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Role of the Type III Secreted Exoenzymes S, T, and Y in Systemic Spread of *Pseudomonas aeruginosa* PAO1 In Vivo

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***Pseudomonas aeruginosa* uses a dedicated type III secretion system to deliver toxins directly into the cytoplasm of host cells. While progress has been made in elucidating the function of type III-secreted toxins in vitro, the in vivo functions of the type III-secreted exoenzymes are less well understood, particularly for the sequenced strain PAO1. Therefore, we have systematically deleted the genes for the three known type III effector molecules (*exoS*, *exoT*, and *exoY*) in *P. aeruginosa* PAO1 and assayed the effect of the deletions, both singly and in combination, on cytotoxicity in vitro and in vivo. We found that the type III secretion system acts differently on different cell types, causing an *exoST*-dependent rounding of a lung epithelial-like cell line in contrast to causing an *exoSTY*-independent but translocase (*popB*)-dependent lysis of a macrophage cell line. We utilized an in vivo competitive infection model to test each of our mutants, examining replication in the lung and spread to secondary sites such as the blood and spleen. Type III mutants inoculated intranasally exhibited only a minor defect in replication and survival in the lung, but *popB* and *exoSTY* triple mutants were profoundly defective in their ability to spread systemically. Intravenous injection of the mutants indicated that the type III secretion machinery is required for survival in the blood. Furthermore, our findings suggest that the effector-independent *popB*-dependent cytotoxicity that we and others have observed in vitro in macrophage cell lines may not be of great importance in vivo.**

Pseudomonas aeruginosa is a gram-negative pathogen noted for its innate resistance to antibiotics and for its ability to cause a wide spectrum of serious opportunistic infections. *P. aeruginosa* is a leading cause of ventilator-associated pneumonia and catheter infections in hospitalized patients (11, 39) and also tends to infect burn victims and immunocompromised patients, such as the elderly and cancer patients being treated with immunosuppressive chemotherapy (26, 29, 39). Perhaps most notoriously, *P. aeruginosa* is also the main pathogen responsible for the severe chronic lung infections in cystic fibrosis patients, which ultimately lead to loss of lung function and death in this patient group (15).

Among its large arsenal of virulence traits, *P. aeruginosa* encodes a type III secretion system (45). The type III secretion machinery is a dedicated system that allows bacteria to inject toxins directly into the cytoplasm of a host cell (20). The presence of a functional type III secretion system is correlated with a poor disease outcome in patients with ventilator-associated pneumonia (16).

There are four known type III secreted toxins in *P. aeruginosa*. Exoenzyme S and exoenzyme T are highly homologous bifunctional proteins with an amino-terminal G-protein-activating protein (GAP) domain and a carboxy-terminal ADP ribosylation domain (2, 12, 14, 22). The GAP domains of either enzyme target small Rho-like GTPases and can cause cytoskeletal rearrangements (14, 22). The ADP ribosylation domain of ExoS targets small Ras-like proteins (1, 9, 10, 28, 44), whereas that of ExoT was recently demonstrated to target CrkI and CrkII (36), two host kinases that regulate focal adhesion and phagocytosis. Exoenzyme U is a phospholipase (32), and exoenzyme Y an adenylate cyclase (46). The complement of effectors varies from strain to strain, but, in general, all strains have *exoT* and about 89% of strains have *exoY*. The presence of *exoS* and that of *exoU* appear to be mutually exclusive (31). Strains expressing *exoU* appear to be more acutely cytotoxic and to lyse target cells in vitro (6). The sequenced *P. aeruginosa* strain PAO1 contains *exoS*, *exoT*, and *exoY* but lacks *exoU* (35). Secretion of all of the exoenzymes into host cells apparently depends on the presence of the *popB* and *popD* gene products, which are believed to form part of the translocation machinery (17, 33, 38).

A growing body of in vitro experiments suggests that the mode of intoxication by the *P. aeruginosa* type III-secreted effectors depends not only on the particular effector but also on the targeted cell type (4). Specifically, when infected by a strain lacking *exoU*, epithelial cells appear to round up but do not lose their membrane integrity until late during the infection process (4). Macrophages, on the other hand, appear to be lysed quite readily, even by strains that do not express *exoU* (4, 5). Based on earlier analyses of type III-mediated toxicity in *Yersinia pseudotuberculosis*, it has been proposed that cells can be killed via pores that are formed in the macrophage membrane by the type III translocation machinery (40).

Although the type III secretion-mediated toxicity of *P. aeruginosa* has been intensively studied in vitro, much less is understood about how the various type III effectors collaborate to promote *P. aeruginosa* infection in vivo. Several animal models of infection have been used to analyze the contribution of type III effectors to virulence. One model assayed lung trauma in rats and rabbits after intranasal infection by monitoring the release of coinoculated radioactive albumin into the

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bloodstream. These experiments helped establish ExoU as a major agent of trauma by *P. aeruginosa* strain PA103 (6, 7, 23). Other experiments demonstrated that strain PAK bacteria lacking *exsA*, the gene encoding the major regulator of the type III secretion regulon, were severely defective for lung colonization and spread to the liver and spleen in adult mice (34). However, when a neonatal mouse model was used to assess the contribution of ExsA to colonization in strain PAO1, no significant defect was found (37). These differences in experimental outcome may be due to the different animal models (infant mice versus adult mice) or, more likely, to differences in strain background, since PAO1 does not express the effectors efficiently (30). Experiments with an *exoU-exoT* double-null strain PA103 mutant also failed to uncover a significant defect in lung colonization, but since these experiments were carried out in competition with the wild type, any colonization defect may have been masked by *trans*-complementation. Interestingly, however, the *exoUT* mutant was impaired in spread to the liver (12). A defect in systemic spread was also observed in early experiments in a mouse burn model using strain PA388, in which a transposon mutant which was unable to produce ExoS displayed a defect in spread to the bloodstream (27). The type III secretion system of PA103, but not that of PAK, was shown to be required for virulence in a corneal-infection model (24). The type III-mediated toxicity of the sequenced strain PAO1 has been less well studied than that of other strains, in part because the type III effectors in this strain are poorly expressed in vitro (30).

In this study, we systematically examined the role of type III secretion in the sequenced reference strain PAO1. We generated in-frame deletions of each of the three known type III-secreted exoenzymes, as well as the double and triple mutants. Using these and additional mutants, we confirmed that the type III secretion system of PAO1 mediates significant but differential toxicity in vitro against both A549 cells and a mouse macrophage-like cell line. We also developed a competitive acute in vivo infection model in which neutropenic mice are infected intranasally and the ability of wild-type and mutant bacteria to spread to the bloodstream and spleen is quantified. Although replication and survival of type III mutants in the lung were not greatly impaired by type III mutations, we found that spread to secondary sites such as the blood and spleen was decreased approximately 30-fold. Moreover, we observed a similar defect when bacteria were injected intravenously, implying that survival in the blood is a critical in vivo function of the type III secretion system.

MATERIALS AND METHODS

Bacterial strains. All *P. aeruginosa* mutants used in this study were derived from strain PAO1 (laboratory stock) (19, 35). Laboratory stocks of *Escherichia coli* DH5α and SM10λpir were used for cloning and mating into *Pseudomonas*, respectively. All strains and plasmids used in this study are listed in Table 1.

Plasmids. Plasmid pP32-*exoS*c was generated by amplifying the *exoS* open reading frame and promoter region with primers PexoS5 (5′-AAAAAggtaccCCGAGTCACTGGAGGCGGCCATTA-3′) and exoS5H (5′-AAAAAaagcttGGTCAGGCCAGATCAAGGCCGCGCAT-3′) (lowercase letters indicate restriction sites) and cloning the resulting PCR product as a KpnI/HindIII fragment into plasmid pPSV32 (30).

The deletion constructs pEXΔ*popB*, pEXΔ*exoS*, pEXΔ*exoT*, and pEXΔ*exoY* were provided to us by Matthew Wolfgang and Stephen Lory prior to publication. Briefly, the deletion plasmids were generated by recombinational cloning with the Gateway kit (Invitrogen). First, two regions flanking each deletion were amplified. The two flanking regions were then spliced together in a second round of PCR with the outer primers from the first round (splicing by overlap extension) (41). The outer primers were tailed with *attB* sites, allowing the spliced deletion PCR products to be cloned into the entry vector pDONR201. The deletion constructs were then recombinationally cloned into the allelic exchange vector pEX-GW (43). The exact primer sequences are available upon request (Matthew Wolfgang, University of North Carolina, Chapel Hill). Deletions were in frame so as to minimize polar effects.

A *lacZ* reporter gene was transferred to the neutral phage attachment site (*attB*) of the *P. aeruginosa* chromosome as follows: 317 nucleotides of the *Vibrio cholerae lacZ* promoter were cloned into the miniCTX-lacZ vector (3), and the resulting plasmid was then transferred to the *P. aeruginosa* chromosome (*attB* site) by mating and selection for tetracycline resistance. The selectable marker was removed by transient expression of the Flp recombinase from plasmid pFLP2 (18), which was then cured by counterselection on sucrose plates. The resulting *P. aeruginosa* strain PAO1 *attB*::*lacZ* was confirmed to grow as well as wild-type PAO1 in competition assays in vitro and in vivo.

TABLE 1. Strains and plasmids

Strain or plasmid	Relevant genotype or description	Reference or source
Strains		
RP289	PAO1, wild type	19
RP436	PAO1 *attB*::*Plac* (*V. cholerae*)-*lacZ*	This study
RP365	PAO1 Δ*popB*	This study
RP550	PAO1 Δ*exoS*	This study
RP555	PAO1 Δ*exoT*	This study
RP562	PAO1 Δ*exoY*	This study
RP580	PAO1 Δ*exoS* Δ*exoT*	This study
RP568	PAO1 Δ*exoS* Δ*exoY*	This study
RP564	PAO1 Δ*exoT* Δ*exoY*	This study
RP576	PAO1 Δ*exoS* Δ*exoT* Δ*exoY*	This study
Plasmids		
pPSV32	Shuttle vector with gentamicin resistance gene (*aacCI*), PA origin, *lacI*^{q}, and the *lac* promoter and MCS[a] of pUC18	30
pP32-*exoS*c	*exoS* gene including promoter region cloned into pPSV32	This study

[a] MCS, multiple cloning sites.

Cell lines. A549 cells (ATCC CCL-185), a human cell line with epithelial morphology, and RAW264.7 (ATCC TIB-71), a mouse monocyte-derived cell line, were grown in RPMI 1640 tissue culture medium (CellGro) supplemented with 2 mM glutamine, 10 mM HEPES, and 10% fetal bovine serum.

Cell rounding assay. A549 cells were seeded in 24-well plates at approximately 8×10^4 cells/well the day prior to the experiment. On the day of the experiment, cells were infected at a multiplicity of infection of 50 (assuming $\sim 10^5$ A549 cells/well) for 4 h. The infection was stopped by removing the medium and fixing the cells with 4% paraformaldehyde solution. The percentage of cells that were rounded was assessed by phase microscopy.

LDH release assay. Necrosis of RAW264.7 macrophages was assayed by monitoring the release of the cytoplasmic enzyme lactate dehydrogenase (LDH). On the day of the experiment, RAW264.7 cells were scraped up, washed once with fresh RP10 medium, resuspended in fresh RP10 without phenol red indicator dye (Invitrogen), and then dispensed into a 96-well plate (Corning) at 10^4 cells/well. The cells were infected with *P. aeruginosa* at a multiplicity of infection of 50. After 300 to 330 min of infection, the extent of LDH release was assayed with the Cytotox96 kit according the manufacturer's instructions (Promega).

Animal experiments. Three days prior to infection, 4- to 8-week-old C57BL/6 mice were injected intraperitoneally with 200 mg of cyclophosphamide (Sigma)/kg of body weight. Some mice were also injected intraperitoneally with 250 μg of the RB6-8C5 monoclonal antibody (RB6; gift from Daniel Portnoy) 2 days prior to infection and then again on the day of infection. This dose of RB6 has been reported to be sufficient to eliminate neutrophils (13, 42). Depletion of leukocytes by the day of infection was confirmed by peripheral blood analysis (see below). On the day of the infection, mice were anaesthetized with a mixture of xylazine and ketamine and infected intranasally or intravenously. For intranasal infections, 20 μl of a suspension of 5×10^8 CFU of *P. aeruginosa*/ml was placed on the nares of an anaesthetized mouse. The mouse was kept upright as the entire inoculum was inhaled into the lungs. For intravenous infections,

anaesthetized mice were infected retro-orbitally with 50 μl of a 10^8-CFU/ml suspension of *P. aeruginosa*. After the infection, the mice were monitored during their recovery. At 21 h postinfection, the mice were sacrificed by CO_2 asphyxiation. Mice failing to survive to 21 h were excluded, though analysis of a sampling of dead mice indicated that their inclusion would not have significantly affected the results. Approximately 200 μl of blood was removed by cardiac puncture and diluted into 5 ml of phosphate-buffered saline with 0.5% Triton X-100. The lungs and spleens were subsequently removed, homogenized for approximately 10 s with a PowerGen 700 homogenizer (Fisher Scientific), and titered for CFU on Luria-Bertani X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) plates (50 μg of X-Gal/ml) and scored both for total CFU and ratio of blue (wild-type) to white (mutant) bacteria.

Peripheral blood counts. Approximately 200 μl of blood was harvested from the tail veins of four C57BL/6 mice prior to and 3 days following intraperitoneal injection with 200 mg of cyclophosphamide/kg. Blood was diluted threefold in heparin–phosphate-buffered saline and analyzed by the clinical blood analysis facility at Children's Hospital (Boston, Mass.).

RESULTS

The role of the effector proteins of strain PAO1 in cytotoxicity against cultured cell lines. We constructed mutants with deletions of the known type III-secreted effectors from *P. aeruginosa* PAO1 and assayed them for their ability to intoxicate A549 cells (Fig. 1A) and RAW264.7 macrophages (Fig. 1B). The rounding of A549 cells depended entirely on exoenzymes S and T, since a mutant lacking both enzymes was unable to cause cell rounding. This defect in cytotoxicity was readily complemented by expressing exoenzyme S in *trans* (Fig. 1C). These results are consistent with previous experiments using *P. aeruginosa* PA103 (12). The rounding is due to the amino-terminal GAP domains of ExoS or ExoT (14, 22).

The PAO1 exoenzyme mutants that we generated were also tested for cytotoxicity against RAW264.7 macrophages (Fig. 1B). In contrast to the results with A549 cells, we found that RAW264.7 cells were overtly lysed (rather than rounded without lysis) by *P. aeruginosa* PAO1 and that, moreover, deletion of all three effectors had only a small effect on the lysis of RAW264.7 cells. Any cytotoxicity attributable to the effector proteins themselves appears to be due to the action of exoenzyme S.

As mentioned above, it has been proposed that macrophage lysis depends on pore formation by the translocation machinery itself (5). Consistent with this observation, a PAO1 *popB* mutant is greatly reduced in cytotoxicity, even against RAW264.7 macrophages. The presence of exoenzyme Y had no apparent effect on phenotype in either assay system. It remains possible that the *popB*-dependent *exoSTY*-independent cytotoxicity against RAW264.7 macrophages that we observe is due to another type III effector, though significant bioinformatic and experimental efforts have not yet identified any other novel type III effector.

The in vitro experiments in Fig. 1 are important for several reasons. The complementation data (Fig. 1C) provide evidence that the triple-effector mutant that we generated did not accumulate a second-site mutation affecting toxicity and that the secretion machinery is in fact still intact. These data also confirm the previous findings that the type III toxicity exhibited by *P. aeruginosa* differentially affects different cell types and underline the important question as to which are the relevant cell types targeted in vivo by the *P. aeruginosa* type III secretion system.

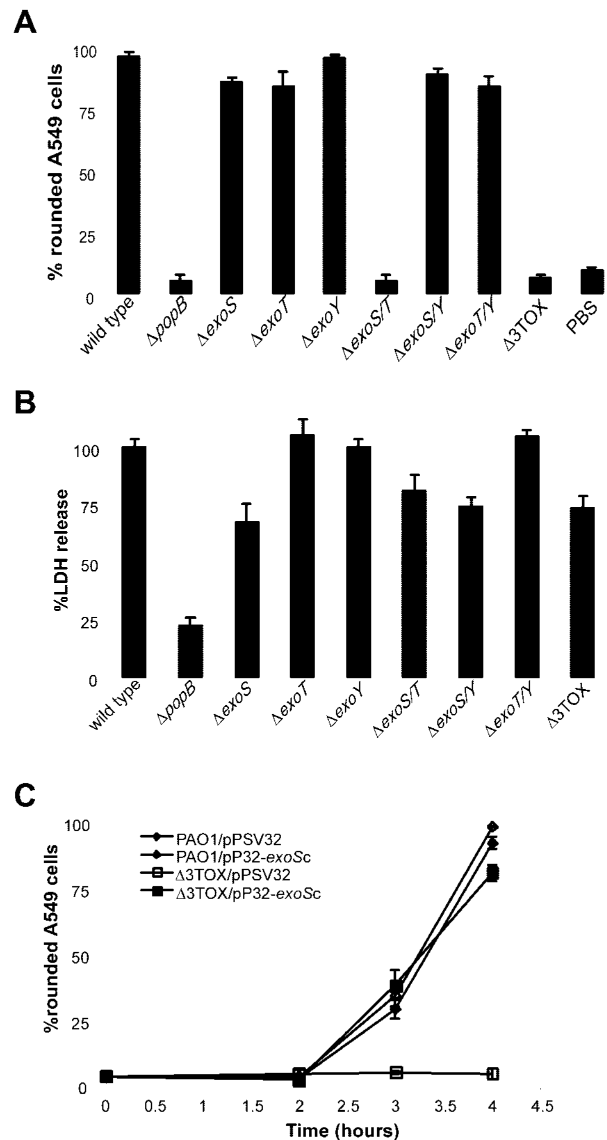

FIG. 1. Differential effects of the type III secretion system on cultured cells in vitro. (A) Rounding of A549 lung epithelial cell-like cells requires exoenzymes S and T. Cells were exposed to wild-type *P. aeruginosa* PAO1 or the indicated PAO1 mutant strains for 4 h before fixation and enumeration of the percentage of cells that had rounded up. Δ3TOX is a PAO1 Δ*exoS* Δ*exoT* Δ*exoY* triple mutant. PBS, phosphate-buffered saline. (B) Lysis of RAW264.7 macrophages requires an intact type III secretion system but does not require any of the known secreted effector proteins. The amounts of LDH released from cells ~5 h after exposure to wild-type *P. aeruginosa* PAO1 or the indicated PAO1 mutant strains are shown. The amount of enzyme released by detergent was set as 100%, and the amount of enzyme released in mock-treated cells was set as 0%. (C) The cell rounding phenotype of the Δ3TOX mutant can be complemented by expression of *exoS* from a plasmid. Wild-type PAO1 or PAO1 Δ3TOX was transformed with an ExoS expression plasmid (pP32-*exoSc*) or an empty vector control plasmid (pPSV32), and the extent of A549 cell rounding was assessed at various time points after infection.

A neutropenic-mouse model of acute infection and systemic spread. To study the in vivo role of the type III secretion system, we needed a relevant animal model. Since there is currently no animal model that faithfully replicates the chronic

TABLE 2. Peripheral blood counts in untreated and immune-depleted mice

Cell type	Blood count (cells/mm^3) ± SD in C57BL/6 mice		
	Untreated (n = 4)	Treated with: Cyclophosphamide (200 mg/kg) (n = 4)	Treated with: Cyclophosphamide and RB6 (250 μg) (n = 4)
Total white blood cells	$(10.6 \pm 0.4) \times 10^3$	$(0.17 \pm 0.10) \times 10^3$	$(0.04 \pm 0.02) \times 10^3$
Red blood cells	$(9.6 \pm 0.4) \times 10^6$	$(6.65 \pm 0.08) \times 10^6$	$(9.5 \pm 2.4) \times 10^6$
Neutrophils	$(0.8 \pm 0.0) \times 10^3$	$(0.06 \pm 0.05) \times 10^3$	$(0.005 \pm 0.01) \times 10^3$ (undetected in 3 of 4 mice)
Lymphocytes	$(9.4 \pm 0.4) \times 10^3$	$(0.085 \pm 0.038) \times 10^3$	$(0.013 \pm 0.017) \times 10^3$ (undetected in 2 of 4 mice)
Eosinophils	$(0.2 \pm 0.0) \times 10^3$	$(0.02 \pm 0.02) \times 10^3$ (undetected in 2 or 4 mice)	$(0.0 \pm 0.0) \times 10^3$ (undetected in 4 of 4 mice)
Monocytes	$(0.13 \pm 0.05) \times 10^3$	$(0.007 \pm 0.006) \times 10^3$	$(0.002 \pm 0.001) \times 10^3$

P. aeruginosa lung infections seen in cystic fibrosis, we decided to examine the role of type III secretion in acute *P. aeruginosa* infections. As mentioned previously, healthy individuals are usually resistant to *P. aeruginosa* pneumonia, whereas clinically significant *Pseudomonas* infections tend to occur in patients with compromised immune systems. These infections are considerably more serious when associated with disseminated bacteremia. We therefore used mice that had been rendered neutropenic with the chemotherapeutic agent cyclophosphamide. We examined the ability of *P. aeruginosa* to spread from the site of inoculation (the lung) to disseminated sites (the blood and spleen). One key technical feature of the experiments is that the infections were performed competitively. Wild-type PAO1 was modified to express the *lacZ* gene from the neutral phage attachment site (*attB*), and these wild-type (*lac*-positive) bacteria were inoculated at a ratio of 1:1 with type III secretion mutants. The relative defect in colonization and systemic spread of the type III secretion mutants was monitored as a relative decrease in *lac*-negative bacteria compared to the *lac*-positive wild type in the output. Competitive infections allowed us to control for the variations in inoculation efficiency that we observed via the intranasal route.

We first characterized the extent of neutropenia induced 3 days after a single dose of cyclophosphamide (200 mg/kg) (Table 2). Peripheral blood analysis of mice prior to and 3 days after cyclophosphamide treatment demonstrated that a single dose of cyclophosphamide resulted in a significant decrease in circulating immune cells. Lymphocytes were affected most severely, decreasing to about 1% of the level seen in untreated animals. The number of monocytes dropped to about 6.5% of the level in control animals, and the number of neutrophils dropped to about 7.5% of the control (Table 2).

Initial experiments compared the survival and systemic spread of wild-type PAO1 to those of a Δ*popB* mutant or a strain lacking the genes for the three known effectors in PAO1, *exoS*, *exoT*, and *exoY* (Δ3TOX) (Fig. 2). While survival in the lung was at most only mildly affected, spread to the blood and spleen was significantly impaired. Both the Δ*popB* mutant and the Δ3TOX mutant were recovered from the spleen at only about 3% of the level of the wild-type strain. The competitive indices (CI) for the blood values were less pronounced (CI of 0.25 for the Δ*popB* mutant and 0.29 for the Δ3TOX strain), but still significant. The fact that the deletion of the three known effector molecules could essentially account for the entire effect of the type III secretion machinery on systemic spread suggested that the translocase complex-mediated cytotoxicity seen with the RAW264.7 macrophage cell line or primary macrophages (4) is not of great importance in vivo.

We next assayed the impact of deleting the three known effectors individually or in combination. Deletion of *exoS*, *exoT*, or *exoY* alone had no significant effect on the ability of *P. aeruginosa* PAO1 to spread and/or survive systemically (Fig. 3A). Of the double-null mutants, the strain lacking both *exoS* and *exoT* was most severely affected (Fig. 3B). Comparing the spleen values for the Δ*exoSY* and the Δ*exoTY* double mutants to those for the *exoS* and *exoT* single mutants, respectively, suggests that exoenzyme Y may contribute to systemic spread in vivo, even though we were not able to demonstrate any significant effect on cytotoxicity in our in vitro models. The contribution appears to be minor, however, since the Δ*exoST* double mutant was not statistically different from the strain lacking all three exoenzymes in our assay.

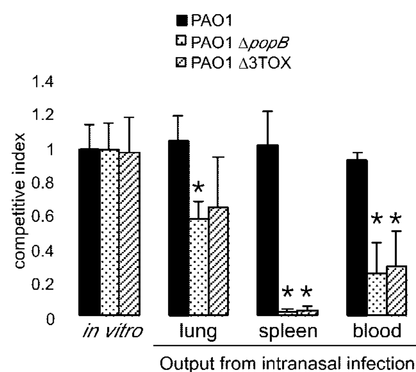

FIG. 2. Type III secretion mutants administered intranasally exhibit a mild defect in replication in the lung but exhibit reduced spread and survival in secondary sites, such as spleen and blood. Wild-type *P. aeruginosa* PAO1 or isogenic Δ*popB* or Δ3TOX mutants (producing white colonies on X-Gal plates) were mixed 1:1 with wild-type *P. aeruginosa* PAO1 carrying *lacZ* at a neutral phage attachment site (*attB*; produces blue colonies on X-Gal plates). The CI was calculated as the ratio of white to blue colonies in the output sample divided by the ratio of white to blue colonies in the input sample. In vitro-grown samples consisted of a 1:1,000 dilution of the inoculum grown overnight in Luria broth. Δ3TOX is a PAO1 Δ*exoS* Δ*exoT* Δ*exoY* triple mutant. At least three mice were analyzed for each genotype. *, $P < 0.02$ versus wild-type PAO1 for each tissue.

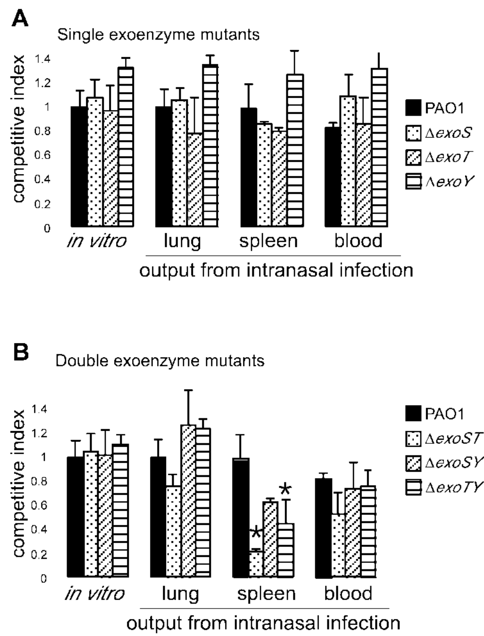

FIG. 3. Systematic analysis of in vivo growth and survival of single and double exoenzyme mutants. (A) PAO1 and isogenic mutants carrying in-frame deletions of exoenzyme S, T, or Y were inoculated intranasally (or grown in vitro) in competition with PAO1 *attB*::*lacZ*. After 21 h, the indicated tissues were homogenized and plated on X-Gal plates and the ratio of white to blue colonies was enumerated. The CI was calculated as the ratio of white to blue colonies in the output sample divided by the ratio of white to blue colonies in the input sample. At least three mice were analyzed for each genotype. (B) Same as panel A, but double mutants were tested. Data from Fig. 2 for PAO1 are shown for comparison. *, $P < 0.02$ versus wild-type PAO1 for each tissue.

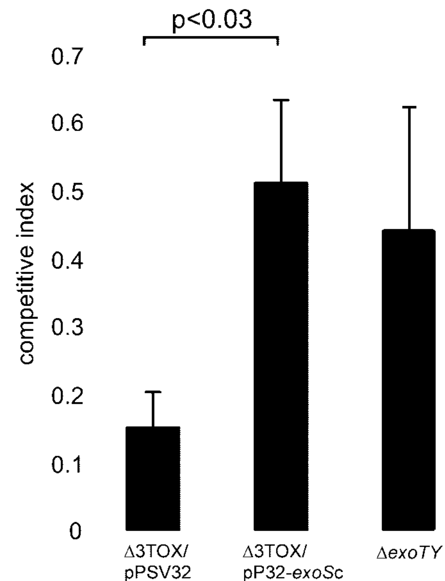

FIG. 4. An *exoS* expression plasmid can complement the growth and survival defect of a Δ3TOX (Δ*exoS* Δ*exoT* Δ*exoY)* mutant. PAO1 Δ3TOX was transformed with a plasmid expressing *exoS* (pP32-*exoSc*) or with an empty vector control plasmid (pPSV32). The two strains were inoculated intranasally, and the CI for the spleen was calculated as for Fig. 2. The testing of individual recovered colonies confirmed that both plasmids were stably maintained during in vivo growth. The CI for the Δ*exoTY* mutant (from Fig. 3) is shown for comparison. Student's *t* test was used to calculate *P* values.

Importantly, the defect in systemic spread could be complemented by expressing *exoS* in *trans*. A Δ3TOX strain expressing ExoS from a plasmid exhibited significantly higher growth and survival in the spleen than a Δ3TOX strain with a control plasmid (Fig. 4; $P < 0.03$). Indeed, complementing the Δ3TOX strain with the *exoS* plasmid restored systemic spread to the levels of the *exoTY* deletion mutant (Fig. 4).

Type III effectors are required for survival in blood. Three hypotheses were considered to explain the requirement for the type III secretion system for systemic spread in vivo: (i) the type III secretion system is required for breaching the epithelial barrier in the lung, (ii) the type III secretion system is required for survival in the blood, and (iii) both are the case. The last possibility is particularly intriguing, since the in vitro data suggested that there are differing mechanisms of intoxication for epithelial cells and macrophages, raising the possibility that the effector proteins may be involved in breaching the epithelial barrier, whereas translocase-mediated cytotoxicity could be involved in lysing circulating phagocytes.

To distinguish these possibilities, we analyzed the survival of our Δ*popB* and Δ3TOX mutants when inoculated intravenously instead of intranasally. As before, we analyzed survival in the blood, spleen, and lungs. Both the Δ*popB* mutant and the Δ3TOX mutant were reduced in number in the lung, blood, and spleen, suggesting that the defect in systemic spread that we observed in the intranasal infections is likely due to the impaired ability of the mutants to survive in the blood (Fig. 5). While the levels of the mutant bacteria in the intravenous route of infection appear to be more severely depressed in the lung than in the blood, the difference is not statistically significant, suggesting that survival in the bloodstream is the main determinant controlling the spread of the bacteria in this model.

It was surprising that *P. aeruginosa* was cleared by host defenses from the blood of cyclophosphamide-treated animals. However, blood analysis of cyclophosphamide-treated animals (Table 2) indicated the presence of residual leukocytes, particularly neutrophils, that might mediate clearance even in cyclophosphamide-treated mice. Therefore, we tested the effect of depleting residual neutrophils from the cyclophospha-

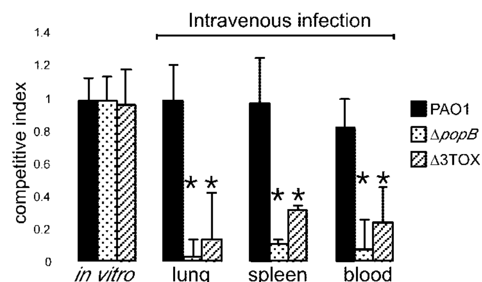

FIG. 5. The type III secretion system is required for survival of *P. aeruginosa* in the blood. The indicated mutant strains were inoculated intravenously, and 21 h later lungs, spleens, and blood were recovered from infected animals. The CI was calculated as for Fig. 2. *, $P < 0.02$ versus wild-type PAO1 for each tissue.

mide-treated mice with the neutrophil-specific monoclonal antibody RB6, as described previously (13, 42). Peripheral blood analysis confirmed that RB6 treatment reduced neutrophils to virtually undetectable levels (Table 2). These cyclophosphamide- and RB6-treated animals were infected intranasally with the *popB* mutant, and the CI for colonization of the spleen was determined. Interestingly, the CI for the Δ*popB* mutant in cyclophosphamide- and RB6-treated mice was less than 0.008 ± 0.005 ($n = 6$ mice), which is slightly but not significantly lower than the CI for the *popB* mutant in mice treated only with cyclophosphamide ($P = 0.06$; Student's t test). These results suggested that residual neutrophils are not required for eliminating type III secretion mutants from the blood of cyclophosphamide-treated mice.

DISCUSSION

A wide variety of pathogens use a type III secretion system to inject toxins directly into host cells. Here we have developed an in vivo competition assay to systematically test the impact of the type III secretion machinery and the secreted effectors on the survival and systemic spread of *P. aeruginosa*.

Prior work has shown that the mode of intoxication can vary with the cell type that *P. aeruginosa* infects, as well as the complement of effector proteins expressed by a given strain. *P. aeruginosa* strains were earlier classified as either cytotoxic or invasive, based on their cytotoxicity against cultured corneal epithelial cells (8). Later, it was discovered that this difference among strains is related to the type III effector proteins expressed by a given strain. Cytotoxic strains express the type III effector protein ExoU, a phospholipase with cytolytic activity (6). Invasive strains, on the other hand, bear the gene for exoenzyme S. In this study we analyzed strain PAO1, an *exoSTY*-positive but *exoU*-negative invasive strain. PAO1 is also the sequenced strain (35) whose type III secretion system, for historical reasons, has not been extensively characterized. We systematically deleted the genes for each of the effector molecules, singly or in combination, and assayed their effect on cytotoxicity in vitro and growth and systemic spread in vivo.

Consistent with previous work for strains PA388 and CHA (4, 5), our results demonstrate that strain PAO1 appears to intoxicate macrophages and epithelial cell-like cells by differing mechanisms. The rounding of epithelial cell-like cells is entirely dependent on the action of exoenzymes S and T, whereas the lysis of macrophages depends on the presence of a functional translocase but apparently does not depend on the individual effector molecules themselves. It has been proposed that translocase-dependent but effector-independent cytotoxicity arises when an "empty" type III secretion translocation apparatus punctures holes in host cell membranes (40). It is impossible to rule out the existence of additional undiscovered effectors, though extensive searches have failed to turn up such molecules. Nevertheless, the above in vitro experiments raised the question of the relevant mode of toxicity in vivo.

To address this question, we assessed the role of the type III effectors, as well as the type III secretion machinery, on survival and systemic spread in vivo. Since healthy C57BL/6 mice readily clear *P. aeruginosa* PAO1 and because we wished for our model to reflect the clinical realities of *P. aeruginosa* infections, we performed our experiments with immunocompromised animals. In the first set of experiments, animals were infected intranasally and the persistence of the bacteria in the lung and spread to blood and spleen were assayed the following day. Compared to wild-type bacteria, mutants lacking either a functional translocase (Δ*popB* mutant) or all three known effectors (Δ3TOX) did not display a severe defect for survival in the lung but were severely affected in their ability to be recovered from the blood or spleen of the infected mice. Of the three effector proteins, ExoS and ExoT appeared to be most important for infection.

On one hand, the reduced recovery of Δ*popB* and Δ3TOX mutants from the blood and spleen could indicate that the type III secretion machinery and effectors are needed to breach the epithelial layer and escape the lung into the bloodstream. On the other hand, the results could also be explained by an inability of the mutants to survive in blood. Performing intravenous infections demonstrated that the Δ*popB* and Δ3TOX mutants had a severe defect in survival in blood. While the Δ3TOX mutant appeared to be slightly more adept than the Δ*popB* mutant at surviving in blood, the difference was not significant. It appears, therefore, that *popB*-dependent but *exoSTY*-independent cytotoxicity (translocase-mediated cytotoxicity) is not as significant for survival in blood in our animal model as it is for intoxication of macrophages and neutrophils in vitro (5). We may have reached a different conclusion for a different animal model, i.e., one in which macrophages were not depleted by cyclophosphamide. Indeed, Lee et al. (25) found that PAK Δ*pscC* mutants were more defective in survival and replication in the spleens and livers of BALB/c mice than were Δ*exoSTY* mutants. However, it is worth noting that other work, comparing PA103 *exsA* and *exoUT* mutants in a corneal-infection model, concluded that translocase-mediated cytotoxicity is not significant in vivo (24).

Although our results highlight a role for the type III secretion system in survival in the blood, our experiments do not rule out the possibility that the type III secretion system might also play a role in helping *P. aeruginosa* breach the lung epithelium and gain access to the bloodstream. Because our experiments were performed competitively, the wild-type bacteria present might have breached the epithelium and allowed type III secretion mutants to access the blood. The potential for *trans*-complementation is one drawback of performing competitive infections. On the other hand, it is interesting that the type III secretion mutants exhibit a defect in our model, despite the potential for *trans*-complementation. Indeed, the fact that type III mutants were defective in systemic spread even when coinoculated with the wild type provided further support to the notion that damage to the epithelium (which should benefit mutant and wild-type bacteria alike) may not be the only function of the type III secretion system in vivo.

In this issue, Lee et al. (25) describe noncompetitive lung infections with another invasive strain (PAK) but were not able to ascertain a clear role for type III secretion in systemic spread. Although Lee et al. found that intranasally inoculated type III mutants were decreased in colonization of the liver and spleen, this decrease may have been due to a similar decrease seen in the lungs. It is possible that our use of competitive infections ameliorated any replication defects of the type III mutants in the lungs, thereby allowing us to uncover a role for type III secretion in spread to the spleen and blood.

On the other hand, the differences between our study and that of Lee et al. (25) may be due to the use of different mouse strains (C57BL/6 versus BALB/c) or *P. aeruginosa* strains (PAO1 versus PAK).

It was surprising that *P. aeruginosa* type III secretion mutants were cleared by host defenses from the circulation of severely leukopenic animals. Even mice treated with cyclophosphamide and further depleted of neutrophils with the RB6 monoclonal antibody were capable of preferentially clearing Δ*popB* mutants from the spleen. It remains possible that the depletion of neutrophils from cyclophosphamide- and RB6-treated mice was incomplete or that the Δ*popB* mutants were eliminated by fresh neutrophils that arose in the mice upon infection. However, we consider the latter possibility unlikely since we injected the animals with a second dose of the RB6 antibody at the time of infection and, based on previous studies, it is expected that adequate levels of RB6 remained in circulation during the 21 h of infection (13, 42). We also considered the possibility that our Δ*popB* mutant had accumulated mutations rendering it serum sensitive. However, we found that the Δ*popB* mutant retained viability in fresh 50% mouse serum, as did the parental PAO1 strain and the Δ3TOX mutant (data not shown). Moreover, our complementation data (Fig. 4) suggest that the exoenzyme mutations and not unlinked secondary mutations account for the phenotype that we observe. We therefore favor the idea that the type III secretion apparatus is required to target a component of host defense present in the circulation of cyclophosphamide-treated mice. Macrophages are one of the cell types that have been previously shown to play a role in *P. aeruginosa* infections (21), and it is possible that the few macrophages remaining in cyclophosphamide-treated mice were sufficient to preferentially clear the Δ*popB* mutant.

We have presented data that demonstrate the importance of the PAO1 type III secretion machinery for survival in blood and systemic spread in vivo. These data are consistent with observations in patients, where the presence of a functional type III secretion system in the infecting strain of *Pseudomonas* is correlated with a higher incidence of death or recurrence of the infection (16). The nature of the host factors that are targeted by the type III secretion machinery and that control the survival of *P. aeruginosa* in the blood remains to be fully elucidated.

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