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Use of the *Galleria mellonella* Caterpillar as a Model Host To Study the Role of the Type III Secretion System in *Pseudomonas aeruginosa* Pathogenesis

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Nonvertebrate model hosts represent valuable tools for the study of host-pathogen interactions because they facilitate the identification of bacterial virulence factors and allow the discovery of novel components involved in host innate immune responses. In this report, we determined that the greater wax moth caterpillar *Galleria mellonella* is a convenient nonmammalian model host for study of the role of the type III secretion system (TTSS) in *Pseudomonas aeruginosa* pathogenesis. Based on the observation that a mutation in the TTSS *pscD* gene of *P. aeruginosa* strain PA14 resulted in a highly attenuated virulence phenotype in *G. mellonella*, we examined the roles of the four known effector proteins of *P. aeruginosa* (ExoS, ExoT, ExoU, and ExoY) in wax moth killing. We determined that in *P. aeruginosa* strain PA14, only ExoT and ExoU play a significant role in *G. mellonella* killing. Strain PA14 lacks the coding sequence for the ExoS effector protein and does not seem to express ExoY. Moreover, using Δ*exoU* Δ*exoY*, Δ*exoT* Δ*exoY*, and Δ*exoT* Δ*exoU* double mutants, we determined that individual translocation of either ExoT or ExoU is sufficient to obtain nearly wild-type levels of *G. mellonella* killing. On the other hand, data obtained with a Δ*exoT* Δ*exoU* Δ*exoY* triple mutant and a Δ*pscD* mutant suggested that additional, as-yet-unidentified *P. aeruginosa* components of type III secretion are involved in virulence in *G. mellonella*. A high level of correlation between the results obtained in the *G. mellonella* model and the results of cytopathology assays performed with a mammalian tissue culture system validated the use of *G. mellonella* for the study of the *P. aeruginosa* TTSS.

The human opportunistic bacterial pathogen *Pseudomonas aeruginosa* has proven to be a particularly versatile pathogen that is capable of causing diseases in plants, nematodes, and insects as well as in mice and humans (13, 31, 36, 47, 51). One particular *P. aeruginosa* strain, PA14, originally isolated from a human burn wound patient, has been used to demonstrate that *P. aeruginosa* virulence-related genes important for mouse pathogenesis can be identified by screening for less virulent mutants in plants or nematodes (36, 48, 52). In general, the use of nonvertebrate model hosts has facilitated the identification of bacterial virulence factors in a number of human bacterial pathogens in addition to *P. aeruginosa* and has led to the identification of new components involved in host innate immune responses (1, 9, 14, 24, 35, 36, 38, 41, 48, 52).

In gram-negative plant and animal pathogens, a highly conserved feature of pathogenesis is the so-called type III secretion system (TTSS) required for the translocation of effector proteins (virulence factors) directly into the cytosol of target eukaryotic cells (22, 29, 34). In mammals, the main targets of the translocated effector proteins include the host cytoskeleton and innate immune response pathways of macrophages and epithelial cells. For example, in *Yersinia* spp. and *P. aeruginosa*, TTSS effector proteins alter the normal actin cytoskeleton and induce apoptosis in infected macrophages, thereby inhibiting phagocytosis (4, 7, 21, 25, 37, 49). Less is known about the mode of action of TTSS effector proteins in plant pathogens. Nevertheless, in all plant pathogens studied to date, the TTSS, which is encoded by the so-called *hrp* and *hrc* genes, is absolutely essential for pathogenesis. For *Pseudomonas syringae*, a relatively large number of putative TTSS effector proteins have been identified (6, 43).

To date, four TTSS effector proteins have been identified for *P. aeruginosa*, ExoS, ExoT, ExoU, and ExoY (18, 30, 54–56). These *P. aeruginosa* effector proteins are responsible for disruption of the actin cytoskeleton in host cells (21, 23, 42, 53), inhibition of DNA synthesis (39), interference with cell matrix adherence (40), production of epithelial cell injury (4, 18, 19, 25, 27, 53), inhibition of internalization (10, 23), and induction of apoptosis (25, 32). Interestingly, however, despite the involvement of the TTSS in pathogenesis in a variety of plant and animal pathogens studied to date, no mutations in TTSS-related genes were identified among a total of 8,200 *P. aeruginosa* strain PA14 random transposon Tn*phoA* insertion mutants screened for decreased virulence in either plants (lettuce) or nematodes (*Caenorhabditis elegans*) as model hosts (36, 48, 52). These results were particularly surprising because TTSS-related genes represent such a large target for Tn*phoA* insertion events. These results suggested either that Tn*phoA* was not targeting TTSS-related genes in PA14 or that the TTSS was not an important feature of *P. aeruginosa* pathogenesis in plants and nematodes.

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Reference or source
Strains		
PA14	Virulent burn wound isolate of *P. aeruginosa*	47
2B3	PA14 *pscD*::Tn*phoA*	Miyata et al., unpublished
2B3R	Reverted PA14 *pscD*::Tn*phoA*	Drenkard and Ausubel, unpublished
2B3R-restored	Reverted PA14 *pscD*::Tn*phoA*$^+$ with a wild-type copy of *pscD*	This study
Δ*pscD*	PA14 Δ*pscD* (1.17-kb in-frame deletion of *pscD*)	This study
Δ*exoT*	PA14 Δ*exoT* (1.3-kb in-frame deletion of *exoT*)	This study
Δ*exoU*	PA14 Δ*exoU* (2.0-kb deletion of *exoU*)	This study
Δ*exoY*	PA14 Δ*exoY* (1.1-kb deletion of *exoY*)	This study
Δ*exoT* Δ*exoU*	PA14 Δ*exoT* Δ*exoU* (*exoT* and *exoU* deletions)	This study
Δ*exoT* Δ*exoY*	PA14 Δ*exoT* Δ*exoY* (*exoT* and *exoY* deletions)	This study
Δ*exoU* Δ*exoY*	PA14 Δ*exoU* Δ*exoY* (*exoU* and *exoY* deletions)	This study
Δ*exoT* Δ*exoU* Δ*exoY*	PA14 Δ*exoT* Δ*exoU* Δ*exoY* (*exoT*, *exoU*, and *exoY* deletions)	This study
PA103	Virulent lung isolate of *P. aeruginosa*	2
Sm10λ*pir*	*E. coli* strain used for mating constructs into *P. aeruginosa; thi thr leu tonA lacY supE recA*::RP4-2-Tc::mu Km^r	15
OP50	*E. coli* strain used as a food source in *C. elegans* assays	36
Plasmids		
pUCP19	Cloning vector that replicates stably in *Pseudomonas;* Amp^r	50
pEX18Ap	Positive selection suicide vector; Amp^r	45
pEX18*pscD1*	pEX18 with a 2.76-kb *Sac*I-*Hin*dIII fragment containing *pscD* from PA14	This study
pEX18*pscD*Δ*1*	pEX18 with a 1.17-kb *Kpn*I-*Hin*dIII fragment containing an in-frame deletion of *pscD*	This study
pEX18*exoT*Δ*4*	pEX18 with a 1.3-kb *Kpn*I-*Hin*dIII fragment containing an in-frame deletion of *exoT*	This study
pEX18*exoU*Δ*5*	pEX18 with a 2.0-kb *Kpn*I-*Hin*dIII fragment containing a deletion of *exoU*	This study
pEX18*exoY*Δ*4*	pEX18 with a 1.1-kb *EcoR*I-*Hin*dIII fragment containing a deletion of *exoY*	This study

Recently, 1,560 *P. aeruginosa* PA14 Tn*phoA* mutants (that had previously been screened in *C. elegans*) were rescreened in the greater wax moth caterpillar *Galleria mellonella* (S. Miyata et al., unpublished data). *P. aeruginosa* PA14 kills *G. mellonella* at a 50% lethal dose (LD_{50}) of approximately 1.0 to 10.0 (depending on experimental conditions) when bacterial cells are injected directly into the *G. mellonella* body cavity (31). This screen led to the identification of a mutation in the TTSS *pscD* gene of strain PA14 (S. Miyata et al., unpublished data), suggesting that in contrast to plants and nematodes, *G. mellonella* and perhaps other insects would be appropriate alternative nonmammalian hosts for identification and study of the components of the *P. aeruginosa* TTSS. Indeed, a recent publication reporting work that was carried out independently from the experiments reported here showed that the *P. aeruginosa* TTSS plays a key role in virulence in *Drosophila melanogaster* (16).

In the present study, we used the *G. mellonella-P. aeruginosa* model system to examine the TTSS and its role in pathogenesis. We show that strain PA14 does not express ExoS and that although none of the other three known effector proteins (ExoT, ExoU, and ExoY) is essential for virulence, both ExoT and ExoU play significant roles in *G. mellonella* killing. Moreover, because a Δ*exoT* Δ*exoU* Δ*exoY* triple mutant was less attenuated in virulence than a Δ*pscD* mutant, we conclude that additional *P. aeruginosa* TTSS virulence components remain to be identified. Finally, we found a high level of correlation between the results obtained with *G. mellonella* and the results of cytopathology assays performed with CHO cells, demonstrating that the *G. mellonella* model system represents a useful tool for identification and study of the components of type III secretion in *P. aeruginosa*.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains and plasmids used in this study are listed in Table 1. Bacterial strains were routinely cultured in Luria-Bertani (LB) broth (3) supplemented with appropriate antibiotics as needed. Antibiotics were used at the following concentrations: ampicillin, 50 μg/ml; carbenicillin, 300 μg/ml; kanamycin, 30 μg/ml for *Escherichia coli* and 200 μg/ml for *P. aeruginosa*; and rifampin, 100 μg/ml.

***G. mellonella* killing assays.** Overnight PA14 cultures grown in LB broth were diluted 1:100 in the same medium and grown to an optical density at 600 nm (OD_{600}) of 0.3 to 0.4. Cultures were centrifuged, and pellets were resuspended in 10 mM $MgSO_4$ to an OD_{600} of 0.1. Serial 10-fold dilutions were made in 10 mM $MgSO_4$ supplemented with 0.5 mg of rifampin/ml. Five-microliter aliquots of the serial dilutions were injected into *G. mellonella* larvae (Van der Horst Wholesale, St. Marys, Ohio) as previously described (31). A final concentration of approximately 10 μg of rifampin per g of larva prevented infection by bacteria naturally present on the surface of the larvae. Ten larvae were injected per dilution, and at least four different serial dilutions were inoculated per strain. Larvae were incubated in 10-cm plates at 37°C, and the number of dead larvae was scored 1 to 4 days after infection. A larva was considered dead when it displayed no movement in response to touch. A mock inoculation was performed in each experiment to monitor the killing due to physical injury or infection by pathogenic contaminants. LD_{50}s were determined by using the SYSTAT program for statistical analysis as previously described (31).

Wild-type *pscD* replacement in mutant 2B3. A 2.76-kb PCR fragment that contained a wild-type copy of the *pscD* gene was amplified from strain PA14 with primers that contained *Sac*I and *Hin*dIII restriction sites. The PCR-amplified fragment was subcloned into the *Sac*I and *Hin*dIII sites of the positive-sucrose-selection suicide vector pEX18Ap (45), generating plasmid pEX18*pscD1*. Plasmid pEX18*pscD1* was used to introduce the wild-type *pscD* sequence into the homologous region of the 2B3 mutant chromosome by allelic exchange as described previously (15), creating "restored" strain 2B3 (2B3-restored). The replacement was confirmed by Southern blotting and sequencing of a PCR fragment that contained the *pscD* gene.

Sequencing of the *exoS* open reading frame and surrounding sequences. For sequence analysis of the PA14 *exoS* open reading frame, a 1.44-kb PCR product from PA14 was generated with primers exoS1 (5′-TACGGTACCTTCTTCGT GCTCAACGTC-3′) and exoS4 (5′-ATCAAGCTTCACGATGGCCTGATTCC AC-3′) and sequenced. To corroborate that *exoS* was not present somewhere else

in the genome of strain PA14, Southern blot analysis was performed with a radiolabeled probe generated by random priming (3) from a 0.23-kb fragment that corresponded to part of the PAO1 *exoS* open reading frame. The probe was used to hybridize *Eco*RI-digested genomic DNA from strains PA14, PAO1, and PAK.

Sequencing of the *exoY* open reading frame and surrounding sequences. For the generation of a PA14 Δ*exoY* mutant, we sequenced the *exoY* open reading frame and surrounding sequences because the region upstream of *exoY* in strain PA14 was different from those in PAO1 and PA388. The PA14 *exoY* gene was cloned by hybridizing a radiolabeled probe generated from a 1.0-kb PCR fragment that contained most of the PAO1 *exoY* open reading frame to a PA14 genomic cosmid library in vector pJSR1 (47). A hybridizing clone identified from the genomic library was then subjected to sequence analysis with PAO1- and PA14-specific primers.

Generation of PA14 Δ*pscD*, Δ*exoT*, Δ*exoU*, Δ*exoY*, Δ*exoT* Δ*exoU*, Δ*exoT* Δ*exoY*, Δ*exoU* Δ*exoY*, and Δ*exoT* Δ*exoU* Δ*exoY* mutants. The oligonucleotide primers used to generate deletion mutations in the PA14 *pscD*, *exoT*, *exoU*, and *exoY* genes were designed based on DNA sequences from strain PAO1, except that the *exoU* downstream primer was based on the PA103 sequence and the *exoY* and *exoU* upstream primers were based on the PA14 sequence. The *pscD* deletion was generated by replacing 2.76 kb of wild-type sequence with a 1.59-kb PCR-amplified fragment that contained a 1.17-kb deletion in the *pscD* open reading frame. The PCR-amplified fragment containing the deletion was subcloned into the *Sac*I and *Hin*dIII sites of pEX18Ap (45), generating plasmid pEX18*pscD*Δ*1*. The resulting construct was used to introduce the deletion of the *pscD* gene into the wild-type PA14 genome by homologous recombination (15), resulting in the Δ*pscD8* mutant. Similar strategies were used to construct Δ*exoT*, Δ*exoU*, and Δ*exoY* mutants as well as Δ*exoT* Δ*exoU*, Δ*exoT* Δ*exoY*, Δ*exoU* Δ*exoY*, and Δ*exoT* Δ*exoU* Δ*exoY* mutants. For the Δ*exoT*, Δ*exoU*, and Δ*exoY* mutants, we replaced 3.0, 3.6, and 2.8 kb of wild-type sequence with 1.7-, 1.6-, and 1.7-kb PCR-amplified fragments that contained 1.3-, 2.0-, and 1.1-kb deletions, respectively. The PCR-amplified fragments that contained the *exoT* and *exoU* deletions were cloned into the *Kpn*I and *Hin*dIII sites of pEX18Ap, and the *exoY* deletion fragment was cloned into the *Eco*RI and *Hin*dIII sites of pEX18Ap, generating plasmids pEX18*exoT*Δ*4*, pEX18*exoU*Δ*5*, and pEX18*exoY*Δ*4*, respectively. The resulting constructs were used to introduce the deletions of the genes into the wild-type PA14 genome, resulting in Δ*exoT1*, Δ*exoU3*, and Δ*exoY1* mutants. The Δ*exoT* Δ*exoU*, Δ*exoT* Δ*exoY*, and Δ*exoU* Δ*exoY* double mutants were constructed by recombining *exoT*Δ*4* into Δ*exoU3*, *exoY*Δ*4* into Δ*exoT1*, and *exoY*Δ*4* into Δ*exoU3*. The Δ*exoT*Δ *exoU* Δ*exoY* triple mutant was constructed by recombining *exoT*Δ*4* into the Δ*exoU* Δ*exoY* double mutant. All of the deletion mutations were confirmed by PCR and/or by Southern blot analysis with radiolabeled probes specific to either upstream or downstream regions. DNA sequence analysis showed that the *pscD* and *exoT* deletions were in frame but that the *exoU* and *exoY* deletions were out of frame.

Analysis of extracellular proteins. Analysis of extracellular proteins from the different *P. aeruginosa* strains was performed as described by Hauser et al. (27). A calcium chelator (nitrilotriacetic acid [NTA]; 10 mM) was added to the incubation medium to induce the TTSS. Proteins were recovered from the supernatant by ammonium sulfate precipitation (final concentration, 85% [wt/vol]). Twenty-microliter samples containing precipitated proteins were analyzed on sodium dodecyl sulfate (SDS)–10% polyacrylamide gels stained with Coomassie brilliant blue (3).

Immunoblot analysis. For detection of the PA14 ExoT and ExoU secreted effectors, extracellular proteins were prepared as described above. Proteins were electrotransferred to nitrocellulose for immunoblot analysis. ExoT and ExoU were detected by using rabbit polyclonal ExoT and ExoU antisera specific for the ExoT and ExoU proteins of strain PA103, respectively (kindly provided by J. Engel [26]). Horseradish peroxidase-linked anti-rabbit immunoglobulin G (IgG) (Amersham) diluted 1:2,000 was used as a secondary antibody. The procedures used for Western blotting were described previously (3).

For the detection of PA14 ExoY, cultures grown under type III expression-inducing conditions were concentrated by using an ammonium sulfate solution saturated to 55%. Rabbit antibody specific for ExoY was used at a dilution of 1:20,000. Mouse monoclonal antibody 166, specific for PcrV, was included in the incubation mixture. Peroxidase-labeled secondary antibodies to rabbit IgG and mouse IgG were used at a dilution of 1:7,000, and reactivity was detected by using chemiluminescence.

Invasion assay. HeLa cells were cultured in MEMα medium (Gibco-BRL) with 10% fetal bovine serum (FBS) (Gibco-BRL). HeLa cells were plated in 12-well tissue culture plates at approximately 10^5 cells/well and incubated for 20 to 24 h at 37°C with 5% CO_2. PA14-derived strains were inoculated into LB broth and incubated overnight at 37°C without shaking as described previously (23). Bacterial cultures were pelleted and resuspended in MEMα medium containing 1% bovine serum albumin (BSA) (Sigma, St. Louis, Mo.) and 20 mM HEPES (Sigma). HeLa cells were washed once with MEMα medium (with 1% BSA and 20 mM HEPES), inoculated in triplicate with 10^7 bacteria, and incubated for 2 h at 37°C without CO_2. Amikacin was added to the infected cells to a final concentration of 400 μg/ml, and the plates were incubated for an additional 2 h at 37°C without CO_2. The number of live cells present in the wells at the end of the incubation period was scored by using a hemocytometer after trypan blue staining (Sigma). The removal of antibiotic-containing medium prior to the release of intracellular bacteria was performed as described previously (23). Infected HeLa cells were lysed with phosphate-buffered saline (PBS) (pH 7.4) containing 1% Triton X-100 and incubated for 30 min at room temperature. Serial dilutions of the lysed cells were plated on LB agar. The number of internalized bacteria per well was divided by the number of live cells per well. The ratios were normalized to the ratio obtained with Δ*pscD*.

Cytotoxicity assays. HeLa cells were cultured in MEMα medium with 10% FBS. HeLa cells were transferred to six-well tissue culture plates and incubated for 20 to 24 h to >80% confluence (~5×10^5 cells/well) at 37°C with 5% CO_2. Overnight cultures of PA14-derived strains were inoculated onto LB agar plates and incubated overnight at 37°C. Bacterial lawns were washed once with PBS and resuspended in MEMα medium containing 1% BSA and 15 mM HEPES. HeLa cell monolayers were infected in duplicate with 10^7 bacteria. Following 3 h of incubation at 37°C without CO_2, the wells were washed once with PBS. HeLa cells were pelleted at 1,200 rpm (Eppendorf centrifuge) for 5 min, resuspended in 200 μl of PBS, and stained with 0.4% trypan blue in PBS (Sigma). After the cells were dislodged, the fraction of dead cells was scored by using a hemocytometer.

HeLa cell rounding assays. HeLa cells were cultured in MEMα medium with 10% FBS. HeLa cells were transferred to 24-well tissue culture plates and incubated overnight at 37°C to >80% confluence in the presence of 5% CO_2. PA14-derived strains were grown in LB broth overnight at 37°C without shaking. HeLa cells were washed once with MEMα medium (with 1% BSA and 20 mM HEPES), inoculated in triplicate with 10^6 bacterial cells, and incubated for 4 to 6 h at 37°C without CO_2. Rounding was assessed by visual inspection of the wells at magnifications of ×100 and ×200 by using a Zeiss IM35 microscope.

Bacterial plaque assays. CHO cells were cultured in Ham's F-12 medium (InVitrogen, Carlsbad, Calif.) containing an organic buffer mixture (BufferAll; Sigma) and 10% heat-inactivated newborn calf serum (InVitrogen). CHO cells were seeded in 60-mm tissue culture dishes at a density of 4×10^6 cells/dish and incubated overnight at 37°C in 5% CO_2. The bacterial inoculum was prepared by resuspending bacteria grown on agar plates in serum-free Ham's F-12 medium and diluting the suspension to a final concentration of 80 CFU per ml. Cell monolayers were rinsed once with Hanks balanced salt solution without phenol red (InVitrogen), and 0.1 ml of the bacterial inoculum (containing about 8 CFU) was added to each well containing CHO cells. The inoculum was covered with 4 ml of a 1% solution of methylcellulose (Sigma) in Ham's F-12 medium containing BufferAll and 2% heat-inactivated newborn calf serum. The dishes were incubated for 16 to 18 h at 37°C in 5% CO_2. Subsequently, methylcellulose was aspirated and the cells were stained with Gram's crystal violet (Becton Dickinson) for at least 5 min. The stain was aspirated, and the dishes were rinsed with water and allowed to dry at room temperature. Plaques, which appeared as circular holes in the monolayers, were counted and measured.

***C. elegans* and *Arabidopsis* pathogenicity assays.** *C. elegans* fast killing assays were carried out as described by Mahajan-Miklos et al. (36). Slow killing assays were performed as described by Tan et al. (51). In both cases, worm mortality was scored over time. A worm was considered dead when it failed to respond to touch. Twenty worms were placed on assay plates, which were then incubated at 25°C. *E. coli* strain OP50 was used as a control for both fast and slow killing assays.

Arabidopsis leaf infiltration assays were carried out as previously described (47) with slight modifications. Five-week-old *Arabidopsis* ecotype Columbia plants were infiltrated with a 1:100 dilution of log-phase PA14 cultures (OD_{600}, 0.2).

RESULTS

The *pscD* TTSS gene is required for virulence in a *G. mellonella*-*P. aeruginosa* model system. *P. aeruginosa* strain PA14 mutant 2B3, which contains a Tn*phoA* insertion in the *pscD* gene, was identified during a screen for mutants that exhibited decreased virulence in *G. mellonella* by using a PA14 Tn*phoA*-mutagenized library. Briefly, 1,560 individual insertion mutants

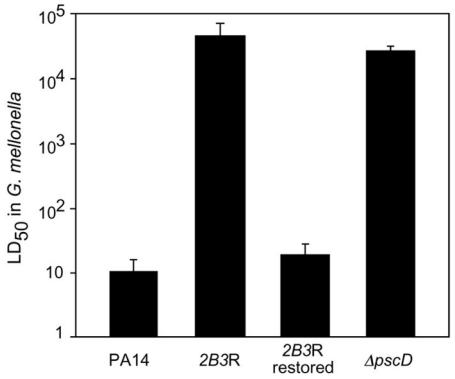

FIG. 1. LD_{50}s of *P. aeruginosa* strains in *G. mellonella* larvae. *G. mellonella* larvae were infected with *P. aeruginosa* strains as described in Materials and Method and incubated for 1 day at 37°C. LD_{50}s were determined by using the SYSTAT program as previously described (31). Bars represent the means and standard deviations of at least three experiments.

from two existing PA14 Tn*phoA* transposon mutant libraries (36, 52) were injected into wax moths and examined after 60 to 72 h of incubation at 25°C. After two rounds of screening, mutant 2B3 and six additional mutants that showed decreased killing compared to the wild type were identified (Miyata et al., unpublished). The identities of the genes disrupted by the Tn*phoA* insertions in the putative avirulent mutants were determined by using PCR amplification and sequencing of the genomic DNA adjacent to the transposon insertion. Analysis of the sequence data obtained from mutant 2B3 revealed that the insertion disrupted a gene that is 97% identical to the *pscD* gene from *P. aeruginosa* strain PA388 (55). To confirm that the virulence defect of mutant 2B3 in *G. mellonella* larvae was due to the Tn*phoA* insertion in the *pscD* gene, we replaced the Tn*phoA* insertion in 2B3 with a wild-type copy of *pscD* (generating strain 2B3-restored) by marker exchange as described in Materials and Methods. These experiments showed that both mutant 2B3 and strain 2B3-restored had motility defects that were not linked to the Tn*phoA* insertion (E. Drenkard and F. Ausubel, unpublished data). The motility defects of strains 2B3 and 2B3-restored, however, were readily reverted to the wild-type phenotype by plating either strain on nonselective medium and incubating the plates for 3 to 5 days. The revertants, referred to as 2B3R and 2B3R-restored, were isolated from the edges of 2B3 and 2B3-restored colonies and tested for motility (Drenkard and Ausubel, unpublished).

In *G. mellonella*, PA14 mutant 2B3R was significantly attenuated in virulence, with an LD_{50} of higher than 40,000, compared to an LD_{50} of 10 for parent strain PA14 (Fig. 1). In contrast, the LD_{50} of strain 2B3R-restored was not significantly different from the LD_{50} of wild-type strain PA14 (Fig. 1), confirming that the mutation in the *pscD* gene is responsible for the avirulent phenotype observed in wax moth caterpillars.

Further characterization of the role of the *pscD* gene in virulence was carried out by using an in-frame internal deletion of *pscD* in PA14 (Δ*pscD*) to ensure that the phenotypes observed were linked to the *pscD* mutation. The deletion was designed to affect the structure of the *pscD* gene product but not to have a polar effect on the expression of downstream genes, and it was generated as described in Materials and Methods. Despite these precautions, it is nevertheless possible that the mutation could have had an effect on some of the genes downstream of *pscD*. As shown in Fig. 1, the LD_{50} of the Δ*pscD* in-frame deletion mutant was not significantly different from the LD_{50} of mutant 2B3*R*.

The *pscD* gene is involved in the secretion of TTSS proteins. The *pscD* gene in *P. aeruginosa* is located within a large cluster of genes whose products are thought to be involved in the formation of the type III secretion apparatus, based on their homology to the Ysc injectisome of *Yersinia pestis* (20, 29). We investigated whether *pscD* of *P. aeruginosa* strain PA14 was involved in the secretion of TTSS proteins by analyzing the profiles of secreted proteins by SDS-polyacrylamide gel electrophoresis (PAGE). We took advantage of the fact that the TTSS can be induced in vitro at 37°C in liquid cultures by growing bacteria in media that contain the calcium chelator NTA (27). When the protein profiles from the Δ*pscD* mutant were compared to those from wild-type strain PA14, several bands corresponding to secreted proteins were absent from the Δ*pscD* mutant (Fig. 2A, lanes 2 and 3). Based on size similarities to proteins from strain PA103 (26, 53), it appears likely that at least some of the proteins absent from supernatants from Δ*pscD* mutant cultures corresponded to TTSS protein ExoU (72 kDa), TTSS translocator proteins PopB (40 kDa) and PopD (31.3 kDa), and TTSS extracellular determinant PcrV (32.2 kDa) (Fig. 2A, lanes 2, 3, and 9). As shown in Fig. 2A, lanes 3 and 4, mutant 2B3R showed the same protein profile as the Δ*pscD* mutant. On the other hand, the secretion of TTSS proteins was restored to a wild-type profile when the Tn*phoA* insertion was replaced by a wild-type copy of *pscD* in strain 2B3R-restored (Fig. 2A, lane 5).

To corroborate whether some of the bands that were absent from the Δ*pscD* mutant corresponded to known *P. aeruginosa* effector proteins (53), we performed immunoblot analysis of supernatants from wild-type strain PA14 and the Δ*pscD* mutant by using antibodies specific for the ExoT and ExoU proteins of *P. aeruginosa* strain PA103 and the ExoY protein of strain PA388. The ExoT and ExoU antisera reacted with secreted proteins in both strain PA14 and strain PA103 (Fig. 2B and C, lanes 1 and 7). In contrast, the ExoT and ExoU antisera did not react with proteins of the expected sizes in the Δ*pscD* supernatant (Fig. 2B and C, lanes 2). The ExoY antiserum detected a band in strain PA103/pUC-PexoY but the band was absent from wild-type strain PA14 (Fig. 2D). Although we were unable to detect the ExoY protein by immunoblot analysis, sequence analysis of the *exoY* region revealed that the *exoY* open reading frame is present in strain PA14 and is 99% identical to that in strain PA388 (along with 0.6 kb of surrounding sequence). The latter data suggest that the ExoY protein is not expressed in PA14 or is expressed at very low levels.

Moreover, sequence analysis allowed us to determine that while the sequences surrounding the *exoS* open reading frame in strain PAO1 were present in strain PA14, the actual *exoS* coding sequence was absent from strain PA14 (data not shown). These data were confirmed by Southern blot hybridization with a PAO1 *exoS* fragment as a probe. The probe specific for the *exoS* sequence did not hybridize to genomic

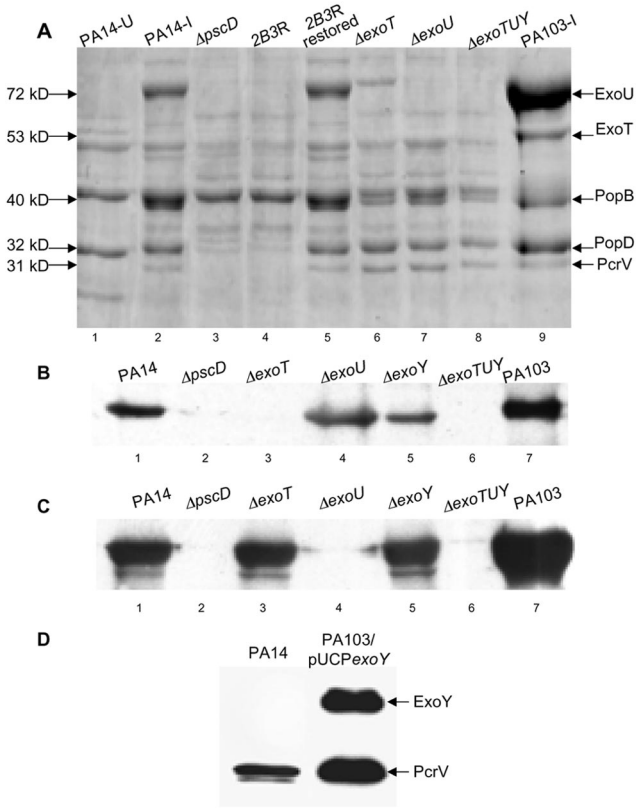

FIG. 2. Extracellular protein profiles of *P. aeruginosa* strains. (A) Coomassie brilliant blue-stained polyacrylamide gel (10%) of concentrated culture supernatants from strains grown in the presence of the calcium chelator NTA (27). Wild-type PA14 was also grown in the presence of Ca^{2+} as a negative control. Secreted proteins were precipitated with ammonium sulfate as described in Materials and Methods. Relative mobilities of the known proteins from PA103 are indicated by arrows. PA14 that was not induced (PA14-U), PA14 that was induced (PA14-I), and PA103 that was induced (PA103-I) by the calcium chelator NTA are shown in lanes 1, 2, and 9, respectively. (B) Immunoblot of polyacrylamide gel probed with antisera reactive to ExoT. (C) Immunoblot of polyacrylamide gel probed with antisera reactive to ExoU. (D) Immunoblot of Coomassie brilliant blue-stained polyacrylamide gel probed with antisera reactive to ExoY and PcrV.

DNA from PA14 but did hybridize to genomic DNA fragments of the expected sizes from *P. aeruginosa* strains PAO1 and PAK (data not shown) that encode ExoS (19).

Construction of *P. aeruginosa* type III secretion effector mutants. To determine which of the effector proteins secreted through the *P. aeruginosa* TTSS are involved in *G. mellonella* killing, we constructed internal deletions in the PA14 *exoT*, *exoU*, and *exoY* genes as described in Materials and Methods. The Δ*exoT* Δ*exoU*, Δ*exoU* Δ*exoY*, and Δ*exoT* Δ*exoY* double mutants and the Δ*exoT* Δ*exoU* Δ*exoY* triple mutant were also constructed as described in Materials and Methods.

To verify that the generated deletion mutations abolished the secretion of the corresponding effector proteins, we analyzed secreted protein profiles by SDS-PAGE and immunoblot analysis after specific induction of type III proteins (see above) and compared them to the profiles of the proteins secreted from strain PA103. Compared to the PA14 control, the Δ*exoU* mutant showed one missing band with a molecular weight that corresponded to that of the PA14 ExoU protein (Fig. 2A, lanes 2 and 7). This result was confirmed by immunoblot analysis (Fig. 2C, lanes 1 and 4). Although no clear differences in extracellular protein profiles were observed for the Δ*exoT* mutant and the wild type in the Coomassie brilliant blue-stained gel (Fig. 2A, lanes 2 and 6), immunoblot analysis revealed a missing protein of the size expected for ExoT in the Δ*exoT* mutant (Fig. 2B, lanes 1 and 3). As stated above, the ExoY antiserum did not detect a band in strain PA14. Profiles of secreted proteins analyzed by SDS-PAGE and immunoblot analysis showed the expected phenotypes for the double mutant (data not shown) and the triple mutant (Fig. 2A, lane 8, and Fig. 2B and C, lanes 6).

The Δ*pscD*, Δ*exoT*, Δ*exoU*, and Δ*exoY* mutants were tested by using a mammalian tissue culture system to corroborate that the phenotypes observed for the PA14 deletion mutants corresponded to the phenotypes reported previously for other *P. aeruginosa* strains (18, 23, 53). We first verified that PA14 ExoT is involved in inhibiting the internalization of *P. aeruginosa* in HeLa cells. These experiments were performed in a Δ*exoU* mutant background because ExoU is cytotoxic and it is difficult to assay bacterial invasion with a cytotoxic strain (23). Figure 3A shows that the ExoT-producing Δ*exoU* mutant is internalized in HeLa cells approximately fourfold less efficiently than the Δ*exoT* Δ*exoU* double mutant, consistent with the proposed role of the ExoT effector protein in inhibiting internalization (23). Moreover, the Δ*exoU* Δ*exoY* double mutant did not show any significant difference in internalization with respect to the Δ*exoU* mutant, indicating that if ExoY is secreted, it does not have an important role in inhibiting internalization (Fig. 3A). We observed that the level of internalization of the Δ*exoT* Δ*exoU* mutant, which has an intact TTSS apparatus, was reproducibly higher than that of the Δ*pscD* mutant (Fig. 3A). A similar finding was reported previously by Garrity-Ryan et al. (23) for strain PA103. These authors attributed the differences in internalization observed between PA103 *pscJ*::Tn5 and PA103 Δ*exoT* Δ*exoU* to the presence of other type III secretion-dependent factors that promote internalization.

The results obtained from a HeLa cell cytotoxicity assay showed that both the Δ*pscD* and the Δ*exoU* strains displayed approximately 13-fold reductions in cytotoxicity compared to wild-type strain PA14 (Fig. 3B), confirming the cytotoxic role of ExoU in strain PA14. On the other hand, the deletion mutations in *exoT* or *exoY* did not cause any difference in cytotoxicity compared to wild-type strain PA14 (Fig. 3B).

Previous reports indicated that ExoS, ExoT, and ExoY are involved in cell rounding and disruption of the actin cytoskeleton in CHO and HeLa cells (23, 53). Rounding assays performed with HeLa cells showed that the Δ*exoU* Δ*exoY* mutant caused dramatic rounding of the cells and confirmed the role of ExoT in disruption of the actin cytoskeleton in strain PA14. On the other hand, little rounding or detachment was observed when HeLa cells were infected with the Δ*pscD* or Δ*exoT* Δ*exoU* Δ*exoY* mutant. Moreover, the appearance of the latter cells was similar to that of uninfected cells. Importantly, HeLa cells infected with the Δ*exoT* Δ*exoU* mutant showed no differences in rounding compared to Δ*pscD* or Δ*exoT* Δ*exoU* Δ*exoY* mutant-infected cells, suggesting that if ExoY is secreted by strain PA14, it may not be functional.

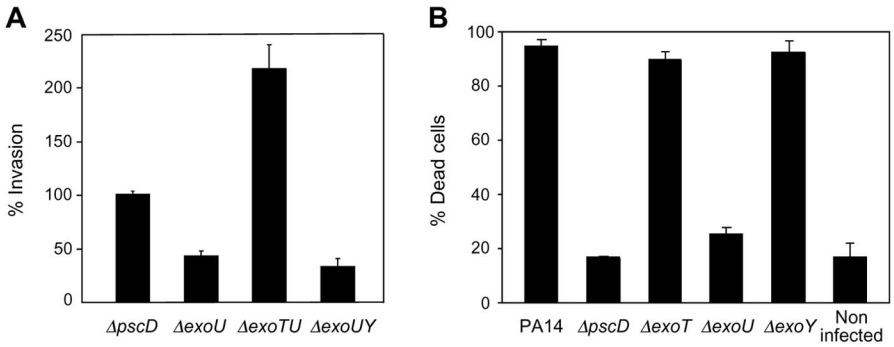

FIG. 3. Effects of secreted effectors ExoT and ExoU in a mammalian tissue culture system. (A) Internalization of PA14 mutants. HeLa cells were infected with the Δ*pscD*, Δ*exoU*, Δ*exoT* Δ*exoU*, and Δ*exoU* Δ*exoY* isogenic mutants of *P. aeruginosa* strain PA14 and incubated for 2 h at 37°C. Internalized bacteria were released as described previously (23). Bars represent the means and standard deviations of three independent experiments performed in triplicate. (B) Cytotoxicity of PA14 mutants. HeLa cells were infected with wild-type PA14 and with the Δ*pscD*, Δ*exoT*, Δ*exoU*, and Δ*exoY* mutants as described in Materials and Methods. At 3 h postinfection, HeLa cells were stained with trypan blue, and the percentage of dead cells was scored with a hemocytometer. Bars represent the means and standard deviations of at least three independent experiments that showed similar results.

Roles of type III effector proteins in *G. mellonella* killing. We analyzed the virulence phenotypes of the Δ*exoT*, Δ*exoU*, and Δ*exoY* mutants in the *G. mellonella* model to determine the roles, if any, of the different effector proteins in *G. mellonella* killing. Figure 4 shows no statistical differences between the LD_{50}s of the Δ*exoT* and Δ*exoY* mutants and the wild-type strain, indicating that neither ExoT nor ExoY is essential for *G. mellonella* killing. On the other hand, the Δ*exoU* mutant exhibited a modest but reproducible attenuation in virulence, suggesting that ExoU plays a more significant role in *G. mellonella* pathogenesis than either ExoT or ExoY. To determine the effects of ExoT, ExoU, and ExoY on wax moth killing when these proteins were translocated individually, we tested Δ*exoU* Δ*exoY*, Δ*exoT* Δ*exoY*, and Δ*exoT* Δ*exoU* double mutants along with a Δ*exoT* Δ*exoU* Δ*exoY* triple mutant. As shown in Fig. 4, the LD_{50} of the Δ*exoU* Δ*exoY* mutant was slightly increased in the wax moth killing assay, whereas the Δ*exoT* Δ*exoY* mutant had the same LD_{50} as wild-type PA14, indicating that individual translocation of either ExoT or ExoU is sufficient to induce high levels of killing in wax moths. Therefore, simultaneous inactivation of both *exoT* and *exoU* was necessary to obtain the high-level attenuated killing phenotype that was observed in the Δ*pscD* mutant (Fig. 4). On the other hand, the data obtained with the Δ*exoT* Δ*exoU* and Δ*exoT* Δ*exoU* Δ*exoY* mutants indicated that ExoY does not play a significant role in killing (Fig. 4).

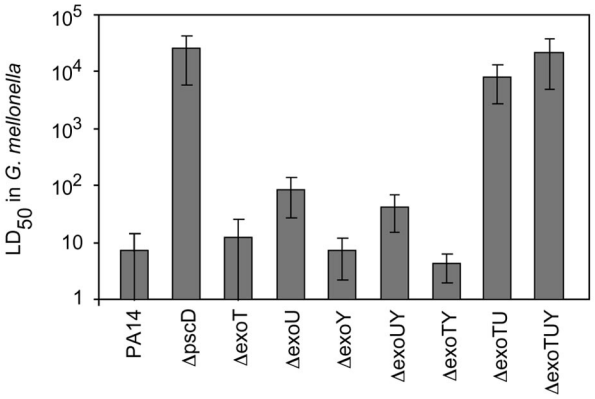

FIG. 4. Killing of *G. mellonella* larvae by PA14 type III secretion mutants. LD_{50}s of *P. aeruginosa* strain PA14 and Δ*pscD*, Δ*exoT*, Δ*exoU*, Δ*exoY*, Δ*exoU* Δ*exoY*, Δ*exoT* Δ*exoY*, Δ*exoT* Δ*exoU*, and Δ*exoT* Δ*exoU* Δ*exoY* mutants are shown. *G. mellonella* larvae were inoculated with *P. aeruginosa* strains as described in Materials and Methods and incubated for 1 day at 37°C. LD_{50}s were determined by using the SYSTAT program as previously described (31). Bars represent the means and standard deviations of bacterial counts from at least three experiments.

Importantly, the results obtained with the *G. mellonella* model correlated with data obtained from cytopathology assays performed with CHO cells. There was no statistical difference between the diameters of the plaques formed on tissue culture cells by the Δ*exoT* Δ*exoU* and Δ*exoT* Δ*exoU* Δ*exoY* mutants, indicating that the ExoY effector protein of PA14 did not have a significant cytopathologic effect on CHO cells (Table 2). Moreover, the Δ*exoT* and Δ*exoU* mutants formed much larger plaques than the Δ*exoT* Δ*exoU* double mutant (Table 2), suggesting that individual translocation of either ExoT or ExoU is sufficient to induce high levels of cytopathologic effects, similar to what we observed in the *G. mellonella* model. Finally, the diameter of plaques formed by the Δ*exoU* mutant was slightly different from that of the wild type, whereas the Δ*exoT* mutant had the same plaque phenotype as wild-type strain PA14; these results correlated with the results obtained in the wax moth model (Table 2 and Fig. 4).

Killing of *G. mellonella* involves additional components se-

TABLE 2. Results of bacterial plaque assays with CHO cells

Strain	Avg plaque diam (mm) for 12 samples
PA14	8.12 ± 1.00
Δ*pscD*	No plaques formed
Δ*exoT*	7.54 ± 0.86
Δ*exoU*	5.62 ± 0.74
Δ*exoY*	8.83 ± 1.05
Δ*exoT* Δ*exoU*	2.71 ± 0.86
Δ*exoT* Δ*exoU* Δ*exoY*	3.0 ± 0.83

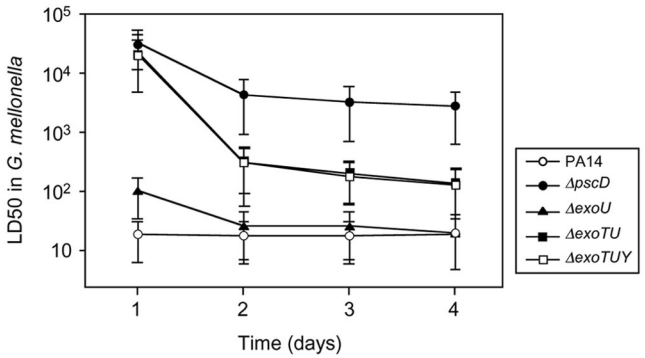

FIG. 5. Time course experiment for strain PA14 and Δ*pscD*, Δ*exoU*, Δ*exoT* Δ*exoU*, and Δ*exoT* Δ*exoU* Δ*exoY* mutants. Infection of *G. mellonella* larvae and determination of LD_{50}s were performed as described in Materials and Methods. Wax moths were incubated for 1 to 4 days at 37°C. Data represent the means and standard deviations of at least three independent experiments that showed similar results.

creted by the TTSS. The results obtained from the cytopathology assays performed with CHO cells also showed that the Δ*exoT* Δ*exoU* Δ*exoY* triple mutant had a phenotype intermediate between those of the Δ*pscD* mutant and wild-type strain PA14 (Table 2). These data suggest the involvement of additional components of type III secretion in PA14 mammalian pathogenesis (Table 2). To determine whether those components could also be detected by using the *G. mellonella* model system, we designed a time course experiment with PA14 and the Δ*pscD*, Δ*exoT*, Δ*exoU*, Δ*exoY*, Δ*exoT* Δ*exoU*, and Δ*exoT* Δ*exoU* Δ*exoY* mutants. Figure 5 shows that although the Δ*exoT* Δ*exoU* and Δ*exoT* Δ*exoU* Δ*exoY* mutants had LD_{50}s similar to that of the Δ*pscD* mutant at 24 h after injection, when the extent of killing was determined at later times (2 to 4 days), the Δ*exoT* Δ*exoU* and Δ*exoT* Δ*exoU* Δ*exoY* mutants exhibited approximately 165-fold decreases in their LD_{50}s with respect to the Δ*pscD* mutant (Fig. 5). Because the Δ*pscD* mutant exhibited a significantly higher LD_{50} than the Δ*exoT* Δ*exoU* and Δ*exoT* Δ*exoU* Δ*exoY* mutants at later time points (Fig. 5), these data suggest that additional type III effector proteins also play an important role in wax moth pathogenesis. Moreover, the data suggest that the unidentified type III component(s) relevant for wax moth killing has a predominant effect later in the infection process. Additionally, the Δ*exoU* mutant also showed a slight decrease in the LD_{50} after 24 h (Fig. 5), whereas the Δ*exoT* and Δ*exoY* mutants showed no statistical differences with respect to the wild type throughout the experiment (data not shown).

The TTSS is not required for virulence in *C. elegans* and *Arabidopsis* model systems. The killing of *C. elegans* by *P. aeruginosa* appears to be mediated by at least two different mechanisms. Fast killing occurs under high-osmolarity conditions in a very short period of time (4 to 24 h) and is toxin mediated (36). Alternatively, PA14 can also accumulate in the lumen of the intestine of *C. elegans* and kill the host over a period of 2 to 3 days. This second type of killing is called slow killing and requires live bacteria (51). To assess whether the TTSS is involved in the killing of *C. elegans*, the Δ*pscD*, Δ*exoT*, and Δ*exoU* mutants were tested in both fast and slow killing assays. In both *C. elegans* killing models, all three mutants showed no difference in slow or fast killing with respect to the wild type (Fig. 6A and B), indicating that type III secretion is not required to kill *C. elegans*.

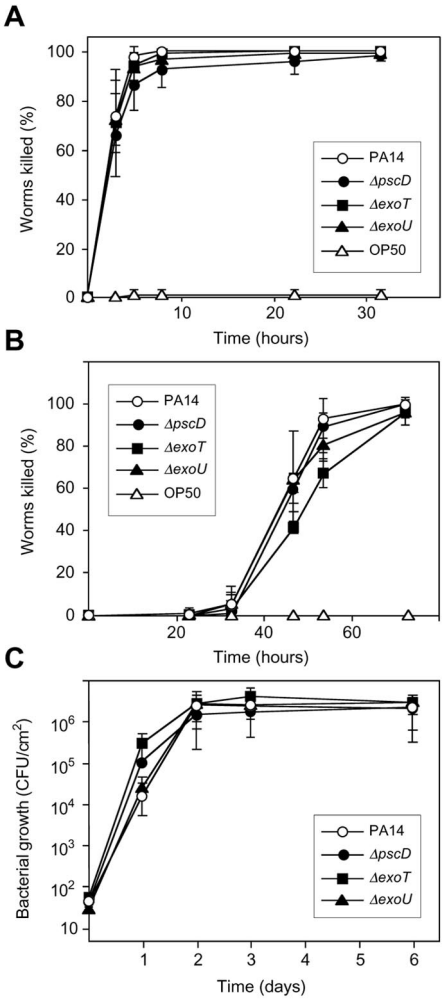

FIG. 6. *C. elegans* and *Arabidopsis* pathogenicity assays. (A) Assay of fast killing of *C. elegans* feeding on wild-type PA14 and Δ*pscD*, Δ*exoT*, and Δ*exoU* mutants. The assay was carried out as described in Materials and Methods. (B) Assay of slow killing of *C. elegans* feeding on wild-type PA14 and Δ*pscD*, Δ*exoT*, and Δ*exoU* mutants. *E. coli* OP50 was used as a negative control in each assay and showed killing close to zero. For both experiments, data represent the means and standard deviations of three replicates. The experiments were repeated at least three times, with similar results. (C) *Arabidopsis* ecotype Columbia leaves were infiltrated with wild-type PA14 and Δ*pscD*, Δ*exoT*, and Δ*exoU* mutants. Bacterial growth within the leaves was monitored for 6 days. Data represent the means and standard deviations of bacterial colony counts from six different leaves, except for time zero (at which point only four leaves were sampled). The experiment was repeated at least three times, with similar results.

Because type III secretion plays a critical role in a variety of plant pathogens, (5, 29, 33), we investigated whether the *P. aeruginosa* PA14 TTSS is relevant for pathogenesis in the model plant *Arabidopsis thaliana*. The Δ*pscD*, Δ*exoT*, and Δ*exoU* mutants infiltrated leaves of the Columbia ecotype of *Arabidopsis*, and bacterial growth in the infected leaves was monitored over a 6-day period. In the *Arabidopsis* model, the Δ*pscD*, Δ*exoT*, and Δ*exoU* mutants showed no differences in

symptom development or growth in leaves with respect to the wild-type strain (Fig. 6C).

DISCUSSION

A variety of *P. aeruginosa* strains are highly virulent in insects, such as *G. mellonella* (28, 31) and *D. melanogaster* (16), when inoculated directly into the hemolymph. The starting point for the work described in this report was the observation that a *P. aeruginosa* strain PA14 *pscD*::Tn*phoA* insertion mutant is highly attenuated in a *G. mellonella* pathogenesis model. Based on its homology to the *Y. pestis* YscD protein, which is required for secretion of the YopM effector protein (44), it is likely that PscD forms part of the *P. aeruginosa* TTSS apparatus (20). Protein profiles obtained with a PA14 Δ*pscD* mutant confirmed the importance of the *pscD* gene in the secretion of TTSS-dependent proteins in strain PA14 (Fig. 2A to C). Some of the proteins present in supernatants from wild-type PA14 cultures, which corresponded to known type III secretion proteins, such as ExoU, PopB, PopD, and PcrV, were not present in supernatants from the Δ*pscD* mutant. Moreover, immunoblotting indicated that the ExoT and ExoU effector proteins were not secreted in Δ*pscD* mutant supernatants.

Four type III effectors have been identified in *P. aeruginosa* to date: ExoS, ExoT, ExoU, and ExoY (18, 30, 54–56). Sequence analysis showed that although PA14 contains the sequences that surround *exoS*, the *exoS* coding sequence is not present in PA14, suggesting that at some point, PA14 may have carried *exoS* but it was deleted. The latter results agree with reports from Feltman et al. (17) and corroborate the inverse correlation that exists between the presence of the *exoS* and *exoU* genes in *P. aeruginosa*. Analysis of more than 100 *P. aeruginosa* isolates showed that all isolates but 2 contained either *exoS* or *exoU* but not both (17).

Interestingly, antibodies raised against the PA388 ExoY effector protein did not react with any of the proteins secreted in the PA14 supernatant. This result suggests that the ExoY protein may not be expressed in strain PA14 even though the *exoY* coding sequence is present in the PA14 genome and the sequences corresponding to *exoY* and 200 bp upstream are 99% identical to those in PA388.

The major goal of the work presented in this report was to determine whether the three known TTSS effector proteins in PA14 (ExoT, ExoU, and ExoY) play a significant role in *G. mellonella* killing. This goal was accomplished by constructing deletions in the *exoT*, *exoU*, and *exoY* genes, thereby generating Δ*exoT*, Δ*exoU*, and Δ*exoY* mutants; Δ*exoT* Δ*exoU*, Δ*exoU* Δ*exoY*, and Δ*exoT* Δ*exoY* double mutants; and a Δ*exoT* Δ*exoU* Δ*exoY* triple mutant. None of the three single mutants showed the high-level attenuated killing that was observed for the Δ*pscD* mutant, indicating that none of the three effectors (ExoT, ExoU, or ExoY) is essential for *G. mellonella* killing.

On the other hand, the results obtained with the Δ*exoU* Δ*exoY* and Δ*exoT* Δ*exoY* double mutants combined with the results obtained with the Δ*exoT* and Δ*exoU* single mutants indicate that both ExoT and ExoU activities play important roles in pathogenesis and that at least one of these activities is required for a high level of virulence. Importantly, similar results were obtained in the cytopathology assays performed with CHO cells (Table 2). Although the absence of a phenotype for the Δ*exoT* mutant in the cytopathology assay would suggest that ExoT does not play a significant role in pathogenesis, the results obtained with the Δ*exoT* Δ*exoU* double mutant indicated that both ExoT and ExoU are important virulence factors in insect and mammalian pathogenesis. As shown in Fig. 4 and Table 2, the Δ*exoT* Δ*exoU* double mutant exhibits a much more severe phenotype than either of the single Δ*exoT* and Δ*exoU* mutants in both *G. mellonella* and CHO cells. The fact that the single and double TTSS mutants generated in this study exhibit corresponding phenotypes in *G. mellonella* killing and CHO cytopathology assays validates the use of the *G. mellonella* model for the identification and study of TTSS components relevant for mammalian pathogenesis.

Interestingly, determining the extent of killing of *P. aeruginosa* in *G. mellonella* over the course of several days showed that the Δ*pscD* mutant was significantly attenuated with respect to the Δ*exoT* Δ*exoU* Δ*exoY* triple mutant, suggesting that an additional type III effector protein(s) or a type III-mediated mechanism that does not involve any of the effector proteins known in strain PA14 is partly responsible for the Δ*pscD* attenuated phenotype. Consistent with these results, recent reports have indicated that an as-yet-unknown type III secretion protein induces apoptosis in macrophages and HeLa cells (25). Moreover, *P. aeruginosa* cystic fibrosis isolates have been shown to cause ExoU-independent rapid cell death resulting from a pore-forming activity dependent on the intact *pcrGVH-popBD* operon (11) in macrophages and polymorphonuclear neutrophils (12). Importantly, the data obtained with the Δ*pscD* mutant and the Δ*exoT* Δ*exoU* Δ*exoY* triple mutant in *G. mellonella* also correlated with the data obtained from the cytopathology assay performed with CHO cells (Fig. 5 and Table 2). The Δ*exoT* Δ*exoU* Δ*exoY* triple mutant showed an intermediate phenotype between the Δ*pscD* mutant and wild-type PA14. These latter data further validate the use of the *G. mellonella* model for the study of the TTSS in *P. aeruginosa*.

Functionally distinct roles have been attributed to the four known effector proteins in *P. aeruginosa*. ExoT and ExoS are involved in inhibiting internalization and clearance by macrophages and other host cells implicated in defense mechanisms (10, 19, 21, 23). Additionally, ExoS is cytotoxic to eukaryotic cells (21, 40) and has been shown to mediate apoptosis (21, 32). ExoS, ExoT, and ExoY are implicated in disruption of the actin cytoskeleton (21, 23, 42, 53, 56), and ExoU has primarily a cytotoxic effect on host cells (18, 27, 53).

Consistent with previous reports (18, 23), we showed that the PA14 ExoT and ExoU effector proteins are involved in inhibiting the internalization (Fig. 3A) and acute cytotoxicity (Fig. 3B) of HeLa cells, respectively. In addition, cell rounding assays confirmed the role of ExoT in disruption of the actin cytoskeleton (23, 53). In contrast to previous reports, however, no involvement in cell rounding was observed for PA14 ExoY. These results were consistent with the observation that the PA14 ExoY protein could not be detected immunologically, even though an intact *exoY* gene appears to be present in the PA14 genome.

Even though the TTSS is conserved in various animal and plant pathogens and is involved in virulence in mammals (8), plants (5, 29, 33), and several nonvertebrate hosts, such as *G. mellonella* (31), *D. melanogaster* (16), and *Dictyostelium discoideum* (46), no *P. aeruginosa* type III secretion mutant had been

identified previously from screens of *P. aeruginosa* mutant libraries in *Arabidopsis* or *C. elegans* (36, 48, 52). Consistent with previous results, the data obtained in this study with the Δ*pscD*, Δ*exoT*, and Δ*exoU* mutants suggest that the TTSS does not play a significant role in the virulence of PA14 in either the *C. elegans* or the *Arabidopsis* model system. The results obtained clearly indicate that neither of the two modes of *C. elegans* killing was dependent on an intact type III secretion apparatus (Fig. 6A and B). Because the expression of the TTSS requires cell-to-cell contact (22, 29, 34) and toxin-mediated fast killing does not require live bacteria (51), the TTSS was not necessarily expected to play a role in *C. elegans* fast killing. However, *C. elegans* slow killing represents a more suitable system with which to detect an attenuated phenotype, because the mutants are in direct contact with the host. Based on the results obtained in this study, we initially hypothesized that the *C. elegans* lumen lacks the appropriate environment necessary to induce the expression of the TTSS. However, the identification of a type III secretion gene, *invH*, during a screen performed with a *Salmonella enterica* Tn*phoA*-mutagenized library for mutants that exhibited decreased virulence in *C. elegans* (A. Aballay and F. Ausubel, unpublished data) suggests that the effect of type III secretion proteins in *P. aeruginosa* may be masked by other virulence factors that play a more predominant role in *C. elegans* pathogenesis.

PA14 has also been shown to infect and cause disease in *Arabidopsis* (47). Plant pathogens, such as *P. syringae*, utilize the TTSS to secrete effector proteins that facilitate disease in susceptible plants and elicit a hypersensitive response in resistant plants (5, 29, 33). While a critical role for type III secretion has been implicated in the full virulence of several bacterial plant pathogens (5, 29, 33), our results show that TTSS mutations had no significant impact on the growth of PA14 in *Arabidopsis* leaves (Fig. 6C). As is the case in *C. elegans*, the effect of TTSS proteins may be masked by molecules, such as phospholipase C and exotoxin A, that have been found to be associated with disease symptoms in *Arabidopsis* leaves (47).

The work described in this report confirmed that *G. mellonella* is an important nonmammalian model host for study of the TTSS in *P. aeruginosa* virulence. Our results are consistent with those of a recent study by Fauvarque et al. (16) that showed the importance of the *P. aeruginosa* TTSS in the induction of the rapid death of *D. melanogaster*, indicating that insects are appropriate alternative nonmammalian hosts for identification and study of the components of the *P. aeruginosa* TTSS.

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