Use of model plant hosts to identify Pseudomonas aeruginosa virulence factors

(gacA/phycocyanin/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 Tpho4 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 Tpho4 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 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 Tpho4 carried on the suicide plasmid pRT731 in E. coli strain SM10 Apir (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 Tpho4 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 Tpho4 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 of the blue color that results from dephosphorylation of XP by periplasmic alkaline phosphatase generated by PhoA* mutants.

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 MgSO₄, resuspended at an optical density of 0.2 (OD₆₀₀ = 0.2) in 10 mM MgSO₄, and diluted 1:100 and 1:1,000 (corresponding to a bacterial density of ~10⁶ and 10⁵ cfu/ml, respectively). Approximately 10 µl of the diluted cells were inoculated with a Pipetman into stems of an ~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 MgSO₄. The midrib of each lettuce leaf was inoculated with three different TphoA-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 MgSO₄ (corresponding to a bacterial density of 10⁷/cm² leaf disk area) and were injected into leaves of 6-week-old *Arabidopsis* plants ecotype Llagoastera 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 MgSO₄ showed no symptom development.

**Mice Mortality Studies.** A 5% total surface area burn was fashioned on the outreached 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 x 10⁵ or 5 x 10⁶ *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 χ² test with Yates’ correction or Fisher’s exact test. Differences between groups were considered statistically significant at P ≤ 0.05.

**DNA Manipulation, Molecular Cloning, and Sequence Analysis of TphoA 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’-AAATATCGCCTTGAGCAGGC-3’ (LGR1), and 5’-AAATACCTACTATGCGCTG-3’ (LGR2) correspond to sequences on opposite strands at the 5’ end of TphoA. The oligonucleotides 5’-CCATCTCATCATGAGGATC-3’ (LGR3) and 5’-CCGAGGATGACCTGCTG-3’ (LGR4) correspond to sequences on opposite strands at the 3’ end of TphoA. LGR1 + LGR2 or LGR3 + LGR4 were used to amplify by inverse PCR (iPCR) DNA sequences adjacent to the sites of TphoA 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 EcoRV 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 TphoA 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 pJ55R1 (11) for a clone containing the gene corresponding to the *pho34B12* mutation. A 3.7-kb EcoRI fragment, identified in cosmid clone pLGR34B12 which corresponded to the *pho34B12* mutation, was subcloned into the EcoRI site of pRR54 (22) after filling-in the ends of both vector and fragment to construct pLGR34B12. The same fragment (made blunt ended) was subcloned into the Smal 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 EcoRI fragment was also subcloned into the EcoRI site of pBlueScript 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’-CGCATCGTGCAAAACGGTTGGGCGCC-3’ and 5’-GCCGATGGCGGAGATCATGCTGCCGAGTC-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’-TGCGAAGCGATACCCGGGCGC-3’ and 5’-GTTCCACCTTCGCAGCGCAGCCC3’ (Reg3) were also used to amplify a 1,659 bp from pBSR34B12 containing ORF1, the oligonucleotide primers 5’-GATTTCCACCTTCGAGACCGACGAGTCTC-3’ and 5’-GCGATGGCGGAGATCATGCTGCCGAGTC-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 TphoA 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 by filling-in the ends of both vector and fragment to construct pLGR34B12 which corresponded to the gene with a wild-type copy as described (23). The 3.7-kb EcoRI fragment was also subcloned into the EcoRI site of pBlueScript SK +/− to construct pBSR34B12 and used for DNA sequence analysis.

**Generation of a Nonpolar gacA Mutation.** A nonpolar gacA mutation in UCBPP-PA14 was constructed by cloning a 3.5-kb *Psl* fragment containing the gacA gene from cosmid plGR43 (11) into the unique BamHI restriction site in the suicide vector pEBGR (27) by using BamHI linkers. A 950-bp EcoRI-HindIII Klenow end-filled fragment containing the kanamycin resistance gene cassette from pUC18K (28) was then cloned into the unique BamHI 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 TphoA 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 elicited 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 TnphoA-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 TnphoA insertion, using as a probe a 1,542-bp Bgl-BamHI fragment containing the kanamycin resistance conferring gene of TnphoA (14). Two of the nine UCBPP-PA14 TnphoA mutants, pho34B12 and pho15, expressed alkaline phosphatase activity suggesting that the genes containing these TnphoA insertions encode membrane-spanning or secreted proteins (14, 29).

The nine TnphoA 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 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 (~10^5-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 TnphoA-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 ≤ 0.05 at both doses except for 25A12 and 16G12, which were not significantly different from wild type at the higher dose of 5 × 10^6 cells. In addition, mutant 33A9 also failed to cause mortality even at a higher dose of 5 × 10^7.

We used DNA blot analysis and DNA sequence analysis to determine whether TnphoA in the nine less pathogenic mutants had inserted into known genes. DNA blot analysis revealed that mutant 1D7 contains a TnphoA 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 TnphoA insertions (20). The IPCR products were cloned and then subjected to DNA sequence analysis. Mutant pho15 contains TnphoA 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 cholerae are required for pathogenesis (33–35). Computer analysis with the program BLASTX showed that when the DNA sequences corresponding to the remaining seven TnphoA 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::TnphoA mutant was identified from

### Table 1. P. aeruginosa mutants obtained by TnphoA mutagenesis

<table>
<thead>
<tr>
<th>Strain</th>
<th>Growth in Arabidopsis leaves</th>
<th>Symptoms elicited in Arabidopsis</th>
<th>% mouse mortality</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>PA14</td>
<td>5.5 × 10^7</td>
<td>Severe</td>
<td>73</td>
<td>Unknown</td>
</tr>
<tr>
<td>33C7</td>
<td>8.3 × 10^7</td>
<td>None</td>
<td>0</td>
<td>Unknown</td>
</tr>
<tr>
<td>1D7</td>
<td>7.5 × 10^4</td>
<td>Weak</td>
<td>0</td>
<td>Unknown</td>
</tr>
<tr>
<td>25A12</td>
<td>1.7 × 10^6</td>
<td>Weak</td>
<td>1</td>
<td>Unknown</td>
</tr>
<tr>
<td>33A9</td>
<td>5.1 × 10^6</td>
<td>Moderate</td>
<td>0</td>
<td>Unknown</td>
</tr>
<tr>
<td>25F1</td>
<td>1.5 × 10^6</td>
<td>Moderate</td>
<td>0</td>
<td>Unknown</td>
</tr>
<tr>
<td>34H4</td>
<td>3.8 × 10^6</td>
<td>Moderate</td>
<td>0</td>
<td>Unknown</td>
</tr>
<tr>
<td>pho34B12</td>
<td>4.0 × 10^6</td>
<td>Moderate</td>
<td>0</td>
<td>Unknown</td>
</tr>
<tr>
<td>pho15</td>
<td>3.9 × 10^4</td>
<td>Moderate</td>
<td>0</td>
<td>unknown</td>
</tr>
<tr>
<td>16G12</td>
<td>2.3 × 10^5</td>
<td>Moderate</td>
<td>0</td>
<td>100</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Growth in Arabidopsis</th>
<th>Symptoms elicited in Arabidopsis</th>
<th>% mouse mortality</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>10^3</td>
<td>5 x 10^3</td>
<td>5 x 10^3</td>
<td>Gene identity</td>
</tr>
</tbody>
</table>

Normal differences were observed 4–5 days after infection. None, no symptoms; chlorosis, chlorosis circumscripting the inoculation site; weak, localized water-soaking and chlorosis of tissue circumscripting 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 ~5 × 10^5 or 5 × 10^6 cells.

BLASTX analysis yielded no encoded proteins with significant homology.
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 TphoA 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::TphoA 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 phoA gene. This mutation, 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* 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* produces a regulator of diverse pathogenicity factors.

To rule out the possibility that a secondary mutation 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* produces a regulator of diverse pathogenicity factors.

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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 correspond 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 of 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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