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Use of model plant hosts to identify *Pseudomonas aeruginosa* virulence factors

(*gacA*/pyocyanin/virulence model)

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ABSTRACT **We used plants as an *in vivo* pathogenesis model for the identification of virulence factors of the human opportunistic pathogen *Pseudomonas aeruginosa*. Nine of nine Tn*phoA* mutant derivatives of *P. aeruginosa* strain UCBPP-PA14 that were identified in a plant leaf assay for less pathogenic mutants also exhibited significantly reduced pathogenicity in a burned mouse pathogenicity model, suggesting that *P. aeruginosa* utilizes common strategies to infect both hosts. Seven of these nine mutants contain Tn*phoA* insertions in previously unknown genes. These results demonstrate that an alternative nonvertebrate host of a human bacterial pathogen can be used in an *in vivo* high throughput screen to identify novel bacterial virulence factors involved in mammalian pathogenesis.**

Pseudomonas aeruginosa is a ubiquitous Gram-negative bacterium isolated from soil, water, and plants (1), and is an opportunistic human pathogen that infects patients who are immunodeficient or otherwise compromised. A variety of *P. aeruginosa* virulence factors have been described, and the majority of these, such as exotoxin A, elastase, and phospholipase C, were first detected biochemically on the basis of their cytotoxic activity (2). Subsequently, the genes corresponding to these factors or genes that regulate the expression of these factors were identified. In general, most pathogenicity-related genes in mammalian bacterial pathogens were first detected by using a biochemical assay. In contrast to mammalian pathogens, simple systematic genetic strategies have been routinely employed to identify pathogenicity-related genes in plant pathogens. Following random transposon-mediated mutagenesis, thousands of mutant clones of the phytopathogen are inoculated separately into individual plants to determine if they contain a mutation that affects the pathogenic interaction with the host (3–8). Comparable experiments with whole-animal mammalian pathogenicity models are not feasible because of the vast number of animals that must be subjected to pathogenic attack.

Reports indicating similarities between plant and animal pathogens (9, 10) prompted us to search for a strain of *P. aeruginosa* that was capable of eliciting disease in both a well-defined plant pathogenesis model and a well-defined animal pathogenesis model. Recently, we described (11) a clinical isolate of *P. aeruginosa*, UCBPP-PA14, that is infectious in both an *Arabidopsis thaliana* leaf infiltration model and in a mouse full-thickness skin thermal burn model. We showed that mutations in three *P. aeruginosa* pathogenicity-related genes, *toxA, plcS,* and *gacA*, caused significant decrease in pathogenicity in both models (11). The utilization of common virulence-related genes by *P. aeruginosa* for infecting animals and plants led us to hypothesize that previously unknown virulence determinants required for *P. aeruginosa* pathogenesis in animals could be identified by screening randomly mutagenized UCBPP-PA14 clones for ones that exhibited decreased virulence in plants. A subset of these mutants would be expected to correspond to genes that encode common virulence determinants for plant and animal pathogenesis. Such a systematic genetic approach should not only lead to a better understanding of the mechanisms of *Pseudomonas* infections, but should also lead to important information concerning the type of virulence strategies that are functionally conserved during evolution.

MATERIALS AND METHODS

Strains, Growth Conditions, and Plasmids. *P. aeruginosa* strain UCBPP-PA14 is a human clinical isolate used in this study for the identification of novel virulence-related genes (11), and *P. aeruginosa* strains PAK (12) and PAO1 (13) have been studied extensively in many laboratories. Luria–Bertani (LB) broth and agar were used for the growth of *P. aeruginosa* and *Escherichia coli* strains at 37°C. Minimal medium (M9) was also used for the growth of *P. aeruginosa*.

Transposon Mutagenesis. Transposon-mediated mutagenesis of UCBPP-PA14 was performed by using Tn*phoA* carried on the suicide plasmid pRT731 in *E. coli* strain SM10 λpir (14). Donor and recipient cells were plated together on LB agar plates and incubated at 37°C for 8–10 h and subsequently plated on LB plates containing rifampicin (100 μg/ml) (to select against the *E. coli* donor cells) and kanamycin (200 μg/ml) (to select for Tn*phoA* containing *P. aeruginosa* cells). Colonies that grew on the rifampicin and kanamycin media were replicated to LB containing ampicillin (300 μg/ml); ampicillin-resistant colonies indicated pRT731 integration into the UCBPP-PA14 genome and were discarded.

Alkaline Phosphatase Activity. A total of 2,500 prototrophic UCBPP-PA14 Tn*phoA* mutants were screened on peptone glucose agar plates (15) containing 40 μg/ml 5-bromo-4-chloro-3-indoly phosphate (XP). Peptone medium was selected because it suppresses the production of the endogenous blue-green pigment pyocyanin and the fluorescent yellow pyoverdin, permitting visualization of the blue color that results from dephosphorylation of XP by periplasmic alkaline phosphatase generated by $PhoA^+$ mutants.

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Abbreviations: LB, Luria–Bertani; IPCR, inverse PCR.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AF031571).

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Growth Conditions and Mutant Isolation Strategy. *P. aeruginosa* strains that were grown to saturation in LB broth at 37°C were washed in 10 mM $MgSO_4$, resuspended at an optical density of 0.2 (OD_{600} = 0.2) in 10 mM $MgSO_4$, and diluted 1:100 and 1:1,000 (corresponding to a bacterial density of $\approx 10^6$ and 10^5 cfu/ml, respectively). Approximately 10 μl of the diluted cells were inoculated with a Pipetman into stems of an $\approx$12-week-old lettuce plants (variety Romaine or Great lake) grown in MetroMix potting soil in a greenhouse (26°C). The stems were washed with 0.1% bleach and placed on 15 cm diameter Petri dishes containing one Whatman filter (Whatman #1) that was impregnated with 10 mM $MgSO_4$. The midrib of each lettuce leaf was inoculated with three different Tn*phoA*-generated *P. aeruginosa* mutants to be tested and the wild-type UCBPP-PA14 strain as a control. The plates were kept in a growth chamber during the course of the experiment at 28–30°C and 90–100% relative humidity. Symptoms were monitored daily for 5 days.

In the *Arabidopsis* leaf infiltration model, *P. aeruginosa* strains grown and washed as above were diluted 1:100 in 10 mM $MgSO_4$ (corresponding to a bacterial density of $10^3/cm^2$ leaf disk area) and were injected into leaves of 6-week-old *Arabidopsis* plants ecotype Llagostera as described for infiltration of *Pseudomonas syringae* (11, 16). Incubation conditions and monitoring of symptoms were the same as in the lettuce experiments. Leaf intercellular fluid containing bacteria was harvested and bacterial counts were determined as described (11, 16). Four different samples were taken by using two leaf discs per sample. Control plants inoculated with 10 mM $MgSO_4$ showed no symptom development.

Mice Mortality Studies. A 5% total surface area burn was fashioned on the outstreached abdominal skin of 6-week-old male AKR/J mice (The Jackson Laboratories) weighing between 25 and 30 g as described (11, 17). Immediately following the burn, mice were injected with 5×10^3 or 5×10^5 *P. aeruginosa* cells, and the number of animals that died of sepsis was monitored each day for 10 days. Animal study protocols were reviewed and approved by the subcommittee on Animal Studies of the Massachusetts General Hospital. Statistical significance for mortality data was determined by using a χ^2 test with Yates' correction or Fisher's exact test. Differences between groups were considered statistically significant at $P \leq 0.05$.

DNA Manipulation, Molecular Cloning, and Sequence Analysis of Tn*phoA* Mutants. *P. aeruginosa* chromosomal DNA was isolated by phenol extraction (18), and DNA blotting and hybridization studies were performed as described (19). The oligonucleotides 5′-AATATCGCCCTGAGCAGC-3′ (LGR1), and 5′-AATACACTCACTATGCGCTG-3′ (LGR2) correspond to sequences on opposite strands at the 5′ end of Tn*phoA*. The oligonucleotides 5′-CCATCTCATCA-GAGGGTA-3′ (LGR3) and 5′-CGTTACCATGTTAGGAG-GTC-3′ (LGR4) correspond to sequences on opposite strands at the of the 3′ end of Tn*phoA*. LGR1 + LGR2 or LGR3 + LGR4 were used to amplify by inverse PCR (IPCR) DNA sequences adjacent to the sites of Tn*phoA* insertion as described (20). Amplified DNA fragments ranging in size from 350 to 650 bp were cloned into pBlueScript SK+/− by filling in the ends of the IPCR products prior to subcloning them into the *Eco*RV site of pBlueScript SK+/−. To determine the sequence of IPCR-amplified products, double-stranded DNA sequencing was performed with the Sequenase 2.0 kit (United States Biochemical). Sequences obtained were compared with the nonredundant peptide sequence databases at the National Center for Biotechnology Information by using the BLASTX program (21).

Isolation and Manipulation of a the Wild-Type Gene Corresponding to the *pho34B12* Mutation. The IPCR product that was generated from UCBPP-PA14 Tn*phoA* mutant *pho34B12* mutant was labeled with a random primed DNA labeling kit (Boehringer Mannheim) and used to probe a genomic library of UCBPP-PA14 chromosomal DNA in pJSR1 (11) for a clone containing the gene corresponding to the *pho34B12* mutation. A 3.7-kb *Eco*RI fragment, identified in cosmid clone pLGR34B12 which corresponded to the *pho34B12* mutation, was subcloned into the *Eco*RI site of pRR54 (22) after filling-in the ends of both vector and fragment to construct pLGRE34B12. The same fragment (made blunt ended) was subcloned into the *Sma*I site of pCVD (23) to construct pLGR34. pLGR34 was used to replace the mutated *pho34B12* gene with a wild-type copy as described (23). The 3.7-kb *Eco*RI fragment was also subcloned into the *Eco*RI site of pBlue-Script SK+/− to construct pBSR34B12 and used for DNA sequence analysis.

A 1,659-bp sequence corresponding to the *pho34B12* insertion that contains two overlapping ORFs (ORF1 and ORF2) on opposing strands was submitted to GenBank and was assigned accession number AF031571. ORF1 is 1,148 bp (nucleotides 361–1,509) and ORF2 is 1,022 bp (nucleotides 1,458–436). The overlap of the two ORFs is from nucleotides 436–1,458. ORF1 contains a second putative translational start site at nucleotide 751 corresponding to a coding region of 758 bp. The oligonucleotide primers 5′-CGCATCGTCGAA-ACGCTGGCGGCC-3′ and 5′-GCCGATGGCGAGATCAT-GGCGATG-3′ were used to amplify a 1,100-bp fragment from pBSR34B12 containing ORF1. Because of the two putative initiation sites present in ORF1, the oligonucleotide primers 5′-TGCGCAACGATACGCCGTTGCCGACGATC-3′ and 5′-GATTCCACCTTCGCAGCGCAGCCC-3′ (Reg3) were also used to amplify a 1,659 bp from pBSR34B12 containing ORF1. The oligonucleotide primers 5′-GATTCCACCTTCG-CAGCGCAGCCC-3′ and 5′-GCCGATGGCGAGATCATG-GCGATG-3′ were used to amplify a 1,302-bp fragment from pBSR34B12 containing ORF2. All primer combinations were designed to contain the putative upstream regulatory elements of each ORF. The PCR products obtained (1,100, 1,659, and 1,302 bp) were cloned into pCR2.1 (Invitrogen) to construct pLE15, pLE1, and pLE2, respectively. All three PCR products were subcloned into pRR54 to construct pRRLE15, pRRLE1, and pRRLE2, respectively.

Enzymatic Activities of Tn*phoA* Mutants. *P. aeruginosa* strains grown for 18 h in LB were used for assays of enzymatic activities. Proteolytic and elastolytic activities were determined as described (24). Quantitation of pyocyanin was determined as described (25). Hemolytic activity was detected following incubation on plates containing Trypticase soy agar (BBL) supplemented with 5% sheep red blood cells (26).

Generation of a Nonpolar *gacA* Mutation. A nonpolar *gacA* mutation in UCBPP-PA14 was constructed by cloning a 3.5-kb *Pst*I fragment containing the *gacA* gene from cosmid pLGR43 (11) into the unique *Bam*HI restriction site in the suicide vector pEGBR (27) by using *Bam*HI linkers. A 950-bp *Eco*RI–*Hin*cII Klenow end-filled fragment containing the kanamycin resistance gene cassette from pUC18K (28) was then cloned into the unique *Bam*HI restriction site (made blunt ended) in *gacA*, such that transcription is maintained and translation of the downstream portion of *gacA* is reinitiated at the 3′ end of the kanamycin cassette. The resultant construct, SW 7-4, containing the kanamycin gene cassette within the *gacA* gene and in the orientation of its transcription, was used to marker-exchange by homologous recombination the disrupted *gacA* gene into the wild-type UCBPP-PA14 genome.

RESULTS

By using the procedures described in *Materials and Methods*, we mutagenized the *P. aeruginosa* UCBPP-PA14 genome with the transposon Tn*phoA* and screened 2,500 prototrophic mutants for impaired pathogenicity in a lettuce stem assay. In our previous publication on UCBPP-PA14, the plant leaf infiltra-

tion model for UCBPP-PA14 utilized *Arabidopsis* (11), but we substituted lettuce in these current studies because several mutants could be tested on a single lettuce stem. Interestingly, we found that lettuce was not only susceptible to infection by UCBPP-PA14 but also was susceptible to the well-characterized *P. aeruginosa* strains PAK (12) and PAO1 (13). Both of these latter strains proliferated in lettuce leaves and elicited disease symptoms similar to those elecited by UCBPP-PA14, characterized by water soaking followed by soft rot 4–5 days postinfection (data not shown). In later stages of infection, all three *P. aeruginosa* strains invaded the entire midrib of a lettuce leaf resulting in complete maceration and collapse of the tissue.

As summarized in Table 1, we identified nine Tn*phoA*-generated mutants of UCBPP-PA14 among the 2,500 prototrophs screened that elicited null, weak, or moderate rotting symptoms on lettuce stems compared with the wild-type strain. Severe maceration of the leaf was not observed with any of the mutants. DNA blot analysis showed that each of the nine mutants contains a single Tn*phoA* insertion, using as a probe a 1,542-bp *Bgl*I–*Bam*HI fragment containing the kanamycin resistance conferring gene of Tn*phoA* (14). Two of the nine UCBPP-PA14 Tn*phoA* mutants, *pho34B12* and *pho15,* expressed alkaline phosphatase activity suggesting that the genes containing these Tn*phoA* insertions encode membrane-spanning or secreted proteins (14, 29).

The nine Tn*phoA* mutants were further tested by measuring their growth rate over the course of 4 days in *Arabidopsis* leaves as a quantitative measure of pathogenicity (11, 16). Although none of the mutants showed any significant differences in their growth rates as compared with the wild-type strain in either rich or minimal medium, the growth rate over time of all nine mutants in *Arabidopsis* leaves was significantly lower than the wild type strain. Table 1 lists the maximal levels of growth reached by each mutant at the fourth day postinfection. In the case of all nine mutants, less severe symptom development reflected reduced bacterial counts in leaves. All of the mutants

Table 1. *P. aeruginosa* mutants obtained by Tn*phoA* mutagenesis

Strain	Growth in *Arabidopsis* leaves*	Symptoms elicited in *Arabidopsis*†	% mouse mortality‡		Gene identity
			5×10^3	5×10^5	
PA14	5.5×10^7	Severe	53	100	
33C7	8.3×10^4	None	0	0	Unknown§
1D7	7.5×10^5	Weak	0	50	*gacA*
25A12	1.7×10^6	Weak	11	87	Unknown
33A9	5.1×10^6	Moderate	0	0	Unknown
25F1	1.5×10^4	Moderate	0	20	Unknown
34H4	3.8×10^6	Moderate	0	33	Unknown
pho34B12	4.0×10^6	Moderate	0	56	Unknown
pho15	3.9×10^4	Moderate	0	62	*dsbA*
16G12	2.3×10^5	Moderate	20	100	Unknown

*Four different samples were taken using two leaf discs/sample. Control plants inoculated with 10 mM $MgSO_4$ showed no symptoms during the course of the experiments. Three independent experiments gave similar results.

†Symptoms observed 4–5 days after infection. None, no symptoms; chlorosis, chlorosis circumscribing the inoculation site; weak, localized water-soaking and chlorosis of tissue circumscribing the inoculation site; moderate, moderate water-soaking and chlorosis with most of the tissue softened around the inoculation site; severe, severe soft-rotting of the entire leaf characterized by a water-soaked reaction zone and chlorosis around the inoculation site at 2–3 days postinfection.

‡All animal experiments were conducted at least twice using 8–10 animals/experiment. Independent experiments showed similar percentage mortality rates. Mice were injected with $\approx 5 \times 10^3$ or 5×10^5 cells.

§BLASTX analysis yielded no encoded proteins with significant homology.

except *33C7* elicited either weak or moderate rot and water soaking symptoms with varying amounts of chlorosis (yellowing) (Table 1). Interestingly, however, as summarized in Table 1, the levels of proliferation of the individual mutants did not directly correlate with the severity of symptoms that they elicited. For example, even though mutant *25A12* grew to similar levels as mutants *33A9*, *pho34B12*, and *34H4*, only 10-fold less than wild-type UCBPP-PA14, mutant *25A12* elicited very weak symptoms. Similarly, mutants *33C7*, *pho15*, and *25F1* all reached similar maximal levels of growth ($\approx 10^3$-fold less than the growth of the wild type); however, only mutant *33C7* failed to cause any disease symptoms (Table 1). The differences observed in the degree of symptoms and proliferation levels among the nine mutants suggest that these mutants may carry insertions in genes that are involved in various stages of the plant infectious process.

The pathogenicity of each of the nine Tn*phoA*-generated mutants that were less pathogenic in the plant leaf assay was measured in a full-thickness skin thermal burn mouse model (11, 17). As shown in Table 1, all nine mutants were significantly different from the wild-type with a $P \leq 0.05$ at both doses except for *25A12* and *16G12*, which were not significantly different from wild type at the higher dose of 5×10^5 cells. In addition, mutant *33A9* also failed to cause mortality even at a higher dose of 5×10^6.

We used DNA blot analysis and DNA sequence analysis to determine whether Tn*phoA* in the nine less pathogenic mutants had inserted into known genes. DNA blot analysis revealed that mutant *1D7* contains a Tn*phoA* insertion in the *gacA* gene (30, 31), which we had shown previously to be an important pathogenicity factor for *P. aeruginosa* in both plants and animals (11). For the other eight mutants we used the IPCR to generate amplified products corresponding to DNA sequences adjacent to the sites of the Tn*phoA* insertions (20). The IPCR products were cloned and then subjected to DNA sequence analysis. Mutant *pho15* contains Tn*phoA* inserted into a *P. aeruginosa* gene (from strain PA01) previously deposited in GenBank (accession no. U84726) that shows a high degree similarity to the *Azotobacter vinelandii dsbA* gene, which encodes a periplasmic disulfide bond forming enzyme (32). Homologues of *dsbA* in the bacterial phytopathogen *Erwinia chrysanthemi* and in the human pathogens *Shigella flexneri* and *Vibrio cholera* are required for pathogenesis (33–35). Computer analysis with the program BLASTX showed that when the DNA sequences corresponding to the remaining seven Tn*phoA* insertions were translated in all possible reading frames, no significant similarities to any known genes were found (Table 1).

We performed a variety of biochemical tests to categorize the nine less pathogenic UCBPP-PA14 mutants on the basis of whether they exhibited defects in previously described primary virulence factors and/or metabolic pathways. All mutants were assayed for protease, elastase and phospholipase activities and for their ability to secrete the secondary metabolite pyocyanin (24–26). Pyocyanin is a redox-active phenazine compound excreted by most clinical strains of *P. aeruginosa* that kills mammalian and bacterial cells through the generation of reactive oxygen intermediates and which has been implicated as a *P. aeruginosa* virulence factor (36–38). Mutants *33C7*, *33A9*, *34H4*, *25F1,* and *16G12* showed no defects in any of the biochemical assays used. Mutant *pho34B12* showed decreased hemolytic activity on blood agar plates, reduced elastase activity (≈50%), and no detectable pyocyanin production. Mutant *pho15* showed only traces of elastase activity and a decrease in proteolytic activity (60–70%) compared with the wild type. Mutant *25A12* showed a 50% decreased elastolytic activity. Finally, mutant *1D7*, which contains an insertion in *gacA*, showed reduced levels of pyocyanin (≈50%) as compared with the wild type. In addition to mutant *1D7* a second independent *gacA*::Tn*phoA* mutant was identified from

the plant screen, mutant *33D11*. This latter mutant also exhibited a similar reduction in pyocyanin production and reduced virulence in both plants and mice.

On the basis of the DNA sequence analysis and biochemical testing of the mutants, the genes targeted by the Tn*phoA* insertions in mutants *1D7* and *pho34B12* were chosen for further analysis. As discussed above, *1D7* contains an insertion in *gacA* that we had shown previously to encode a virulence factor in *P. aeruginosa* (11). Recently a *gacA*-like gene has also been shown to be an important virulence factor for *Salmonella typhimurium* (39). However, the two *gacA*::Tn*phoA* insertions *(1D7* and *33D11*) isolated in this study, the *gacA* insertion mutant that we constructed previously (11) and an independently constructed *P. aeruginosa gacA* mutation that affects the production of several known virulence factors (36), all exert a polar effect on at least one gene, a homologue of the *E. coli uvrC* gene immediately downstream of *gacA* (11, 30, 40). To provide definitive evidence that the loss of pathogenicity phenotypes of the *gacA* mutants constructed in our laboratory was caused by the disruption of the *gacA* ORF *per se* rather than caused by a polar effect on a gene downstream of *gacA*, we constructed, as described in *Materials and Methods*, a nonpolar *gacA* mutation in UCBPP-PA14 with a DNA cassette encoding a gene that confers kanamycin resistance. Importantly, the nonpolar *gacA* mutant exhibited the same diminished level of pathogenicity in the mouse assay (50% mortality) and in the *Arabidopsis* assay (growth to 3×10^5 cfu/cm^2 after 4 days) as the *gacA*::Tn*phoA* mutant *(1D7),* but did not exhibit the UV sensitivity of the polar *gacA* mutants. Like *1D7*, the nonpolar *gacA* mutant also excreted lower levels of pyocyanin (50%) compared with the wild type.

Mutant *pho34B12* was chosen for further analysis for the following reasons. First, the insertion in *pho34B12* is situated directly downstream of the *P. aeruginosa* pyocyanin biosynthetic genes *phnA* and *phnB* (25) in a previously uncharacterized region of the *P. aeruginosa* genome. Second, the *pho34B12* insertion causes a pleiotropic phenotype that includes reduced elastase and hemolytic activities, suggesting that the gene in which the *pho34B12* Tn*phoA* insertion is situated may encode a regulator of diverse pathogenicity factors.

To rule out the possibility that a secondary mutation in *pho34B12* was responsible for the loss of pathogenicity phenotype rather than the Tn*phoA* insertion, we replaced the *pho34B12*::Tn*phoA* mutation by homologous recombination with the corresponding wild-type gene. This replacement resulted in restoration of the pathogenicity defect in both plants and animals as well as restoration of hemolytic and elastolytic activity and pyocyanin production to wild-type levels (Table 2). These results show that the Tn*phoA* insertion in *pho34B12* is the cause of the pleiotropic phenotype of this strain, including the loss of pathogenicity phenotype. The fact that no putative ORFs are in the next 500 bp downstream of the stop codon following the *pho34B12*::Tn*phoA* insertion (see below) makes it unlikely that Tn*phoA* exerts a polar effect on a downstream gene that is responsible for the phenotype of mutant *pho34B12*. Genetic complementation analysis of *pho34B12* with a plasmid (pLGRE34B12) containing a 3.7-kb insert that includes *pho34B12* and part of the *phnAB* region resulted in restoration of the elastase and hemolytic activities to wild-type levels and more than 10-fold overproduction of pyocyanin (Table 2). However, the impaired pathogenicity phenotype of *pho34B12* in both *Arabidopsis* and mice was not complemented by pLGRE34B12 (Table 2), most likely because of the presence of multiple copies of the wild-type gene corresponding to *pho34B12*.

Table 2. Complementation analysis of *pho34B12*

Strain	Growth in *Arabidopsis* leaves	Symptoms elicited in *Arabidopsis*	% mouse mortality, 5×10^5	% pyocyanin
PA14	5.5×10^7	Severe	100	100
pho34B12	4.0×10^6	Moderate	56	≤1
pho34B12 Reconstructed to wild type	3.9×10^7	Severe	100	120
pho34B12 +*pLGRE34B12*	6.1×10^5	Moderate	0	600
pho34B12 +pRRLE2	7.0×10^5	Moderate	13	40
pho34B12 +pRRLE1	5.0×10^5	Moderate	13	1,400
pho34B12 +pRRLE15	1.0×10^5	Moderate	22	1,360

Further DNA sequence analysis showed that the region containing the *pho34B12* mutation encodes two almost completely overlapping ORFs (ORF1 and ORF2) that are transcribed in opposite directions. Moreover, ORF1 has two potential methionine start codons (designated OFR1-S and ORF1-L). The predicted proteins encoded by ORF1-S and ORF1-L, which are transcribed in the same direction as the *phnA*, *phnB*, and *phoA* genes, contain a consensus motif that corresponds to a lipid attachment site found in a variety of prokaryotic membrane lipoproteins (41). These membrane lipoproteins are synthesized with a precursor signal peptide, providing an explanation for the Pho$^+$ phenotype of the *pho34B12* insertion (41). The predicted protein encoded by ORF2 contains an N-terminal helix-turn-helix DNA-binding motif similar to the helix-turn-helix motif found in the *LysR* family of transcriptional regulators (42, 43). This class of proteins includes regulators involved in both mammalian and plant pathogenesis (44). The existence of two functional almost completely overlapping ORFs is unusual in bacterial genomes.

To determine which of the ORFs encoded in the *pho34B12* region are functional, additional complementation analysis was carried out by using plasmids that contained PCR products corresponding to ORF1-S, ORF1-L, and ORF2. The production of both pyocyanin and elastolytic activity was restored to 20–40% of wild-type levels by the plasmid synthesizing the protein encoded by ORF2 (pRRLE2). Similarly, the hemolytic ability of this complemented strain was partially restored. Complementation of *pho34B12* with plasmids pRRLE1 and PRRLE15, corresponding to ORF1-S and ORF1-L, respectively, also restored the hemolytic, pyocyanin, and elastolytic activities. Interestingly, however, the presence of plasmids pRRLE1 and PRRLE15 resulted in a 10-fold higher production of pyocyanin and a 2-fold higher level of elastase activity. Neither pRRLE1, pRRLE15 or pRRLE2 complemented the loss of pathogenicity phenotypes of mutant *pho34B12* in either plants or animals (Table 2). Further characterization of this region including site-directed mutagenesis is necessary to determine which of the three ORFs is (are) required for pathogenicity in plants and animals.

DISCUSSION

The data presented in this paper clearly demonstrate that previously unknown *P. aeruginosa* virulence factors (genes) that play a significant role in mammalian pathogenesis can be readily identified by screening random *P. aeruginosa* mutants for ones that display attenuated pathogenic symptoms in plants. This result is consistent with our previous study in which we demonstrated that at least three *P. aeruginosa* genes encode virulence factors involved in both plant and animal pathogenesis (11). On the other hand, we did not expect to find that all nine of the mutants that were isolated because of decreased virulence in plants would also be less virulent in mice. The simplest interpretation of this result is that *P.*

aeruginosa pathogenesis in plants and animals is the result of a substantially overlapping set of genes that may be considered to be basic virulence genes. Another possible interpretation is that some of the identified genes may encode regulatory proteins (i.e., *pho34B12*), that control different effector molecules, a subset of which may be specific for either plants or animals. We also did not expect that the majority of mutants that would be identified in this study (seven of nine) would corresponded to previously unknown genes. By using the Poisson distribution, a genome size for *P. aeruginosa* of 5.9 Mb and an average gene size of 1.1 kb, we calculated that the 2,500 mutants tested represents 25% of the total number that needs to be tested to give ≈95% probability of testing each gene in the assay. Therefore, because our screen for *P. aeruginosa* virulence mutants is not nearly saturated, it is likely that many additional *P. aeruginosa* genes with important roles in pathogenicity await discovery.

Importantly, at least two of the previously known virulence-related factors (genes) identified in our model as being important in plant pathogenesis are not only important virulence factors for *P. aeruginosa* in a mouse burn model, but have also been described as important virulence factors in other Gram-negative pathogens. These latter pathogenicity factors (genes) include *dsbA,* and *gacA* (33–35, 39). This finding makes it likely that many of the previously unknown factors identified in *P. aeruginosa* will be generally relevant for Gram-negative pathogenesis.

Another important conclusion from this study is that the high throughput *in vivo* screening method that we have developed can lead to the identification of pathogenicity factors that do not correlate with obvious biochemical defects. Mutants *33C7*, *33A9*, *34H4, 25F1*, and *16G12* exhibited no detectable defects in several known *P. aeruginosa* pathogenicity factors and, two of these mutants *33C7* and *33A9* were among the most debilitated in the mouse model. Moreover, even though mutants *pho34B12* and *25A12* did exhibit diminished production of known virulence factors, the genes corresponding to these mutants have not been identified previously, most likely because the biochemical defects in these mutants cannot be readily identified in a simple high throughput screen. This finding attests to the sensitivity of our screen for loss of pathogenicity phenotypes.

In the last few years, other high throughput screens for identifying bacterial pathogenicity factors have been described. The IVET (*in vivo* expression technology) identifies promoters that are specifically activated during pathogenesis (45, 46), STM (signature-tagged transposon method) identifies genes that are required for survival in a host (47), and DFI (differential fluorescence induction) utilizes green fluorescent protein and fluorescence-activated cell sorting to identify genes that are activated under specific conditions or in specific host cell types (48). These approaches are complimentary with the one that we have described in this paper; each approach has advantages and disadvantages. One advantage of our screening procedure in a nonvertebrate host is that it directly measures pathogenicity whereas the IVET and DFI methods measure pathogenicity-associated gene expression. One disadvantage of the STM procedure is that it only identifies genes whose function cannot be complemented in trans by the mixed population of bacterial mutants used for the inoculum, whereas our screen in a nonvertebrate involves testing each mutant clone separately.

Our laboratory is currently expanding the *P. aeruginosa* multi-host system to include additional nonvertebrate hosts including the nematode *Caenorhabditis elegans* (M.-W.T., S. Mahajan, and F.M.A., unpublished data) and insects, *Drosophila melanogaster* (S. Mahajan and F.M.A., unpublished data) and *Galleria mellonella* (G. Jander and F.M.A., unpublished data). *P. aeruginosa* is an affective pathogen of each of these organisms. It also seems likely that the multi-host pathogenesis model can be extended to other pathogens as well (49).

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1. Palleroni, J. N. (1984) in *Bergey's Manual of Systematic Bacteriology*, ed. Bergey, J. G. Holt ed. (Williams & Wilkins, Baltimore, MD), pp. 141–172.
2. Fink, R. B., Jr. (1993) *Pseudomonas aeruginosa the Opportunist: Pathogenesis and Disease* (CRC, Boca Raton, FL).
3. Boucher, C., Barberis, P., Trigalet, A. & Demery, D. (1985) *J. Gen. Microbiol.* **131,** 2449–2457.
4. Boucher, C. A., Van Gijsegem, F., Barberis, P. A., Arlat, M. & Zischek, C. (1987) *J. Bacteriol.* **169,** 5626–5632.
5. Comai, L. & Kosuge, T. (1982) *J. Bacteriol.* **149,** 40–46.
6. Lindgren, P. B., Peet, R. C. & Panopoulos, N. J. (1986) *J. Bacteriol.* **168,** 512–522.
7. Rahme, L. G., Mindrinos, M. N. & Panopoulos, N. J. (1991) *J. Bacteriol.* **173,** 575–586.
8. Willis, D. K., Hrabak, E. M., Rich, J. J., Barta, T. M., Lindow, S. E. & Panopoulos, N. J. (1990) *Mol. Plant-Microbe Interact.* **3,** 149–156.
9. Cho, J. J., Schroth, M. N., Kominos, S. D. & Green, S. K. (1975) *Phytopathology* **65,** 425–431.
10. Elrod, R. P. & Braun, A. C. (1942) *J. Bacteriol.* **44,** 633–645.
11. Rahme, L. G., Stevens, E. J., Wolfort, S. F., Shao, J., Tompkins, R. G. & Ausubel, F. M. (1995) *Science* **268,** 1899–1902.
12. Ishimoto, K. S. & Lory, S. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 1954–1957.
13. Holloway, B. W., Krishnapillai, V. & Morgan, A. F. (1979) *Microbiol. Rev.* **43,** 73–102.
14. Taylor, R. K., Manoil, C. & Mekalanos, J. J. (1989) *J. Bacteriol.* **171,** 1870–1878.
15. Ostroff, R. M., Vasil, A. I. & Vasil, M. L. (1990) *J. Bacteriol.* **172,** 5915–5923.
16. Dong, X., Mindrinos, M., Davis, K. R. & Ausubel, F. M. (1991) *Plant Cell* **3,** 61–72.
17. Stevens, E. J., Ryan, C. M., Friedberg, J. S., Barnhill, R. L., Yarmush, M. L. & Tompkins, R. G. (1994) *J. Burn Care Rehabil.* **15,** 232–235.
18. Strom, M. S. & Lory, S. (1986) *J. Bacteriol.* **165,** 367–372.
19. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. & Struhl, K. (1996) *Current Protocols in Molecular Biology* (Wiley, New York).
20. Ochman, H., Medhora, M. M., Garza, D. & Hartl, D. L. (1990) in *PCR Protocols: A Guide to Methods and Applications*, eds. Innis, M. A., Gelfand, D. H., Sninsky, J. J. & White, T. J. (Academic, San Diego), pp. 219–227.
21. Gish, W. & States, D. J. (1993) *Nat. Genet.* **3,** 266–272.
22. Roberts, R. C., Burioni, R. & Helinski, D. R. (1990) *J. Bacteriol.* **172,** 6204–6216.
23. Donnenberg, M. S. & Kaper, J. B. (1991) *Infect. Immun.* **59,** 4310–4317.
24. Toder, D. S., Gambello, M. J. & Iglewski, B. H. (1991) *Mol. Microbiol.* **5,** 2003–2010.
25. Essar, D. W., Eberly, L., Hadero, A. & Crawford, I. P. (1990) *J. Bacteriol.* **172,** 884–900.
26. Ostroff, R. M. & Vasil, M. L. (1987) *J. Bacteriol.* **169,** 4597–4601.
27. Akerley, B. J., Cotter, P. A. & Miller, J. F. (1995) *Cell* **80,** 611–620.
28. Menard, R., Sansonetti, P. J. & Parsot, C. (1993) *J. Bacteriol.* **175,** 5899–5906.
29. Manoil, C. & Beckwith, J. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 8129–8133.
30. Laville, J., Voisard, C., Keel, C., Maurhofer, M., Défago, G. & Haas, D. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 1562–1566.
31. Gaffney, T. D., Lam, S. T., Ligon, J., Gates, K., Frazelle, A., DiMaio, J., Hill, S., Goodwin, S., Torkewitz, N., Allshouse, A. M., Kempf, H.-J. & Becker, J. O. (1994) *Mol. Plant-Microbe Interact.* **7,** 455–463.
32. Bardwell, J. C. A., McGovern, K. & Beckwith, J. (1991) *Cell* **67,** 581–589.
33. Shevchik, V. E., Bortoli-German, I., Robert-Baudouy, J., Robinet, S., Barras, F. & Condemine, G. (1995) *Mol. Microbiol.* **16,** 745–753.
34. Peek, J. A. & Taylor, R. K. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 6210–6214.
35. Watarai, M., Tobe, T., Yoshikawa, M. & Sasakawa, C. (1995)

Proc. Natl. Acad. Sci. USA **92,** 4927–4931.
36. Hassett, D. J., Charniga, L., Bean, K., Ohman, D. E. & Cohen, M. S. (1992) *Infect. Immun.* **60,** 328–336.
37. Kanthakumar, K., Taylor, G., Tsang, K. W., Cundell, D. R., Rutman, A., Smith, S., Jeffery, P. K., Cole, P. J. & Wilson, R. (1993) *Infect. Immun.* **61,** 2848–2853.
38. Miller, R. A., Rasmussen, G. T., Cox, C. D. & Britigan, B. E. (1996) *Infect. Immun.* **64,** 182–188.
39. Johnston, C., Pegues, D. A., Hueck, C. J., Lee, C. A. & Miller, S. I. (1996) *Mol. Microbiol.* **22,** 715–727.
40. Reimmann, C., Beyeler, M., Latifi, A., Winteler, H., Foglino, M., Lazdunski, A. & Haas, D. (1997) *Mol. Microbiol.* **24,** 309–319.
41. Hayashi, S. & Wu, H. C. (1990) *J. Bioenerg. Biomembr.* **22,** 451–471.
42. Henikoff, S., Haughn, G. W., Calvo, J. M. & Wallace, J. C. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 6602–6606.
43. Viale, A. M., Kobayashi, H., Akazawa, T. & Henikoff, S. (1991) *J. Bacteriol.* **173,** 5224–5229.
44. Finlay, B. B. & Falkow, S. (1997) *Microbiol. Mol. Biol. Rev.* **61,** 136–169.
45. Wang, J., Mushegian, A., Lory, S. & Jin, S. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 10434–10439.
46. Mahan, M. J., Slauch, J. M. & Mekalanos, J. J. (1993) *Science* **259,** 686–688.
47. Hensel, M., Shea, J. E., Gleeson, C., Jones, M. D., Dalton, E. & Holden, D. W. (1995) *Science* **269,** 400–403.
48. Valdivia, R. H. & Falkow, S. (1996) *Mol. Microbiol.* **22,** 367–378.
49. Chadwick, J. S. (1967) *Fed. Proc.* **26,** 1675–1679.