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Pseudomonas aeruginosa killing of *Caenorhabditis elegans* used to identify *P. aeruginosa* virulence factors

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ABSTRACT **We reported recently that the human opportunistic pathogen *Pseudomonas aeruginosa* strain PA14 kills *Caenorhabditis elegans* and that many *P. aeruginosa* virulence factors (genes) required for maximum virulence in mouse pathogenicity are also required for maximum killing of *C. elegans*. Here we report that among eight *P. aeruginosa* PA14 Tn*phoA* mutants isolated that exhibited reduced killing of *C. elegans*, at least five also exhibited reduced virulence in mice. Three of the Tn*phoA* mutants corresponded to the known virulence-related genes *lasR*, *gacA*, and *lemA*. Three of the mutants corresponded to known genes (*aefA* from *Escherichia coli*, *pstP* from *Azotobacter vinelandii*, and *mtrR* from *Neisseria gonorrhoeae*) that had not been shown previously to play a role in pathogenesis, and two of the mutants contained Tn*phoA* inserted into novel sequences. These data indicate that the killing of *C. elegans* by *P. aeruginosa* can be exploited to identify novel *P. aeruginosa* virulence factors important for mammalian pathogenesis.**

Until recently, systematic genetic analysis of the pathogenic interaction between a eukaryotic host and a bacterial pathogen has been hampered by the lack of a genetically tractable system in which both the pathogen and host are amenable to low-cost, high-throughput genetic screens. Ideally, the eukaryotic host should be a model genetic organism such as *Caenorhabditis elegans, Drosophila melanogaster*, or *Arabidopsis thaliana* for which a complete genome sequence is available or will be available in the near future. Similarly, it would be advantageous if the genome sequence of the bacterial pathogen were also available. A final advantageous feature of a model pathogenesis system would be the use of a bacterial pathogen in the model that is also an important human pathogen.

We recently developed a *C. elegans–Pseudomonas aeruginosa* pathogenesis system that has all of the features described above (1, 2). The genome sequence of *C. elegans* is essentially complete (3), and the *P. aeruginosa* strain PA01 is available at http://www.pseudomonas.com. *P. aeruginosa* is a ubiquitous Gram-negative bacterium that is an important opportunistic human pathogen that is capable of causing disease in humans whose immune system has been compromised, who have sustained major trauma, or who are inflicted with cystic fibrosis (4). *P. aeruginosa* also has been reported to cause disease in plants, insects, and a variety of vertebrates (5–8). Interestingly, we showed that a clinical isolate of *P. aeruginosa* strain PA14 is not only a pathogen of mice but also kills *C. elegans* (1, 2), and we identified two distinct modes of PA14-mediated killing of *C. elegans* that appear to be mechanistically distinct. When PA14 is grown on low-nutrient media, *C. elegans* killing occurs over the course of several days and is referred to as "slow killing." In contrast, when PA14 is grown on high-osmolarity media, *C. elegans* killing occurs over the course of several hours and is referred to as "fast killing." Slow killing requires live bacteria and is correlated with the accumulation of PA14 in the *C. elegans* gut (2), whereas fast killing is mediated at least in part by low-molecular-weight toxins, including phenazines, and does not require live bacteria (1).

In previous publications, our laboratory showed that *P. aeruginosa* strain PA14 also causes disease in a variety of plants including lettuce and *A. thaliana* (8, 9). Using a plant–*P. aeruginosa* pathogenesis system, we identified a set of PA14 mutants that is less pathogenic in a plant leaf infiltration assay; remarkably, most of these mutants also were less pathogenic in a mouse burn model (9). Importantly, we showed that 6 of 10 genes identified as encoding important virulence factors for mouse and *A. thaliana* pathogenesis are also required for effective killing of *C. elegans* (2).

In this paper we demonstrate how the *C. elegans–P. aeruginosa* slow-killing model can be exploited to identify previously unknown *P. aeruginosa* virulence-associated factors and to identify virulence-associated systems that appear to be universal among Gram-negative animal and plant bacterial pathogens. These results validate our strategy of using a simple nonvertebrate host to scan the entire *P. aeruginosa* genome for pathogenesis-related genes.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, Media, and Bacterial Genetics. The *P. aeruginosa* strain PA14 (8), the *E. coli* strains OP50 (10) and DH5α (Bethesda Research Laboratories), and the PA14 Tn*phoA* mutants *25F1* and *pho15* (9) have been described. Complete media for bacterial culture and maintenance were Luria broth and King's broth (11, 12). Minimal medium was M9 (12). The broad host-range cloning vector pUCP18 (13), the *E. coli* cloning vector pCR2.1 (Invitrogen), and the plasmid pKDT17, which contains the *P. aeruginosa lasR* gene (14), have been described. Plasmid pCH3, which contains the *E. coli dsbA* cloned into pBAD18, was a gift from C. Guilhot (Harvard Medical School, Boston). *E. coli* DH5α was used for plasmid constructions and plasmids were introduced into *P. aeruginosa* PA14 by electroporation (15).

Screening for PA14 Tn*phoA* Mutants with *C. elegans* Slow-Killing Defects. *P. aeruginosa* PA14 was mutagenized with Tn*phoA* as described (9). Individual PA14::Tn*phoA* clones were inoculated into 200 μl King's B medium in microtiter plates

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Abbreviations: NG, nematode growth; IPCR, inverse PCR.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database [accession nos. AF116282 (*pho15*), AF116283 (*35A9*), AF116284 (*25F1*), AF116285 (*50E12*), AF116277 (*12A1*), AF116278 (*41C1*), AF116279 (*44B1*), AF116280 (*48D9*), and AF116281 (*41A5*)].

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containing rifampicin and neomycin at 100 and 200 μg/ml, respectively. Ten microliters of an overnight culture was spread on nematode growth medium (NG; modified from NGM as described in ref. 16; 0.35% peptone instead of 0.25%) in 5.5 cm Petri plates and incubated at 37°C for 24 h. After 8–24 h at room temperature (23–25°C) each plate was seeded with two L4-stage hermaphrodite *C. elegans* strain N2 (Bristol) (10). Plates were incubated at 25°C and examined for live worms after 5 days. On plates seeded with a nonpathogenic mutant, thousands of progeny worms were present at day 5 and the bacterial lawn was completely consumed, whereas very few or no live worms were found on the plates seeded with the wild-type strain and the bacterial lawn remained intact. Putative nonpathogenic or attenuated mutants identified in the preliminary screen were retested and subjected to a virulence assay to determine the kinetics of *C. elegans* killing.

***C. elegans* Slow- and Fast-Killing Assays.** Slow-killing kinetics of *C. elegans* by PA14 and its derivatives were determined by using the same procedure as for the mutant screen except that each NG plate was seeded with 40–50 L4 *C. elegans.* Plates were scored every 4–6 h, and three to four replicates per trial were performed. A worm was considered dead when it no longer responded to touch. Worms that died as a result of getting stuck to the wall of the plate were excluded from the analysis. Fast-killing kinetics were determined in the same way as for slow killing except that high-osmolarity peptone/glucose/sorbitol (PGS; 1% Bacto-Peptone/1% NaCl/1% glucose/0.15 M sorbitol/1.7% Bacto-Agar) medium was used instead of NG medium and the plates were seeded with 30–40 worms.

***Arabidopsis* and Mouse Pathogenicity Assays.** An *Arabidopsis* leaf-infiltration assay using ecotype Llagostera and a mouse full-thickness skin burn assay using inbred mice (strain AKR/J; The Jackson Laboratory) were performed as described (8, 9).

Molecular Analysis of Tn*phoA* Mutants. Standard DNA manipulation protocols were performed as described in ref. 17. The generation and sequencing of inverse PCR (IPCR) sequence tags corresponding to Tn*phoA* insertions were carried out as described (1, 9). IPCR sequence tags were used to identify corresponding sequences in the *P. aeruginosa* PA01 genome database (http://www.pseudomonas.com) by using the program BLASTN (18). PA01 sequence was used to design oligonucleotide primers corresponding to mutants *25F1, pho15, 50E12*, and *35A9*, which then were used to amplify a region from the wild-type PA14 genome corresponding to each mutant. Each of these amplified fragments from PA14 was sequenced directly (using the Sequenase PCR Product sequencing Kit, United States Chemical) or cloned into pCR2.1 and sequenced using standard dideoxy-sequencing methods. The sequences were deposited in the GenBank database and assigned the accession numbers AF116282 (*pho15*), AF116283 (*35A9*), AF116284 (*25F1*), and AF116285 (*50E12*). The IPCR sequence tags were also deposited in GenBank and assigned the following accession numbers: AF116277 (*12A1*), AF116278 (*41C1*), AF116279 (*44B1*), AF116280 (*48D9*), and AF116281 (*41A5*). The predicted translation products of the identified ORFs in each of the sequences were compared with the GenBank database by using BLASTX (19).

Genetic Complementation Analysis of Mutants *pho15*, *25F1*, and *50E12*. *pho15(dsbA).* Primers TMW8 (5′-GCACTGATCGCTGCGTAGCACGGC-3′) and TMW9 (5′-TGACGTAGCCGGAACGCAGGCTGC-3′) were used to amplify a 1,126-bp fragment from PA14 genomic DNA containing the PA14 *dsbA* coding region plus 176 bp of sequence upstream of the translational start. This fragment was cloned into pCR2.1 by using the TA cloning kit (Invitrogen) to generate pCRdsbA. A 1.2-kb *Sac*I/*Xba*I fragment containing the *dsbA* ORF was subcloned from pCRdsbA into *Sac*I/*Xba*I-digested pUCP18 to construct pPAdsbA, placing the transcription of *dsbA* under the control of a constitutive *E. coli lacZ* promoter. A 700-bp *Kpn*I/*Sph*I fragment containing the *E. coli dsbA* was subcloned from pCH3 into *Kpn*I/*Sph*I-digested pUCP18 to generate pEcdsbA, placing the *E. coli dsbA* under the control of a constitutive *E. coli lacZ* promoter.

25F1. Mutant *25F1* contained Tn*phoA* inserted into the first ORF of a three-gene operon, *orf338*, *orf224*, and *orf252*. A 1.8-kb PCR fragment containing 482 bp of upstream promoter sequence, the entire *orf338*, and a truncated *orf224* was amplified from PA14 genomic DNA by using primers F2327 (5′-CGAGGAATCCAGTCGAGGTG-3′) and R4180 (5′-GCAAGATGCAGCCGAGAGTAG-3′). The product was cloned directly into pCR2.1 to construct plasmid pMT403. A *Sac*I/*Xba*I fragment from pMT403, which contained the *orf338* PCR product and 482 bp of upstream sequences, was cloned into the *Sac*I/*Xba*I site of pUCP18 to construct pORF338, placing *orf338* under the control of its native promoter. A 3.6-kb genomic fragment containing *orf338*, *orf224*, *orf252*, and upstream sequences was amplified from genomic PA14 DNA using primers RIF3115 (5′-GTCAGAATTCTCAGCTTGACGTTGTTGCCC-3′) and RIR6757 (5′-GTCAGAATTCGACTTCTATTACCGCGACGCC-3′), each containing an *Eco*RI site (underlined). The *Eco*RI digest of the PCR product was cloned into the *Eco*RI site of pUCP18 to construct plasmid p3-ORFs, which contains *orf338, orf224*, and *orf252* under the control of their native promoters.

50E12. Mutant 50E12 contained Tn*phoA* inserted into a gene homologous to *ptsP*. Promoter prediction by neural network analysis (http://www-hgc.lbl.gov/projects/promoter.html) suggests that the PA14 *ptsP* homologue is cotranscribed with the upstream ORF, *orf159. orf159* encodes a putative 159 residue polypeptide that is closely related to proteins with a core MutT domain of unknown function found in *Haemophilus influenzae* (GenBank accession no. Q57045). A 4.3-kb PCR fragment, containing the PA14 *ptsP* homologue ($ptsP_{Pa}$), *orf159*, and 1.2-kb upstream sequences, with an *Eco*RI site introduced at each end, was amplified from genomic PA14 DNA by using the primers RIF1698 (5′-GTCAGAATTCGATGTTCCAGTCCCAGATCCC-3′) and RIR6002 (5′-GTCAGAATTCCAGTAGACCACCGCCGAGAG-3′). This fragment was cloned into the *Eco*RI site of pUCP18 to generate p206-lac and p206-nat. In p206-lac, the transcription of *orf159* and $ptsP_{Pa}$ is under the control of both a constitutive *E. coli lacZ* promoter and their native promoters. In p206-nat, *orf159* and $ptsP_{Pa}$ are only under the control of their native promoters.

Biochemical and Physiological Characterization of PA14 Tn*phoA* Mutants. Supernatants from log-phase Luria broth cultures were tested for proteolytic and elastolytic activities as described (20). Pyocyanin was assayed by absorbance at 520 nm in acidic solution after growth in King's A broth (11) modified by the addition of 100 μM $FeCl_3$ (21). Phospholipase C was assayed by using *p*-nitrophenylphosphorylcholine as a substrate, as described (22).

RESULTS

Identification of PA14 Mutants Defective in *C. elegans* Slow Killing. A total of 2,400 prototrophic *P. aeruginosa* PA14 Tn*phoA* insertion mutants were screened individually using the *C. elegans* slow-killing assay for mutants that failed to kill or exhibited attenuated killing (see *Materials and Methods* for details). Eight mutants (*12A1, 35H7, 35A9, 44B1, 41A5, 41C1, 48D9*, and *50E12*), which consistently gave a lower rate of *C. elegans* killing relative to the parental PA14 strain, were chosen for further study. DNA blot analysis showed that each Tn*phoA* mutant contained a single transposon insertion (data not shown). All of the mutants grew at the same rate as the wild-type strain in NG medium and in minimal M9 medium, indicating that the attenuated pathogenicity phenotypes observed were not simply a result of growth defects of the mutants.

To determine whether bacterial virulence genes (factors) mediating slow killing are relevant to pathogenesis in other hosts, the eight mutants obtained by screening in the nematode slow-killing assay were tested for their virulence in an *Arabidopsis* leaf-

infiltration assay and in a mouse full-thickness burn model (8, 9). As summarized in Table 1, six *(12A1, 35H7, 48D9, 50E12, 41C1,* and *41A5)* of the eight mutants were less pathogenic in *Arabidopsis*, and at least five *(12A1, 48D9, 50E12, 35A9*, and *44B1)* were less pathogenic in mice. Mutant *35H7* (which contains a Tn*phoA* insertion in *gacA*) was not tested in the mouse burn model because we showed previously that other PA14 *gacA* mutants exhibited reduced virulence in this model (8, 9). Mutant *41C1* also may be reduced in virulence in the mouse burn model, but a test at a lower-inoculation dose is required to reach a definitive conclusion. These data confirmed our previous conclusion that there is a large amount of overlap between the PA14 virulence factors required for pathogenesis in mice and plants and for slow killing of *C. elegans* (2).

The killing of *C. elegans* by four representative PA14 Tn*phoA* slow-killing mutants and the growth of these same mutants in *Arabidopsis* leaves are shown in Fig. 1. Mutants *35A9* and *44B1* exhibited reduced pathogenicity in nematodes but grew to wild-type levels in *Arabidopsis* (Fig. 1 *A* and *B*). Mutants *48D9* and *50E12* exhibited reduced pathogenicity in both plants and nematodes (Fig. 1 *C* and *D*). All four of these mutants are less pathogenic in mice.

In a separate publication, we described the isolation of six additional Tn*phoA* PA14 mutants (*1G2, 3E8, 6A6, 8C12, 23A2*, and *36A4*) that exhibit reduced killing in the *C. elegans* fast-killing assay (1). Interestingly, although at least four of these mutants also exhibited reduced pathogenicity in the mouse burn model, only one [*36A4,* a *hrpM* homologue (1, 23)] exhibited a phenotype in the *C. elegans* slow-killing assay (data not shown). All eight mutants described in this paper that were isolated in the slow-killing assay killed *C. elegans* as fast as wild type under the high-osmolarity fast-killing conditions (see *Materials and Methods*, data not shown). These observations confirmed the conclusion reported elsewhere that slow and fast killing of *C. elegans* are mechanistically distinct (2).

Genetic Analysis of Tn*phoA* Target Sites and Biochemical and Physiological Characterization of PA14 Tn*phoA* Mutants. To determine which genes were disrupted in the eight PA14::Tn*phoA* slow-killing mutants, IPCR was used to amplify DNA sequences adjacent to the sites of the Tn*phoA* insertions in each of the mutants. DNA fragments corresponding to the IPCR products were identified, sequenced, and analyzed as described in *Materials and Methods*.

The DNA sequence analysis, summarized in Table 1, showed that both novel and known genes were identified. In addition to the eight mutants identified in the nematode screen, two PA14 mutants isolated previously in the plant infection model as less pathogenic, *pho15* and *25F1* (9), were subjected to additional analysis because they are also attenuated in *C. elegans* slow killing. We reported previously that *pho15* contains a Tn*phoA* insertion in a homologue of the *E. coli dsbA* gene (9) and that the insertion in *25F1* did not correspond to a known gene. With further sequence analysis, we confirm the assignment of *pho15* as *dsbA* and report that *25F1* has a Tn*phoA* insertion in an ORF with homology to *orfT* from *Chlorobium tepidum* (see below).

A detailed description of the DNA sequence analysis and biochemical analysis of each of the eight mutants that correspond to previously described genes [*12A1* (*lasR*), *35H7* (*gacA*), *48D9* (*lemA*), *pho15* (*dsbA*), *25F1* (*orfT*), *50E12* (*pstP*), *35A9* (*mtrR*), and *41C1* (*aefA*)] is presented in the following sections.

Mutant 12A1. The Tn*phoA* insertion in *12A1* is inserted into codon 154 of the previously described *lasR* gene of *P. aeruginosa* PA01 (27). The phenotype of *12A1*, as with other known *lasR* mutants, is pleiotropic and includes decreased elastase and protease production (27, 28). In contrast to known *lasR* phenotypes, however, *12A1* produces two to three times more pyocyanin than the parent PA14 strain does at stationary phase. Previously, we showed that PA14 accumulated in the lumen of *C. elegans* during the slow-killing assay in contrast to *E. coli* or a PA14 *gacA* mutant, which failed to accumulate (2). The *lasR* mutant expressing green fluorescent protein from plasmid pRR54GFP19–1 (2) also failed to accumulate in the worm gut (data not shown).

Fig. 2*A* shows that the defective slow-killing phenotype of *12A1* was restored completely to wild-type killing levels when the *P. aeruginosa lasR* gene under the control of the constitutive *lacZ* promoter was expressed *in trans* in strain *12A1* (pKDT17). Elastase production, but not the overproduction of pyocyanin (data not shown), also was restored to wild-type levels in *12A1* (pKDT17), suggesting that *12A1* may harbor a second mutation that results in the up-regulation of pyocyanin production that is not related to the attenuated pathogenicity phenotype.

Mutant 35H7. There is a Tn*phoA* insertion within codon 188 of the previously described GacA protein in *P. aeruginosa* strain PA14 (8) in mutant *35H7*. Mutant *35H7* was severely defective in killing *C. elegans* under slow-killing conditions, similar to two other *gacA* mutants that we had tested previously (2). We also tested a derivative of one of these latter *gacA* mutants, described in ref. 8, in which the mutated *gacA* gene had been replaced with a 2.4-kb DNA fragment containing the wild-type *gacA* gene. The restored wild-type strain killed *C. elegans* as efficiently as PA14 (data not shown).

Mutant 48D9. Tn*phoA* is inserted between codon 491 and 492 of a gene encoding a 925-aa homologue of the *P. syringae* LemA protein, a sensor kinase belonging to a family of bacterial two-component regulators (26). The cognate response regulator of LemA in *P. syringae* is GacA (29), and GacA + LemA have been shown to affect the expression of a variety of virulence factors in animal and plant pathogens (8, 30, 31).

Mutant pho15. Fig. 2*B* shows that the pathogenicity-defective phenotype of *pho15* in *C. elegans* was fully restored by constitutive expression of the *E. coli* or the PA14 *dsbA* gene *in trans*.

Mutant 25F1. In *25F1,* Tn*phoA* is inserted within codon 100 of a putative gene (*orf338*) that encodes a 338-aa protein, the first gene of a putative 3-gene operon. The predicted downstream genes (*orf224* and *orf252*) encode 224- and 252-aa proteins, respectively. *orf338* is 28.5% identical (37.7% similar) to *orfT* of

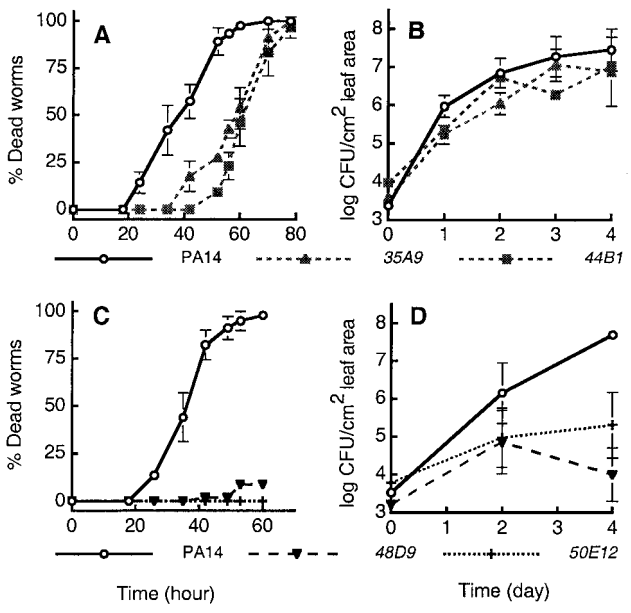

FIG. 1. Pathogenicity phenotypes of representative *P. aeruginosa* PA14 Tn*phoA* mutants on *C. elegans* and *Arabidopsis* relative to the wild-type strain. *C. elegans* mortality rates under slow-killing conditions mediated by *P. aeruginosa* PA14 Tn*phoA* mutants are shown in *A* and *C*, and growth of the same mutants in *Arabidopsis* leaves are shown in *B* and *D*. (*A* and *B*) Mutants *35A9* (▲) and *44B1* (■) exhibited reduced pathogenicity in nematodes but grew the same as the parental strain, PA14 (○) in *Arabidopsis*. (*C* and *D*) Mutants *48D9* (▼) and *50E12* (+) exhibited reduced pathogenicity in both plants and nematodes compared with PA14 (○).

Table 1. Pathogenicity of *P. aeruginosa* PA14 mutants on various hosts

Strain Isolation Number	Pathogenicity phenotypes: *C. elegans* killing LT_{50}, h*	Pathogenicity phenotypes: Growth in *Arabidopsis* leaf†	Pathogenicity phenotypes: % Mouse mortality (n) 5×10^5‡	Gene in which Tn*phoA* was inserted or closest published homologue (organism; GenBank accession no.)
PA14	39.6 ± 2.8	2.9×10^7	100 (>16)	
12A1	>90	1.7×10^6	50 (8)	*lasR* (*P. aeruginosa*; P25084)
35H7[d]	>90	1.2×10^4	NT§	*gacA* (*P. aeruginosa; Q51373)*
48D9	>90	1.0×10^4	50 (8)	*lemA* (*P. syringae*; P48027)
50E12	>90	2.0×10^5	0 (16)	*ptsP* (*A. vinelandii*; Y14681)
35A9	61.2 ± 0.8	7.6×10^6	53 (17)	*mtrR* (*N. gonorrhoeae*; P39897)
44B1	62.7 ± 2.3	1.0×10^7	56 (18)	No matches
41C1	51.1 ± 0.3	2.4×10^5	81 (16)	*aefA* (*E. coli*; P77338)
41A5	46.2 ± 0.4	1.3×10^4	100 (8)	No matches

*A mutant is considered attenuated in nematode pathogenicity if the mean time required to kill 50% of the worms feeding on it (LT_{50} from three to four replicates) is 2 SDs less than the LT_{50} of parental PA14 in the same experiment; for calculations of LT_{50} see ref. 2. A $LT_{50} > 90$ is given to mutant strains that kill less than 30% worms at 90 h.

†Colony-forming unit per cm^2 leaf area of bacterial counts at 4 days postinoculation of 10^3 bacteria; means of four to five samples. Mutants are defined as less pathogenic when the mean value of bacterial counts is 2 SDs lower relative to wild type within the same experimental set.

‡Six-week-old male inbred strain mice (from The Jackson Laboratories), weighing between 20 and 30 g, were injected with 5×10^5 bacterial cells. n, total number of mice tested. The number of animals that died of sepsis was monitored each day for 7 days.

§Another *gacA* mutant *1D7* was isolated independently from a plant screen. Mutant *1D7* has been tested on mice and showed 50% mortality (9).

C. tepidum (GenBank accession no. U58313), the function of which is unknown. *orf224* has significant homology to mannose-1-phosphate guanylyltransferases (MPGs) from eukaryotes, archaeobacteria, cyanobacteria, and mycobacteria, but it is not clear whether *orf224* encodes a functional MPG because all known MPGs consist of 359–388 aa residues, whereas *orf224* encodes a 224-aa polypeptide. *orf252* encodes a homologue of *E. coli* DjlA (ref. 32; GenBank accession no. P31680), which may play a role in the correct assembly, activity, and/or maintenance of a number of membrane proteins, including the two-component histidine kinase signal-transduction systems (33).

As shown in Fig. 2*C*, pORF338 and p3-ORFs, which express *orf338* and all three ORFs (*orf338*, *orf224*, and *orf252*) from their native promoters, respectively, both partially complemented the slow-killing phenotype of *25F1*. These results indicate that the Tn*phoA* insertion in *orf338* likely is responsible for the pathogenicity phenotype but do not rule out the possibility of a polar effect of Tn*phoA* insertion on downstream genes or that the downstream genes *orf224* and *orf252* also may play a role in PA14 virulence.

Mutant 50E12. The Tn*phoA* insertion in *50E12* is inserted within codon 39 of a predicted 759-aa protein that is 86.7% identical (90.4% similar) to the *Azotobacter vinelandii* PtsP protein (25), which is predicted to encode Enzyme I^{Ntr}, a presumptive transcriptional regulator of RpoN-dependent operons (34). The *A. vinelandii* PtsP protein is required for accumulation of poly-β-hydroxybutyrate (25).

As shown in Fig. 2*D*, plasmid p206-lac, which expresses *orf159* and $ptsP_{Pa}$ under the control of the constitutive *lacZ* promoter, and plasmid p206-nat, in which transcription of *orf159* and $ptsP_{Pa}$ is controlled only by their native promoter (see *Materials and Methods*), both partially complemented *50E12*. In both cases, wild-type killing levels were restored, although it took longer for the complemented strains to kill 100% of the worms than PA14. Importantly, partial complementation also was observed in the burned-mouse assay using p206-nat. Mouse mortality was 39% for the complemented strain compared with 100% and 0% mortality when infected by the wild-type strain and 50E12, respectively (data not shown).

Mutant 35A9. The Tn*phoA* in *35A9* is inserted in the first codon of a putative 210-aa protein (encoded by *orf210*) that is most closely related (31.5% identity) to the *N. gonorrhoeae* MtrR protein (24) that belongs to the TetR family of helix–turn–helix-containing bacterial transcriptional regulators (Prosite: PS01081). *orf210* is adjacent to and divergently transcribed from three genes that are homologous to components of the energy-dependent efflux system in *P. aeruginosa*. Analyses of sequences from *P. aeruginosa* PA01 show that together, these four genes define a new energy-dependent efflux system in *P. aeruginosa* (M.-W.T. and F.M.A., unpublished data). The other energy-dependent efflux systems in *P. aeruginosa* described previously are the *mexR, mexA-mexB-oprK* system, the *nfxB, mexC-mexD-oprJ* system, and the *nfxC, mexE-mexF-oprN* system, which function as multidrug efflux pumps (35–37).

Mutant 41C1. Tn*phoA* is inserted in a homologue of the putative *E. coli* integral membrane protein AefA, which is a member of the UPF0003 protein family (Prosite: PS01246). The function of these proteins is not known.

DISCUSSION

By screening 2,400 Tn*phoA* mutants of *P. aeruginosa* strain PA14, we have identified 8 *P. aeruginosa* mutants that exhibit reduced "slow" killing of *C. elegans*. Importantly, six of these mutants are less pathogenic in an *Arabidopsis*–leaf infiltration model, and at least five are less virulent in a mouse full-thickness skin burn model. These data demonstrate that the *C. elegans* slow-killing model can be used to efficiently identify *P. aeruginosa* virulence genes (factors) required for mammalian and plant pathogenesis.

An important feature of the *C. elegans* slow-killing screen is its ability to identify *P. aeruginosa* mutants that are only slightly impaired in their ability to kill *C. elegans*. This high degree of sensitivity is a consequence of the following features of slow killing. First, the longer it takes for hermaphrodite worms to be killed, the more progeny are produced. Second, early larval stages are apparently more resistant to killing by *P. aeruginosa*. Thus, attenuated bacterial mutants that delay killing only by a few hours result in the production of partially resistant progeny by the survivors, which effectively "amplifies" a weak defect into a readily observable phenotype. That is, on plates containing attenuated PA14::Tn*phoA* mutants, two seeded L4-stage hermaphrodites will produce hundreds of progeny worms. Severely debilitated PA14 mutants allow the production of thousands of progeny worms that completely consume the bacterial lawn, whereas no live worms or very few worms are found after seeding onto plates containing wild-type PA14. In contrast to the *C. elegans* screen, it would be extremely difficult to identify mildly

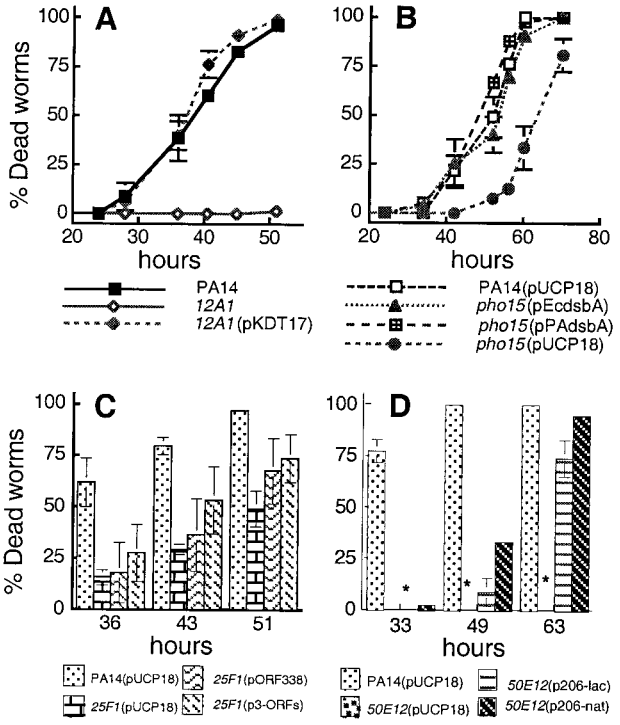

FIG. 2. Complementation analysis of the ability of *P. aeruginosa PA14* mutants to mediate slow killing of *C. elegans*. (*A*) Slow killing of 1-day-old adult *C. elegans* by wild-type PA14, *12A1*, and *12A1* (pKDT17; expressing PAO1 *lasR* under the control of a constitutive *E. coli lacZ* promoter). (*B*) Slow killing of L4 larval-stage *C. elegans* by PA14 (pUCP18; vector control), *pho15* (pUCP18; vector control), *pho15* (pEcdsbA; expressing *E. coli dsbA* under the control of the constitutive *lacZ* promoter), and *pho15* (pPAdsbA; expressing *P. aeruginosa dsbA* under the control of a constitutive *E. coli lacZ* promoter). (*C*) Slow killing of L4 larval-stage *C. elegans* by PA14 (pUCP18; vector control), *25F1* (pUCP18; vector control), *25F1* (pORF338; expressing *orf338* from its native promoter), and *25F1* (p3-ORFs; expressing *orf338-orf224-orf252* from their native promoters). (*D*) Slow killing of L4 larval-stage *C. elegans* by *50E12* (pUCP18; vector control), PA14 (pUCP18; vector control), *50E12* (p206-lac; expressing the putative *orf159-ptsP*$_{Pa}$ operon under the control of a constitutive *E. coli lacZ* promoter), and 50E12 (p206-nat; expressing the putative *orf159-ptsP*$_{Pa}$ operon under the control of its native promoter). *, Zero mortality was observed for worms feeding on *50E12*(pUCP18). For all experiments shown, each data point represents means ± SD of three to four replicates. At least two independent experiments were performed for each analysis.

attenuated *P. aeruginosa* mutants using a vertebrate model because of the large number of animals that would have to be tested to observe a statistically significant reduction in pathogenicity. Interestingly, some of the PA14 mutants that were only moderately attenuated in *C. elegans* killing, such as *35A9* and *44B1*, were markedly less pathogenic in mice.

A variety of biochemical assays revealed that a majority of the PA14 mutants that were obtained in the *C. elegans* slow-killing screen were indistinguishable from the wild-type parent and would not have been identified by screening solely on the basis of a defect in a known pathogenicity-related factor. Consistent with this observation, many of the genes identified by the mutant screen are either completely novel or have not been defined previously as virulence factors in *P. aeruginosa* or other pathogenic bacteria.

The analysis of eight Tn*phoA* mutants identified in this paper, nine Tn*phoA* mutants isolated in a plant screen (9), six Tn*phoA* mutants isolated in a *C. elegans* fast-killing screen (1), and three PA14 mutants that had been constructed by marker exchange in the *toxA, plcS,* and *gacA* genes (8) indicated that the mutants fall into several categories. In creating these categories, we have combined mutants that affect either fast or slow *C. elegans* killing for the sake of simplicity. As summarized in Table 2, 15 class I mutants corresponding to 12 different genes are less pathogenic in all 3 hosts, 2 class II mutants are less pathogenic in nematodes and mice, 5 class III mutants are less pathogenic in plants and mice, 3 class IV mutants are less pathogenic in plants and nematodes, and 1 class V mutant is less pathogenic on nematodes. Remarkably, among 23 different genes that, when mutated, are less pathogenic in nematodes or plants, 19 were identified as playing a significant role in pathogenesis in a mouse burn model. Moreover, some of the mutants that did not show a significant decease in pathogenicity in the mouse burn model, such as *41C1* (see Table 1), might have shown a defect if they had been tested at a lower-inoculation dose. Importantly, mutations in none of these genes affect the growth of PA14 *in vitro* in either minimal or rich medium.

The summary of mutant phenotypes shown in Table 2 highlights the advantages of using several nonvertebrate hosts in screens for mutants involved in mammalian pathogenesis. As exemplified by class II mutants that are less pathogenic in nematodes and mice but not in plants and by class III mutants that are less pathogenic in plants and mice but not in nematodes, a significant proportion of the mutants that exhibited attenuated virulence in the mouse model would have been missed if only one screen in a single nonvertebrate host had been used.

To date, we have screened a total of 2,500 Tn*phoA*-generated mutants of PA14 in a plant pathogenesis assay (9), 2,400 in the *C. elegans* slow-killing pathogenesis assay (this paper), and 3,300 in the *C. elegans* fast-killing pathogenesis assay (1). This represents approximately 25% of the total number that needs to be tested to give a 95% probability of testing each gene in each of the assays (9). Among the genes isolated from the plant and nematode screens, *37H7* and *1D7* are both alleles of *gacA* and *3E8* and *6A6* are both alleles of *phzB*. The high proportion of previously uncharacterized virulence-related genes isolated from these multihost screens and the low number of multiple alleles isolated indicate that many *P. aeruginosa* genes that are functionally important in pathogenicity remained to be identified.

Given the relatively large number of *P. aeruginosa* PA14 mutants involved in this study, it was not feasible to demonstrate for each of the mutants that the Tn*phoA* insertion is the cause of

Table 2. Phenotypes of PA14::Tn*phoA* mutants

Class	Phenotype	Mutant:
I	Less pathogenic in nematodes, plants, and mice	*toxA, gacA, ID7* (*gacA*), *35H7* (*gacA*), *25F1 (orfT), 34H4, pho15* (*dsbA*), *pho34B12, 12A1* (*lasR*), *48D9* (*lemA*), *50E12* (*pstP*), *3E8* (*phzB*), *6A6* (*phzB*) *8C12, 36A4* (*hrpM*)
II	Less pathogenic in nematodes and mice	*35A9* (*mtrR*), *44B1*
III	Less pathogenic in plants and mice	*plcS, 33A9, 33C7, 25A12, 16G12*
IV	Less pathogenic in plants and nematodes;	*41A5, 23A2, 41C1* (*aefA*)
V	Less pathogenic in nematodes only	*IG2*

For most of the mutants, virulence in mice was determined at an inoculum of 5×10^5 cells, a relatively high dose that results in 100% mortality with PA14 wild type. It is conceivable that more of the mutants would be classified as being attenuated in mouse pathogenesis if a lower-inoculation dose had been used.

the pathogenicity-related phenotype(s) and that it is the disruption of the ORF interrupted by Tn*phoA* that causes the mutant phenotype(s) rather than disruption of downstream gene expression due to polar effects of the transposon insertion. Nevertheless, five mutants corresponding to three known virulence factors (*gacA*, *lasR*, and *dsbA)* and two novel virulence factors [*orf338 (25F1)* and *ptsP (50E12)*] were chosen for additional genetic analysis. The results, some of which are presented in Fig. 2, show that at least in the context of the *C. elegans* slow-killing model, the mutant phenotypes associated with insertions in these five genes could be correlated directly with the impaired killing phenotype. In two cases (*dsbA* and *lasR*), the insertions in these genes could be complemented *in trans* by plasmids that expressed only the mutated gene. In the case of *gacA*, a nonpolar insertion in *gacA* was shown to cause the same phenotype as a polar insertion (2) and a reconstructed wild-type *gacA* gene was shown to have the same level of *C. elegans* killing as wild-type PA14 (data not shown). In two cases, an operon containing the mutated gene (the *orf159, ptsP*$_{PA}$ operon corresponding to *50E12* and the *orf338, orf224*, and *orf252* operon corresponding to *25F1*) was able to partially complement the impaired killing phenotype. Moreover, in the case of *50E12*, partial complementation also was obtained in the mouse burn model.

Among known virulence factors, the *C. elegans* screen identified LasR, LemA, and GacA as important for pathogenicity in nematodes, plant, and mice (Table 2, class I). Interestingly, these proteins form a hierarchical cascade of interacting proteins, regulating the transcription and export of virulence factors, including ToxA (another class I factor) (28), via the type II secretion machinery (38, 39). LasR has been identified previously in *P. aeruginosa* strain and, together with LasI, is one of two quorum-sensing systems found in *P. aeruginosa*. The *lasR-lasI* system is a global regulator of many virulence-associated genes (40). GacA, together with its cognate sensor, LemA, are members of a two-component signaling pathway. GacA functions upstream of the *lasR-lasI* modulon in a complex, cell-density-dependent signal-transduction pathway regulating several exoproducts and virulence factors (41). Thus, we show that the *C. elegans* screen is efficient in identifying several interacting virulence-associated systems that appear to be universal among Gram-negative animal and plant bacterial pathogens. In addition, we show that *gacA* and *lasR* mutants fail to accumulate in the gut of *C. elegans*, suggesting that the establishment and/or proliferation of bacteria within the host also may be dependent on this regulatory cascade. Further screening using plants and nematodes as host likely will identify other components of this complicated virulence-related pathway as well as the effector molecules that are under the regulation of these genes.

From an evolutionary perspective, the requirement for functional *lasR*, *lemA*, and *gacA* gene products as pathogenicity factors in plants, nematodes, and mice suggests that quorum sensing and regulated export of proteins are general features of pathogenesis in all hosts. The *lasR, lemA*, and *gacA* genes are present in many plant and animal bacterial pathogens as well as in saprophytes. We speculate it is likely that LasR, LemA, and GacA initially served as master regulators, enabling ancestral prokaryotic organisms to adapt to their environment. Later, with the appearance of eukaryotes, LasR, LemA, and GacA evolved to regulate a variety of genes that allowed prokaryotes to invade and establish residency inside eukaryotes.

In conclusion, the work presented here in combination with two other recent publications from our laboratory (1, 2) show that the *C. elegans–P. aeruginosa* pathogenesis model that we have developed has a variety of useful features currently not found in any other pathogenesis models, including those utilizing *A. thaliana* (42) and *D. melanogaster* (43) as model genetic hosts, in which genetic dissection is limited largely to the host because genome sequencing of their pathogens have not been initiated. These features include the ability to carry out high-throughput genetic identification of both pathogen and host genes involved in the pathogenic interaction between a pathogen and a host for which complete genome sequences are now available.

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