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Pseudomonas aeruginosa Disrupts *Caenorhabditis elegans* Iron Homeostasis, Causing a Hypoxic Response and Death

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SUMMARY

The opportunistic pathogen *Pseudomonas aeruginosa* causes serious human infections, but effective treatments and the mechanisms mediating pathogenesis remain elusive. *Caenorhabditis elegans* shares innate immune pathways with humans, making it invaluable to investigate infection. To determine how *P. aeruginosa* disrupts host biology, we studied how *P. aeruginosa* kills *C. elegans* in a liquid-based pathogenesis model. We found that *P. aeruginosa*-mediated killing does not require quorum-sensing pathways or host colonization. A chemical genetic screen revealed that iron chelators alleviate *P. aeruginosa*-mediated killing. Consistent with a role for iron in *P. aeruginosa* pathogenesis, the bacterial siderophore pyoverdin was required for virulence and was sufficient to induce a hypoxic response and death in the absence of bacteria. Loss of the *C. elegans* hypoxia-inducing factor HIF-1, which regulates iron homeostasis, exacerbated *P. aeruginosa* pathogenesis, further linking hypoxia and killing. As pyoverdin is indispensable for virulence in mice, pyoverdin-mediated hypoxia is likely to be relevant in human pathogenesis.

INTRODUCTION

Pseudomonas aeruginosa is a ubiquitous Gram-negative bacterium capable of causing disease in myriad hosts (Chand et al., 2011; Rahme et al., 1995; Tan et al., 1999a). Its ability to infect diverse taxa is attributed to multiple virulence factors: a variety of secreted toxins, siderophores, a quorum-sensing system, and biofilm formation (Costerton et al., 1995; Smith and Iglewski, 2003; Tang et al., 1996). Human *P. aeruginosa* infections result in serious complications in burns and eye lesions, and infections can become systemic in immunodeficient patients. Additionally, *P. aeruginosa* establishes life-long infections in the lungs of patients with chronic obstructive pulmonary disease, diffused panbronchitis, or cystic fibrosis (CF) (Govan and Deretic, 1996; Høiby, 1994; Lieberman and Lieberman, 2003; Lyczak et al., 2000). Finally, this organism remains a stubborn etiological agent responsible for many nosocomial infections (Rosenthal et al., 2010). *P. aeruginosa* shows high levels of innate antibiotic resistance (De Kievit et al., 2001; Fisher et al., 2005), and outbreaks caused by multidrug resistant strains are on the rise (Obritsch et al., 2005). Exacerbating this, only a few antipseudomonal compounds are currently in development (Bumann, 2008). These factors illustrate the importance of determining the mechanisms of *P. aeruginosa* virulence and of identifying treatments that may help prevent disease.

Despite ongoing research efforts, the virulence mechanisms underlying many *Pseudomonas* infection models remain elusive. Unfortunately, no single model, including those in mammals, has succeeded in recapitulating all of the features of *P. aeruginosa* virulence relevant to human disease, whether chronic or acute. We have utilized *Caenorhabditis elegans* as a host to develop infection assays for diverse bacterial species, including *P. aeruginosa* (Powell and Ausubel, 2008). Several features of *C. elegans* make it desirable for studying host-pathogen interactions, including the ability to easily carry out forward, reverse, and chemical genetic screens, its small size and rapid generation time, and susceptibility to human pathogens. *C. elegans-P. aeruginosa* infection models are particularly useful, as many *P. aeruginosa* virulence-related factors are conserved across widely divergent taxa from nematodes to plants to mammals (Kim and Ausubel, 2005; Rahme et al., 1995, 1997; Tan and Ausubel, 2000). In addition, the human innate immune system shares many characteristics with that of *C. elegans*, despite the relatively simple immune response pathways of the latter.

Here we report that *P. aeruginosa*-mediated killing of *C. elegans* in a liquid pathogenesis format requires the siderophore pyoverdin and the phosphatase activity of the bacterial dual-function, two-component sensor KinB. Unlike other *C. elegans* pathogenesis assays, known quorum-sensing pathways, intestinal colonization, and phenazines are dispensable for killing in the liquid assay. A library of known bioactive chemicals was used to identify virulence inhibitors. One hit, the iron-chelating compound ciclopirox olamine, implicated iron and the siderophore pyoverdin in virulence, demonstrating the value of querying host-pathogen interactions in the context of a high-throughput, whole-organism approach. Importantly, we show that PA14 triggered a lethal hypoxic crisis in *C. elegans* that

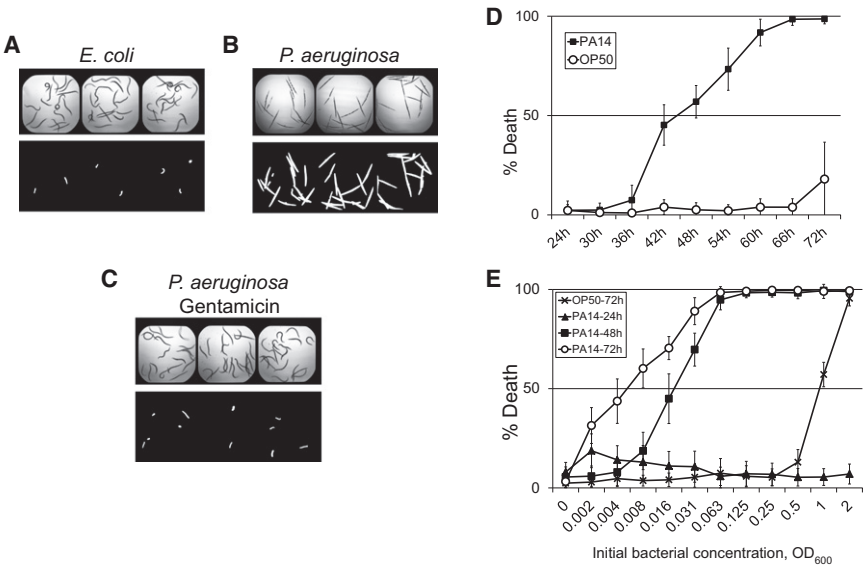


Figure 1. The *C. elegans-P. aeruginosa* LK Assay Shows Robust, Virulence- and Time-Dependent Killing

(A–C) *C. elegans* were fed a normal, nonpathogenic *E. coli* OP50 food source (A) or *P. aeruginosa* PA14 (B and C). In (C), gentamicin, an anti-*Pseudomonas* antimicrobial, was added. In (A)–(C), the upper panels are bright-field images and the lower panels show fluorescence from Sytox Orange absorbed by dead worms.

(D) Killing by PA14 is time dependent, with most death occurring between 36 and 60 hpi (squares). OP50 shows virtually no killing (circles).

(E) Time course with PA14 or OP50 over varying initial bacterial concentrations.

Error bars in (D) and (E) show the SD. See also Figure S1.

requires the *h*ypoxia-*i*nducing *f*actor HIF-1 for host defense. This hypoxic response is at least partially dependent upon pyoverdin. Combined, these data demonstrate a previously unknown role for pyoverdin in *P. aeruginosa* virulence that is likely reflected in mammalian infection.

RESULTS

Development of a Robust *C. elegans*-PA14 Liquid Killing Assay

To study the factors driving *P. aeruginosa* strain PA14 virulence, and to facilitate high-throughput screening (N.V.K. and F.M.A., unpublished data), we established a liquid-based killing (LK) assay using *C. elegans* as a host (described in detail in the Experimental Procedures and summarized in Figure S1 available online). Virtually no host death was observed in the LK assay, as shown by Sytox Orange staining (Moy et al., 2009), when worms were exposed to the normal laboratory food *E. coli* OP50 (Figures 1A and 1D), consistent with previous observations that *C. elegans* can be maintained and grown in liquid medium (Stiernagle, 2006). PA14, on the other hand, killed most worms within 48 hr postinoculation (hpi) (Figures 1B and 1D). Host death was contingent upon the presence of live PA14, as most worms survived the incubation period when gentamicin was added (Figure 1C). Almost no killing was observed within the first 24 hpi regardless of the initial bacterial inoculum, but a relatively low starting titer of PA14 was sufficient to cause host death by 48 or 72 hpi (Figure 1E), suggesting that time is necessary for the lethal interaction between the host and the pathogen to be established. In contrast to PA14, very high initial concentrations of OP50 were necessary to kill (Figure 1E). Combined, these data show that the LK assay is robust and that killing requires exposure to live *P. aeruginosa*.

The Liquid Killing Assay Does Not Utilize Most *P. aeruginosa* Virulence Factors Involved in Other *C. elegans* Killing Assays

To determine whether LK shares *P. aeruginosa*-encoded virulence determinants with previously described *C. elegans* killing assays, we first compared it to agar-based intoxication, referred to as "fast killing," which takes place on rich media (Mahajan-Miklos et al., 1999). Fast killing occurs within 24 hr, and the key determinant is the production of phenazines, a family of toxic small molecules (Cezairliyan et al., 2013; Mahajan-Miklos et al., 1999). Therefore, we tested PA14*Δphz* (Dietrich et al., 2006), a strain devoid of phenazine biosynthetic enzymes that is dramatically attenuated in fast killing (Cezairliyan et al., 2013). This strain showed no attenuation in liquid (Figure 2A), indicating that liquid killing and fast killing utilize different mechanisms.

Cyanide-based lethal paralysis is another well-studied virulence mechanism observed during infection of *C. elegans* with *P. aeruginosa* PAO1 (Darby et al., 1999). We ruled out the involvement of cyanide in LK by determining that cyanide production under LK assay conditions was negligible for PAO1, MPAO1, and PA14 strains (Figure S2A). Like PA14, both PAO1 and MPAO1 killed *C. elegans* in the LK assay (Figure S2B). In addition, PA14, PAO1, and MPAO1 strains with lesions in cyanide production genes were indistinguishable from their wild-type parents (Figure S2B).

Agar-based "slow killing" (SK) takes place on modified *C. elegans* NGM media (Tan et al., 1999a) and is the most commonly used *C. elegans-P. aeruginosa* assay. Although the mechanisms underlying pathogenesis in this assay remain unclear, multiple virulence factors, including quorum sensing, are required. An extended panel of PA14 mutants attenuated in SK, both canonical (e.g., *gacA*, *lasR*, *pqsE*, and *mvfR*) and recently described (e.g., *kinB*, *clpA*, etc.) was tested in our liquid assay (Table S1 and Figure 2A) (Feinbaum et al., 2012; Rahme et al., 1995; Tan et al., 1999b). Of these mutants, only *kinB* exhibited reduced virulence in LK. This distinguishes our liquid killing assay from a previously published liquid-based *C. elegans-P. aeruginosa* killing assay, in which *gacA* mutants showed strong attenuation (Garvis et al., 2009).

Colonization of the host intestinal tract is thought to be a key pathogenic determinant in SK (Tan et al., 1999a). Mutations in *C. elegans* that increase resistance to PA14 infection also exhibit reduced intestinal colonization (Evans et al., 2008; Garsin et al., 2003). To investigate whether mutants attenuated in SK show

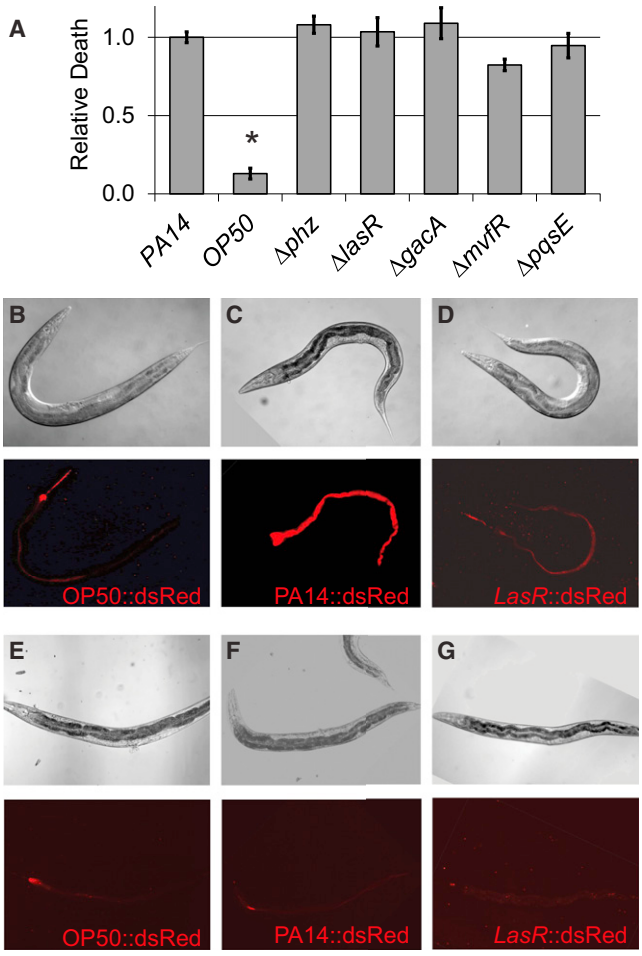


Figure 2. LK Involves Different Virulence Mechanisms than Plate-Based Assays

(A) PA14Δ*phz* and mutants exhibiting attenuation in SK were statistically indistinguishable from PA14 in liquid. Exposure time was 44 hr, and killing was normalized to PA14. Error bars represent the SE, and asterisks indicate $p < 0.01$.

(B–D) DIC (top) and fluorescence (bottom) images show whether worms are colonized by OP50-dsRed (B), PA14-dsRed (C), or PA14Δ*lasR*-dsRed (D) in SK.

(E–G) No colonization was observed in worms fed OP50-dsRed (E), PA14-dsRed (F), or PA14Δ*lasR*-dsRed (G) in a parallel liquid assay.

See also Figure S2 and Table S1.

decreased intestinal colonization, we used derivatives of OP50, PA14, and PA14Δ*lasR* engineered to express dsRed. LasR is a transcriptional regulator mediating a key PA14 quorum-sensing system (Williams and Cámara, 2009). As expected, OP50-dsRed showed no intestinal accumulation under SK conditions (Figure 2B), whereas wild-type PA14-dsRed displayed strong colonization (Figure 2C). PA14Δ*lasR*-dsRed, which is significantly impaired in SK (data not shown), displayed a marked reduction in intestinal colonization compared to wild-type PA14 (Figure 2D). We used the same bacterial reporters to query colonization in liquid, but none of the strains displayed significant intestinal colonization (Figures 2E–2G). Colonization was quantified for SK, and LK assays and confirmed these results (Figures S2C and S2D). Together, these data suggest that significantly different mechanisms underlie pathogenesis in the SK and LK assays. This may be partially explained by the much higher bacterial density in SK, where worms are exposed to a saturated lawn of bacteria.

The Phosphatase Activity of KinB Is Required for Pathogenesis in Liquid Killing

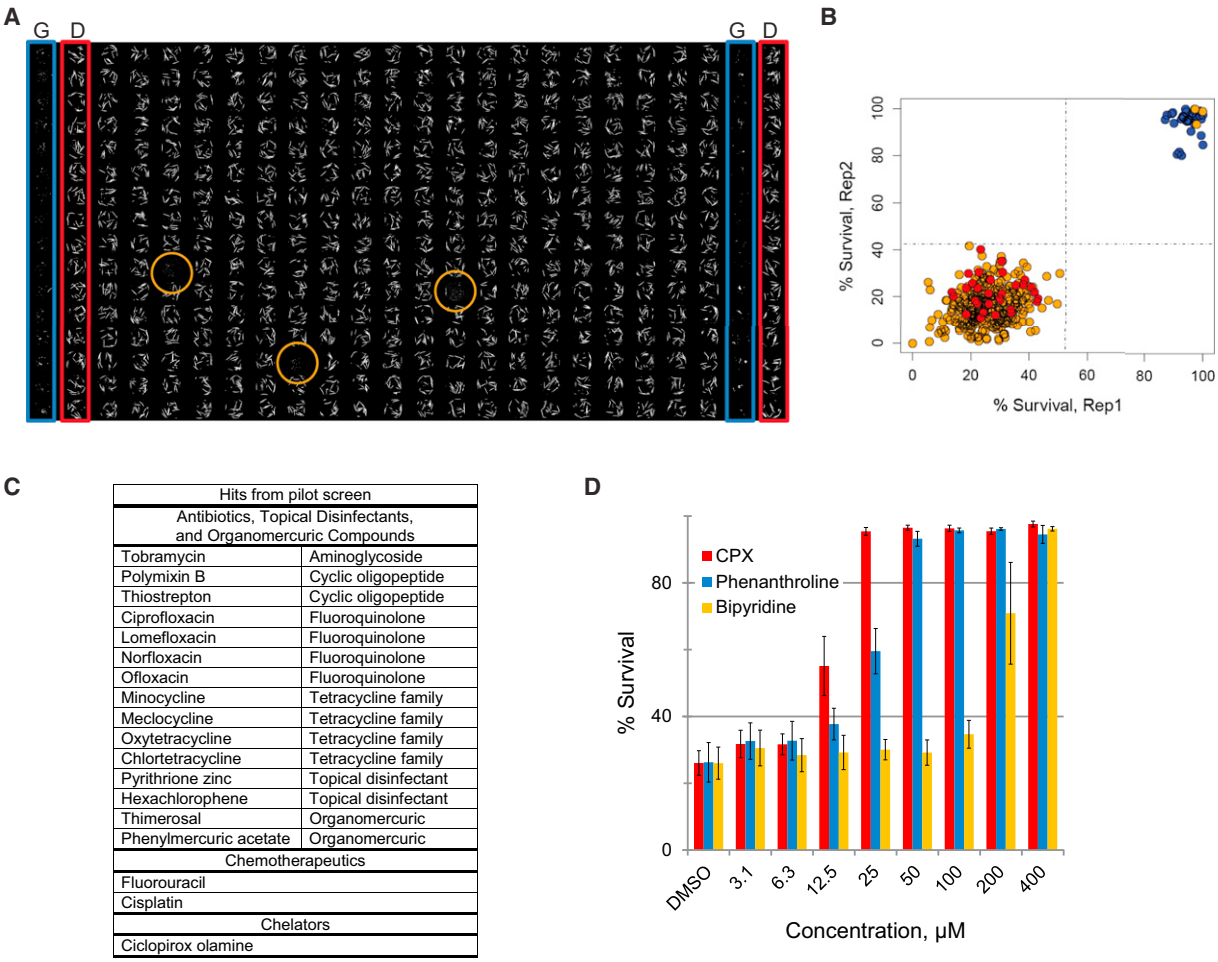
KinB, a two-component sensor histidine kinase that regulates motility, virulence factor production, and biofilm formation, is a key determinant for *P. aeruginosa* infection of zebrafish (Chand et al., 2011) and is important for virulence in a murine acute pneumonia model (Damron et al., 2012). Intriguingly, zebrafish studies suggested that neither the kinase activity of *kinB* nor its canonical partner, *algB*, were required for acute virulence (Chand et al., 2011) but that the phosphatase activity of KinB was critical (Chand et al., 2012). To investigate the role of *kinB* in LK, we first carried out a time-course study to confirm that PA14Δ*kinB* was attenuated in both LK (Figure S2E) and SK (Figure S2F). Next, we demonstrated that neither *algB* deletion nor abrogation of KinB kinase activity had a significant effect on pathogenicity in LK (Figure S2G). In contrast, the phosphatase activity of KinB was indispensable for wild-type virulence in LK, as loss of critical catalytic residues strongly ameliorated pathogenesis. These data suggest that PA14 utilizes an acute, rather than a chronic, virulence mechanism for LK.

A Screen of Small Molecules Reveals the Importance of Iron in the Liquid Killing Assay

As an additional method to gain insight into the pathogenic mechanisms involved in PA14-mediated LK, we used an unbiased chemical genetics approach. We tested 1,600 known bioactive molecules for their ability to attenuate virulence and obtained 18 hits (Figure S3). The hits included antibiotics (11), topical disinfectants (two), organomercuric compounds (two), chemotherapeutics (two), and a chelating agent (one). The presence of antibiotics among the hits was expected, as PA14 is susceptible to the families of antibiotics identified. Similarly, the chemotherapeutics (5-fluorouracil and cisplatin) interfere with DNA replication and might function as nonspecific antimicrobials. The identification of the ferric iron-chelating agent ciclopirox olamine (CPX) was unexpected and suggested that iron molecules might serve an important role in the host-pathogen interaction. We tested whether two additional iron-chelating agents, 1,10-phenanthroline (Phe) and 2,2′-bipyridine, could alleviate PA14 pathology in LK. Phe can chelate both ferric and ferrous iron, while bipyridine (which exhibits the weakest rescue) binds mostly ferrous iron. All three chelators exhibited dose-dependent rescue (Figure 3D), confirming that iron plays an important role in the killing process.

Pyoverdin, an Important Virulence Determinant, Is Required for Liquid Killing

Many microbes synthesize siderophores, small polypeptides with high affinity for oxidized iron, to promote acquisition of this critical nutrient (Crosa, 1989; Schalk, 2008). *P. aeruginosa* is known to produce two major siderophores, pyoverdin and pyochelin. Siderophore biosynthesis and iron acquisition are known to be required for *P. aeruginosa* virulence in both plant



Hits from pilot screen	
Antibiotics, Topical Disinfectants, and Organomercuric Compounds	
Tobramycin	Aminoglycoside
Polymixin B	Cyclic oligopeptide
Thiostrepton	Cyclic oligopeptide
Ciprofloxacin	Fluoroquinolone
Lomefloxacin	Fluoroquinolone
Norfloxacin	Fluoroquinolone
Ofloxacin	Fluoroquinolone
Minocycline	Tetracycline family
Meclocycline	Tetracycline family
Oxytetracycline	Tetracycline family
Chlortetracycline	Tetracycline family
Pyrithrione zinc	Topical disinfectant
Hexachlorophene	Topical disinfectant
Thimerosal	Organomercuric
Phenylmercuric acetate	Organomercuric
Chemotherapeutics	
Fluorouracil	
Cisplatin	
Chelators	
Ciclopirox olamine	

Figure 3. A High-Throughput Screen Identified 18 Compounds that Rescue LK

(A) Fluorescence micrographs of each well of a 384-well plate with small molecules screened for curing in this assay. Columns outlined in blue and red are gentamicin (positive) and DMSO (negative) controls, respectively. Circled in orange are three compounds that showed significant rescue, as demonstrated by the absence of stained worms.

(B) A scatter plot shows the percent survival in each of the two replicates for the example plate. Circles colored red represent wells containing DMSO, circles shaded blue depict gentamicin wells, and test compounds are shown as orange-colored circles. Three compounds showing significant rescue are at the upper right.

(C) A list of the compounds, and the classes to which they belong, is also shown.

(D) Dose dependence of rescue by chelating molecules was tested. Ciclopirox olamine (CPX), 1,10-phenanthroline, or 2,2′-bipyridine was added to LK assay in serial 2-fold dilutions, and death was measured after 48 hr of infection. Error bars represent the SE of three biological replicates.

See also Table S2.

and mammalian infections (Meyer et al., 1996; Nadal Jimenez et al., 2010; Takase et al., 2000). We hypothesized that disruption of siderophore biosynthesis would mimic the effects of adding a strong chelating molecule, such as CPX or Phe, and would block LK by PA14.

We tested a panel of previously generated PA14 transposon-insertion mutants (Liberati et al., 2006) that affected either pyoverdin or pyochelin biosynthesis. Disruption of pyoverdin but not pyochelin biosynthesis resulted in virulence attenuation (Figure 4A). Although *pvd* mutants were attenuated in LK, prolonged exposure still resulted in substantial *C. elegans* death (Figure 4C), suggesting multiple independent virulence mechanisms. None of the *pvd* mutants tested showed attenuation in SK (Figure 4D). These data suggest that pyoverdin plays a crucial role in *P. aeruginosa* virulence that is specific to LK.

Deletion of *pvdA*, but not *pchD*, in *P. aeruginosa* strain PAO1 causes reduced virulence in several murine infection models (Takase et al., 2000). Similarly to the PA14 *pvd* and *pch* mutants, both PAO1Δ*pvdA* and PAO1Δ*pvdF* were attenuated in LK, whereas PAO1Δ*pchBA*, which has been shown to produce virtually no pyochelin (Reimmann et al., 1998), displayed wild-type virulence (Figure 4B).

Iron Chelators Prevent the Growth of PA14 in Liquid Killing

The simplest explanation for the ability of the three iron chelators to attenuate PA14-mediated LK is that they inhibit PA14 growth. Indeed, the minimal inhibitory concentrations (MICs) for CPX, Phe, and bipyridine in LK media were 25, 50, and 400 μM, respectively, which were about the same as their effective doses

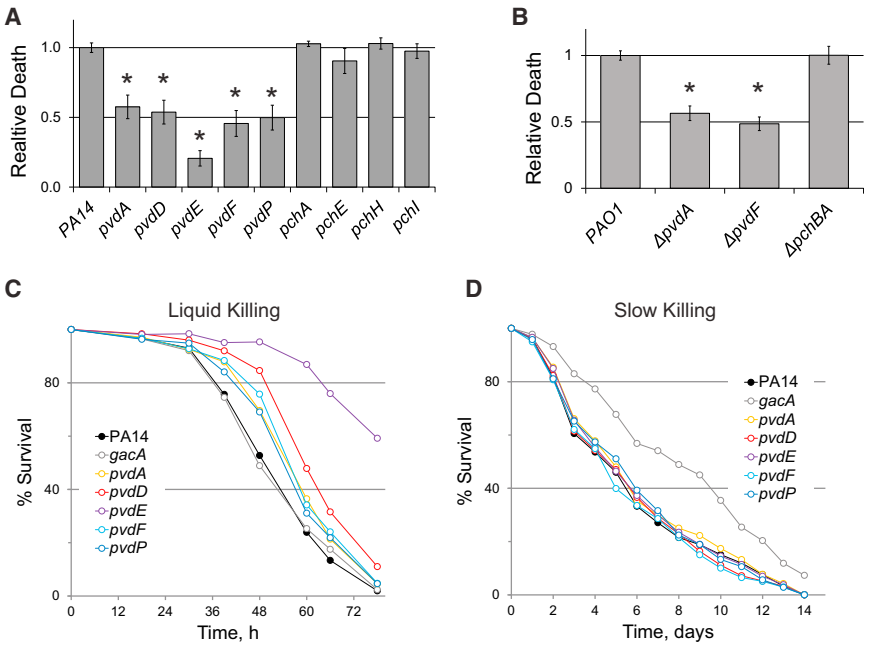


Figure 4. Pyoverdin Biosynthesis Is Important for Virulence in LK

Pyoverdin, but not pyochelin, biosynthesis mutants in PA14 (A) and PAO1 (B) showed attenuated virulence in liquid. Exposure length was 44 hr, and killing was normalized to PA14 (A) or PAO1 (B). Error bars represent standard error, and asterisks indicatep < 0.01. Killing assays were performed for a panel of PA14 pyoverdin biosynthesis mutants and PA14Δ*gacA* in LK (C) and SK (D). All *pvd* mutants were significantly attenuated in liquid ($p < 0.01$) but not in SK conditions. See also Table S3.

in the LK assay (Table S2). These data suggested that the reduced virulence of *pvd* mutants might be a consequence of poor growth of the mutants under LK assay conditions due to iron deficiency. However, the *pvd* mutants exhibited wild-type growth kinetics and final growth densities in both nutrient-rich and nutrient-poor conditions (data not shown). An alternative explanation for chelator-mediated rescue is that nutrient-poor media like LK nonspecifically sensitize PA14 to adverse environmental conditions. This seems unlikely, however, because PA14 was at least as resistant to antibiotics in LK as in LB (Table S2).

Iron Chelation Triggers a Hypoxic Response and Death in *C. elegans*

While we observed that treatment with metal-chelating compounds rescued worms at low concentrations (Figure S4A), we also noticed that significantly increasing the concentrations of these molecules resulted in host death (Figure S3A for Phe, and data not shown for CPX and bipyridine). As the lethal Phe dosage was more than 20-fold higher than the MIC for PA14, it seemed probable that worm killing at this high dose was due to Phe toxicity, not bacterial virulence. Indeed, high concentrations of Phe caused worm killing, even in the absence of PA14 (Figure S3B). These data suggest that iron sequestration causes host death by hijacking oxidized iron required for normal biological functions.

A critical iron-dependent process in aerobically respiring organisms is oxidative phosphorylation. Importantly, there is a direct connection between iron homeostasis and the HIF proteins, a family of hypoxia-inducible transcription factors in both *C. elegans* and mammals (Peyssonnaux et al., 2007; Romney et al., 2011). HIF-1 is the *C. elegans* ortholog of the mammalian HIF-1α transcription factor and is crucial for worm survival during hypoxia (Jiang et al., 2001).

Reasoning that treatment with a strong iron chelator like Phe could induce a hypoxic response in *C. elegans*, we used quantitative RT-PCR (qRT-PCR) to measure expression of the ten most upregulated genes that are dependent on HIF-1 and the ten most upregulated HIF-1-independent genes induced during hypoxia (Shen et al., 2005). Treatment of worms with a lethal dose of Phe in the LK assay with OP50 for 16 hr resulted in an average 60.6-fold upregulation of hypoxia-responsive genes compared to an untreated control (Figure 5A). Mock treatment, or treatment with a low concentration of Phe, did not result in significant upregulation (1.4- and 3.7-fold on average, Figure 5A and data not shown). Combined, these data suggest that treatment with a high concentration of an iron-chelating compound is sufficient to induce a hypoxic response in *C. elegans*.

PA14 Phenocopies Iron Loss in a Pyoverdin-Dependent Manner

The shared molecular function of pyoverdin and Phe (i.e., iron binding) suggested that pyoverdin synthesized by PA14 might also cause a hypoxic response in LK. qRT-PCR analysis of worms 16 hpi with PA14 showed significant (40.2-fold, on average) upregulation of hypoxic response genes (Figure 5A). *C. elegans* infected on solid media, however, showed little change in these transcripts, demonstrating the specificity of this hypoxic response to LK. Furthermore, the hypoxic response was mitigated when a pyoverdin biosynthesis mutant was substituted for PA14 (Figure 5A for PA14*pvdF* and data not shown for PA14*pvdE* and PA14*pvdP*). These data support a model in which pyoverdin production disrupts host iron homeostasis and triggers a hypoxic response.

hif-1 Mutation Enhances Susceptibility to PA14-Mediated Liquid Killing

As HIF-1 is necessary for a portion of the hypoxic response observed in LK, we tested whether *hif-1* mutation affects sensitivity to PA14 in LK and SK assays. *hif-1(ia4)* mutants were more susceptible than wild-type worms in the liquid assay ($p = 1.91 \times 10^{-7}$; Figure 5C) but were more resistant than wild-type in the SK assay ($p = 1.61 \times 10^{-6}$; Figure 5D). To test whether *hif-1* mutants were nonspecifically susceptible to liquid conditions, we incubated them with OP50 instead of PA14. After 3 weeks of incubation, less than 10% of the worms were dead, which was comparable with wild-type N2 worms (data not shown). This suggests

A

		Liquid Infection				Solid
		OP50	OP50/chelator	PA14	*pvdF*	PA14
Flavin-containing monoxygenase	*fmo-2*	2.8	13.8	127.9	29.1	-7.5
O-acyltransferase	*oac-54*	-2.2	8.0	6.9	2.3	0.3
Uncharacterized gene	F22B5.4	18.2	24.1	162.8	54.5	4.1
HIF-1 Regulator	*rhy-1*	-5.4	9.9	4.5	3.6	-1.2
Collagen P4H subunit	*phy-2*	-1.9	2.4	10.9	7.8	-0.7
Uncharacterized gene	Y44A6C.1	2.6	5.4	6.3	4.6	2.3
Ca/calmodulin-dependent kinase	*efk-1*	6.3	6.5	14.9	4.4	1.5
Nuclear hormone receptor	*nhr-57*	0.5	8.7	2.6	3.2	1.3
Uncharacterized gene	F59B10.4	0.4	7.3	13.7	7.4	2.3
Proline hydroxylase, inhibits HIF-1	*egl-9*	-0.6	-2.0	5.9	3.5	1.3
Uncharacterized gene	F26A3.4	0.2	0.4	12.3	7.2	1.5
NURF complex component	*nurf-1*	-0.1	-1.3	-0.2	-1.3	0.1
HSP70 family protein	*hsp-70*	1.1	263.2	96.2	55.1	5.1
HSP16 family protein	F08H9.4	2.5	41.5	20.9	14.5	2.5
HSP70 family protein	F44E5.5	-2.5	432.3	165.3	106.9	4.5
Mnk MAPK-interacting kinase	*mnk-1*	1.0	0.8	2.9	1.3	0.9
HSP70 family protein	F44E5.4	-1.3	376.5	114.8	83.6	3.5
Uncharacterized gene	F45D3.4	6.7	8.3	24.9	6.0	8.3
Uncharacterized gene	W02D9.10	-0.2	5.3	6.8	5.9	1.2
MATH domain protein	*bath-44*	0.4	0.3	4.2	3.1	1.8
	Average	1.4	60.6	40.2	20.1	1.6

B

		Filtrate			
		OP50	OP50/Fe	PA14	PA14/Fe
	fmo-2	0.3	0.2	7.1	4.4
	oac-54	-3.8	-3.7	1.9	-1.0
	F22B5.4	2.7	2.5	55.0	21.5
	rhy-1	-3.1	-5.3	-0.6	-2.1
	phy-2	-3.2	-3.0	2.9	-0.3
	Y44A6C.1	3.5	2.9	3.9	2.8
	efk-1	4.1	3.9	7.9	4.5
	nhr-57	0.2	-0.6	-0.6	-1.4
	F59B10.4	2.5	2.9	9.6	2.2
	egl-9	-0.1	-0.1	0.5	-1.2
	F26A3.4	1.5	0.6	2.8	1.3
	nurf-1	0.9	0.3	0.3	-0.4
	hsp-70	5.9	7.2	75.2	5.9
	F08H9.4	2.6	4.7	4.7	1.5
	F44E5.5	6.4	7.4	83.2	6.0
	mnk-1	1.0	0.8	1.6	0.4
	F44E5.4	5.2	6.1	84.9	5.6
	F45D3.4	2.4	4.5	3.5	3.1
	W02D9.10	0.1	-0.1	1.9	0.0
	bath-44	0.0	0.2	2.9	-0.9
Average		1.5	1.6	17.4	2.6

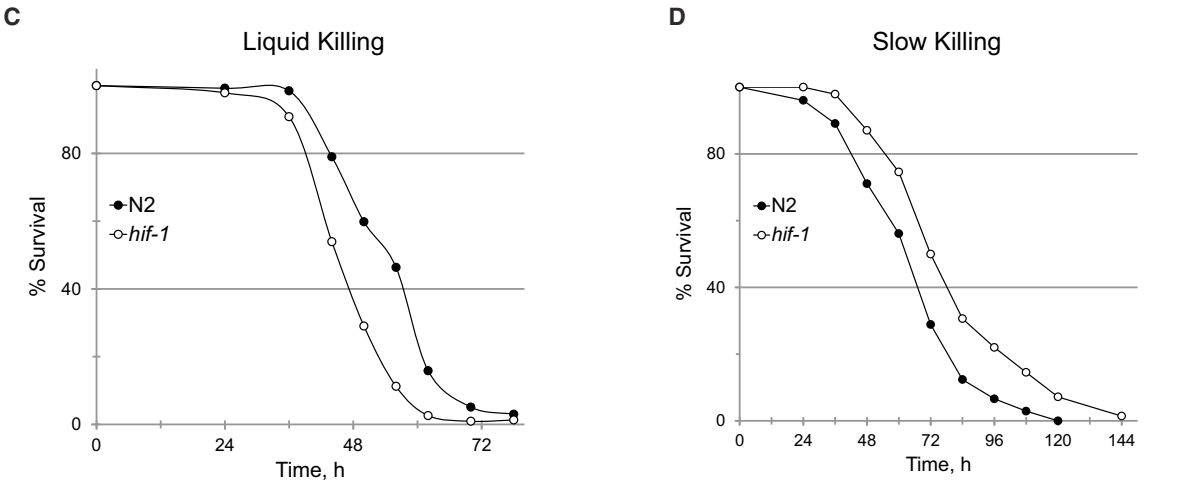


Figure 5. Exposure to PA14 or Pyoverdin Triggers a Hypoxic Response

(A and B) A panel of 20 hypoxia-upregulated genes was assayed by qRT-PCR under various conditions, including exposure to OP50 in liquid, OP50 with 1.2 mM Phe, PA14 or PA14*pvdF* in liquid, and PA14 infection in SK (A) or filtrates from OP50 or PA14 with or without iron supplementation during growth (B). Values were normalized to untreated conditions. Fold changes are color coded as follows: blue, <−5; white, between −5 and 2; pale yellow, between 2 and 5; yellow, between 5 and 10; orange, between 10 and 100; red, >100. HIF-1-dependent gene names are shaded gray.

(C and D) *cdc-25.1(RNAi)* (called N2) and *cdc-25.1(RNAi);hif-1(ia4)* (called *hif-1*) survival was compared in LK (C) and SK (D) conditions. *cdc-25.1(RNAi);hif-1(ia4)* exhibited enhanced susceptibility in liquid and enhanced resistance on agar ($p < 0.001$).

See also Figure S3.

that the difference in susceptibility is not merely a consequence of hypoxic conditions in the assay. We also saw no difference between *hif-1* mutants and wild-type N2 when infected with *Enterococcus faecalis* in liquid (data not shown) using a previously described *C. elegans-E. faecalis* liquid infection assay (Moy et al., 2009). These data, along with the qRT-PCR results

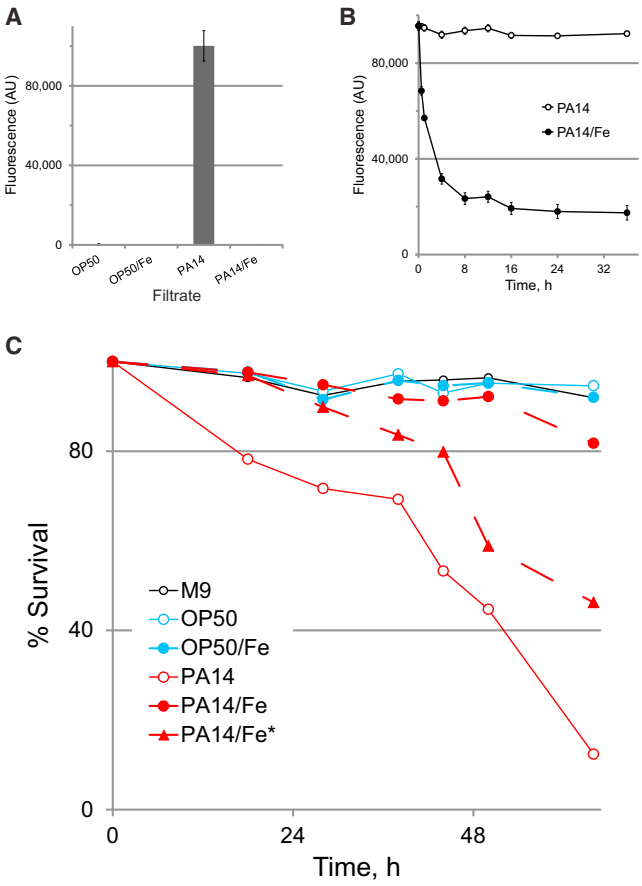


Figure 6. Pyoverdin Exposure Causes Host Death

(A) Relative pyoverdin concentration in filtrates was measured by fluorescence emission at 460 nm.

(B) PA14 filtrate was incubated with (PA14/Fe) and without (PA14) iron, and fluorescence emission was assayed.

(C) Survival of OP50-fed worms exposed to various filtrates in LK. PA14 filtrate shows significant killing (PA14, $p = 0$) that is ameliorated by growing the pathogen with 100 μM iron supplementation (PA14/Fe, $p = 0$, compared to PA14 filtrate) or by overnight incubation of PA14 filtrate with 100 μM iron (PA14/Fe* $p < 10^{-6}$).

Error bars in (A) and (B) represent the SE. See also Figure S4.

described above, suggest that the hypoxic response is specific to LK and that the HIF-1-dependent hypoxic response serves a protective role against *P. aeruginosa* but not *E. faecalis*.

Pyoverdin-Mediated Iron Sequestration Induces a Hypoxic Response and Lethality

As pyoverdin is a secreted nonribosomal polypeptide, we hypothesized that cell-free filtrates from PA14 cultures would also induce a hypoxic response in the LK assay. Indeed, PA14 filtrates from cells grown to saturation in iron-poor M9 medium (which induces pyoverdin biosynthesis), but not from OP50 cultures, were sufficient to trigger a hypoxic response (Figure 5B). The level of pyoverdin measured in LK media after 44 hpi was comparable to its level in 50% PA14 filtrate, which was used for experiments (data not shown). Pyoverdin biosynthesis is abrogated under iron-replete conditions (Cornelis et al., 2009; Ochsner et al., 1995), and any pyoverdin or other siderophore produced under conditions of iron excess would likely bind the iron present in the medium, limiting the pathogenic potential of the siderophore. Consistent with the hypothesis that pyoverdin is the active factor in the filtrates causing worm death, PA14 filtrate produced from iron-supplemented cultures contained no pyoverdin (Figure 6A) and elicited virtually no hypoxic response (Figure 5B).

We also tested whether the pyoverdin level in filtrates was sufficient to mediate lethality in the LK assay. As expected, little or no death was observed when unconditioned M9 or OP50 filtrates were added to the assay (Figure 6C). In contrast, PA14 filtrate showed significant killing. Addition of exogenous iron during PA14 growth significantly attenuated ($p = 0$) killing by this filtrate, demonstrating that the iron-chelating property of pyoverdin was critical for toxicity.

Pyoverdin also promotes the production of several *P. aeruginosa* virulence factors, including exotoxin ToxA and the protease PrpL (Lamont et al., 2002). It is possible that iron supplementation during overnight growth diminished production of these pathogenic determinants, thereby decreasing killing. To test this, we split a filtrate produced from an overnight PA14 culture and incubated half with 100 μM iron overnight. We reasoned that exogenous iron would bind to the pyoverdin, decreasing its ability to trigger hypoxia without affecting ToxA, PrpL, or other pyoverdin-dependent virulence factors. Iron acquisition by pyoverdin was verified by monitoring a time-dependent decrease in fluorescence (Figure 6B). This filtrate, PA14/Fe*, which was supplemented with iron after filtration, showed reduced killing in the liquid assay ($p = 9.21 \times 10^{-7}$) compared to untreated filtrate (Figure 6C). Taken together, our data demonstrate that pyoverdin-mediated sequestration of host iron is sufficient to induce a hypoxic response and lethality.

P. aeruginosa encodes multiple virulence pathways that may act simultaneously in pathogenesis. To examine the importance of iron sequestration in the context of an active interaction between PA14 and *C. elegans*, we tested whether iron supplementation had an effect. Addition of 100 μM ferric chloride to the LK assay significantly attenuated host killing ($p = 0.004$), confirming the biological significance of pyoverdin toxicity (Figure S4).

DISCUSSION

We report that *P. aeruginosa* causes *C. elegans* killing in a liquid assay. Unlike previously reported *C. elegans-P. aeruginosa* pathogenicity assays (Garvis et al., 2009; Mahajan-Miklos et al., 1999; Tan et al., 1999b), LK is independent of phenazine production, known quorum-sensing pathways, and intestinal colonization. Using a high-throughput screen, we identified a role for iron in PA14-mediated LK, which was confirmed by the attenuated virulence of mutants defective in pyoverdin biosynthesis. As host iron is crucial for multiple biological processes, we reasoned that pyoverdin might be directly functioning as a worm-killing toxin, similar to treatment with a lethal concentration of chelator (Figure 7). qRT-PCR analysis showed strong induction of hypoxic response genes in LK with PA14, which was abolished when pyoverdin mutants were used. Consistent with this, *hif-1* mutants, which are sensitized to hypoxia,

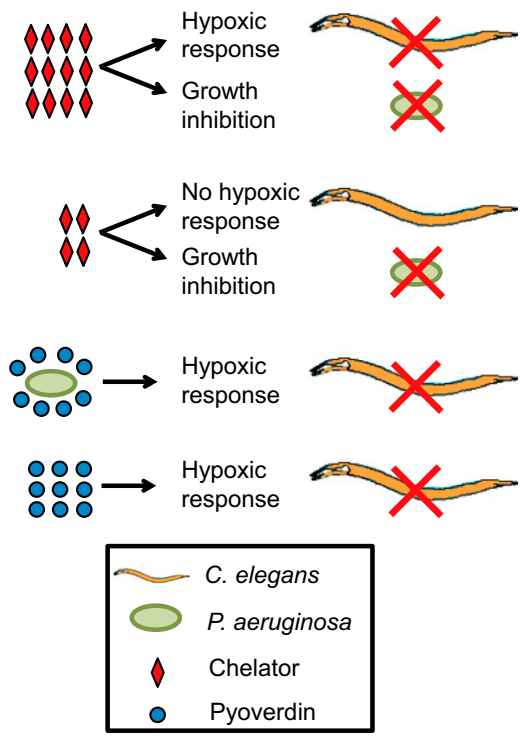


Figure 7. A Schematic of Iron-Scavenger Toxicity in Liquid
Exposure to a high concentration of chelator, PA14, or pyoverdin present in filtrate triggers a hypoxic response and subsequent worm death. A low concentration of chelator precludes bacterial growth but does not induce a hypoxic response, thereby preventing death.

exhibited increased death in LK but not in SK or during infection with *E. faecalis*. Finally, we used cell-free filtrates to determine that pyoverdin is sufficient to induce a hypoxic response and killing in the absence of PA14. Filtrate produced from an iron-supplemented culture of PA14 (a condition known to limit pyoverdin production) did not kill the host. Incubation with iron after filtration also alleviated LK. Combined, these data demonstrate that PA14 causes pyoverdin-dependent disruption of host iron homeostasis, triggering a hypoxic response and death. Although pyoverdin is sufficient to mediate LK, as discussed below, other factors are also required for wild-type levels of virulence.

Pyoverdin is critical for *P. aeruginosa*, as iron acquisition is typically required both for normal growth and for pathogenesis (Meyer et al., 1996; Takase et al., 2000). In addition, iron acquisition by pyoverdin or pyochelin was required for the "red death" phenomenon observed in *C. elegans* infected with *P. aeruginosa* strain PAO1 (Zaborin et al., 2009). Pyoverdin biosynthesis is negatively regulated by the presence of intracellular iron (Cornelis et al., 2009), and pyoverdin autoregulates its own production in a feed-forward loop until sufficient iron is acquired. We note that the PA14*pvdE* mutant, which showed the greatest attenuation of the *pvd* mutants tested, produces a small amount of fluorescence that is characteristic of pyoverdin (Table S3). We speculate that translation of the truncated PA14*pvdE* transcript might result in a partially functional protein with a dominant-negative phenotype. Unfortunately, little is known about the function of the *pvdE* gene product, precluding a definitive explanation of the *pvdE* mutant phenotype. It is also worth noting that it is unlikely that heme metabolism is altered in this assay, as heme-responsive genes (Severance et al., 2010) showed no significant transcriptional changes by qRT-PCR (data not shown).

Consistent with the hypothesis that pyoverdin may hijack host-assimilated iron in mammals, pyoverdin stimulates bacterial growth in media supplemented with human serum or plasma (Cox and Adams, 1985), suggesting that it can use the ferriproteins in these fluids as a source of iron. Pyoverdin has also been shown to directly remove iron from transferrin (Meyer et al., 1996). The removal of iron from normal iron transport proteins can also cause iron toxicity due to the production of hydroxide radicals (Baldwin et al., 1984; Coffman et al., 1990). Whether pyoverdin might directly cause damage to mammalian tissues by iron removal remains an intriguing and important question.

Observations that *P. aeruginosa* and other pathogens can elicit a HIF-1-associated hypoxic response in mice and humans are also consistent with a potential role for direct pyoverdin toxicity in mammalian infections. For example, human HIF-1 stabilization has been observed after exposure to *P. aeruginosa* in intestinal epithelial cells and in bronchial epithelial cells derived from a CF patient (Koury et al., 2004; Legendre et al., 2011). Recent work has also suggested that HIF-1 transcription factor activity is important for optimal innate immune function during bacterial infections in mice (Nizet and Johnson, 2009). HIF-1 activity seems to play an important role, as a tug-of-war takes place during the host-pathogen interaction. Under normoxic conditions, HIF-1 is targeted for degradation by EGL-9 family hydroxylases (Epstein et al., 2001). When iron, an essential EGL-9 cofactor, is depleted (i.e., by treatment with a chelating molecule or siderophore), HIF-1 is stabilized. In turn, *P. aeruginosa* appears to have developed a mechanism to counteract HIF-1 stabilization by targeting HIF-1α for degradation using the 26S proteasome and its own quorum-sensing machinery (Legendre et al., 2012). Either *egl-9(sa307)* or *egl-9(n586ts)* was insufficient to increase survival in the presence of PA14 in LK, as was *vhl-1(ok161)*, the E3 ligase targeting HIF-1 for degradation (N.V.K., unpublished data). It is currently unclear whether this is due to HIF-1 stabilization alone being insufficient to promote survival under these conditions, observed pleiotropy for *egl-9* alleles during infection (Luhachack et al., 2012), or HIF-1 inhibition via a VHL-1-independent mechanism (Shao et al., 2010). Therefore, the role of HIF-1 stabilization in response to *P. aeruginosa* infection requires further investigation.

P. aeruginosa pyoverdin mutants might also exhibit reduced virulence in mammalian infection models because they are deficient in virulence factor production. Pyoverdin regulates the production of a variety of virulence factors, including ToxA and the protease PrpL, in addition to its own biosynthesis (Lamont et al., 2002; Vasil and Ochsner, 1999). Interestingly, PrpL is capable of mediating degradation of several secreted proteins, including lactoferrin and transferrin (Wilderman et al., 2001), which might mediate iron release for siderophore-mediated acquisition. Such degradation has been reported in patients with chronic lung conditions, including CF patients (Britigan et al., 1993). In this way, pyoverdin regulates the production of virulence factors that cause the release of iron from host proteins in addition to taking iron directly from them.

In contrast to pyoverdin, disruption of pyochelin, the other major siderophore of *P. aeruginosa*, had little effect on LK. This is consistent with observations in mammals, where pyochelin disruption alone does not result in attenuation but does show a synthetic interaction when combined with pyoverdin removal (Takase et al., 2000). Unfortunately, double mutants defective in both pyoverdin and pyochelin biosynthesis exhibited severe growth defects in LK medium (data not shown), limiting the conclusions that could be drawn. One possible explanation that has been put forth for this synthetic interaction is that both siderophores play roles in virulence, but that pyoverdin is more important (Ankenbauer et al., 1985; Takase et al., 2000).

Although we have shown that pyoverdin is an important virulence factor in the *C. elegans* LK model, PA14 *pvd* mutants are still able to kill the nematodes, suggesting that other virulence factors remain to be discovered. An important *P. aeruginosa* virulence factor in mammalian models is alginate, which serves as a prognosticator for outcomes in CF patients (Ramsey and Wozniak, 2005). A key regulator in alginate production is KinB, which has been implicated in *C. elegans* SK, zebrafish, and mouse infection models (Chand et al., 2011; Damron et al., 2012; Feinbaum et al., 2012) and is also required for LK (this study). KinB's role in alginate production, however, does not appear to be relevant for LK. Alginate is more commonly involved in chronic infections and is not thought to be necessary for acute virulence (Yorgey et al., 2001), and a recent report suggests that KinB might be key in mediating the transition between acute and chronic *P. aeruginosa* infections (Chand et al., 2012). Also, it is unlikely that KinB plays a major role in regulating pyoverdin production in LK since *kinB* mutants synthesize normal levels of pyoverdin (data not shown). Further characterization of KinB in *P. aeruginosa* virulence will be necessary to identify its role(s) in pathogenesis, both in *C. elegans* and in vertebrates.

P. aeruginosa represents a serious risk to patients who have CF, immunodeficiencies, or are in hospital settings. The ubiquity of *P. aeruginosa* ensures that this bacterium will remain an obstacle to human health, and its propensity to develop antimicrobial resistance demonstrates that increased understanding of its virulence is critical and that novel treatments are necessary. The identification of novel drug leads requires the development of assays that are amenable to high-throughput screens, similar to the LK assay presented here. Model systems like this are of limited value, however, unless the mechanisms underlying virulence are understood. Our development of a PA14 liquid killing platform, and the demonstration that *C. elegans* killing is directly mediated by pyoverdin toxicity, should prove useful for *Pseudomonas* researchers and will further our understanding of complex host-pathogen interactions.

EXPERIMENTAL PROCEDURES

Additional detailed methods are presented in the Supplemental Experimental Procedures available online.

Liquid Killing Assay

glp-4 worms were synchronized by hypochlorite isolation of eggs from gravid adults, followed by hatching of eggs in S-basal. L1 larvae were transferred to NGM plates seeded with OP50 and incubated at 15°C for 16 hr and then transferred to 25°C for 48 hr. Worms were rinsed from the plates and washed in S-basal. An overnight PA14 LB culture (350 μl) was spread on an SK plate and incubated at 37°C for 24 hr then at 25°C for 24 hr. Bacteria were scraped from SK plates and resuspended in S-basal supplemented with 5 μg/ml cholesterol. The titer was determined by spectrometry and adjusted to a final OD_{600} = 0.03. SK media (17 μl) and resuspended bacteria (33 μl) were added to each well. Eighteen worms were dispensed into each well of a 384-well plate with a COPAS BioSort robot (Union Biometrica). Plates were sealed with gas-permeable membranes (Diversified Biotech) and incubated at 25°C without agitation at 80%–85% humidity for 44–48 hr. Subsequently, worms were washed five times with a BioTek ELx405 microplate washer, and Sytox Orange (Invitrogen) was added to a final concentration of 0.7 μM. After 18–24 hr, plates were imaged in both bright-field and Cy3 fluorescence channels with an IXMicro automated microscope (Molecular Devices). Worm survival was scored by automated image analysis with CellProfiler (Kamentsky et al., 2011; Moy et al., 2009). The pipeline consisted of illumination correction (to flatten lighting abnormalities), adaptive intensity thresholding (to identify worms in bright-field images), and normalization of fluorescence area to bright-field area. Size- and contrast-based filters were used to exclude debris and other artifacts.

For assays using N2 or *hif-1(ia4)* mutants, worms were prepared via hypochlorite egg preparation as above, but L1 larvae were transferred to NGM plates seeded with RNA interference targeting the cell-cycle gene *cdc-25.1* to ensure the sterility of adults used for LK assays.

High-Throughput Chemical Screen

Screening was performed at the National Screening Laboratory for the Regional Centers of Excellence in Biodefense and Emerging Infectious Diseases (NSRB) at Harvard Medical School. Plates (384-well; Corning #3712) were filled with 25 μl media (see above) with a WellMate Microplate Dispenser (Thermo Scientific). Compound (0.3 μl) in DMSO was pin transferred into each well with an Epson Compound Transfer Robot system, and PA14 and nematodes were added. Each plate had DMSO- and gentamicin-treated wells to serve as negative and positive controls, respectively (Figure 3). Compounds were tested at 20 μg/ml, and chemicals that exhibited rescue in duplicate plates (more than three SD from DMSO control) were considered hits.

qRT-PCR

For RNA collection, LK assay was performed as described above, except that volumes were adjusted to 150 μl, and the assay was performed in 96-well plates with 40 worms per well. Worms from two 96-well plates were combined 16 hpi (all conditions except filtrates) or 24 hpi (filtrates) and washed twice with S-basal. RNA purification and qRT-PCR were performed as previously described (McEwan et al., 2012), except that fold changes were calculated with a ΔΔCt method. Primer sequences are available upon request.

Filtrate Production and Pyoverdin Production Determination

Bacteria were inoculated into M9 medium as described above and grown 18–20 hr at 37°C with agitation. Bacteria were pelleted by centrifugation at 4,000 g for 30 min. Supernatants were decanted and sequentially filtered through 0.45, 0.45, and 0.20 μm filters. Pyoverdin production was determined spectrophotometrically by measurement of emission at 460 nm with an excitation of 400 nm. Filtrates were stored at 4°C for up to 24 hr prior to use.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures, three tables, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.chom.2013.03.003.

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