PCN and synthetic immunostimulatory molecule R24 bind to the ligand-binding domain of NHR-86

To determine if PCN and R24 are ligands of NHR-86, we performed biophysical assays. We expressed and purified the binding domain of NHR-86 (Figure 3A). We expressed and purified the binding domain of NHR-86 (Figure 3A) in a dose-dependent manner. The equilibrium dissociation constant (K\textsubscript{d}), which characterizes the affinity of PCN and R24 for the NHR-86(LBD), are 24.24 and 5.53 μM, respectively (Figures 3A and 3B).

As an orthologous means to demonstrate that PCN and R24 bind to NHR-86, we utilized a cellular thermal shift assay (CETSA), a technique based on the principle that the binding of a ligand to its target stabilizes the protein complex against denaturing and aggregating at higher temperatures.\textsuperscript{55} For these studies, we used CRISPR-Cas9 to insert a 3xFLAG tag at the N terminus of the NHR-86 protein. As a control, we used a strain
expressing a transgene that contains a 3xFLAG-labeled NHR-12 protein. 56 Which is the closest nematode paralog of NHR-86. 30 Using these strains, we probed for either NHR-86 or NHR-12 in whole-cell lysates using an anti-FLAG antibody. PCN and R24 treatments each led to the thermal stabilization of NHR-86 over a range of temperatures (Figures 3C–3E and S3B). We quantified the area under the curve from biological replicates and found that treatment with PCN and R24 each increased the thermal stability of NHR-86 (Figures 3E and S3B). The thermal stabilization of NHR-86 by PCN was reproducible, significant, and more subtle than by R24. Importantly, R24 and PCN each failed to thermally stabilize NHR-12 (Figures 3F, 3G, and S3C). Consistent with the intrinsic tryptophan fluorescence assays (Figure 3A), the negative control phenazine metabolite PCA did not thermally stabilize NHR-86 (Figures 3C–3E and S3B).

To further characterize the binding of R24 and PCN to NHR-86, we modeled the three-dimensional structure of the protein in silico (Figure 4A). HNF-4α, the mammalian homolog of C. elegans NHR-86 forms a stable homodimer. 67 Thus, we used this conformation to model NHR-86. We docked PCN, R24, and PCA into a potential ligand-binding pocket identified in the NHR-86(LBD) (Figure 4A) and used molecular dynamics simulations to calculate the free energy of binding for these molecules. We found that R24 and PCN each bind stably to NHR-86(LBD), whereas PCA does not (Figure 4B; Video S1). These calculations also predicted that R24 has an increased affinity for the NHR-86(LBD) compared with PCN, a finding that was confirmed experimentally in both the intrinsic tryptophan fluorescence quenching biophysical assays (Figures 3A and 3B) and the CETSA thermal stabilization (Figures 3C–3E). Consistent with these data, R24 caused a more robust induction of anti-pathogen effector genes than PCN at equimolar concentrations (Figures 4C–4F).

Examination of both PCN (Figure 4G) and R24 (Figure S4A) docked in silico within the ligand binding pocket of NHR-86(LBD) revealed that the phenylalanine (F) at residue 379 interacts with each of these ligands. We used CRISPR genome editing to mutate this amino acid to histidine (F379H) in C. elegans
Figure 4. The phenylalanine at position 379 of NHR-86 is required for the binding of PCN and R24

(A) In silico molecular modeling of full-length apo NHR-86 as a homodimer. The identified ligand-binding pocket is indicated in red.

(B) Average free energy of ligand-binding for PCA, PCN, and R24 calculated using the molecular mechanics/generalized born surface area (MM/GBSA). See also Video S1.

(C–F) qRT-PCR analysis of wild-type animals exposed to either solvent control (1% DMSO) or 100 μM R24 or 100 μM PCN. Data are the average of biological replicates (n = 3) with error bars giving SEM. *equals p < 0.05 (Brown-Forsythe and Welch ANOVA with Dunnett’s multiple comparisons test).

(G) An in silico model of PCN bound to the identified binding pocket in the NHR-86(LBD). The interaction of phenylalanine 379 (F379) (cyan) and PCN (white) is shown.

(H) A representative immunoblot of a CETSA experiment using an anti-FLAG antibody that probed whole-cell lysates from C. elegans 3xFLAG::NHR-86 and 3xFLAG::NHR-86F379H strains treated with indicated conditions.

(I) A representative densitometric quantification from a CETSA experiment that characterized the interaction of solvent control, PCN, and R24 with 3xFLAG::NHR-86 and 3xFLAG::NHR-86F379H (n = 3).

(J) The area under the curve was quantified from each biological replicate for the experiment described in (I) and normalized to the solvent control condition of 3xFLAG::NHR-86 (n = 3). All biological replicates for this experiment are shown in Figure S4B. Data are the average of all biological replicates with error bars giving SEM. *equals p < 0.05 (two-tailed, unpaired t test with Welch’s correction).

(K) Intrinsic tryptophan fluorescence intensity of the purified LBD of wild-type NHR-86 and NHR-86 containing the F379H mutation treated with the indicated concentrations of PCN and R24, each normalized to the solvent control-treated samples. Curves represent a non-linear regression fit of the scaled fluorescence intensity.
3xFLAG::nhr-86 animals. Importantly, PCN and R24 were not able to thermally stabilize 3xFLAG::NHR-86<sup>F379H</sup> in CETSA experiments performed as described above (Figures 4H–4J and S4B). We confirmed that NHR-86<sup>F379H</sup> protein was translated at wild-type levels (Figure S4C). Additionally, we expressed and purified from E. coli the NHR-86(LBD)<sup>F379H</sup> mutant protein (Figure S3A). The NHR-86(LBD)<sup>F379H</sup> mutation attenuated the quenching of the intrinsic tryptophan fluorescence by both PCN and R24 compared to the wild-type NHR-86(LBD) protein (Figure 4K). Thus, F379 in NHR-86 is required for the binding of PCN and R24 to the LBD of NHR-86. Importantly, the nhr-86<sup>F379H</sup> mutation blunted immune effector induction in C. elegans following PCN treatment (Figures 4L–4P).

In summary, these data demonstrate that PCN directly binds to the LBD of NHR-86.

### The bacterial metabolite PCN is a pattern of pathogenesis sensed by C. elegans NHR-86 to activate innate immunity

Phenazine metabolites rapidly kill C. elegans in a model of acute pathogen toxicity (also called the “fast kill” assay) and are required for the full virulence potential of P. aeruginosa in mice. As previously observed, phenazine toxins secreted into the agar by P. aeruginosa rapidly killed wild-type C. elegans (Figures 5A, 5B, and S5). Exposure to exogenous PCN protected C. elegans from phenazine-mediated killing in this assay (Figures 5A and 5B), data that agree with our hypothesis that C. elegans uses PCN as a recognition signal to protect itself from P. aeruginosa intoxication. Furthermore, post-embryonic degradation of C. elegans NHR-86:AID protein by auxin supplementation abrogated the protection conferred by PCN against phenazine-mediated killing (Figures 5A and 5B).

Using a pathogenesis assay that examines intestinal infection by P. aeruginosa (the “slow kill” assay), we previously observed that the synthetic immunostimulatory small molecule R24 protects C. elegans from P. aeruginosa infection in a manner dependent on nhr-86. Consistent with these data and the observation that PCN activates the transcription of infection response genes, such as <i>irg-4</i> (Figure 2D) and <i>irg-5</i> (Figure 2E), PCN treatment also extended the lifespan of C. elegans infected with P. aeruginosa (Figure S5B). nhr-86(RNAi) abrogated the protection from <i>P. aeruginosa</i> killing conferred by PCN treatment. Of note, the PCN-mediated lifespan extension during <i>P. aeruginosa</i> infection was more subtle than that conferred by R24 treatment (Table S2). These data are consistent with the observation that R24 bound more tightly to the LBD of NHR-86 (Figure 3) and more potently activated the transcription of anti-pathogen effectors (Figures 4C–4F) than PCN.

We assessed the toxic effects of PCN itself (i.e., in the absence of a pathogen) by examining the development of C. elegans in the presence or absence of this phenazine (Figures 5C and 5D). PCN was mildly toxic to wild-type worms. However, PCN treatment was deleterious to the growth and survival of C. elegans with degraded NHR-86:AID protein (Figures 5C and 5D). Thus, NHR-86 mobilized a host response that counteracted the toxicity of PCN. We conclude that the toxic bacterial metabolite PCN is a pattern of pathogenesis sensed by <i>C. elegans</i> NHR-86 to activate protective anti-pathogen defenses.

### C. elegans sense PCN to assess the relative threat of virulent <i>P. aeruginosa</i> but not other pathogenic bacteria

In its natural habitat, <i>C. elegans</i> encounter <i>Pseudomonas</i> sp. that likely encode the phenazine biosynthetic operon. We therefore hypothesized that <i>C. elegans</i> senses PCN to assess the relative threat of virulent <i>P. aeruginosa</i> in its environment. We found that the amount of PCN, as quantified by LC/MS/MS, in strains of <i>P. aeruginosa</i> with varying degrees of virulence potential (PA14, PAO1, and PAK) correlated with the production of the other toxic phenazines in these strains (PCA [Figure 6A], 1-HP [Figure S6A], and PYO [Figure S6B]). These data are noteworthy considering that NHR-86 sensed only PCN and not PCA (Figures 3 and 4) or the other phenazines (Figures 1D and 1E) to activate anti-pathogen defenses. Accordingly, the <i>P. aeruginosa</i> strains that produced more PCN had enhanced pathogenicity (Figure 6B) and more robustly induced the <i>C. elegans</i> anti-pathogen effectors <i>irg-4p::gfp</i> and <i>cyp-35C1p::gfp</i> (Figure S6C).

We drove phenazine production in <i>P. aeruginosa</i> PAO1, a strain that naturally produces fewer phenazines (Figure 6A) and is less pathogenic than PA14 (Figure 6B), by overexpressing <i>pqsE</i>, a pseudomonal gene necessary for phenazine production by the <i>rhf</i> quorum-sensing pathway. Overexpressing <i>pqsE</i> in <i>P. aeruginosa</i> PAO1, and also in PA14, increased phenazine production, including PCA and PAO1 (Figures 6C, 6D, S6D, and S6E), enhanced the induction of <i>C. elegans irg-4p::gfp</i> (Figure 6E), and augmented the pathogenicity of these strains (Figures 6F and 6G). Furthermore, the quantity of PCN produced in these <i>P. aeruginosa</i> overexpression strains directly correlated with their virulence potential toward <i>C. elegans</i> (Figure 6H). These data established a direct connection between phenazine production in <i>P. aeruginosa</i>, pathogen virulence potential, and the activation of anti-pathogen defenses in nematodes.

The transcriptional signature of <i>C. elegans</i> exposed to PCN specifically marks infection with <i>P. aeruginosa</i> but not other bacterial pathogens (Figures 6I–6L). We compared the <i>C. elegans</i> genes induced during infection with five gram-negative (<i>P. aeruginosa</i>, <i>Serratia</i> marcescens, <i>Photorhabdus</i> luminescens, <i>Erwinia</i> carotovora, and <i>Shigella</i> flexneri) and two gram-positive (<i>Enterococcus faecalis</i> and <i>Staphylococcus aureus</i>) bacterial pathogens with those that are differentially expressed following exposure to PCN. Only the genes that were upregulated during <i>P. aeruginosa</i> treatment intensity data points for each condition. Data are the average of biological replicate samples (n = 3) with error bars giving SEM. *equals p < 0.05 (paired t test with Welch’s correction) for equilibrium dissociation constant (K<sub>D</sub>) between the wild-type NHR-86(LBD) and the NHR-86<sup>F379H</sup> mutant protein. SDS-PAGE analysis of purified NHR-86(LBD)<sup>F379H</sup> is shown in Figure S3A. (L) Images of indicated <i>C. elegans irg-4p::gfp</i> animals grown on media that was supplemented with PCN (448 μM) or solvent control, as indicated (scale bar, 200 μm). (M–P) qRT-PCR analysis of the indicated innate immune genes in wild-type and NHR-86<sup>F379H</sup> animals exposed to either solvent control or PCN (448 μM) in the absence of infection. Data are the mean of biological replicates (n = 3) with error bars giving SEM. *equals p < 0.05 (two-way ANOVA with Tukey’s multiple comparisons test). Source data for this figure are in Table S3. See also Figure S4.
Although it is well-established that C. elegans coordinates inducible immune defenses to provide protection during pathogen infection, the identification of immune receptors that are directly involved in pathogen recognition in nematodes has been elusive. Here, we demonstrated that a C. elegans nuclear hormone receptor is a bona fide pattern recognition receptor that detects the pathogen-derived metabolite PCN. We showed that PCN bound to the LBD of NHR-86, which then directly activated anti-pathogen defenses that provided protection from P. aeruginosa. In addition, we found that PCN is sensed in C. elegans to assess the relative threat of virulent P. aeruginosa specifically but not other pathogenic bacteria. Thus, we conclude that PCN is a pattern of pathogenesis sensed by C. elegans to detect an individual bacterial pathogen in a specific manner from among its bacterial food.

Pattern recognition of pathogen-derived metabolites is a distinct model of immune sensing in the bacterivore C. elegans, an organism that does not use canonical pattern recognition receptors, such as TLRs, to activate innate immunity. We speculate that C. elegans lost canonical MAMP/PAMP-driven mechanisms of pattern recognition because these microbial elements are ubiquitous in the natural habitat of nematodes and thus are insufficient to distinguish disease-causing pathogens from microbial food sources. Sensing of pathogen-specific metabolic signatures by host nuclear hormone receptors is reminiscent of the immune response in plants in which specific host-encoded resistance (R) proteins evolved to sense individual pathogen-derived virulence determinants (so-called R gene-effector pairs). C. elegans encode 274 nuclear hormone receptors. Thus, decoding the metabolic signatures of bacterial pathogens by these ligand-activated transcription factors is an evolutionarily adaptable mechanism that allows nematodes to distinguish a broad range of pathogens from non-pathogenic bacterial food. Further studies are needed to identify additional nuclear hormone receptor/pattern of pathogenesis pairs.

Bacteria are the only known natural producers of phenazine metabolites. In addition to Pseudomonas sp., diverse environmental bacteria, such as Burkholderia sp., Streptomyces sp., and Nocardioida sp., encode the phenazine biosynthetic operon and synthesize these molecules. In P. aeruginosa, the production of phenazines is controlled by quorum-sensing pathways that are activated when bacteria reach high cellular density, such as during growth in biofilms. These molecules contribute to pseudomonal pathogenesis during infection, likely by interrupting electron transport in mitochondria. In addition, phenazines (PCN, in particular) are predominant in P. aeruginosa biofilms where they help to maintain redox balance within the relatively anoxic environment of the biofilm interior.

The phzH gene, which encodes the enzyme that synthesizes PCN, is not located in the phenazine biosynthetic operon and may be exclusively expressed in Pseudomonas sp. Thus, PCN production may be specifically associated with P. aeruginosa that are in a disease-causing growth state and mark strains that elaborate toxic phenazines—one aspect of virulence in a bacterial species with multiple mechanisms of pathogenesis.

Multiple transcriptional regulators control the expression of overlapping sets of immune effectors in C. elegans. For example, the transcription factor ATF-7 functions downstream of the p38 PMK-1 immune pathway to control the basal, or resting, expression of innate immune genes. During P. aeruginosa infection or PCN exposure, many of these immune genes are induced by NHR-86 in a manner independent of p38 PMK-1/ATF-7 signaling.
C. elegans sense PCN to assess the relative threat of virulent P. aeruginosa but not other pathogenic bacteria

(A–C) High-performance liquid chromatography-ultraviolet (HPLC-UV) spectroscopy was used to quantify the individual phenazines in the indicated strains. (A) PCN production was compared with the pathogenicity of vector control. See Figure S6 for a comparison of PCA (Figure S6D) and PCN (Figure S6E) production in these strains.

(B) PCN production with 1-HP (Figure S6A) and PYO (Figure S6B) in these strains. Pearson correlation coefficient (r) from biological replicates is significant (p < 0.05, n = 3). See also Table S3 for the HPLC-UV and LC-MS/MS phenazine expression is significant (p < 0.05, log-rank test, n = 3). Data are representative of three biological replicates.

(C) Survival data at 2 h for strains of the indicated genotypes are shown for the experiment described in (F). Data are the average of three biological replicates each containing three trials with error bars showing SEM (n = 9). *equals p < 0.05 (two-way ANOVA with Tukey’s multiple comparisons test). Sample sizes, 2 h survival, and p values for each replicate are shown in Table S2.

(D) HPLC-UV spectroscopy data showing the comparison of PCN production versus PCA production in biological replicates of the indicated strains. Pearson correlation coefficient (r) is significant (p < 0.05, n = 3).

(E) Images of C. elegans irg-4::gfp animals infected with the indicated P. aeruginosa strains (scale bar, 200 μm).

(F) Phenazine toxicity assay with wild-type strains. Pearson correlation coefficient (r) is significant (p < 0.05, n = 3). See Figure S6F for a comparison of PCA (Figure S6G) and PCN (Figure S6H) production in these strains.

(G) Comparison of PCN production in the indicated P. aeruginosa genotypes with their pathogenicity toward C. elegans in the phenazine toxicity assay is presented. Pearson correlation coefficient (r) from biological replicates is significant (p < 0.05, n = 3). See also Table S3 for the HPLC-UV and LC-MS/MS phenazine retention times and abundance for the data shown in (A–D) and (F).

(legend continued on next page)
Our group and others have shown that the basal activity of the p38 PMK-1 pathway is adjusted in response to micronutrient scarcity, changing environmental conditions, and inputs from chemosensory neurons.\textsuperscript{8–14} We have proposed that immune effector priming in this manner is a mechanism to anticipate threats during periods of relative vulnerability to pathogen infection.\textsuperscript{15} In this context, bacterial patterns of pathogenesis are sensed by nuclear hormone receptors to further augment immune effector expression in a manner that provides pathogen- or pathogen effector-specific protection.

Importantly, phenazines also activate innate immunity in mammals through interaction with the aryl hydrocarbon receptor (AhR), a protein that recognizes a diverse array of ligands, including environmental toxins and endogenous ligands.\textsuperscript{16,75} Thus, the interpretation of bacterial metabolites as a mechanism to direct host defenses toward potential pathogens may be among the most primordial forms of immune sensing in all metazoans.

Limitations of the study
Sensing of the pathogen-derived phenazine metabolite PCN by NHR-86 activated protective host defenses that enabled \textit{C. elegans} to survive the challenge with \textit{P. aeruginosa}. In addition, we found that \textit{C. elegans} with depleted \textit{nhr-86} protein were not more susceptible to phenazine-mediated pathogenesis in the fast kill assay, findings that are consistent with our prior report.\textsuperscript{39} There are several possible explanations that could account for the observed lack of a pathogen-susceptibility phenotype in the \textit{nhr-86}-depleted animals. The amount of PCN produced by pathogenic strains of \textit{P. aeruginosa} in the conditions tested was generally lower than the \(K_d\) of the PCN-NHR-86 binding equilibrium. Previous studies have found that \textit{P. aeruginosa} can produce greater quantities of PCN under other growth conditions.\textsuperscript{25} Additionally, the pathogen-mediated killing of \textit{C. elegans} may occur too rapidly in the fast kill assay to resolve hypersusceptibility phenotypes. It is also possible that other signaling pathways in \textit{C. elegans} can compensate for the loss of \textit{nhr-86}.

STAR\textsuperscript{\#}METHODS
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SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.immuni.2023.01.027.

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AUTHOR CONTRIBUTIONS
REFERENCES

28. Studer, A.E., Mathews, S.W., Hough, D., Yin, V.P., and Maina, C.V. (1999). The nuclear receptor superfamily has undergone extensive proliferation and diversification in nematodes. Genome Res. 9, 103–120.


### KEY RESOURCES TABLE

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Critical commercial assays

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Deposited data

| Raw and analyzed mRNA-Seq data | This study | GEO: GSE202258 |

Experimental models: Organisms/Strains

| C. elegans: Strain: N2 (Bristol) | Brenner77 | WB Cat# WBStrain00000001; RRID: WB-STRAIN:WBStrain00000001 |
| C. elegans: Strain: AU307 agls44[irg-4p::gfp::unc-54-3'UTR;myo-2p::mCherry] | Pukkila-Worley et al.11 | N/A |
| C. elegans: Strain: AY101 acl101[pDB09.1 (irg-5p::gfp); pRF4(rol-6(su1006)))] | Bolz et al.61 | WB Cat# WBStrain000000322; RRID: WB-STRAIN:WBStrain000000322 |
| C. elegans: Strain: VL491 nhr-86(tm2590) | Arda et al.50 | WB Cat# WBStrain00040127; RRID: WB-STRAIN:WBStrain00040127 |
| C. elegans: Strain: MG167 sid-1(qf9); aktls9 [vha-6p::sid-1::SL2::GFP] | Melo et al.19 | N/A |
| C. elegans: Strain: RPW137 nhr-86(ums12) | Peterson et al.39 | N/A |
| C. elegans: Strain: RPW99 nhr-86(tm2590);agls44 [irg-4p::gfp::unc-54-3'UTR;myo-2p::mCherry] | Peterson et al.39 | N/A |
| C. elegans: Strain: RPW106 nhr-86(tm2590);acl101 [pDB09.1 (irg-5p::gfp); pRF4(rol-6(su1006))] | Peterson et al.39 | N/A |
| C. elegans: Strain: RPW165 nhr-86(ums12); agls44 [irg-4p::gfp::unc-54-3'UTR;myo-2p::mCherry] | Peterson et al.39 | N/A |
| C. elegans: Strain: CA1200 ieSi57 [eft-3p::TIR1::mRuby::unc-54 3'UTR + Cbr-unc-119(+)] | Zhang et al.51 | WB Cat# WBStrain0004055; RRID: WB-STRAIN:WBStrain0004055 |
| C. elegans: Strain: OP318 unc-119(ed3); wgs1318 [nhr-12::TY1::EFGFP::3xFLAG[32C12]+unc-119(+)] | Gerstein et al.56 | WB Cat# WBStrain00030124; RRID: WB-STRAIN:WBStrain00030124 |
| C. elegans: Strain: RPW423 umsEx88[cyp-35C1p::gfp::unc-54-3'UTR; myo-2p::mCherry] | This study | N/A |
| C. elegans: Strain: RPW348 nhr-86(ums64[3xFLAG::NHR-86::AID]); ieSi57 [eft-3p::TIR1::mRuby::unc-54 3'UTR + Cbr-unc-119(+)] | This study | N/A |
| C. elegans: Strain: RPW424 nhr-86(ums65[3xFLAG::NHR-86::AID]); ieSi57 [eft-3p::TIR1::mRuby::unc-54 3'UTR + Cbr-unc-119(+)] | This study | N/A |
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| C. elegans: Strain: RPW191 nhr-86(ums14[3xFLAG::NHR-86::AID]); agls44 [irg-4p::gfp::unc-54-3'UTR;myo-2p::mCherry] | This study | N/A |
| C. elegans: Strain: RPW401 nhr-86(ums14[3xFLAG::NHR-86::AID]); agls44 [irg-4p::gfp::unc-54-3'UTR;myo-2p::mCherry] | This study | N/A |
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Experimental models: Media

- Bacto peptone: Thermo Fisher Scientific Cat# 211677
- BD Bacto agar: BD Cat# 214030

Oligonucleotides

See Table S4

Recombinant DNA

- phERD30T: Qiu et al. 83 NovoPro Cat# V005565
- pSMT3: Yunus et al. 84 N/A
- pPD95.75: This study N/A
- pHER30T::pqsE: This study N/A
- pSMT3::nhr-86 ligand-binding domain: This study N/A
- pSMT3::nhr-86 F379H ligand-binding domain: This study N/A
- pPD95.75::cyp-35C1::gfp: This study N/A

Software and algorithms

- Fiji/ImageJ: Schindelin et al. 85 RRID:SCR_002285
- CHOPCHOP: Labun et al. 86 RRID:SCR_015723
- Zen 2.3: Zeiss RRID:SCR_013672
- Gen5: BioTek Instruments RRID:SCR_017317
- BGISEQ-500 platform: BGI Americas Corp RRID:SCR_017979
- OASIS 2: Han et al. 87 RRID:SCR_014450
- R Console (Version 3.5): The R Foundation RRID:SCR_001905
- FastQC (Version 0.11.5): https://www.bioinformatics.babraham.ac.uk/projects/fastqc/ RRID:SCR_014583
- Kallisto (version 0.45.0): Bray et al. 88 RRID:SCR_016582
- Sleuth (version 0.30.0): Pimentel et al. 89 RRID:SCR_002555
- GSEA (version 4.2.3): Subramanian et al. 90 RRID:SCR_003199
- pheatmap (version 1.0.12): https://cran.r-project.org/web/packages/pheatmap/index.html RRID:SCR_016418
- WormCat 2.0: Holdorf et al. 91; Higgins et al. 92 N/A
- GraphPad Prism 9: https://www.graphpad.com/scientific-software/prism/ RRID:SCR_002798
- AlphaFold-Multimer: Evans et al. 93 N/A
- Protein Preparation Wizard, SiteMap, LigPrep, Glide, and Desmond (Schrödinger v.19-4): https://www.schrodinger.com RRID:SCR_014879
- PyMOL (v. 2.3.4): http://www.pymol.org/ RRID:SCR_003005
- OPLS3: Harder et al. 94 N/A
- VMD (v. 1.9.4): http://www.ks.uiuc.edu/Research/vmd/ RRID:SCR_001820

Other

- 0.22 µm cellulose Spin-X columns: Thermo Fisher Scientific Cat# 07-200-386
- HPLC screw-top vials with fixed inserts: Agilent Technologies Cat# 5188-6592

(Continued on next page)
RESOURCE AVAILABILITY

Lead contact
Further information requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Read Pukkila-Worley (read.pukkila-worley@umassmed.edu).

Material availability
Strains and reagents generated in this study are available upon request.

Data and code availability
- The mRNA-seq datasets have been deposited at NCBI Gene Expression Omnibus and are publicly available as of the date of publication. Accession numbers are listed in the key resources table. All other data are available in the manuscript and the accompanying Table S3, which contains all source data and statistical tests used.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

C. elegans strains
The previously published C. elegans strains used in this study were: N2 Bristol, AU307 agls44 [irg-4p::gfp::unc-54-3'UTR; myo-2p::mCherry]45, AY101 acl101 [pDB09.1[irg-5p::gfp]; pRF4[rol-6[su1006]]], VL491 nhr-86[tm2590]55, VL648 unc-119[ed3] III; wvls22[nhr-86p::nhr-86ORF::gfp unc-119(+)10], RPW137 nhr-86[ums12]19, RPW99 nhr-86[tm2590]; agls4419, RPW106 nhr-86[tm2590]; acl10119, RPW165 nhr-86[ums12]; agls4419, SJ4100 zcls13 [hsp-6::gfp + lin-15(+)]55, CA1200 ieSi57 [eft-3p::TIR1::mRuby::unc-54 3'UTR + Cbr-unc-119(+)]55, OP318 unc-119[ed3]; wgls318[nhr-12::TY1::EGFP::3xFLAG(92C12)+unc-119(+)]56, MGH167 sid-1(qt9); alx19 [vha-6p::sid-1::SL2::GFP]19. The strains developed in this study were: RPW423 umsEx88[cyp-35C1p::gfp::unc-54-3'UTR; myo-2p::mCherry], RPW348 nhr-86[ums64[NHR-86::AID]]; ieSi57, RPW424 nhr-86[ums65[3xFLAG::NHR-86]]; ieSi57, RPW427 nhr-86[ums66[3xFLAG::NHR-86::AID]]; ieSi57, RPW191 nhr-86[ums14[3xFLAG::NHR-86]]; agls44, RPW430 nhr-86[ums67[3xFLAG::NHR-86[F379H]]]; agls44.

C. elegans growth conditions
C. elegans strains were maintained on standard nematode growth medium (NGM) plates [0.25% Bacto peptone, 0.3% sodium chloride, 1.7% agar (BD Bacto), 5 µg/mL cholesterol, 25 mM potassium phosphate pH 6.0, 1 mM magnesium sulfate, 1 mM calcium chloride] with E. coli OP50 as a food source, as described.77

Bacterial strains
Bacteria used in this study were Escherichia coli (E. coli) OP50, E. coli DH5x, E. coli HT115(DE3), and Pseudomonas aeruginosa strains PA14,79 PAO1,73 PAK,73 PA14 ΔphzA1-G1 ΔphzA2-G2 (Δphz),49 PA14 ΔgacA,7 PA14 transposon mutants,96 PA01 overexpressing pqsE (this study), and PA14 overexpressing pqsE (this study). PA14 ΔrhlR, PA14 ΔlasR and PA14 ΔpqsR were obtained from Fred Ausubel.

Bacterial growth conditions
E. coli OP50 were grown in LB broth supplemented with 0.175 mg/mL streptomycin at 37°C for 16-18 hours at 250 rpm. P. aeruginosa strains were grown in LB broth at 37°C for 14 hours at 250 rpm. LB was supplemented with gentamycin at a final concentration of 50 µg/mL where indicated.

METHODS

Feeding RNAi NHR screen
Knockdown of target genes was performed by feeding C. elegans E. coli HT115 expressing dsRNA targeting the gene of interest, as previously described.78,95,36 In brief, HT115 bacteria expressing dsRNA targeting genes of interest were grown in Lysogeny broth (LB) Lennox medium containing 50 µg/mL ampicillin overnight with shaking (250 rpm) at 37°C. Overnight cultures were seeded
onto NGM plates containing 5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and 50 μg/mL carbenicillin and incubated at 37 °C for 16 hours, after which synchronized L1 animals were transferred to bacterial lawns and allowed to grow until the L4 stage.

We identified 274 nuclear hormone receptors that contained either an NHR zinc finger domain or an NHR ligand-binding domain in the most recent release of the C. elegans genome (WS228) that were likely transcribed into protein. RNAi clones for 190 of these 274 genes were obtained from a previously characterized library that was shared with us as a gift from Albertha J.M. Walhout. 73 RNAi clones were obtained from either the Ahringer, Ahringer Supplemental, or Vidal RNAi libraries. 39,96,97 For 9 other RNAi clones, either the entire coding region of the gene or the largest exon for each gene was amplified by PCR using C. elegans coding DNA or genomic DNA as the template, respectively (see Table S4 for the primer list). PCR products were cloned into the RNAi expression vector L4440 using NEEBuilder HiFi DNA Assembly (New England Biolabs #E2621), transformed into E. coli HT115, and selected on LB containing 5 μg/mL tetracycline and 50 μg/mL ampicillin, as previously described. 45 Transformants were then grown in LB containing 50 μg/mL ampicillin and frozen in a 96-well plate at 15% glycerol. Immediately prior to performing the screen, RNAi clones were stamped from frozen 96-well plates onto LB agar plates containing 50 μg/mL ampicillin and 5 μg/mL tetracycline. The source of the RNAi clones is summarized in Table S5. All clones were confirmed by Sanger sequencing.

For the RNAi screen, C. elegans irg-4p::gfp transcriptional reporter strains were grown from the L1 to L4 stage on E. coli HT115 expressing dsRNA targeting 271 of 274 C. elegans NHR genes in the genome. In brief, each well in a 24-well plate, containing RNAi agar medium, was seeded with 50 μL of a 5X concentrated overnight culture of each RNAi clone (in M9 buffer). Seeded RNAi plates were then incubated overnight at 37 °C. Approximately 50 L1 synchronized C. elegans irg-4p::gfp transcriptional reporter animals were then dropped onto each bacterial clone and grown until the L4 stage. Animals were then transferred by washing with M9 to 24-well plates containing 25 μg/mL (112 μM) PCN and seeded with 50 μL E. coli OP50 for 20 hours. GFP induction was assessed by two independent observers.

RNAi clones corresponding to two nhr genes (nhr-86 and nhr-12) abrogated the induction of irg-4p::gfp by PCN and displayed no defects in growth or development. Three RNAi clones were identified that suppressed irg-4p::gfp induction by PCN and had negative pleiotropic effects on worm growth and development, and, for this reason, were not chosen for further study. A subsequent qRT-PCR analysis revealed that irg-4 induction by PCN was not affected in the nhr-12(tm1038) mutant, indicating that nhr-12 was a false positive hit in this screen (Figure S2Q). PCN-mediated induction of C. elegans irg-4p::gfp was abrogated in nhr-86(tm2590) and nhr-86(ums12) mutants (Figure 2C), and degradation of NHR-86 protein abrogated the induction of irg-4 by PCN in a qRT-PCR experiment (Figure 2D). Therefore, nhr-86 was selected for further study. NHR-12, the closest related paralog to NHR-86, was used as a negative control in the CETSAX experiment (Figures 3F and 3G).

C. elegans and P. aeruginosa strain construction

Strain construction by CRISPR/Cas genome editing

All CRISPR genome editing was performed as previously described. 100,101 CRISPR-Cas9 editing with single-stranded oligodeoxy-nucleotide (ssODN) homolog-directed repair was used to tag nhr-86 with an auxin-inducible degron (AID) tag in animals carrying the iessS7 transgene, which expresses the TIR1 protein in all somatic cells. Animals containing the NHR-86595551 mutation were generated in nhr-86(ums14[3xFLAG::NHR-86])::agsl44 animals using CRISPR-Cas12 directed editing with ssODN homolog-directed repair. All CRISPR reagents were purchased from Integrated DNA Technologies. Target guide sequences were selected using the CHOPCHOP web tool. 102 ssODN repair templates contained indicated edits, deletions or insertions with 35 bp flanking homology arms. Cas9- and Cas12a-crRNA guide and ssODN sequences are listed in Table S4. The F1 progeny were screened for Rol phenotypes 3 to 4 days after injection and then for edited alleles using PCR and Sanger sequencing. Primer sequences used for genotyping are listed in Table S4.

Construction of cyp-35C1p::gfp transgenic reporter animals. Animals carrying the umsEx88 transgene were constructed as previously described. 31 Briefly, the region 1000 bp upstream of the cyp-35C1 5’UTR was PCR amplified, digested with HindIII and XbaI, and ligated into the gfp-containing vector pPDF95.75. Young adult N2 animals were microinjected with 25 ng/μL umsEx88 construct along with 5 ng/μL myo-2p::mCherry co-injection marker. Primer sequences are listed in Table S4.

Construction of P. aeruginosa pqsE overexpression strains. P. aeruginosa pqsE was amplified by PCR from P. aeruginosa PA14 DNA and cloned into the broad host range vector pHERD30T using NEBuilder HiFi DNA Assembly (New England Biolabs). Recombinant plasmids were propagated in E. coli DH5α cells and maintained with 50 μg/mL gentamycin selection. P. aeruginosa strains were transformed with pqsE constructs by electroporation and selected on LB agar containing 50 μg/mL gentamycin, as previously described. 102 Primer sequences are listed in Table S4.

Studies with C. elegans GFP-based transcriptional reporters

Immune and detoxification transcriptional reporter assays were performed as previously described. 3,39 We previously observed that induction of GFP in the transcriptional reporter irg-4p::gfp was more robust when the nematode strains were grown on NGM without supplemented cholesterol. 4 Thus, for the studies that utilized C. elegans irg-4p::gfp animals, NGM was prepared without cholesterol supplementation, and 0.1% ethanol was added to maintain an equivalent ethanol concentration. Single colonies of P. aeruginosa strains PA14, PA14 Δpjh, PA14 transposon mutants, and pqsE overexpression strains were grown in 3 mL of LB (for PA14 and PA14 Δpjh) or LB containing 50 μg/mL gentamicin (for PA14 transposon mutants and pqsE overexpression strains) at 37 °C for 14 hours at 250 rpm. 10 μL of culture were then seeded onto “slow-kill” agar (0.35% Bacto-peptone, 0.3% sodium chloride, 1.7% agar, 5 μg/mL cholesterol, 25 mM potassium phosphate, 1 mM magnesium sulfate, 1 mM calcium chloride), allowed to dry.
and incubated at 37 °C for 24 hours followed by 25 °C for 24 hours. *E. coli* OP50 was the uninfected control. Phenazines were added to cooled media at the following final concentrations in 1% DMSO, unless otherwise noted: PCA (112 μM, 25 μg/mL), PCN (112 μM, 25 μg/mL), PYO (119 μM, 25 μg/mL), and 1-HP (25 μM, 5 μg/mL). Of note, 1-HP was lethal to *C. elegans* when supplemented at a similar concentration as the other phenazines. Thus, we performed 1-HP supplementation with the highest concentration that did not affect animal survival in our assay. *P. aeruginosa* Δphz or *E. coli* OP50 were directly seeded onto phenazine-supplemented plates and dried. For *P. aeruginosa* Δphz, lawns were grown at 37 °C for 24 hours, followed by 25 °C for 24 hours. Around 50–100 *C. elegans* transcripational reporter animals at the L4 stage were transferred to each bacterial lawn, prepared as described above. Images were taken to 24 hours post-exposure, as described below.

**Microscopy and image analysis**

Nematodes were mounted onto 2% agarose pads, paralyzed with 50 mM tetramisole (Sigma) and imaged using a Zeiss AXIO Imager Z2 microscope with a Zeiss AxioCam 506 mono camera and Zen 2.3 (Zeiss) software. GFP fluorescence in the *arg-4p::gfp* transcriptional reporters after infection with *P. aeruginosa* mutants was quantified using the Lionheart FX Automatic Microscope (BioTek Instruments) under a 4X objective. After infection for 24 hours, ~50 animals were washed three times in M9 buffer containing 0.01% Triton X-100 and transferred to black-sided clear bottom 96-well plates containing 200 μL of 50 mM tetramisole. Animals were allowed to settle for 5 minutes. Individual animals were identified in each well, and mean GFP fluorescence intensity was quantified per animal using the GenS software (BioTek Instruments).

**Gene expression analyses and bioinformatics**

RNA-sequencing and data analysis were performed as previously described. Briefly, synchronized N2 L1 stage *C. elegans* were grown to the L4 stage on NGM plates seeded with *E. coli* OP50 and transferred by washing with M9 to *P. aeruginosa*, *P. aeruginosa* Δphz, or *E. coli* OP50 for 4 hours. For the NHR-86 RNA-seq experiment, synchronized L1 stage N2 wild-type animals were grown to L4 on either HT115 L4440 Control RNAi bacteria or HT115 nhr-86(RNAi) bacteria. L4 stage animals were then transferred to *E. coli* OP50-seeded agar plates containing solvent control (0.5% DMSO) or 25 μg/mL PCN for 4 hours. For both RNA-seq experiments, animals were harvested by washing with M9, RNA was isolated using TriReagent (Sigma-Aldrich), column purified (Qiagen), and analyzed by 100 bp paired-end mRNA-sequencing using the BGISEQ-500 platform (BGIAmericasCorp) with >20 million reads per sample. The quality of raw sequencing data was evaluated by FastQC (version 0.11.5), and clean reads were aligned to the *C. elegans* reference genome (WBcel235) and quantified using Kallisto (version 0.45.0). Differentially expressed genes were identified using Sleuth (version 3.0.30). Pearson correlation statistical analysis was performed using Prism 9.0. Heatmaps of differentially expressed genes were generated using heatmap (version 1.0.12). Gene set enrichment analysis of RNA-seq was performed using Gene Set Enrichment Analysis (GSEA) (version 4.2.3) for assessing the mitochondrial transcriptional signature in the RNA-seq experiment with PCN.

Gene set enrichment analysis of RNA-seq was performed using GSEA (version 4.2.3) with a custom gene set database of *C. elegans* genes induced during infection with pathogens (*S. aureus*, *E. faecalis*, *E. carotovora*, *P. luminescens*, *S. marcescens*, and *S. flexneri*).

For the qRT-PCR studies, RNA was reverse transcribed to cDNA using the iScript gDNA Clear cDNA Synthesis Kit (Bio-Rad) and analyzed using a CFX384 machine (Bio-Rad) using previously published primers. All values were normalized against the geometric mean of control genes *snb-1* and *act-3*. Relative expression was calculated using the Pfaffl method.

**Chromatin immunoprecipitation qPCR**

ChIP-qPCR was performed as previously described with modification. Briefly, 80,000-100,000 synchronized L1 N2 or VL648 NHR-86::GFP* were grown to the L4 stage on NGM plates seeded with 20x *E. coli* OP50. Animals were transferred by washing with M9 to either solvent control (1% DMSO) or PCN (100 μg/mL) seeded plates with *E. coli* OP50 for 4 hours at 25 °C. Animals were harvested in M9, washed in M9 three times to remove bacteria, washed with PBS once, frozen as small droplets in liquid nitrogen, and placed at -80 °C until processing. Animals were mechanically disrupted by grinding frozen droplets to a fine powder in a mortar and pestle that was pre-chilled in liquid nitrogen. The powder was suspended and crosslinked in 1% formaldehyde (Thermo Fisher Scientific, #28908) (20,000 animals/mL) for 10 minutes at room temperature and quenched with 125 mM glycine. Samples were washed with PBS, resuspended in ChIP lysis buffer (50 mM HEPES–KOH pH 7.5, 300 mM NaCl, 1 mM EDTA, 1% (v/v) Triton X-100, 0.1% (v/v) sodium deoxycholate, 0.5% (v/v) N-Lauroylsarcosine, and 1 mM HALT protease inhibitor), and chromatin sheared using a Bioruptor UCD-200 for 15 cycles (30 s on, 30 s off) to obtain 500–1000 bp DNA fragments. 50 μL of input sample was removed from sheared lysates. Sheared lysates (2 mg) were immunoprecipitated with 5 μg/mL anti-GFP antibody (Thermo Fisher Scientific, #11814460001) bound to protein G Dynabeads (Invitrogen, #10004D) at 4 °C overnight. Immune complex bound beads were washed with ChIP lysis buffer twice, ChIP lysis buffer containing 800 mM NaCl once, ChIP wash buffer (10 mM Tris–HCl pH 8.0, 250 mM LiCl, 0.5% (v/v) sodium deoxycholate, 1 mM EDTA) twice, and TE containing salt (10 mM Tris–HCl pH 8.0, 1 mM EDTA, 50 mM NaCl) once. Chromatin was eluted off the beads with ChIP elution buffer (50 mM Tris–HCl pH 8.0, 1 mM EDTA, 1% SDS), and cross-links were reversed by incubating samples at 65 °C overnight. DNA was treated with 10 μL RNase A (100 mg/mL) (Qiagen, #191010) for 2 hours at 37 °C, 10 μL Proteinase K (20 mg/mL) (New England BioLabs) for 1 hour at 55 °C, and extracted with phenol/chloroform:isooamyl alcohol, ethanol precipitated, and resuspended in elution buffer (EB) (Qiagen). qPCR was performed on input and
immunoprecipitated samples using primers designed upstream of the transcription start site and at an intergenic region. All data are presented as percent of input DNA. Primer sequences used for qPCR are listed in Table S4.

Immunoblot analyses

Protein lysates for cellular thermal shift assay (CETSA) experiments were prepared as described below. For all other immunoblots, protein lysates were prepared using a Teflon Dounce homogenizer from 2,000 C. elegans grown to the L4 larval stage on NGM plates seeded with E. coli OP50, as previously described. 

LDS Sample Buffer (Thermo Fisher Scientific) was added to a concentration of 1X with 1% β-mercaptoethanol. All samples were incubated at 70 °C for 10 minutes. Total protein from each sample was resolved on NuPage Bis-Tris 4–12% gels (Life Technologies), transferred to 0.2 μm nitrocellulose membranes (Bio-Rad), and blocked with 5% milk in 1X TBS + 0.2% Tween-20 for one hour. Blots were then probed with a 1:1000 dilution of mouse monoclonal anti-FLAG M2 (Sigma, #F1804), mouse monoclonal anti-alpha-Tubulin (Sigma, #T5168), or rabbit monoclonal anti-Actin (Abcam, #ab179467) overnight at 4 °C. Anti-mouse IgG-HRP (Abcam, #ab6789) or anti-rabbit IgG-HRP (Cell Signaling Technology, #7074) secondary antibodies were used at a dilution of 1:10,000 to detect the primary antibodies. Blots were then developed with the addition of SuperSignal™ West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific) and visualized using a ChemiDoc MP Imaging System (Bio-Rad). Band intensities were quantified using ImageJ (Fiji).

NHR-86 ligand-binding domain expression and purification

The NHR-86 ligand-binding domain (LBD) mutant containing the F379H mutation was introduced by PCR amplification with primers (Table S4) containing the F379H mutation and the pSTM3::NHR-86(LBD) construct as the template (0.5 ng/μL). Template DNA was digested and PCR ligated using the Q5 Site-Directed Mutagenesis Kit (New England BioLabs) with room temperature incubation for 10 minutes. 1 μL of ligations were transformed into chemically competent E. coli BL21(DE3) cells and maintained with 50 μg/mL kanamycin selection. For protein expression, a single colony was inoculated into 25 mL LB containing 50 μg/mL kanamycin and grown overnight. Overnight cultures were subcultured to an OD_600 of 0.05 in Terrific Broth (2.4% yeast extract, 2% bacto tryptone, 0.4% glycerol, 17 mM KH_2PO_4, 72 mM K_2HPO_4 containing kanamycin and grown at 37 °C with 180 rpm shaking until an OD_600 of 0.6–0.8. Cells were then plated on ice for 15 minutes. After cooling, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM, and cultures were incubated for 18 hours at 16 °C with shaking at 180 rpm. Cultures were harvested by centrifugation at 4,000 rpm for 20 minutes at 4 °C, resuspended in binding buffer [50 mM NaH_2PO_4 pH 8.0, 500 mM NaCl, 0.001% Tween20, 5 mM β-mercaptoethanol, 10% glycerol (w/v), 5 mM imidazole], flash-frozen in liquid N_2, and placed at -80 °C until purification.

To purify the NHR-86(LBD) and NHR-86(LBD)_F379H, samples were thawed and sonicated on ice with a Qsonica Q700 microtip sonicator at an amplitude of 30 for 20 seconds (1 sec on, 1 sec off) followed by 20 seconds off for 12 cycles total. Crude lysate was centrifuged at 10,000 rpm for 30 minutes at 4 °C. The soluble fraction was filtered through a 0.45-μm filter and bound to a pre-equilibrated Ni-NTA resin (Qiagen, #30210) by incubating at 4 °C for 1 hour. The bound resin was placed in a column and allowed to flow by gravity. The column was washed with 20 column volumes of wash buffer [50 mM NaH_2PO_4 pH 8.0, 500 mM NaCl, 0.001% Tween20, 5 mM β-mercaptoethanol, 10% glycerol (w/v), 20 mM imidazole], and protein was eluted with 5 column volumes of elution buffer [50 mM NaH_2PO_4 pH 8.0, 500 mM NaCl, 0.001% Tween20, 5 mM β-mercaptoethanol, 10% glycerol (w/v), 250 mM imidazole]. Protein was dialyzed overnight with 50 mM NaH_2PO_4 pH 8.0, 300 mM NaCl, 10% glycerol (w/v). His6-SUMO tag was removed by incubating 7 units of Ulp1 protease (Sigma, #SAE0067) per mg protein with 0.5 mM DTT overnight at 4 °C. Ulp1 protease and His6-SUMO tag were removed by applying protein digestion to a pre-equilibrated Ni-NTA (Qiagen, #30210) column and collecting the flow-through, which was concentrated, dialyzed overnight, flash-frozen in liquid N_2, and stored at -80 °C.

Protein biophysical assays

Cellular Thermal Shift Assay (CETSA)

For each assay, approximately 100,000–200,000 L4 3xFLAG::NHR-86 or 3xFLAG::NHR-86_F379H animals were resuspended in 1–2 mL of PBS supplemented with HALT protease inhibitor cocktail and lysed using a Teflon Dounce homogenizer on a rotor until all animals were visibly lysed. Cellular debris was removed by centrifugation at 16,000 rpm for 20 minutes at 4 °C. Protein in the clarified whole-cell lysate was quantified using the DC Protein Assay (Bio-Rad) and adjusted to 10 mg/mL. The whole-cell lysate was then divided into 1.5-mL microcentrifuge tubes and treated with either 1–2% DMSO, 400–500 μM PCA, 400–500 μM PCN, or 70 μM R24 for 15–60 minutes at room temperature. While incubating, 50 μL of lysate from each condition was distributed into PCR tube strips and exposed to increasing temperatures (25–65 °C) for 3 minutes on a Bio-Rad C1000 Touch Thermal Cycler, cooled to room temperature for 3 minutes, and immediately placed on ice. Samples were transferred to 1.5-mL microcentrifuge tubes and spun at 20,000 g for 20 minutes at 4 °C to remove precipitated proteins. The supernatants for each temperature and condition were carefully transferred to new tubes – without disturbing the pellet or touching the sides of the tubes – containing LDS Sample Buffer (Thermo Fisher) and 1% β-mercaptoethanol. Samples were then assayed for the presence of 3xFLAG::NHR-86 or 3xFLAG::NHR-86_F379H using immunoblot analysis, as described above.

Please cite this article in press as: Peterson et al., Non-canonical pattern recognition of a pathogen-derived metabolite by a nuclear hormone receptor identifies virulent bacteria in C. elegans, Immunity (2023), https://doi.org/10.1016/j.immuni.2023.01.027
**Intrinsic tryptophan assays**

Measurement of NHR-86(LBD) and NHR-86(LBD)$_{F379H}$ tryptophan fluorescence was performed as previously described with modification. Briefly, 2 μM NHR-86(LBD) or NHR-86(LBD)$_{F379H}$ protein was incubated with either DMSO (1% final) or increasing concentrations of R24, PCN, or PCA in a 20 μL final volume. Samples were incubated at room temperature for 1 hour in 584 black-walled, round-bottom plates (Corning, #3676). Tryptophan fluorescence was measured using a Molecular Devices SpectraMax iD5 instrument with the following settings: excitation at 295 nm, emission at 340 nm, photomultiplier tubes (PMT) low, integration 100 ms.

To correct for non-specific tryptophan quenching, each compound was simultaneously incubated with 10 μM N-acetyl-L-tryptophanamide (NATA) (Sigma, #A6501), a tryptophan analog. The fraction of fluorescence decrease at each compound concentration in NATA was multiplied by the protein solvent control condition, and the measured protein fluorescence at each corresponding compound concentration was then corrected by this factor. Data points for each compound were fit using the following non-linear curve fitting equation:

\[
Y = \frac{Y_0 \left(1 - \left(\frac{K_d + Pt}{Pt + X}\right)^2 - 4 \cdot Pt \cdot X\right)^{0.5}}{2 \cdot Pt} + Y_f\left(\frac{K_d + Pt}{Pt + X}\right)^2 - 4 \cdot Pt \cdot X\left(\frac{K_d + Pt}{Pt + X}\right)\]

\[Y_0 = \text{protein fluorescence intensity with solvent control}\]
\[Pt = \text{protein concentration}\]
\[X = \text{concentration of ligand}\]
\[K_d = \text{equilibrium dissociation constant}\]

**NHR-86 depletion by auxin-inducible degron**

For NHR-86 depletion using the auxin-inducible degron, wild-type and NHR-86::AID animals were treated with 50 μM auxin naphthaleneacetic acid (NAA) (PhytoTech Labs) from the L1 to the L4 larval stage and during all experimental conditions. For these studies, a transgenic C. elegans strain was used that expresses TIR1 in all tissues under the elt-3 promoter. To avoid NAA impacts on bacterial growth and metabolism, NAA was added on top of bacterial lawns and allowed to diffuse into plates for 2 hours prior to use. We confirmed that NHR-86::AID was degraded during auxin treatment by using CRISPR genome editing to introduce a 3X FLAG tag into the NHR-86::AID strain and immunoblotting for 3xFLAG::NHR-86::AID with an anti-FLAG antibody as described below (Figure S2C).

**C. elegans pathogenesis and development assays**

“Fast-killing” P. aeruginosa infection experiments were performed as previously described. In brief, a single colony of P. aeruginosa was inoculated into 3 mL of LB Lennox medium and allowed to incubate at 37 °C for 14 hours at 250 rpm. 5 μL of this culture was spread in the center of 35-mm tissue culture plates containing 4 mL of fast-kill agar (i.e., PSG agar) (1% Bactopeptone, 1% glucose, 1% sodium chloride, 150 mM sorbitol, 1.7% Bacto-agar). Plates were incubated for 24 hours at 37 °C followed by 24 hours at 25 °C. Approximately 40 L4 larval-stage nematodes were transferred to the pseudomonal lawns on fast-kill plates. Dead nematodes were scored at 2, 4, 8 or 24 hours by assessing movement after tapping on the heads with a platinum wire. For the “fast kill” assays with PCN supplementation, agar plates were prepared as above. Following a previously described protocol, the bacterial lawn was scraped from the plates after 48 hours of P. aeruginosa growth, the agar was melted, and 100 μg/mL PCN or DMSO (1% final) was added to the liquid media. The plates were then re-poured. 20 μL of 20x E. coli OP50 was added to plates and allowed to dry. L4 C. elegans were then washed with M9 and transferred to NGM plates containing 100 μg/mL PCN or DMSO (1% final), prepared as described for studies with transcriptional reporters, for 2 hours before being picked to supplemented fast-kill plates. Three trials of each pathogenesis assay were performed. Sample sizes, four-hour survival, and p-values for all trials are shown in Table S2.

“Slow-killing” P. aeruginosa infection experiments (Figure S5B) were performed as previously described. In brief, P. aeruginosa was grown as described above and 10 μL overnight culture was spread onto the center of 35-mm tissue culture plates containing 4 mL slow-kill agar (0.35% Bactopeptone, 0.3% sodium chloride, 1.7% agar, 5 μg/mL cholesterol, 25 mM potassium phosphate, 1 mM magnesium sulfate, 1 mM calcium chloride). Plates were then incubated for 24 hours at 37 °C followed by 24 hours at 25 °C. Wild-type and nhr-86(rnaI) C. elegans pre-treated with 1% DMSO or 100 μg/mL PCN for 2 hours at the L4 stage were then transferred to P. aeruginosa slow-kill plates. Dead animals were scored twice daily until completion. Three trials of the assay were performed. Sample sizes, mean survival and p-values for all trials are shown in Table S2.

Assays assessing the growth of C. elegans were performed as described with some modifications. Briefly, CA1200 and NHR-86::AID animals were grown in the presence of 50 μM auxin until the L4 (Figure 5C) or gravid (Figure 5D) stage. Animals were then transferred onto NGM plates with 50 μM auxin and without exogenous cholesterol. Animals were photographed after 96 hours (Figure 5C) or scored for the percent of live animals after 72 hours (Figure 5D). Lawn occupancy assays were performed as previously described.
Quantification of phenazines

High-performance liquid chromatography-ultraviolet spectroscopy and liquid chromatography/mass spectrometry

Quantification of phenazines was performed as previously described. In brief, agar plates grown with each pseudomonal strain were diced into 5-10 mm cubes and transferred to 50 mL polypropylene tubes containing 5 mL HPLC-grade methanol. Samples were incubated overnight to extract phenazines from both the agar and bacterial biofilms.

Supernatants from methanol-extracted samples were filtered through 0.22-μm cellulose Spin-X columns (Thermo Fisher Scientific 07-200-386), and the filtrates were stored at -80°C until HPLC-UV analysis. On the day of HPLC-UV analysis, 100 μL of supernatant was transferred to HPLC screw-top vials with fixed inserts (Agilent Technologies 5188-6592). Phenazines were quantified using the Agilent 1260 Infinity HPLC with a biphenyl column (Kinetex 00F-4622-E0, 4.6 x 150 mm, 2.6 μm) and a 20 μL injection volume. A gradient method was used, as described previously. Phenazines were quantified by integrating the peaks observed at an absorbance of 366 nm, and phenazines were identified by comparing the retention times to the phenazine standards. All retention times and phenazine quantifications can be found in Table S3.

For Figures S1M and S1P, *P. aeruginosa* PA14 wild-type and *phzH::Tn* mutants were grown as described above. Bacteria were scraped off the surface of the agar and OD600 values were quantified. The agar for each strain was cut up into small pieces and flash-frozen directly in liquid nitrogen. Samples were then pulverized on a Mixer Mill MM 400 (Retsch) under cryogenic conditions at 30 Hz for 90 seconds. Pulverized agar samples were stored at -80°C until LC-MS/MS analysis.

Phenazines were extracted using methanol and chloroform. The organic phase was dried under nitrogen gas and resuspended in methanol. Samples were filtered through a 0.2-μm PVDF filter and assessed on a Thermo Scientific Ultimate 3000 HPLC system coupled with a Thermo Scientific TSQ Quantiva triple quadrupole mass spectrometer with a Waters Acquity BEH C18 Column and Waters Acquity BEH C18 VanGuard pre-column. The sample injection volume was 2 μL. Mobile phase A consisted of 0.1% formic acid in water, and mobile phase B consisted of 0.1% formic acid in acetonitrile. The gradient started at 35% B for 1.5 min and increased to 99% B over the course of 7 min at a flow rate of 0.25 mL/minute. MS analysis was performed with an electrospray ionization source with a capillary voltage of +3.7 kV. The following was used for the sheath gas: 40 Arb; Aux gas: 10 Arb, vaporizer temperature: 250°C, ion transfer tube temperature: 325°C. The multiple reaction monitoring (MRM) parameters were the following: duty cycle time 0.3s, collision-induced dissociation (CID) gas pressure 1.5 mTorr, Q1 resolution (full width at half maximum, FWHM) 0.7, Q3 resolution (FWHM) 0.7. Quantification of phenazines can be found in Table S3.

Molecular modeling and molecular dynamics simulations

AlphaFold-Multimer was used to predict the homodimeric structure of full-length NHR-86. The model was optimized using Protein Preparation Wizard (Schrödinger v.19-4) to determine protonation states at pH 6.0 and optimize the hydrogen bonding network. A restrained minimization was performed using the OPLS2005 force field within a root-mean-square deviation (RMSD) of 0.3 Å. To determine an optimal binding pocket, SiteMap (Schrödinger v.19-4) was used with default settings, and the final binding pocket was chosen based on the size, hydrophobicity, and hydrophilicity. Each ligand of interest was converted to an energy-minimized 3D molecular structure using LigPrep (Schrödinger v.19-4) and docked within the binding pocket using Glide. Each complex was solvated in a cubic box with at least 15 Å between any solute atom and the periodic boundaries using the TIP3P water model. Charges were neutralized using sodium and chloride ions, and additional counterions were added up to a concentration of 0.15 M. Molecular mechanics generalized Born surface solvation (MM/GBSA) calculations were carried out using 100 frames of the simulation using a custom script. Structural figures and movies were generated using PyMOL (v. 2.3.4) and VMD (v. 1.9.4).

QUANTIFICATION AND STATISTICAL ANALYSIS

Differences in the survival of C. elegans in the P. aeruginosa pathogenesis assays were determined with the log-rank test after survival curves were estimated for each group with the Kaplan-Meier method. OASIS 2 was used for these statistical analyses. Statistical hypothesis testing was performed with Prism 9 (GraphPad Software) using methods indicated in the figure legends. Table S3 contains all source data and statistical analysis methods and results. Sample sizes, survival, and p-values for all trials are shown in Table S2.