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 ExoU effector protein and does not seem to express ExoY. Moreover, using ΔexoU ΔexoY, ΔexoT ΔexoY, and ΔexoT ΔexoU 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 ΔexoT ΔexoU ΔexoY triple mutant and a ΔpscD 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 TnphoA 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 TnphoA insertion events. These results suggested either that TnphoA 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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Recently, 1,560 *P. aeruginosa* PA14 TnphoA 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 (LD50) 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 ΔexoT ΔexoU ΔexoY triple mutant was less attenuated in virulence than a ΔpscD 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 (OD600) of 0.3 to 0.4. Cultures were centrifuged, and pellets were resuspended in 10 mM MgSO4 to an OD600 of 0.1. Serial 10-fold dilutions were made in 10 mM MgSO4 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 rifampicin 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. LD50s 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 *SacI* and *HindIII* restriction sites. The PCR-amplified fragment was subcloned into the *SacI* and *HindIII* sites of the positive-sucrose-selection suicide vector pEX18Ap (45), generating plasmid pEX18pscD1. Plasmid pEX18pscD1 was used to introduce the wild-type *pscD* sequence into the homologous region of the *G. mellonella* 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′-TACGTACCTCATTTCCTGTCGCTAACGTCG-3′) and exoS4 (5′-ATCAAGCTTCAAGGCTGATTCCAC-3′) and sequenced. To corroborate that exoS was not present somewhere else

### TABLE 1. Bacterial strains and plasmids used in this study

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<th>Strain or plasmid</th>
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<td><strong>Strains</strong></td>
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<td>Positive selection suicide vector; Amp′</td>
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For statistical analysis as previously described (31).
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 PA01 exo open reading frame. The probe was used to hybridize EcoRI-digested genomic DNA from strains PA01, PA04, and PAK.

Sequencing of the exo open reading frame and surrounding sequences. For the generation of a PA14 ΔexoY mutant, we sequenced the exo open reading frame and surrounding sequences because the region upstream of exoY in strain PA14 was different from those in PA01 and PA388. The PA14 exoY gene was cloned by hybridizing a radiolabeled probe generated from a 1.0-kb PCR fragment that contained the PA01 exo open reading frame to a PA14 genomic cosmid library in vector pSB1 (47). A hybridizing clone identified from the genomic library was then subjected to sequence analysis with PA01- and PA14-specific primers.

Generation of PA14 ΔexoD, ΔexoT, ΔexoU, ΔexoC, ΔexoT ΔexoU, ΔexoC ΔexoT, ΔexoU ΔexoC, ΔexoD ΔexoU, and ΔexoT ΔexoD ΔexoU 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 PA01, except that the exoU downstream primer was based on the PA01 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 SacI and HindIII sites of pEX18Ap (45), generating plasmid pEX18pΔsci. 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 ΔexoD88 mutant. Similar strategies were used to construct ΔexoT, ΔexoU, and ΔexoY mutants as well as ΔexoT ΔexoU, ΔexoT ΔexoY, ΔexoU ΔexoY, and ΔexoT ΔexoU ΔexoY mutants. For the ΔexoT, ΔexoU, and ΔexoY 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 KpnI and HindIII sites of pEX18Ap, and the exoY deletion fragment was cloned into the EcoRI and HindIII sites of pEX18Ap, generating plasmids pEX18pΔExoT, pEX18pΔExoUS, and pEX18pΔExoY, respectively. The resulting constructs were used to introduce the deletions of the genes into the wild-type PA14 genome, resulting in ΔexoT1, ΔexoU3, and ΔexoY1 mutants. The ΔexoT ΔexoU, ΔexoT ΔexoY, and ΔexoU ΔexoY double mutants were constructed by recombining exoTΔU into ΔexoU3, exoUΔT into ΔexoT1, and exoUΔY into ΔexoU3. The ΔexoT ΔexoU ΔexoY triple mutant was constructed by recombining exoTΔU into the ΔexoU ΔexoY 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 (nitritoltriacetic acid [NTA]; 10 mM) was added to the induction medium to inhibit the TTSS. Proteins were recovered from the supernatant by ammonium sulfate precipitation (final concentration, 85% [wt/wt]). Twenty-microliter samples containing precipitated proteins were analyzed on sodium dodecyl sulfate (SDS)-10% polyacrylamide gels stained with Coomassie brilliant blue.

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. ExoS and ExoU were detected by using rabbit polyclonal ExoS and ExoU antisera specific for the ExoS 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 1:200. 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 MEMs medium (Gibco-BRL) with 10% fetal bovine serum (FBS) (Gibco-BRL). HeLa cells were plated in 12-well multiwell culture dishes at approximately live cells/well and incubated overnight at 37°C without shaking. Plaques, which appeared as opaque circular holes in the monolayers, were counted and measured.

RESULTS

The pscD TTSS gene is required for virulence in a G. mel- lonella-P. aeruginosa model system. P. aeruginosa strain PA14 mutant 2B3, which contains a TnphoA insertion in the pscD gene, was identified during a screen for mutants that exhibited decreased virulence in G. mellonella by using a PA14 TnphoA mutagenized library. Briefly, 1,560 individual insertion mutants.
from two existing PA14 TnphoA 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 TnphoA 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 TnphoA insertion in the pscD gene, we replaced the TnphoA insertion in 2B3 with a wild-type copy of pscD (generating strain 2B3R-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 TnphoA 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 LD50 of greater than 40,000, compared to an LD50 of 10 for parent strain PA14 (Fig. 1). In contrast, the LD50 of strain 2B3R-restored was not significantly different from the LD50 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 (ΔpscD) 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 LD50 of the ΔpscD in-frame deletion mutant was not significantly different from the LD50 of mutant 2B3R.

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 ΔpscD mutant were compared to those from wild-type strain PA14, several bands corresponding to secreted proteins were absent from the ΔpscD 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 ΔpscD 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 ΔpscD mutant. On the other hand, the secretion of TTSS proteins was restored to a wild-type profile when the TnphoA 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 ΔpscD mutant corresponded to known P. aeruginosa effector proteins (53), we performed immunoblot analysis of supernatants from wild-type strain PA14 and the ΔpscD 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 ΔpscD 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 PA01 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 PA01 exoS fragment as a probe. The probe specific for the exoS sequence did not hybridize to genomic

FIG. 1. LD50s 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. LD50s were determined by using the SYSTAT program as previously described (31). Bars represent the means and standard deviations of at least three experiments.
To determine which of the effector proteins secreted from the PA14 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 ΔexoT 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 ΔpscD, ΔexoT, ΔexoU, and ΔexoY 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 ΔexoU 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 ΔexoU mutant is internalized in HeLa cells approximately fourfold less efficiently than the ΔexoT ΔexoU double mutant, consistent with the proposed role of the ExoT effector protein in inhibiting internalization (23). Moreover, the ΔexoU ΔexoY double mutant did not show any significant difference in internalization with respect to the ΔexoU 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 ΔexoT ΔexoU mutant, which has an intact TTSS apparatus, was reproducibly higher than that of the ΔpscD 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 pscD::Tn5 and PA103 ΔexoT ΔexoU 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 ΔpscD and the ΔexoU 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 decrease 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 ΔexoU ΔexoY 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 ΔpscD or ΔexoT ΔexoU ΔexoY mutant. Moreover, the appearance of the latter cells was similar to that of uninfected cells. Importantly, HeLa cells infected with the ΔexoT ΔexoU mutant showed no differences in rounding compared to ΔpscD or ΔexoT ΔexoU ΔexoY mutant-infected cells, suggesting that if ExoY is secreted by strain PA14, it may not be functional.

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 ΔexoT ΔexoU, ΔexoU ΔexoY, and ΔexoT ΔexoY double mutants and the ΔexoT ΔexoU ΔexoY 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 ΔexoU 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 ΔexoT 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 ΔexoT 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).

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.
Roles of type III effector proteins in *G. mellonella* killing. We analyzed the virulence phenotypes of the ΔexoT, ΔexoU, and ΔexoY mutants in the *G. mellonella* model to determine the roles, if any, of the different effector proteins in *G. mellonella* killing. Figure 3 shows no statistical differences between the LD50s of the ΔexoT and ΔexoY mutants and the wild-type strain, indicating that neither ExoT nor ExoY is essential for *G. mellonella* killing. On the other hand, the ΔexoU 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 ΔexoU ΔexoY, ΔexoT ΔexoY, and ΔexoT ΔexoU double mutants along with a ΔexoT ΔexoU ΔexoY triple mutant. As shown in Fig. 4, the LD50 of the ΔexoU ΔexoY mutant was slightly increased in the wax moth killing assay, whereas the ΔexoT ΔexoY mutant had the same LD50 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 ΔpscD mutant (Fig. 4). On the other hand, the data obtained with the ΔexoT ΔexoU and ΔexoT ΔexoU ΔexoY mutants indicated that ExoY does not play a significant role in killing (Fig. 4).

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 ΔexoT ΔexoU and ΔexoT ΔexoU ΔexoY mutants, indicating that the ExoY effector protein of PA14 did not have a significant cytopathologic effect on CHO cells (Table 2). Moreover, the ΔexoT and ΔexoU mutants formed much larger plaques than the ΔexoT ΔexoU 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 ΔexoU mutant was slightly different from that of the wild type, whereas the ΔexoT 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-

![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 ΔpscD, ΔexoU, ΔexoT ΔexoU, and ΔexoU ΔexoY 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 ΔpscD, ΔexoT, ΔexoU, and ΔexoY 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.](image)

![FIG. 4. Killing of *G. mellonella* larvae by PA14 type III secretion mutants. LD50s of *P. aeruginosa* strain PA14 and ΔpscD, ΔexoT, ΔexoU, ΔexoY, ΔexoU ΔexoY, ΔexoT ΔexoY, ΔexoT ΔexoU, and ΔexoT ΔexoU ΔexoY 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. LD50s 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.](image)

<table>
<thead>
<tr>
<th>Strain</th>
<th>Avg plaque diam (mm) for 12 samples</th>
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<tbody>
<tr>
<td>PA14</td>
<td>8.12 ± 1.00</td>
</tr>
<tr>
<td>ΔpscD</td>
<td>No plaques formed</td>
</tr>
<tr>
<td>ΔexoT</td>
<td>7.54 ± 0.86</td>
</tr>
<tr>
<td>ΔexoU</td>
<td>5.62 ± 0.74</td>
</tr>
<tr>
<td>ΔexoY</td>
<td>8.83 ± 1.05</td>
</tr>
<tr>
<td>ΔexoT ΔexoU</td>
<td>2.71 ± 0.86</td>
</tr>
<tr>
<td>ΔexoT ΔexoU ΔexoY</td>
<td>3.0 ± 0.83</td>
</tr>
</tbody>
</table>
creted by the TTSS. The results obtained from the cytopathology assays performed with CHO cells also showed that the ΔexoT ΔexoU ΔexoY triple mutant had a phenotype intermediate between those of the ΔpscD 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 ΔpscD, ΔexoT, ΔexoU, ΔexoY, ΔexoT ΔexoU, and ΔexoT ΔexoU ΔexoY mutants. Figure 5 shows that although the ΔexoT ΔexoU and ΔexoT ΔexoU ΔexoY mutants had LD₅₀ similar to that of the ΔpscD mutant at 24 h after injection, when the extent of killing was determined at later times (2 to 4 days), the ΔexoT ΔexoU and ΔexoT ΔexoU ΔexoY mutants exhibited approximately 165-fold decreases in their LD₅₀ with respect to the ΔpscD mutant (Fig. 5). Because the ΔpscD mutant exhibited a significantly higher LD₅₀ than the ΔexoT ΔexoU and ΔexoT ΔexoU ΔexoY 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 ΔexoU mutant also showed a slight decrease in the LD₅₀ after 24 h (Fig. 5), whereas the ΔexoT and ΔexoU 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 ΔpscD, ΔexoT, and ΔexoU 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.

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 ΔpscD, ΔexoT, and ΔexoU 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 ΔpscD, ΔexoT, and ΔexoU 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::TnphoA 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 ΔpscD 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 ExoS, PopB, PopD, and PcrV, were not present in supernatants from the ΔpscD mutant. Moreover, immunoblotting indicated that the ExoT and ExoU effector proteins were not secreted in ΔpscD mutant supernatants.

Four type III effector 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 ΔexoT, ΔexoU, and ΔexoY mutants; ΔexoT ΔexoU, ΔexoU ΔexoY, and ΔexoT ΔexoY double mutants; and a ΔexoT ΔexoU ΔexoY triple mutant. None of the three single mutants showed the high-level attenuated killing that was observed for the ΔpscD 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 ΔexoU ΔexoY and ΔexoT ΔexoY double mutants combined with the results obtained with the ΔexoT and ΔexoU 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 pheno-

type for the ΔexoT mutant in the cytopathology assay would suggest that ExoT does not play a significant role in pathogenesis, the results obtained with the ΔexoT ΔexoU 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 ΔexoT ΔexoU double mutant exhibits a much more severe phenotype than either of the single ΔexoT and ΔexoU 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 ΔpscD mutant was significantly attenuated with respect to the ΔexoT ΔexoU ΔexoY 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 ΔpscD 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 ΔpscD mutant and the ΔexoT ΔexoU ΔexoY 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 ΔexoT ΔexoU ΔexoY triple mutant showed an intermediate phenotype between the ΔpscD 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 ΔpscD, ΔexoT, and ΔexoU 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 TphoA-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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